THE GENETICS OF THE DOG

2ND EDITION

Edited by
Elaine Ostrander and Anatoly Ruvinsky

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The Genetics of the Dog, 2nd Edition

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Preface

The second edition of *The Genetics of the Dog* has been prepared for publication 10 years after the original version. This relatively short period of time has heralded an extraordinary degree of progress in canine genetics, and has seen our community move from the early stages of map building to the completion of large linkage and genome-wide association studies. The latter have allowed us to identify genes for disease susceptibility, behavioural traits and morphological features. In the last 10 years, a reference sequence of the Boxer dog was produced and dozens of genomes of other breeds are currently being sequenced using state-of-the-art high-throughput approaches. This progress in both genetics and genomics has led to the implementation of powerful laboratory methods which, together with the further advancement of bioinformatics, has produced the plethora of data available to students of mammalian biology and comparative genetics. For millennia, dogs have served humans in numerous ways. Most recently, however, the dog has become the model system of choice for the study of many human genetic disorders. In this edition, we have made an attempt to take all of these new developments into consideration. In doing so, not surprisingly, the chapter topics and the authors have changed significantly; the majority of chapters have been written by different authors. We believe that the final output is an improved product, with clarity of structure and updated content.

Recent data suggest that wolf domestication commenced more than 30,000 years ago, and is characterized by several independent events. No other domesticated animal had such an extensive and intertwined history with humans as the dog. It should also be noted that no other mammalian species shows such an enormous range of phenotypic variation as the dog. Multiple domestication events might contribute to this phenomenon. However, other factors, such as intensive selection for both behavioural and morphological traits have contributed to the variation we see today. Since the time of Charles Darwin, it has been apparent that the dog is the best object for studying domestication. Hopefully this book provides readers with interesting insights into different aspects of the domestication process.

The main purpose of the book is to present a comprehensive description of all modern topics related to dog genetics in a single place. The first five chapters discuss systematics, phylogeny, domestication and single gene traits. Chapters 6–10 present the latest data on immunogenetics and genetic aspects of disease. Gene mapping and genome structure are considered in Chapters 11 and 12. The next three chapters cover genetic aspects of behaviour, reproduction and development. Chapters 16 and 17 are devoted to morphological traits and olfactory genetics. Chapters 18–20 deal with genotype testing, pedigree analysis, quantitative genetics and complex traits. Three final chapters discuss the application of dog genetics to medical research, genetic aspects of performance in working dogs and genetic nomenclature. The second edition of this book is sharply different from the first edition by the choice of topics; 11 chapters out of the 23 are entirely new, and others are significantly or completely rewritten. The team of authors has also changed considerably; 25 new researchers were attracted to this project.

The authors of this book have made every attempt to highlight the key publications in the area of dog genetics during the last several decades, with emphasis on the most recent papers, reviews and books. However, we realize that omissions and errors are unavoidable and we wholeheartedly apologize for any omissions or mistakes. The book is addressed to a broad audience, including researchers, lecturers, students, dog breeders, veterinarians, kennel clubs and all those who are interested in modern domestic dog biology and genetics. *The Genetics of the Dog* is a continuation of a series of monographs on mammalian genetics published by CAB International. Other books deal with major domestic species such as the pig, cattle, the horse and sheep (http://www.cabi.org).

This book is a result of truly international cooperation. Scientists from the USA, Europe, Australia and Russia have contributed generously to the project. The editors are very grateful to all of them. We hope that the second edition of *The Genetics of the Dog* will support further consolidation and progress in this field of science and become an indispensable handbook for those interested in the genetics and genomics of man's best friend.

Elaine A. Ostrander
Anatoly Ruvinsky
October 2011

1 Canid Phylogeny and Origin of the Domestic Dog

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Introduction

The domestic dog (*Canis familiaris*) is the most phenotypically diverse mammal species known and ranges in size and conformation from the diminutive Chihuahua to the gargantuan Great Dane. The difference in size and conformation among dog breeds exceeds that among species in the dog family Canidae (Wayne, 1986a,b). Differences in behaviour and physiology are also considerable (Hart, 1995; Coppinger and Coppinger, 2001). An obvious question, therefore, is whether this diversity reflects a diverse ancestry or if it appeared after domestication. Darwin (1871) suggested that, considering the great diversity of dogs, they were likely to have been founded from more than one species. This sentiment has been periodically revisited by researchers (e.g. Lorenz, 1954; Coppinger and Schneider, 1995). Knowledge of the evolutionary history of domestic dogs and of their relationships to wild canids provides insight into the mechanisms that have generated the extraordinary diversity of form and function in the dog. In this chapter, we discuss the evolutionary history of dogs and their relationship to other carnivores as inferred from molecular genetic studies. We also address the mechanisms that may have been involved in the origin of modern breeds.

Evolutionary Relationships of the Domestic Dog

The modern carnivore families originated about 60 million years ago (Flynn and Galiano, 1982, Eizirik *et al.*, 2010). The domestic dog belongs to the family Canidae which, in turn, is classified within the suborder Caniformia and the order Carnivora. Therefore, seals, bears, weasels and raccoon-like carnivores, which are also within the suborder Caniformia, are more closely related to canids than are cats, hyenas and mongooses, which constitute the suborder Feliformia (Fig. 1.1). The Canidae family is the most phylogenetically ancient lineage within

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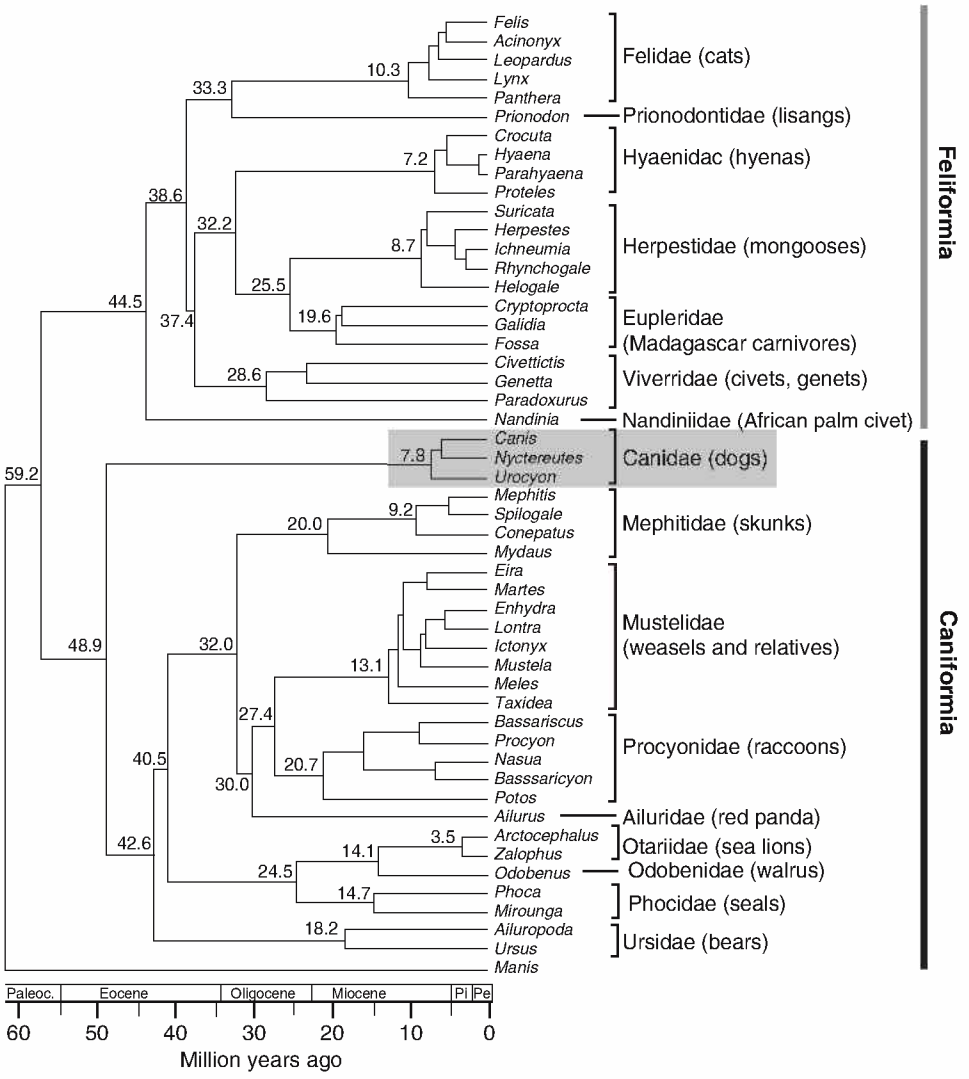

Fig. 1.1. Evolutionary tree of carnivores constructed with partial sequences of 14 genes (7765 base pairs) (based on Eizirik *et al.*, 2010). Suborder and family groupings are indicated. Numbers above nodes are divergence ages in millions of years ago.

the suborder Caniformia, having diverged from other carnivores about 50 million years ago.

Three subfamilies of canids have been recognized. The subfamily Hesperocyoninae includes the oldest and most primitive members of the family (Wang, 1994). This Oligocene to Miocene Age subfamily includes small- to medium-sized predators and lasted for over 20 million years. In the middle Miocene, the Hesperocyoninae were replaced by the Borophaginae, large bone-crushing dogs that are often the most common predators in late Tertiary deposits, but went extinct by the mid Pliocene, about 4 million years ago (Wang *et al.*, 1999). The third subfamily, Caninae, includes all living representatives of the family and first appeared in the Oligocene (Tedford *et al.*, 1995, 2009).

Although canids belong to an ancient lineage, the 36 extant species (Table 1.1) are all very closely related and diverged only about 10 million years ago, probably in North

Table 1.1. Extant species of the family Canidae based on Wilson and Mittermeier (2009), and including the domestic dog.

Taxon	Common name
Family Canidae	
Subfamily Caninae	
Genus: *Canis*	
C. lupus	Grey wolf[a]
C. familiaris	Domestic dog[b]
C. rufus	Red wolf[c]
C. latrans	Coyote
C. simensis	Ethiopian wolf
C. aureus	Golden jackal
C. adustus	Side-striped jackal
C. mesomelas	Black-backed jackal
Genus: *Cuon*	
C. alpinus	Dhole
Genus: *Lycaon*	
L. pictus	African wild dog
Genus: *Chrysocyon*	
C. brachyurus	Maned wolf
Genus: *Speothos*	
S. venaticus	Bush dog
Genus: *Cerdocyon*	
C. thous	Crab-eating fox
Genus: *Atelocynus*	
A. microtis	Short-eared dog
Genus: *Pseudalopex*	
P. culpaeus	Culpeo
P. fulvipes	Darwin's fox
P. griseus	South American grey fox
P. gymnocercus	Pampas fox
P. sechurae	Sechuran fox
P. vetulus	Hoary fox
Genus: *Urocyon*	
U. cinereoargenteus	Northern grey fox
U. littoralis	Island fox
Genus: *Nyctereutes*	
N. procyonoides	Raccoon dog
Genus: *Otocyon*	
O. megalotis	Bat-eared fox
Genus: *Alopex*	
A. lagopus	Arctic fox
Genus: *Vulpes*	
V. velox	Swift fox
V. macrotis	Kit fox
V. vulpes	Red fox
V. corsac	Corsac fox
V. ferrilata	Tibetan fox
V. bengalensis	Indian fox
V. pallida	Pale fox
V. rueppellii	Rüppell's fox
V. chama	Cape fox
V. cana	Blanford's fox
V. zerda	Fennec fox

[a] Some authors include the Great Lakes wolf as a separate species, *C. lycaon*.
[b] Considered by Wilson and Mittermeier (2009) as included in *C. lupus*.
[c] Some studies suggest that the red wolf might represent a lineage produced by hybridization between grey wolves and coyotes.

America (Fig. 1.2). Multiple studies on the evolutionary relationships among the members of the group have taken place using diverse molecular genetic approaches (e.g. Wayne and O'Brien, 1987; Wayne *et al.*, 1997; Bardeleben *et al.*, 2005; Lindblad-Toh *et al.*, 2005). Based on about 15 kb of nuclear DNA sequence (Lindblad-Toh *et al.*, 2005), four distinct groups can be identified within the extant Canidae; these include the North American grey foxes (the most divergent group, which includes the northern grey fox and the island fox), red fox-like canids (e.g. red, kit and Arctic foxes, among others), the South American foxes (e.g. grey and Pampas foxes, bush dog, maned wolf), and the wolf-like canids (the domestic dog, grey wolf, coyote, African hunting dog, dhole, Ethiopian wolf and jackals).

Evolutionary relationships among the canids are also suggested by chromosome similarity. Chromosome number and structure vary widely among canid species (see Chapter 11): from 36 metacentric chromosomes in the red fox to 78 acrocentric chromosomes in wolves, coyotes and jackals (Fig. 1.2). However, the wolf-like canids and South American canids all have high diploid chromosome numbers and acrocentric chromosomes, and are closely related (Fig. 1.2). Similarly, fox-like canids have low chromosome numbers and metacentric chromosomes, and share a common ancestry (Fig. 1.2). This high degree of variation contrasts with most other carnivore families in which chromosome number and structure are well conserved (Wurster-Hill and Centerwall, 1982). All wolf-like canids have 78 chromosomes and can hybridize to produce fertile offspring (Gray, 1954). These are the species that have been considered throughout history as possible ancestors of domestic dogs.

Origin of the Domestic Dog

The origin of domesticated species is seldom well documented. The number, timing and geographical origin of founding events may be difficult to determine from the patchy archaeological record (Zeder *et al.*, 2006). This problem is well exemplified by the domestic dog, for which data are consistent with both single and multiple origins from the grey wolf alone

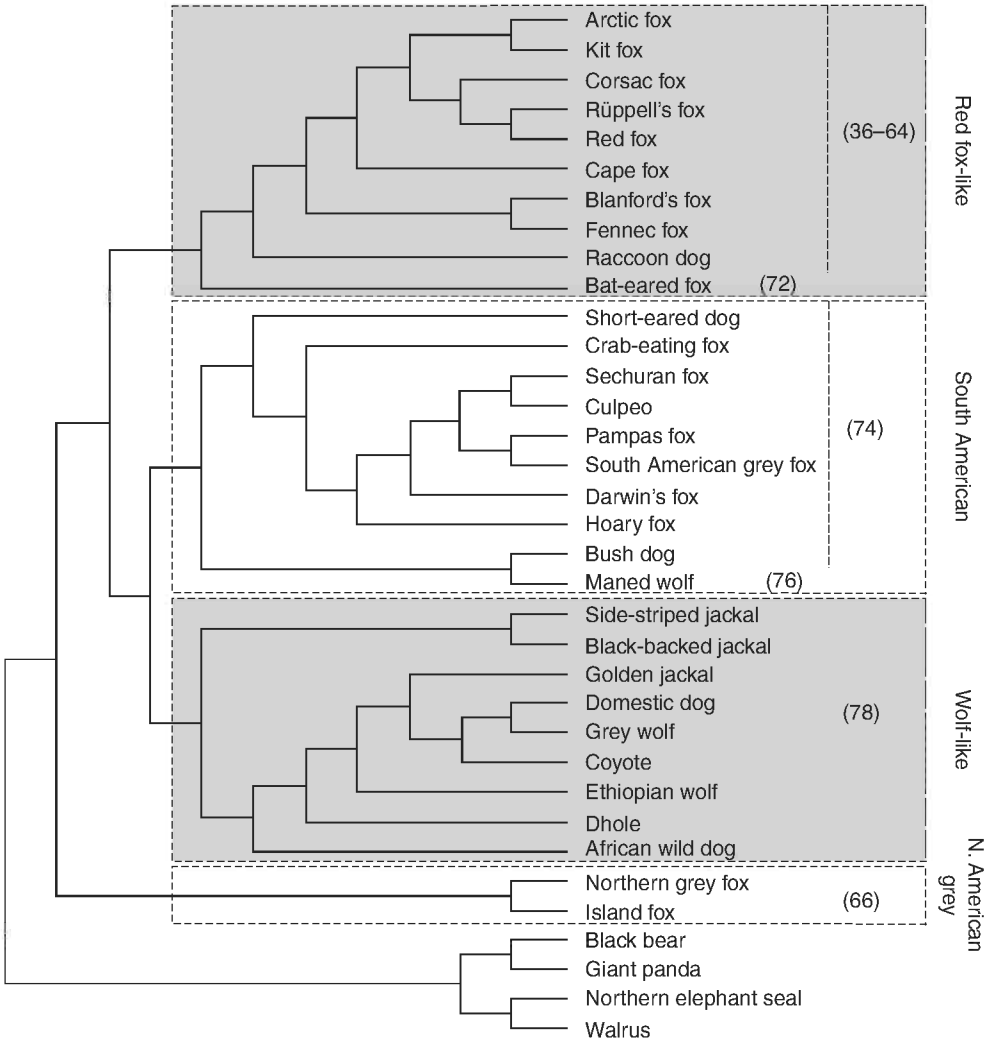

Fig. 1.2. Phylogenetic tree of canids based on analysis of about 15,000 base pairs of exon and intron DNA sequence (based on Linblad-Toh *et al.*, 2005). Boxes indicate main clades (red fox-like, South American, wolf-like and North American grey fox clades). Chromosome numbers are indicated in parentheses for species or groupings of canids (Wurster-Hill and Centerwall, 1982; Wayne *et al.*, 1987).

or, additionally, from the golden jackal, *Canis aureus* (Olsen, 1985; Clutton-Brock, 1995, 1999). Also, very few domestic dog remains have been identified at archaeological sites; remains are often fragmentary and the differentiation from wolf bones can be difficult. Even for the several hundred extant dog breeds that have been developed in the last few hundred years, the specific crosses that led to their establishment are often not known (Dennis-Bryan and Clutton-Brock, 1988). The only criterion traditionally used to differentiate between dog and wolf remains from archaeological sites was skeletal morphology. Most modern dogs are morphologically differentiated from both wolves and jackals (Olsen, 1985), and these differences were used to discriminate species at archaeological sites (e.g. Sablin and Khlopachev, 2002). Consequently, only morphologically differentiated dogs could be distinguished, and

the initial stages of dog domestication, when the morphological differentiation was probably small, might have passed unnoticed. However, increased effort and molecular tools are now being used to identify archaeological canid remains (Germonpré *et al.*, 2009; Losey *et al.*, 2011). The genetic diversity of the founding population is essential knowledge for understanding the immense phenotypic diversity of dogs. A heterogeneous origin would suggest that gene diversity is critical to phenotypic evolution, whereas a limited founder population would imply that developmental variation and novel changes in the genome are more important in breed diversity (e.g. Wayne, 1986a,b).

The ancestor of the dog

Molecular genetic data consistently support the origin of dogs from grey wolves. Dogs have allozyme alleles in common with wolves (Ferrell *et al.*, 1978; Wayne and O'Brien, 1987), share highly polymorphic microsatellite alleles (García-Moreno *et al.*, 1996; Muñoz-Fuentes *et al.*, 2010) and have mitochondrial and nuclear DNA sequences similar or identical to those found in grey wolves (Wayne *et al.*, 1992; Gottelli *et al.*, 1994; Vilà *et al.*, 1997a; Bardeleben *et al.*, 2005). An extensive survey of several hundred grey wolves and dogs found that the two species had only slightly divergent mitochondrial DNA control region sequences (Vilà *et al.*, 1997a). For example, the average divergence between dogs and wolves for this highly variable region was about 2% compared with 7.5% between dogs and coyotes, their next closest kin. The average divergence between dogs and wolves is inside the range of genetic variability observed for wild wolves (Vilà *et al.*, 1997a, 1999). The comparison of close to 15,000 base pairs of DNA sequences in introns and exons distributed over the entire genome represents a better estimate of the genome-wide sequence divergence between species, and confirmed that the grey wolf and the domestic dog are most closely related, with 0.04% and 0.21% sequence divergence in nuclear exon and intron sequences, respectively (Lindblad-Toh *et al.*, 2005). The coyote, golden jackal and Ethiopian wolf showed lower similarity, even though these species occasionally hybridize with dogs in the wild. More recently, the analysis of tens of thousands of genetic markers distributed over the entire genome revealed that they were polymorphic in dogs and wolves, thus confirming their overall similarity, and indicated that the other canid species were more distantly related (vonHoldt *et al.*, 2010).

When dogs were domesticated

More controversial is the exact number of domestication events, their timing and location. Until recently, the oldest domestic dog remains came from the archaeological record from the Middle East and dated from about 12,000–14,000 years ago. Hence, this was considered to be the place and time of domestication (Olsen, 1985; Clutton-Brock, 1995, 1999). However, additional archaeological research showed the existence of remains of the same age or slightly older in Europe (Nobis, 1979; Sablin and Khlopachev, 2002). Nevertheless, the first appearance in the fossil record of domestic dogs, as indicated by their morphological divergence from wolves, may be misleading. Early dogs may have been morphologically similar to wolves for a considerable period of time (Vilà *et al.*, 1997a,b), and the appearance of distinct-looking dogs in the archaeological record may be due to a change in the selection regime and not an indication of the time of domestication.

The possibility of a significantly older date for the domestication of dogs was first strongly supported by the genetic comparison of wolf and dog mitochondrial DNA sequences (Vilà *et al.*, 1997a). A genetic assessment of dog domestication based on control region sequence data found four divergent sequence clades (Fig. 1.3). The most diverse of these clades contained sequences that differ by as much as 1% in DNA sequence (Fig. 1.3, Clade I). After applying a molecular clock, the researchers concluded that the origin of this clade could have been about 135,000 years ago. Although this was a very rough estimate and did not provide confidence intervals, the molecular results implied an ancient origin of domestic dogs from wolves, well before the domestication of

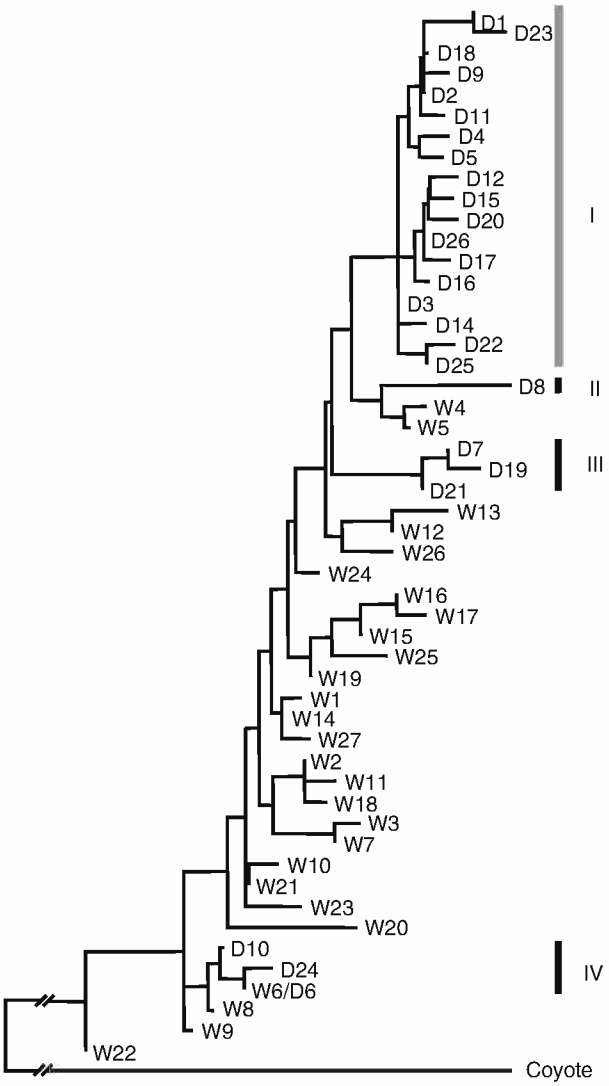

Fig. 1.3. Neighbour-joining relationship tree of wolf (W) and dog (D) mitochondrial DNA control region sequences (Vilà *et al.*, 1997a). Dog haplotypes are grouped in four clades, I to IV.

any other animal or plant, and were in conflict with previous archaeological research. Some subsequent studies were also carried out with similar mitochondrial data, but these studies assumed that Clade I had a polyphyletic origin, and one of the subclades supported the date indicated by the archaeological findings, i.e. around 14,000 years ago (Savolainen *et al.*, 2002; Pang *et al.*, 2009). However, the recent recovery in central Europe of dog remains that date back to more than 30,000 years ago (Germonpré *et al.*, 2009) confirms the notion of a very old domestication. Further, some mathematical simulations based on the patterns of linkage disequilibrium observed in the dog genome after comparing multiple breeds suggested that the pattern found fits well with a model involving the origin of dogs around 27,000 years ago (Lindblad-Toh *et al.*, 2005).

Where dogs were domesticated

The analyses of mitochondrial DNA sequences mentioned above suggested that at least four origination or interbreeding events are implied

in the origin of dogs because dog sequences are found in four distinct groupings or clades, each with a unique ancestry to wolves (Fig. 1.3; Vilà *et al.*, 1997a). The basic structure of the sequence tree has been independently confirmed by many other researchers, with all dog sequences clustering in four to six clades (Okumura *et al.*, 1996; Tsuda *et al.*, 1997; Randi *et al.*, 2000; Savolainen *et al.*, 2002).

A first approach to identifying where dogs had been domesticated could be the comparison of the dog sequences with those found in different wolf populations, with the assumption that dog sequences should be most similar to those in wolves from the places where the domestication had taken place. For many species, patterns of variation in DNA sequences clearly show association with geography in such a way that specific sequences appear to be tightly associated with certain geographical areas. Unfortunately, mitochondrial diversity in wolves is not clearly partitioned and very similar sequences are found in very distant populations (Vilà *et al.*, 1999). Even though the phylogeographic patterns in wolves are not finely defined, it is possible to use this method to exclude the Indian subcontinent and North America as the location of the population ancestral to domestic dogs, as wolves in these areas have well-differentiated mitochondrial sequences. Africa can be excluded because it seems that grey wolves never lived in this continent; a wolf-like mitochondrial lineage has recently been observed in northern Africa (see Rueness *et al.*, 2011), but this is very differentiated from the sequences found in dogs. These observations still leave open most of Eurasia as the possible location of domestication.

A second approach could be to investigate the mitochondrial DNA diversity in extant dog populations. This diversity could be expected to be higher and to include more divergent haplotypes in the areas where the domestication took place because the dog population in those areas would have had a longer time to evolve and diversify. This approach was used by Savolainen *et al.* (2002) and Pang *et al.* (2009) to suggest that the domestication took place in East Asia. However, because the sample analysed was biased towards pure-bred dogs in other areas of the world, and these are likely to represent only a small fraction of the original diversity (Irion *et al.*, 2005; Lindblad-Toh *et al.*, 2005), it is possible that the diversity estimates were also biased. Furthermore, the genetic composition of extant dog populations can be very different from their past composition. For example, the composition of the dog population in the Americas was greatly affected by the arrival of Europeans and their dogs. Thus, the comparison of mitochondrial DNA sequences found in pre-contact dogs revealed the presence of a clade of sequences not observed in modern animals (Leonard *et al.*, 2002; Castroviejo-Fisher *et al.*, 2011).

As mitochondrial DNA is maternally inherited, using this marker alone to try to infer centres of domestication offers a biased view. Vilà *et al.* (2005) showed that the extreme diversity present in the canine major histocompatibility complex (MHC) could suggest that many more wolves contributed to the origin of modern dogs than those inferred from the small number of mitochondrial lineages. This could indicate an important contribution of male wolves to the current diversity in dogs that has passed unnoticed in studies based on maternally inherited genetic markers. For this reason, genome-wide approaches are likely to reflect better the origin of domestic dogs. In this sense, vonHoldt *et al.* (2010) studied 48,000 single nucleotide polymorphisms (SNPs) spread across the genome of dogs and wolves, and suggested that Middle Eastern wolves had been a critical source of genome diversity for dogs, although interbreeding between dogs and local wolf populations had also been important in the early history of dogs. In line with this, Gray *et al.* (2010) showed that the *IGF1* (insulin-like growth factor 1 gene) small dog haplotype is more closely related to those in Middle Eastern wolves.

Origin of Breeds

Many dog breeds are thought to have existed as such for very long periods of time; that is, for thousands of years (American Kennel Club, 1997). This notion probably derives from the similarity between ancient descriptions or artistic representations and modern breeds. The Romans were probably first to develop breeds of dogs that differed dramatically in

conformation and size, though some morphologically divergent dogs were already depicted by the ancient Egyptians and in western Asia 4000 years ago (Clutton-Brock, 1999). Mastiffs and greyhounds were among these dogs, even though multiple surveys fail to show particularly lower diversity in these breeds (Morera *et al.*, 1999; Zajc and Sampson, 1999; Irion *et al.*, 2003; Parker *et al.*, 2004). Similarly, dogs that could be associated with certain modern-day breeds appear to be represented in paintings by Peter Paul Rubens or Diego Velázquez during the first half of the 17th century, but these do not seem to be especially inbred or depleted of diversity either, which suggests that they might not have been isolated from each other since their origin. Most breeds probably did not become reproductively isolated from each other until the closing of their stud books, starting in the mid-19th century.

However, a possible exception to this recent isolation of breeds may be some dog lineages that have been geographically isolated for a very long time. Dingoes and singing dogs were introduced to Australia and New Guinea by ancient travellers as early as 6000 years ago (Corbett, 1995), and this long period of isolation and the small founding population size has translated into limited genetic differentiation (Wilton *et al.*, 1999). Similarly, the genetic analysis of dog remains from the New World that dated from before the arrival of Europeans confirmed that those dogs had an Old World origin but had evolved in isolation for many thousands of years (Leonard *et al.*, 2002). A thorough examination of modern American dogs has revealed that these lineages have practically been wiped out – without leaving a descending population (Leonard *et al.*, 2002; Castroviejo-Fisher *et al.*, 2011).

Despite their recent isolation, modern dog breeds show reduced intra-breed genetic diversity and remarkable differentiation from each other, mostly due to differences in allelic frequencies (e.g. Pihkanen *et al.*, 1996; Zajc and Sampson, 1999; Irion *et al.*, 2003; Parker *et al.*, 2004). This contrasts with the results obtained when maternal (mitochondrial DNA) and paternal (Y chromosome) lineages are investigated in the same breeds: haplotypes are shared between very distinct breeds that do not seem to have much in common (Sundqvist *et al.*, 2006). These results reflect the recent origin of many breeds from a diverse founding stock from which founders were chosen (independently of their maternal and paternal lineages, admixed during thousands of years), followed by a long period of isolation. During the time in isolation, founder effects, selection, inbreeding and random genetic drift led to the uniformization of the breed and to progressive differentiation from other breeds. This within-breed uniformity has made the dog a very valuable model organism in biomedicine (Sutter and Ostrander, 2004), although increasing selective pressures by breeders and owner preferences are leading to further fragmentation in some of them (Björnerfeldt *et al.*, 2008).

A comparison of the diversity found within breeds for mitochondrial DNA and for the Y chromosome, with that found in wild wolf populations, reveals also an interesting pattern (Sundqvist *et al.*, 2006). There is a strong reduction in the diversity on the Y chromosome in dog breeds as compared to wolf populations. As similar numbers of males and females are expected to participate in reproduction in a wolf population, the results are compatible with a stronger selection on male dogs and the existence of popular sires. Selection for a specific trait can be more efficient if that trait is in a male than in a female because a single male can be used to sire a large number of litters every year. This implies that the formation of breeds represents a deep disturbance of the mating patterns observed in wolf populations. Also, the study indicated that breeds belonging to the same functional group, as recognized by the World Canine Organization (Fédération Cynologique Internationale), are more likely to have been involved in the creation of a new breed.

Research Implications

The availability of so many new genetic and genomic tools, including multiple whole genome sequences and commercially available SNP chips, has catapulted studies of the dog and dog evolution into the genomics age. The particular mutations responsible for many

traits have been identified which, in turn, has allowed selection on these traits to be studied at the genetic level (e.g. Anderson *et al.*, 2009). The study of expression profiles in the canine brain (Saetre *et al.*, 2004; Lindberg *et al.*, 2005) and genome-wide comparisons (Cruz *et al.*, 2008) offer a window to understand the domestication process. These tools enable research on the origin of dogs and the remarkable morphological and behavioural diversity in them to be studied in new ways. For many researchers, the study of the origin of the canine diversity is a model to understand how biodiversity appears in the wild. Thus, this study can be the basis for research on the genotype–phenotype relationships in ecological model systems.

The field of ancient DNA has also matured and expanded in the last few years and, in combination with genomic tools and archaeological research, will enable us to address where, when, how and, perhaps, why dogs were domesticated.

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2 Experimental Studies of Early Canid Domestication

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Introduction

What the major evolutionary force might be that drives domestication has long been a debatable issue. The question was: how might contemporary domestic dogs, so very diverse today, have evolved from a uniform wild-type ancestor (Herre, 1959; Belyaev, 1969; 1979; Hemmer, 1990; Clutton-Brock, 1997; Coppinger and Schneider, 1997; Wayne and Ostrander, 1999; Diamond, 2002)? It is well known that certain dog breeds differ in body size and proportions much more than do species, or even genera. Putting it another way, domestication has given rise to drastic morphological and physiological changes in the dog at a rate significantly exceeding what is usually observed in natural populations. If one accepts the classic notion that mutations are rare and random alterations of individual genes, serious doubt is cast on the idea that the changes which occurred during dog domestication over a short span of time were only of a mutational nature. Even making allowance for saltatory events (Eldredge and Gould, 1972), it is, indeed, incomprehensible how all the mutations needed for the creation of the now existing diversity could be accommodated during the millennia that have elapsed since the time the earliest dog appeared (Coppinger and Schneider, 1997).

Mitochondrial and nuclear DNA sequences date the split of the dog from the wolf about 14,000–15,000 BP. However, the process of real dog domestication is distinguished from that of unconscious domestication (proto-domestication), which probably started about 35,000 BP (Galibert *et al.*, 2011; see also Chapter 1). It should be stipulated that mutations were accumulated for the whole of these dozens of millennia. Furthermore, there are data in the literature which indicate that certain mutations, for example, those causing evolutionary changes in characters under pressure of sexual selection, which can eventually set up reproductive barriers, can possibly accumulate

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very rapidly (Civetta and Singh, 1999; Gavrilets, 2000). Certainly, new mutations have kept arising during domestication too. Circumstantial evidence though indicates that their accumulation is not the only source of morphological and physiological changes in dogs.

In fact, an evolutionary consequence of dog domestication is the fundamental reorganization of the reproductive function, which is imperative for evolutionary survival. Dogs fulfil the primary biological task – to reproduce – differently from their wild counterparts. They have lost monoestricity and their seasonal breeding pattern, and have acquired the capacity to breed any time of the year – biannually and more often. It seems very unlikely that this change in the reproductive function, which is the integral result of the complex interaction of many neuroendocrine responses, might have occurred as a single mutational event. It is worth remembering that not only dogs, but also other domestic animals, have lost breeding seasonality. The parallelism of the morphological and physiological variability patterns is nowhere more conspicuous than under conditions of domestication. True, species of domesticated animals are members of distant taxonomic groups (not only of genera and families, but even of orders); but variability in many of their characters is remarkably similar. It appears unlikely that this variability was caused by homologous mutations in homologous genes in the domesticated species. There is more straightforward evidence that mutations did not accumulate rapidly in domestication conditions. Studies on the protein products of more than 50 loci have shown, for example, that dogs and wolves share alleles in common (Wayne and O'Brien, 1987).

The role of founder effects has been emphasized with reference to the evolutionary events occurring during dog domestication (Moray, 1994; Clutton-Brock, 1997; Coppinger and Schneider, 1997; Wayne and Ostrander, 1999). On the basis of mitochondrial DNA (mtDNA) studies, it has been suggested that there initially existed small founder groups, and that these inbred and were repeatedly influenced by genetic drift (Vilà *et al.*, 1997; Savolainen *et al.*, 2002). In contrast, the variety of alleles of the nuclear loci of the major histocompatibility complex (MHC) show the existence of several hundreds of founders, as well as continuous backcrossing of these founders with wild ancestors during proto-domestication (Vilà *et al.*, 2005). It is also important to emphasize that domestication was not an isolated event that took place in a few discrete locations. It was a common phenomenon and started in different places and at different times (Dobney and Larson, 2006; Galibert *et al.*, 2011). All of this indicates that the role of founder effect and inbreeding during the earliest domestication is somewhat exaggerated. The inbreeding event was most common not in the first founders of domestic dogs, but in the founders of breed varieties. The stage of breed formation was the one to pass through the narrow bottleneck. It was recently shown, using the extent of linkage disequilibrium (LD) as an indicator of the effective population size, that the most severe contractions in effective population occurred precisely at this stage, and that as a result of continued inbreeding, 35% of nucleotide diversity was lost at this period. In comparison, the diversity loss at early stages of domestication was estimated at only 5% (Gray *et al.*, 2009).

However, the diversity of the domestic dog is more often discussed in the light of neoteny – or the retention of juvenile traits to adulthood – as a major trend of the changes in development brought about by domestication. It has frequently been noted that many adult dogs are allied to and morphologically similar to wolf puppies. It has even been thought that some characters arrested in a developmental stage may underlie the formation of breeds (Wayne, 1986; Coppinger *et al.*, 1987). Indeed, it was recognized that genetic variability of developmental patterns is the source of rapid and extensive changes at the organismic level (Gould, 1982; Raff and Kauffman, 1983; McDonald, 1990; Pennisi, 1998). Because this variability is of importance, there must be a mechanism that safeguards it from the direct action of selection. For this reason, retarded development in the domestic dog is hardly a consequence of selection for developmental rates. Several authors have considered neoteny as a consequence of direct selection for earlier sexual maturation (Clutton-Brock, 1997; Coppinger and Schneider, 1997; Wayne and Ostrander, 1999), although the efficiency of

this type of selection is very doubtful, as all reproductive traits, including the timing of sexual maturation, have minimum additive genetic diversity (Bronson, 1988). Neoteny might though have arisen as a result of selection for traits that mark developmental rates. Such markers might plausibly have been infantile behavioural traits that have facilitated the adaptation of animals to humans (Coppinger and Schneider, 1997). If this were the case, then it must be considered that delayed development of social behaviour is correlated with the developmental rates of other physiological and morphological characters. This could mean that selection, vectorized for social behaviour, actually works as selection for developmental rates at the level of the whole organism.

The Russian geneticist D.K. Belyaev has pondered over the nature and origin of changes brought about by domestication and over the role of the regulatory developmental mechanisms in these changes (Belyaev, 1969, 1979). His vantage point for viewing evolutionary problems was out of the ordinary at that time. Belyaev believed that the rates of evolutionary transformations, in certain situations, depended not only on the force of selection pressure, but as much on its directionality or vector, i.e. on the intrinsic properties of the genetic systems on which the selection acts. So when the key regulatory loci coordinating the entire process of development happen to be targeted by selection forces, this may lead to explosion of variability on several levels. This process might have created specific conditions at the organism level that gave rise to variability.

The data in the literature supporting Belyaev's idea have been partly reviewed earlier (Trut, 1993). The regulatory mechanisms were probably subjected to strongest selection when conditions became extremely challenging and demanded high tension of the general adaptive systems. The view was expressed that the genome, under such conditions, functions as a specific responsive system and evolves towards increasing genetic variability. The possible molecular mechanisms of this behaviour of the genome have also been discussed (Lenski and Mitler, 1993; Pennisi, 1998; Siegal *et al.*, 2007; Ruvinsky, 2010). The earliest domestication, when animals encountered a man-made environment for the first time, was a drastic replacement of the surroundings. It was, indeed, a violent upheaval, and one that produced a host of variations of such magnitude that the animal kingdom might never have experienced before. The significant fact remains that a new vector was brought into play – the combined action of the natural and the unconscious, artificial selection for particular behavioural traits that favoured animals' ability to coexist with human beings. Belyaev believed that the specificity of evolutionary events under these conditions was determined by selection of this kind, and that the morphological and physiological transformations were primarily patterned by the genetic changes taking place during behavioural reorganization. His unified view of the evolutionary past of the domestic dog needed experimental verification and support. This prompted him with the idea of reproducing a documental scenario of early domestication. The domestication experiment has been carried out at the Institute of Cytology and Genetics of the Siberian Department of the Russian Academy of Sciences for over 50 years. The species under domestication has been the silver fox (*Vulpes vulpes*), a taxonomically close relative of the dog. The experiment has recreated an evolutionary situation of the strongest selection acting on behavioural traits in conditioning the success of adaptation to human beings.

The Domestic Fox in its Making

When the domestication experiment was started, the silver fox had been bred in fur farms for more than 50 years. It may be thought that it had overcome the barrier of natural selection during its alienation from nature, and its caging and breeding in captivity, but it had retained its standard phenotype, strict seasonality of biological functions and the relatively wild behaviour (Fig. 2.1). A genetically determined polymorphism for the expression of aggressive and fear responses to humans was revealed in the farmed fox populations. There might have been, quite plausibly, such polymorphism in the type of defensive responses to humans in the initial natural populations of wolves. Some of the farmed foxes

manifested aggressive responses very weakly. About 10% of the farm-bred foxes were such individuals (Fig. 2.2). The weak responders were selected to become the parental generation to start the experiment. The total number taken from fur farm populations to serve as the initial generation in the experiment was 100 females and 30 males. The number of foxes of reproductive age was minimal (93) in the second generation, and maximal (600) throughout the twentieth to thirty-fifth generations.

The selected foxes yielded more than 52,000 offspring that were tested for amenability to domestication (tameability), and more than 15,800 foxes were used as parents throughout the experiment. The capacity for domestication was tested at different times during development, from 2 weeks of age onwards. Pups interacted with humans for a scheduled time. The experimenter handed food to pups, and attempted to handle and fondle them. The behaviour of the tested pups was scored for various parameters (Trut, 1999). The score for tameability, or amenability to domestication, was the major criterion for selecting animals. Selection was strict: only about 10% of females and not more than 3–5% of males were taken from a preceding generation to produce the next. The apparent effectiveness of selection, the selection process and everything relevant to the establishment of the experimental population has been dealt with elsewhere (Belyaev, 1979; Trut, 1980a,b, 1999).

Fig. 2.1. A strongly aggressive fox of the farm-bred population unselected for behaviour.

Selection was ongoing for more than 50 generations. Behaviour changed during the course of selection, illustrating its effectiveness. Most offspring of the selected population were assigned to the domestication elite. They behaved in many respects like domestic dogs. They did not flee from humans; they yearned for human companionship. When begging for human attention, they wagged their tails, tried to lick like dogs and whined (Fig. 2.3). In addition, specific vocal markers of affiliating behaviour towards humans were revealed in elite tame foxes (Gogoleva *et al.*, 2008, 2010). It is noteworthy that tame foxes

Fig. 2.2. This fox shows a weak aggressive response to attempts to touch it.

from the 'elite of domestication', as well as domestic dogs, are able to read human social signals (point and gaze cues) and react adequately to them (Hare *et al.*, 2005). The early behavioural elites appeared at the sixth generation selected for tameness. Elite in this context means 'impeccable', or tamed to the highest degree. By the 20th generation, 35% of the offspring already selected for tameness were elites. At this time, elite pups made up 70–80% of the experimental population. Many responded to their pet names. When competing for human attention, they growled and snarled at each other (Fig. 2.4). When released from their cages for a while, they acted in a dog-like manner and were submissive towards their master/mistress (Fig. 2.5). Thus, a unique population of silver foxes showing unusual, rather dog-like behaviour was established through long-standing selection for tameability. This was the first among many effects demonstrated during this experimental domestication.

Fig. 2.3. The dog-like behaviour of foxes is noteworthy. It is the result of breeding for tame behaviour.

What could be the mechanisms of the domestication that made dogs and foxes feel more 'at home' in the new social surroundings near man? It is known that in dogs the sensitive period for this adaptation (or primary socialization) during postnatal development starts with the functional maturation of the sensory systems and locomotor activity providing awareness of the environment and

Fig. 2.4. One fox driving another from its mistress and growling like a dog.

response to it. The appearance of the fear response to unknown stimuli is thought to be a factor that does not arrest the exploration of the environment and social adaptation, but rather complicates it (Scott, 1962; Serpell and Jagoe, 1997). It was found that the selection of foxes for domestication accelerated full eye opening and the establishment of the early auditory response (Fig. 2.6). This selection concomitantly retarded the formation of the fear response during early postnatal development and, as a result, offspring of the domesticated population showed no attenuation of exploratory activity in an unfamiliar situation, as the offspring of the farm-bred population did (Fig. 2.7). In fox pups of the population unselected for behaviour, the fear response formed, on the average, by 45 days of life. At this age, the parameters of exploratory activity decreased considerably, but this did not occur even in tame pups aged 60 days because they did not exhibit the fear response at this age.

Fig. 2.5. When released from their cages, elite foxes follow the master/mistress faithfully.

These alterations in the rates of receptor–behavioural development prolonged the sensitive period of social adaptation and increased its efficiency (Belyaev *et al.*, 1985). It is noteworthy that 45 days is not only when the sensitive socialization period ends, it is also the age when glucocorticoids in the peripheral blood rise sharply in offspring of the farm-bred population (Fig. 2.7). In contrast, in offspring of the domesticated population, not only was the fear response not, as yet, manifested and exploration not reduced, glucocorticoids did not rise either. Based on the above considerations, it may be inferred that selection for tame behaviour affected the activity of some genes with significant influence on developmental rates. One of the genetic systems determining the activity of the pituitary–adrenal axis – which is involved in the regulation of the developmental rate – was likely to be a primary point of this selection. This inference will be examined below.

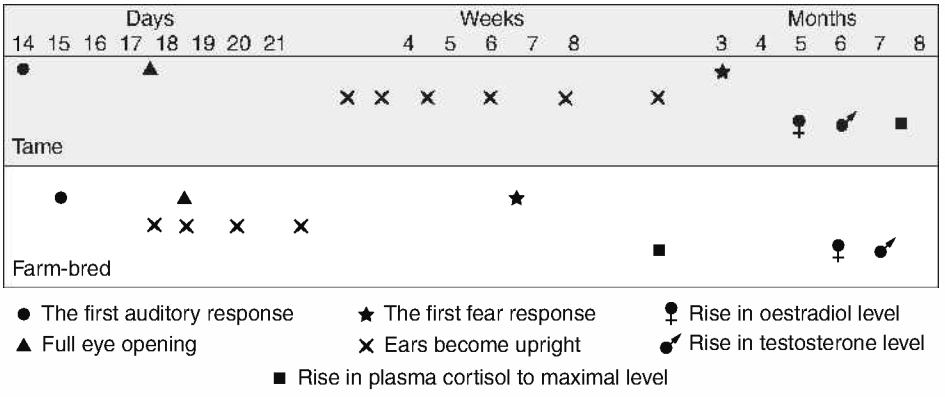

Fig. 2.6. Time appearance of certain characters during postnatal development.

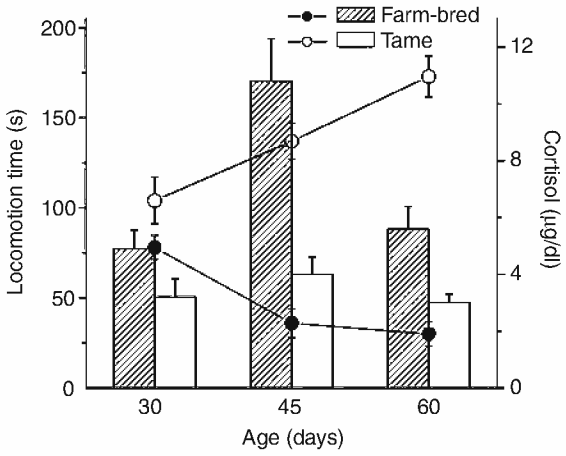

Fig. 2.7. Total time of locomotion, an indicator of exploratory behaviour, and plasma cortisol levels in farm-bred and tame foxes at the age of 30–60 days: locomotion is plotted on the graph; plasma cortisol level is represented as bars.

Phenotypic Novelties

As indicated in the Introduction to this chapter, the view was generally held that the dog has been under domestication for probably about 35,000 years (Galibert *et al.*, 2011), although phenotypic changes started to appear only 10,000–15,000 years ago. However, the generally accepted conclusion of domestication researchers (Herre, 1959; Zeuner, 1963; Wayne, 1986; Clutton-Brock, 1997; Coppinger and Coppinger, 2001; Price, 2002; Dobney and Larson, 2006; Zeder *et al.*, 2006; Galibert *et al.*, 2011) was that the primary increase in diversity was achieved very rapidly. Then, a stasis followed and no changes occurred in the dog during the course of domestication history. The second step of striking explosion of variation came in more recent times with the development of breeding methods during the past 300–400 years (Parker *et al.*, 2004; Wayne and Ostrander, 2007).

Morphological changes started to arise in foxes that had been subjected to selection for tameness for eight to ten generations. Many changes were concordant with those not only of dogs, but also of other domestic animals (Figs 2.8–2.12). As in the dog (Hemmer, 1990), changes in standard coat colour were the first to arise in the fox. Seemingly distinct elements of animal biology, such as behaviour and pigmentation, were altered in an integrated manner. It is now known that the genetic systems of pigmentogenesis are, indeed, involved in neuroendocrine physiology (Tsigas *et al.*, 1995; Barsh, 1996; Schmutz and Berryere, 2007; Ducrest *et al.*, 2008). Thus, there is evidence that the *E* (extension of black) locus in mice encodes the receptor for melanocyte-stimulating hormone; there is also reason to suggest that the *A* (agouti) locus codes for its binding antagonist which, in turn, binds to the receptor (Jackson, 1993; Barsh, 1996).

Fig. 2.8. Specific lack of pigmentation determined by the homozygous state (*SS*) of the incompletely dominant autosomal *Star* (*S*) mutation. The *Star* mutation is one of the earliest phenotypic novelties.

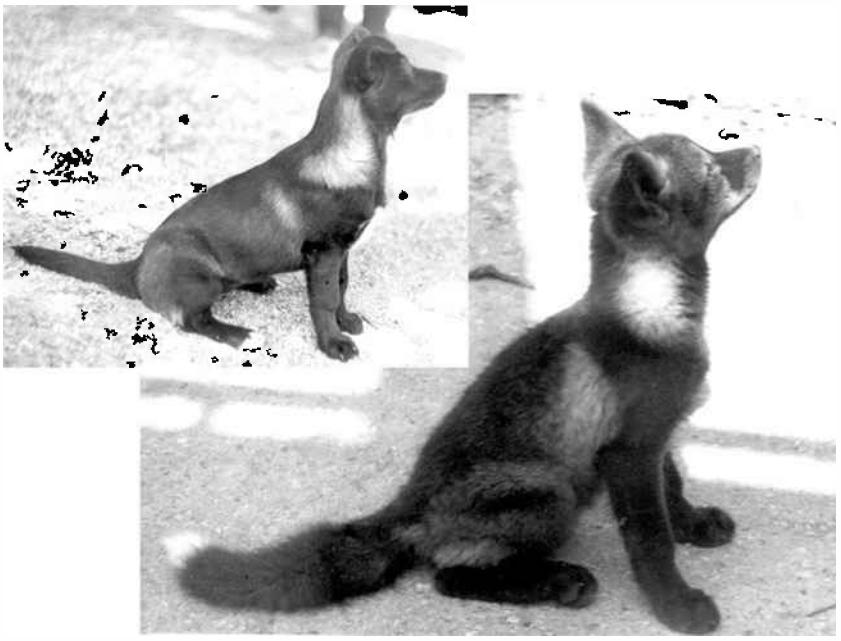

Fig. 2.9. Brown mottling (*bm*) is located on neck, shoulders, flank and hips. There is a phenotypic similarity between *bm* in foxes and the colour trait in dogs possibly caused by the allele of the *Agouti* locus. The *bm* phenotype is determined by an autosomal recessive mutation.

Fig. 2.10. Floppy ears. Ears remain floppy for the first months of life in some domestic foxes, more rarely through life. This aberrant character does not show clear Mendelian segregation, although it recurs in some lines.

The A protein can act as an antagonist in other hormone–receptor interactions, for example, with adrenocorticotrophic hormone (ACTH). It is also of interest that melanocyte-stimulating hormone (MSH), which is involved in the regulation of melanin synthesis, has a receptor not only in the melanocytes; it has other kinds of receptors, one of which expresses exclusively in brain tissues, and at high concentrations in the hippocampus and the hypothalamus (Tsigas *et al.*, 1995), which are the structures that regulate exploratory and emotional behaviour. With this in mind, it is not at all surprising that selection for behaviour gave rise to primarily correlated changes in coat colour.

In farmed foxes, aberrants with the *Star* white marking and curly tails were born at an impressively high frequency of 10^{-1}–10^{-2}. Short-tailed pups and those with floppy ears appeared at a significantly lower frequency (10^{-3}). Some phenotypic changes, such as a curly tail and piebaldness, started to arise in the farm-bred fox populations several years later. It should be noted that in farm populations bred under human control for about 100 years both

natural and artificial selection for domestication proceeded hand in hand. Surely the intensity of this selection was not commensurate with that to which the experimental fox population was subjected; nor were the frequencies of occurrence of aberrant animals in the two populations similar.

Fig. 2.11. Short tail. The number of tail vertebrae is normally 15 in foxes, but their number is reduced to eight to nine in aberrant short-tailed forms. The inheritance pattern of this characteristic is not clear.

What did the increased frequencies of phenotypic novelties in the domesticated population reflect? The increased frequency may be a consequence of random processes and inbreeding whose roles have been highlighted in discussions of early canid domestication (Moray, 1994; Clutton-Brock, 1997; Wayne and Ostrander, 1999). In estimation of the role of inbreeding in the reorganizations brought about by domestication in foxes, it should be emphasized that most, if not all, of the domesticated fox population, from the start of its establishment, was raised in an outbreeding regime. Moreover, effective population size, N_e (where $N_e = 4N_mN_f/N_m + N_f$, and N_m is the number of males, N_f, that of females) did not reduce to less than 93 individuals in the second selected generation, and it considerably increased in the successive generations. At this effective population size, the probability of occurrence of aberrant phenotypes due to homozygotization of recessive mutations appeared to be low (Falconer, 1981). The inbreeding coefficients in the outbred part of

Fig. 2.12. Tail carriage: tail rolled into a circle or a semicircle. Curly tail is the most frequently arising aberration in domesticated foxes. It does not show Mendelian segregation. The genetic basis of the character is, probably, different in different lines of these foxes.

the population, as estimated on the basis of effective population size (Trut, 1980a; Trut *et al.*, 2004) and using a set of randomly chosen polymorphic microsatellite markers (Kukekova *et al.*, 2004; Trut *et al.*, 2009), always fluctuated within the range of 2–7%. However, several fox lines were deliberately maintained in a regime of inbreeding. The level of homozygotization within these lines reached 40–60% (Trut, 1980a). An important factor was that the frequencies of occurrence of phenotypic changes in the offspring of these inbred foxes did not exceed those in the offspring of the outbred foxes. It should be also noted that such a novel phenotype as the *Star* white marking is determined by incompletely dominant mutations and that the heterozygous phenotype is reliably marked (Belyaev *et al.*, 1981). In other words, there are grounds for believing that the emergence of phenotypic novelties was unrelated to inbreeding and homozygotization of identical-by-descent mutations in the domesticated fox population. In that case, might the changes that have arisen be regarded as classical correlated consequences of selection for just any quantitative character? In fact, it is known that strong selection pressure acting on a quantitative character, especially on one of adaptive significance, results in less integrated genetic systems (Falconer, 1981). The harmonious genetic system created by stabilizing selection is set out of balance, and any increase in the value of the selected character is achieved at the expense of a breakdown of genetic homeostasis. For this reason, selection of quantitative characters may lead to the appearance of deviants from the stabilized phenotypic norm. Such classic correlated responses to selection depend, as a rule, on the genetic pool of the starting population, and this renders predicting and reproducing them difficult. Each selection experiment is unique and none can be replicated in terms of the attendant correlated responses. As to the morphological and physiological consequences of domestication, their reproducibility is amazing. To illustrate, dogs and many animals have been repeatedly domesticated at different times and sites throughout their history, and each domestication event recurred and so did the same domestication changes. The changes observed in the experimental fox population are mirror reflections of the morphological novelties arising in other animals under domestication. Taking the remarkable concordance of evolutionary transformations into account, it is hard to regard the changes as just trivial cases of correlated selection responses.

Possibly, the specificities of the emergence of morphological and physiological novelties in domesticated foxes may shed light on the nature of the changes that usually occurred in the domestication elite. Various aberrants were recorded in the same litter of standard tame parents, or parents showing a certain morphological change occasionally had offspring exhibiting quite different characters. These specificities are difficult to interpret when conceding that each morphological novelty resulted from a single specific mutational change. Classic breeding studies also indicated that many of the morphological novelties observed in domestic foxes were not due to segregation in simple Mendelian fashion; some morphological novelties were determined, for example, by single mutational events, such as the *Star* mutation – although this mutation showed peculiar behavioural features, suggesting that the phenomenon of genetic activation–inactivation was possibly behind its emergence and inheritance (Belyaev *et al.*, 1981). In the current literature, many cases of gene silencing, including in the coat colour genes, have been adduced. Silencing is thought to have perhaps resulted from the passage of a modified DNA methylation pattern through meiosis (Morgan *et al.*, 1999; Jablonka and Lamb, 2005; Cuzin *et al.*, 2008; Franklin *et al.*, 2010).

The phenomenon of inherited changes in the activity of genes might have been also involved in morphological and physiological reorganizations in the dog. Hall (1984) has described a pertinent case in his review. As known, the number of 'fingers' of the dog foreleg is five; it is four in the hind leg in all representatives of the Canidae. The fifth hind leg finger was lost some 10–15 million years ago. However, a result of wolf domestication was that the fifth finger, once missing, is now well developed in certain extant dog breeds. This is strongly suggestive that the phenotypic changes in dogs that have arisen during the course of domestication might have been due not only to specific mutational changes, but

also (and to a greater extent) to changes in regulatory embryonic interactions and gene activity.

Craniological Changes

During the past few decades, developments in DNA-based research have contributed much to variability studies. Nevertheless, traditional craniological studies still remain pertinent in the exploration of evolutionary processes (Hanken and Hall, 1993). In some foxes, the shape and size of the skull sharply deviate from normal (Fig. 2.13). In others, the upper jaw is shortened and the tooth bite becomes abnormal (Fig. 2.14). Comparative analysis of farm-bred and domesticated populations revealed that changes in craniological dimensions were most prominent in males. The changes were associated with shortening and widening of the face skull and a decrease in the width and height of the cerebral skull. Moreover, tame males became smaller in almost all the cranial proportions and, as a result, the sexual dimorphism existing in the farm-bred population decreased in the domesticated foxes (Trut *et al.*, 1991). Similar changes in the sexual dimorphism pattern, as judged by cranial measurements, have also been revealed in farm-bred minks when compared with wild mink populations (Lynch and Hayden, 1995). Two mechanisms producing this effect have been implicated: the abolition of sexual selection under farm conditions and enhancement of selection for increased total body size in males and females. These mechanisms can hardly account for the decrease in sexual dimorphism of craniological dimensions in the domesticated fox population. As to sexual selection, its effect was also abolished in the population not selected for behaviour (the control) with which the experimental population was compared. Also, selection for increased total body size was abolished in the domesticated, but not in the control, fox populations.

It is noteworthy that during early domestication, the facial area of the skull became shorter and wider in dogs, as in foxes (Clutton-Brock, 1997; Wayne and Ostrander, 1999).

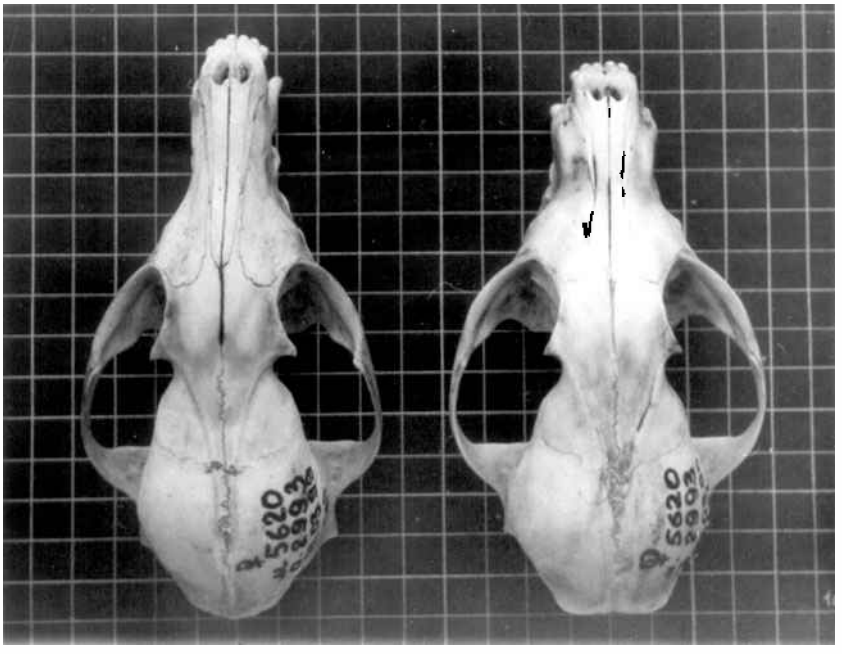

Fig. 2.13. The skulls of 8-month-old female foxes: normal (left), foxes with abnormally shortened and widened skulls (right).

Fig. 2.14. The abnormal tooth bite (underbite) in a fox with a shortened upper jaw.

Evolutionary changes in craniological traits, as in any others, should though be discussed in a genetic context. Until recently, little was known about the relation of certain craniological traits with specific genetic changes. There are the traditional estimates of the heritability of certain dimensions in rats and mice (Atchley *et al.*, 1981). The quantitative genetics of the mandible has been studied in mice (Atchley, 1993), and the effects of certain pigmentation genes on skull shape were identified in the American mink (Lynch and Hayden, 1995). Questions that arise when studying craniological variability though concern the developmental mechanisms more (Atchley and Hall, 1991; Hanken and Hall, 1993; Fondon and Garner, 2007). Thus, it is known that one of the sources of changes in the size and shape of the skull is alterations in the allometric interactions between growth rates. It has been proved that some of the changes in the craniological characters of foxes are explicable by precisely these alterations (Trut *et al.*, 1991). The genes controlling allometric interactions determine either the time when a structure appears or its growth rate. Allometry changes during development. It seems that its genetic determination also changes at different stages. A crucial role was assigned to changes in developmental rate in surveys of the morphological evolution of the dog. In turn, the important role of selection for decreased body size and reproductive timing was recognized in discussions of the nature of changes in allometry (Moray, 1994; Clutton-Brock, 1997; Wayne and Ostrander, 1999). Possibly, the wolf was selected naturally or artificially for smaller body size during early domestication. But the sole selective criterion for modelling the domestication of foxes was behaviour; total body size was an irrelevant character. The body size of domesticated foxes was compared with that of farm-bred foxes only at certain steps of selection (the F_{15-17} and the F_{25-26} generations). The comparisons revealed no correlated decrease in body size. Moreover, total body length tended to increase in tame males, and it was precisely in these males that a decrease in craniological proportions and changes in the face skull were most expressed. The effect of direct selection for reproductive timing (sexual maturation timing) on the described skull changes is also very doubtful, as was noted in the Introduction. However, changes in these reproductive characters did occur in foxes as correlated responses to selection for behaviour (Logvinenko *et al.*, 1978, 1979; Trut, 2007). There are also ample reasons to believe that changes in allometric interactions are the correlated consequences of selection vectorized for domestication. This is evidence that such selection leads to profound genetic changes in the regulation of developmental processes.

Reorganization of the Seasonal Reproduction Pattern

It should be re-emphasized that a major evolutionary consequence of domestication was a fundamental reorganization of reproduction. Dogs lost the reproductive seasonality pattern, and they became able to reproduce in any season and more than once a year. It is of importance that, in the domesticated fox population, the functional activity of the reproductive system was recorded both in females and males at times beyond the fox breeding season as stabilized by natural selection (Belyaev and Trut, 1983). The mating season in foxes normally lasts from the first decade of January to the end of March. Males are in a state of sexual activity during the whole of this period. Mating

entirely depends on when the females are in oestrus. Variability in the mating time during the seasonal time interval is determined mainly by environmental factors, and direct selection for this trait is ineffective. It is very important that some (tame) vixens showed oestral activity both in the autumn and spring, i.e. that biannual oestruation tended to form, although fertile extra-seasonal matings are extremely rare (Fig. 2.15). Pedigree analysis indicated that there did indeed occur an inherited reorganization of the seasonal rhythm of breeding: 300 females in which extra-seasonal sexual activity was recorded in the course of the experiment belonged to 20 unrelated families, i.e. extra-seasonal breeding arose in 20 female founders. Two of these, on referral to the domestication elite, transmitted this ability to the numerous offspring of the following generations. Seventy females showing extra-seasonal mating activity derived from a single female founder and 49 from the others. Thus, among highly domesticated foxes, there is a tendency to lose strict seasonality of reproduction. Such an observation can be regarded as an indication of correlated response to selection for amenability to domestication.

Selection and Developmental Rates

There are then strong grounds for believing that the morphological and physiological transformations in silver foxes are the mirror images of the historical pathway of the domestic dog development. As already noted, discussions of the nature of the transformations brought about by dog domestication have centred on developmental processes and their rates. Neoteny is widely accepted as a mechanism by which the dogs became diversified (Wayne, 2001), and, as an evolutionary trend, it is an appealing notion (Raff and Kauffman, 1983; McDonald, 1990). Thus, it has been postulated that certain breed-specific locomotor and behavioural features are actually retarded juvenile responses (Coppinger *et al.*, 1987). Wayne (1986) and Wayne and Ostrander (1999) extended the general idea to craniological characters.

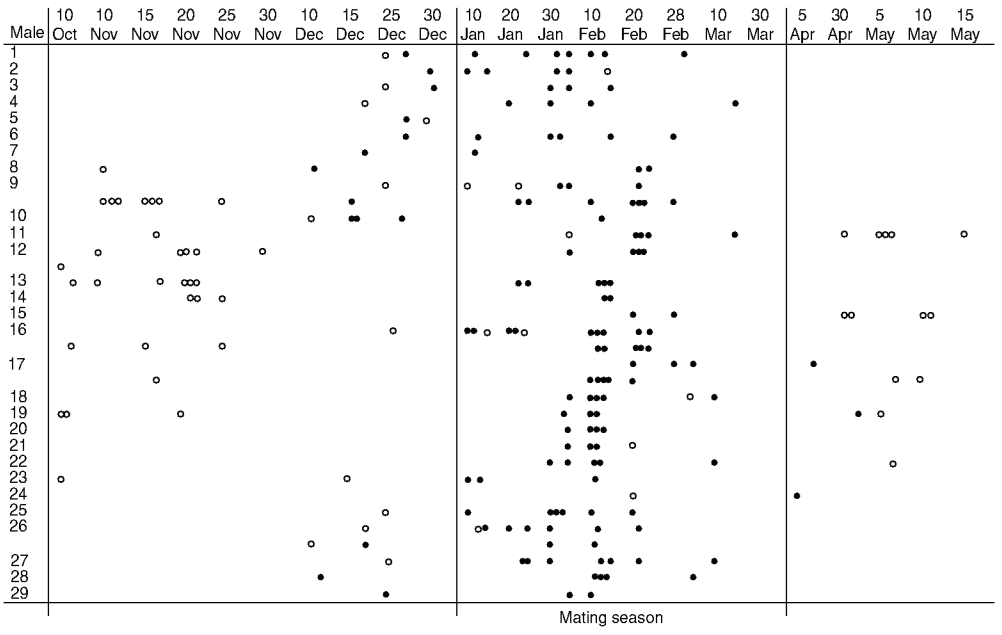

Fig. 2.15. The time course for matings in the tame foxes that showed extra-seasonal sexual activity. The circles in line with the order number of males indicate their matings in years when they mated out of season. Key: white circles, sterile matings; black circles, fertile matings.

Furthermore, there is reason to suppose that some of the differences in the distributions and amounts of brain neurotransmitters (dopamine, for example) between breeds also reflect those in the development of the neurotransmitter system (Arons and Shoemaker, 1992).

To reiterate the crucial question posed in the Introduction: what genetic changes made domestic animals similar to the juvenile forms of their ancestors? Or, in other words, what evolutionary processes have led to neoteny? The results obtained in the course of the study of fox domestication may possibly shed light on the primary cause of changes in developmental rates. It is likely that changes in the rates of the ontogenetic processes underlie the emergence of many new characters in domesticated foxes (Fig. 2.6). It has been suggested, for example, that reorganization of behaviour from the relatively wild to the more docile was achieved through changes in the timing of maturation that set the boundaries of the sensitive period of socialization. As a result, the duration of this period in domesticated foxes became prolonged and similar to that in dogs (Scott, 1962; Belyaev *et al.*, 1985). The developmental rates of certain morphological traits changed too. A typical dog-like trait, such as floppy ears, is nothing other than a retained infantile feature. Ears are floppy during the early postnatal period in all fox pups. They became upright at the age of 2–3 weeks in offspring of the farm-bred population and at 3–4 weeks in the domesticated population. However, in some pups, ears remain floppy up to the third or even fourth months of life, and floppiness was lifelong in exceptional cases. Even certain changes in coat colour were due to shifts of developmental rates. As noted, the *Star* white marking was one of the earliest correlated responses to selection. It was found that the *Star* mutation causes piebaldness. The mutation affects the developmental rate of the primary melanoblasts, the embryonic precursors of the melanocytes. It delays their migration as they travel from the neural crest (the embryonic structure from which the melanoblasts derive, see Chapters 4 and 15), as well as their proliferation. The earliest melanoblasts normally appear in the epidermis of fox embryos on day 28 of development, while in carriers of the *Star* mutation they reach the epidermis on day 30 (Prasolova and Trut, 1993); they arrive too late to the potentially unpigmented areas, so they cannot enter the hair follicle at the appropriate time. For this reason, there are no melanocytes in the areas devoid of pigment. The changes described in craniological dimensions in domesticated foxes are also determined by shifts in the temporal parameters of development. Analysis of the pattern of intracranial allometry demonstrated that selection for behaviour shifts the time of the appearance of the cranial structures and their growth rates (Trut *et al.*, 1991).

Changes in the establishment rates of hormonal status in the experimental foxes appeared to be of importance. Thus, the pattern of embryonic and early postnatal establishment of the functional parameters of the pituitary–adrenal axis was altered in the tame foxes (Plyusnina *et al.*, 1991; Oskina, 1996). As is well known, hormones have multiple tissue and function targets. The presence of a particular hormone at the right time and in the appropriate concentration creates the critical conditions for normal tissue and organ development and functioning. If all the conditions or some of them are not met, a particular developmental process, and its rate, can become destabilized.

Effect of Selection on the Hormonal and Neurotransmitter Systems

Along with the genetic transformation of behaviour, selection in foxes has led to significant changes in the neuroendocrine system and, above all, in the hypothalamic–pituitary–adrenal (HPA) axis – the basic system of adaptation and stress. D.K. Belyaev believed that the new social–anthropogenic environment was a strong stressful factor in the first stages of domestication, and that selection for tolerant behaviour towards humans was accompanied by correlated changes in stress reactivity. Studies of the HPA axis in the silver fox as an object of experimental domestication have begun. A decrease in cortisol levels is observed in tame silver foxes starting from the tenth selected generation (Trut *et al.*, 1972; Naumenko and Belyaev, 1980). By the

twentieth generation, basal cortisol levels in tame foxes were almost twice as low as in the farm-bred. Cortisol level under stress in tame foxes was 30% lower than in farm-bred control population. In the 45th selected generation, the difference between tame and farm-bred foxes increased by 3–5 times; similar changes were observed in the levels of ACTH – the main regulator of adrenal function (Fig. 2.16). No statistically significant differences in ACTH stress levels were observed in the 20th generation of selection, while in the tame foxes of the 45th generation plasma ACTH levels in stress condition were 4–5 times lower than in farm-bred animals (Oskina *et al.*, 2008). Therefore, the functional activity of the HPA axis decreases during selection for tame behaviour. We also found that cortisol levels and adrenal production

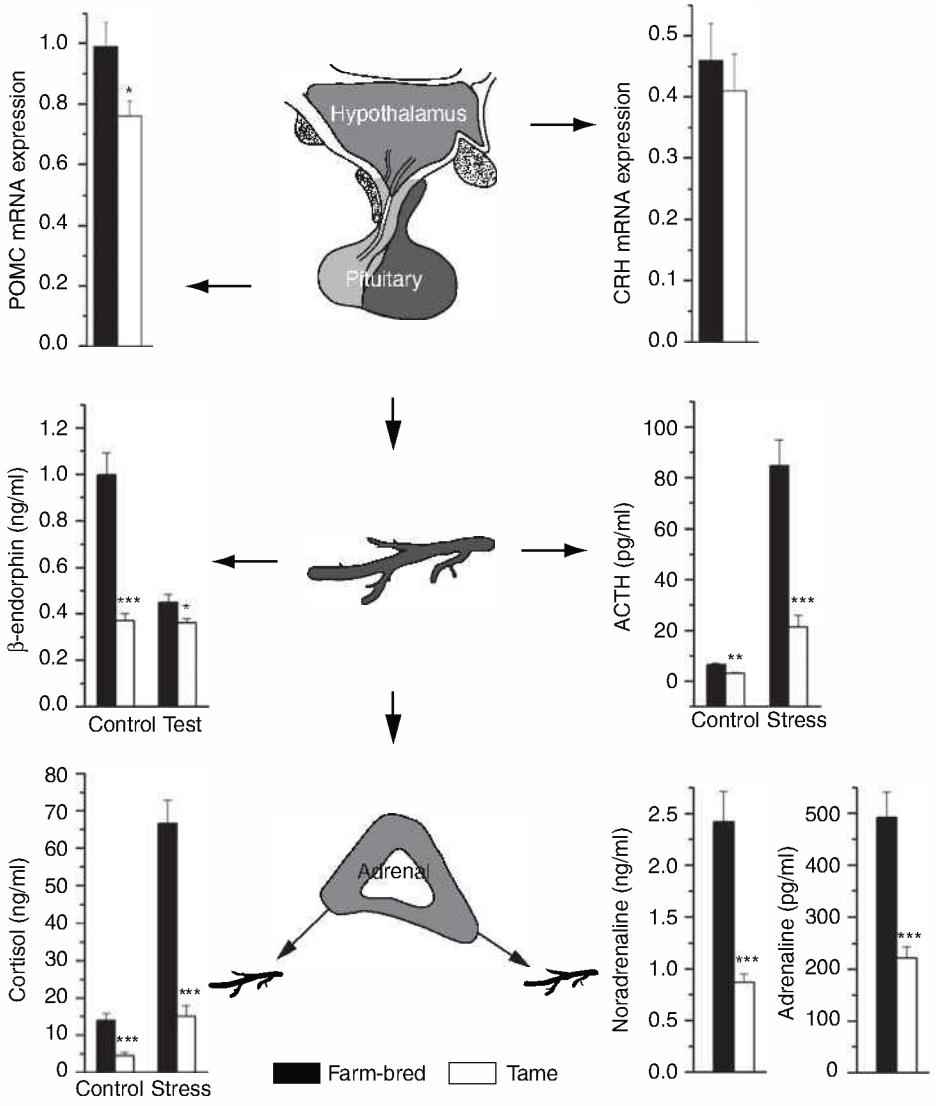

Fig. 2.16. Activity of the hypothalamic–pituitary–adrenal and the sympathetic–adrenal systems in farm-bred and tame foxes. Key: ACTH, adrenocorticotrophic hormone; CRH, corticotrophin-releasing hormone; POMC, propiomelanocortin.

correlated inversely with the advancement of domestication in animals from the same selection generation (Oskina, 1996). It should be noted that no differences were observed between farm-bred foxes and those selected for the enhancement of aggressive behaviour towards humans. Putting it another way, only the selection for positive emotional reaction towards humans correlated closely with hormonal activity of the HPA axis. The connection between behaviour and hormonal response to stress was also shown in dogs. Fearful dogs had increased plasma levels of several hormones (cortisol, progesterone and endorphin) in stress conditions compared with fearless ones (Hydbring-Sandberg *et al.*, 2004).

In foxes, the expression of genes taking part in regulation of the HPA axis also changes during selection. The expression of the gene encoding corticotrophin-releasing hormone (CRH), which stimulates ACTH synthesis in the pituitary, tends to decrease in tame foxes, while the expression of the propiomelanocortin gene (*POMC*) reduces significantly compared with farm-bred foxes (Gulevich *et al.*, 2004). It is known that in the pituitary POMC is a precursor not only of ACTH but also of other biologically active peptides, including β-endorphin, which also enters the bloodstream (Castro and Morrison, 1997). β-endorphin belongs to the family of opioid peptides and acts as a neuropeptide in the central nervous system (CNS), while at the periphery it functions as a hormone and changes under different environmental conditions (Vaanholt *et al.*, 2003). Endorphins have different functions and one of their main effects is analgesia during stress (Bodnar and Klein, 2004). They are also involved in the regulation of different types of behaviour: processes related to emotional state, learning, memory and the system of internal reinforcement and reward (Bodnar and Klein, 2004). The important role of the endogenous opioid system in the integration of behavioural and hormonal response to stress has been shown recently (Bilkei-Gorzo *et al.*, 2008). Preliminary data from our studies show, in tame foxes, that there is no effect of exposure such as contact with humans during a standard testing procedure for plasma β-endorphin levels, although in the control population the concentration decreases by more than twice (Fig. 2.16). At the same time, basal β-endorphin levels measured in comfort conditions (the absence of human exposure) were significantly lower in tame foxes. This could probably be due to the decrease in expression of the gene *POMC* during domestication. It is possible that the reduction of β-endorphin levels observed in control farm-bred foxes after the testing procedure is associated with negative emotions, caused by close contact with humans, that are not observed in tame foxes. So genetic systems controlling the activity of the HPA axis, which has great adaptive significance under extreme environmental conditions, are exposed to substantial indirect selective pressure in foxes during domestication. Knowledge of the fact that in tame foxes the overall pool of circulating glucocorticoids significantly decreases during pregnancy and lactation is essential for understanding the role of the HPA axis under domestication (Fig. 2.17). As a consequence of this, all embryonic and early postnatal development occurs under a low level of maternal glucocorticoids (Trut, 2007; Oskina *et al.*, 2010). It is difficult to overestimate the significance of this fact for morphogenesis, as the maturation and 'programming' of various systems in the early period of development are impossible without glucocorticoids (Demir and Demir, 2001; Owen *et al.*, 2005; Seckl and Meaney, 2006).

Along with the HPA axis, the sympathetic–adrenal system plays an important role in response to a changing environment. Noradrenaline and adrenaline levels in blood were significantly (more than twice) lower in tame than in farm-bred foxes (Fig. 2.16). It is possible that a similar decline can be observed in other domestic animals. In any case, the activity of this system is decreased in domestic guinea pigs compared with their wild ancestors (Kunzl and Sachser, 1999).

Genetic systems regulating the production of neurotransmitters are primarily under the pressure of intensive behavioural selection. The activities of the serotonin, noradrenaline and dopamine transmitter systems in specific brain regions, which are implicated in the regulation of the selected emotional defensive responses, were also altered in tame foxes (Popova *et al.*, 1991, 1997; Trut *et al.*, 2000; Popova, 2006). One must pay special attention to the brain serotonin system during

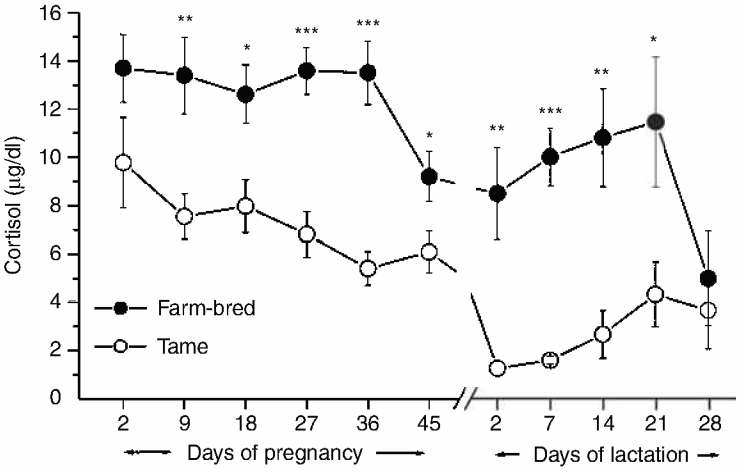

Fig. 2.17. Plasma cortisol level in farm-bred and tame vixens during pregnancy and lactation.

domestication. Numerous pharmacological and neurochemical studies suggest that the brain serotonin system is involved in the inhibition of various types of aggressive behaviour (Miczek *et al.*, 1989; Popova, 1999; Vishnivetskaya *et al.*, 2001). The role of the serotonin system in the inhibition of aggressive behaviour in animals, including foxes, has been thoroughly discussed in the review by Popova (2006). Studies of this system showed elevated levels of serotonin and of its main metabolite, 5-hydroxinol acetic acid, in a number of brain structures in tame foxes. Tame and farm-bred foxes were also different in the level of activity of a main enzyme in serotonin catabolism, monoamine oxidase, as well as in the activity of the key enzyme in serotonin biosynthesis, tryptophan hydroxylase. These changes show increased serotonin concentrations in the brain structures of tame foxes, and agree with data of the inhibitory effect of serotonin on aggression. Our preliminary results, obtained recently, suggest that the expression of the serotonin receptor HTR2C in the prefrontal cortex is significantly higher in tame foxes than in aggressive animals (Kukekova *et al.*, 2011b). As for dogs, different kinds of aggression have a secondary origin, i.e. they arose during the process of breed formation; ambiguous assessments of the serotonin system in dog aggression can be associated with this fact. van den Berg *et al.* (2008) did not show an association between the serotonin receptor genes (*HTR1A, HTR1B, HTR2A*) and human-directed aggressive behaviour in the Golden Retriever. At the same time, the association of serotonin receptor genes (*HTR1D, HTR2C*) and the dopamine receptor gene (*DRD1*) with an aggressive phenotype was found in the English Cocker Spaniel (Vage *et al.*, 2010). In any case, it is difficult to draw any parallels for the role of the serotonin system in the historic domestication of dogs and the experimental domestication of foxes.

It is very important too to emphasize that the serotonin system plays an essential role in the regulation of early development. Although the role of neurotransmitters was discussed in the past, this discussion was recently reignited in a peer review by Levine *et al.* (2006). The involvement of the serotonin system in the regulation of embryogenesis has recently been described in rodents (Cote *et al.*, 2007). These recent (as well as earlier) publications show that neural transmitters are multilateral signalling molecules that play an important role in developmental processes and are able to induce a cascade of gene activations.

Molecular Genetic Implications of Domestication of the Fox

The stunning progress which has occurred over the past decade in the field of genome technologies has attracted great attention of

researchers to the problem of domestication as an evolutionary process. Numerous investigations shed light on the molecular basis of behavioural, morphological and complex physiological traits for different breeds of dog (Lindblad-Toh *et al.*, 2005; Sutter *et al.*, 2007; Spady and Ostrander, 2008; Cadieu *et al.*, 2009; Boyko *et al.*, 2010; Shearin and Ostrander, 2010). However, with results only obtained from comparisons of dogs and wolves from modern populations, our understanding of the molecular mechanisms of early domestication is still limited. Domesticated foxes are a unique model for studying the key molecular changes in domestic animals that occur at this stage. The uniqueness of this model is that both domestic and parental farm-bred fox populations are exactly contemporary and maintained at the same place under the same conditions. We are presently carrying out molecular studies on foxes within the framework of a tripartite collaboration with Cornell University and the University of Utah.

It is worth reiterating that the most intriguing result of dog domestication, as well as of the experimental domestication of foxes, is that under intensive selection for behaviour phenotypic variability is increasing. What is the most amazing is changes in animal size and shape, which are determined by parameters of the skeletal system. Therefore, a fundamental evolutionary question is whether properties of behaviour and morphology are integrated at the genome level, i.e. are there regions in the genome that co-regulate both of these aspects? At the experimental farm of the Siberian Institute of Cytology and Genetics, foxes are selected not only for the elimination of aggressiveness and for domestication, but also in the opposite direction – to preserve and strengthen the expression of aggressive behaviour, which has created a population of aggressive foxes. This population, together with the domesticated one, is a valuable resource for molecular and genetic studies of early domestication.

Needless to say, the most necessary condition for analysis of the molecular nature of any changes is knowledge of the genomic structure of the object under study. It is nucleotide sequencing that provides the most complete information about the structural organization of the genome, but this is not yet available for the fox, although a meiotic linkage map of the fox genome has already been constructed (Kukekova *et al.*, 2007). Despite the dog and the fox being close taxonomically, their karyotypes differ in the number of chromosomes. While the dog has 78 mostly acrocentric chromosomes, the fox has 34 metacentric chromosomes and up to eight additional microchromosomes. Understanding the homology of chromosomal segments in these two species allows us to draw parallels between the genomes. The closeness of dog and fox genomes has already allowed us to adapt canine microsatellite markers for constructing vulpine linkage groups (Kukekova *et al.*, 2004). At present, 385 microsatellite markers are localized on all fox chromosomes (Kukekova *et al.*, 2007, 2011a).

In relation to the fox, the critical factor in mapping such complex quantitative traits as behaviour, and parameters of the skeletal system, is the availability of phenotypes that are suitable for molecular analysis (see also Chapter 20). To search for such phenotypes, the same approach has been used in studying the structure of phenotypic variation in behaviour as in parameters of the skeletal system. Principal component analysis (PCA) has revealed sets of correlated parameters (i.e. principal components, PCs). One of the behavioural components (PC1), which accounts for about 50% of behaviour variability, distinctly differentiates experimental populations in their level of domestic behaviour (Kukekova *et al.*, 2008) (Fig. 2.18). In other words, in backcross (F_1 × tame) and intercross (F_1 × F_1) segregants (F_1 = first filial generation of tame × aggressive cross), these PC1 sets act as individual phenotypes. This indicates the suitability of such phenotypes for molecular analysis. Interval mapping of PC1 behavioural phenotypes of foxes from the backcross population and F_2 revealed that several loci are involved in their formation (Kukekova *et al.*, 2011a). It is important that two of these loci – which are localized on fox chromosome 12 – are found inside the region that overlaps the corresponding region of dog chromosome 5. The involvement of this region in dog domestication was recently demonstrated by vonHoldt *et al.* (2010).

The set of correlated morphological parameters (principal components) by which

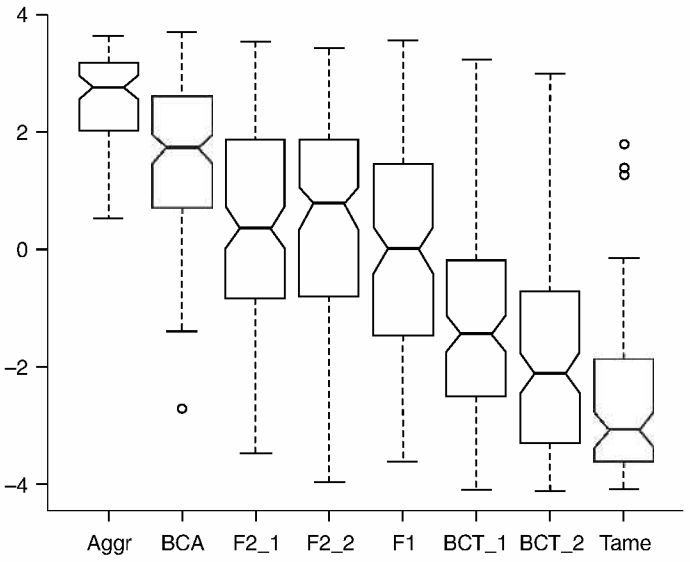

Fig. 2.18. Box plots of the behavioural principal component 1 (PC1) values in fox populations. Horizontal bars within the boxes indicate the population median. Confidence intervals for the medians are shown as notches such that two distributions with non-overlapping notches are significantly different ($P = 0.05$). The bottom and top edges of the boxes indicate the 25 and 75 percentiles. The whiskers indicate the range of data up to 1.5 times the interquartile range. Outliers are shown as Individual circles (Kukekova *et al.*, 2011a). Key: Aggr, aggressive phenotype; BCA, backcross (F_1 × aggressive); F2_1 and F2_2, generation from F_1 × F_1 intercross; F_1, first filial generation of tame × aggressive cross; BCT_1 and BCT_2, backcross (F_1 × tame); Tame, tame phenotype.

populations of dogs and foxes are differentiated has been revealed (Trut *et al.*, 2006). It was found that dogs and foxes have a similar variability in structure in parameters of the skeletal system, i.e. they have corresponding principal components (Kharlamova *et al.*, 2007). The analysis of backcross segregants (F_1 × tame foxes) revealed a statistically significant correlation between the level of domestication and one of the main morphological components (PC2), which reflects the reciprocal ratio of length and width measurements in bones (Trut *et al.*, 2006). Apparently, this component represents the rate of transition from the juvenile form, which is characterized by wider and shorter bones, into the adult form. The same can be said of behavioural components that represent the rate of reduction of infantile affiliative behaviour to a human throughout ontogeny or its retention up to adulthood. In other words, this allows us to suppose that even if there are loci that co-regulate behaviour and morphology, as already noted, they probably have a more general function as rate regulators in development. The search for loci in the fox genome that co-regulate these two phenotypes is one of our main current interests.

It is well documented that during the evolutionary divergence of the dog from the wolf, significant alterations in the expression of primarily brain-specific genes took place. Saetre *et al.* (2004) demonstrated alterations in the expression a few hypothalamic genes (*CALCB* and *NPY*) with multiple functions in the dog, in comparison with wild canids, and assumed these alterations can be caused by strong selection on dogs for behaviour during domestication. The first studies of brain-specific gene expression in foxes selected for tameness were conducted in Sweden (Lindberg *et al.*, 2005). The research was performed on foxes from three groups: descendants of tame foxes exported in 1996 from the Siberian Institute of Cytology and Genetics, farm-bred foxes kept under the same conditions and wild foxes provided by hunters. Differences in gene

expression were detected between these three groups, but the strongest differences were shown between wild and tame foxes, as well as between wild and farm-bred foxes. Statistically significant differences between tame and farm-bred foxes were found for a few genes. Surprisingly, many of these genes are related to haemoproteins (Lindberg *et al.*, 2007).

In contrast, preliminary results from recent transcriptome analysis (Kukekova *et al.*, 2011b) indicate statistically significant differences between the tame and aggressive foxes in the expression of a whole set of genes in the frontal cortex. About 100 genes differ in their expression by more than twice. This means that the selection of foxes for behaviour is also associated with alterations in gene expression in the brain. Of course, these changes aren't as dramatic as those in the dog, which has been diverging from the wolf for thousands of years. Tame foxes still have a long evolutionary process to go through, during which more significant changes in the pattern of genetic expression may occur.

Conclusion: from Tame Foxes to Domestic Dogs

It will probably never be absolutely certain what course evolving dogs might have followed. One can only – with approximations – come closer to a better understanding of the pathways and factors guiding evolving dogs. It is hoped that the domestication experiments with foxes would shed some light on this long-disputed issue. However, the conditions of the experimental recreation of domestication in the modern day do not, even in rough outline, truly illuminate the start of this ancient process. The task of the grand-scale experiment was to reproduce the major factor (as initially suggested) in the first steps of domestication: the intensive selection pressure on behaviour. All animals, from the very start of domestication, were challenged by the same evolutionary situation of the pressure of selection on specific behavioural traits favouring adaptation to the novel social factor of humans.

How are the lessons from long-term selection of foxes for tameness helping us to make clearer judgements about the evolutionary genetic mechanisms of dog domestication? In the light of the results described in this chapter, Hemmer's view (Hemmer, 1990) on the transformation of dog behaviour becomes hardly tenable. He believed that selection for decreased sensitivity of the receptor systems (sense organs), which started to act at the earliest steps of domestication, reorganized dog behaviour. As a result of this 'underreception', exploratory behaviour, stress responsiveness and the fear response all attenuated, and docility formed. To the contrary, foxes selected for domestication were characterized by an earlier establishment of the first auditory response, earlier opening of the eyes and a higher level of exploratory behaviour. Later development of the fear response and, owing to this, lengthening of the sensitive period of socialization were the mechanisms of adaptation to humans and of tame behaviour. To put it another way, transformation of behaviour towards the domestic affected primarily the genes determining the rate of development of sense organs, motor activity and fear response, not the reception level. It was a different matter when morphological mutations exerting a pleiotropic effect on the reception level arose, but these were not the particular mutations that determined the formation of domestic behaviour and the success of social adaptation to a man-made environment.

The experiment with fox domestication demonstrated that, under conditions of strong selection pressure on the behavioural genetic systems, there occurred an increase, in the shortest timespan (at the eighth to tenth generations), in morphological and physiological changes. This disagrees with the view that the dog remained unaltered for a long time. This view was expressed when examining the possibilities for reconsideration of the timing of dog domestication (Vilà *et al.*, 1997). The data on fox domestication are consistent with the classic view that the first increase in diversity occurred explosively from the earliest step in the course of the historical domestication of the dog (Herre, 1959; Zeuner, 1963). Our experimental data suggest that the accumulation of new chance mutations and their homozygotization from inbreeding did not play a major role. Most probably, the phenotypic

changes that have arisen in the course of domestication were caused by changes in a few genes. However, these genes affected the entire development of the dog and hence may have a systemic effect. Their function (mission) was to integrate entire development as a whole and, for this reason, they occupied the highest level in the hierarchical structure of regulation of genome expression. Even small genetic changes of regulators at this high level could produce a cascade of changes in gene activity and, as a consequence, rapid and extensive changes in the phenotype. Many changes in fox phenotype, under conditions of their experimental domestication, had resulted from shifts in the rates at which the relevant developmental processes proceeded. Developmental shifts in tame foxes had, as in dogs, pedomorphic features: a trend towards accelerated sexual maturation on a background of retarded development of somatic characters. The retarded development gave rise to adults showing characters arrested in a developmental stage (neoteny). The role of direct selection for accelerated sexual maturation as of an evolutionary mechanism of the emergence of neoteny has often been examined. Our data strongly suggest that in this case the mechanism is selection for tameness, which affects the genetic system and the function of regulators of the rate of development at the level of the whole organism. As for the acceleration of sexual maturation in foxes, this is also a correlated response to such selection.

Taken together, all the considerations indicate that the concordant behavioural–morphological and physiological transformation in the fox and dog, as well as the similar changes in their developmental timing, may result from the same genetic changes as are provoked by addressed directional selection. Clearly, the experiment with fox domestication has demonstrated what tremendous evolutionary potential may be released by selection vectorized for behaviour. Some important milestones of the evolutionary pathway of dogs under domestication were reproducible over the short span of 50 years by the strongest selection for the genetic systems controlling the specific behavioural trait of tameability. The results of our recent studies of molecular determinants of the tame behaviour of foxes may serve as an additional argument supporting this viewpoint. It is noteworthy that the locus we identified on fox chromosome 12 (*VVU12*), which is closely associated with tame behaviour, appeared to have synteny to the region that was found on dog chromosome 5 (CFA5), for which involvement was recently shown in dog origin as a result of wolf domestication (vonHoldt *et al.*, 2010). This selection may be regarded as the key and universal mechanism of the evolutionary transformation of animals during their domestication.

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3 The History and Relationships of Dog Breeds

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Introduction

The domestic dog has recently gained recognition in the popular arena of genome-wide association studies (GWAS). Dozens of new GWAS studies have been released in the 3 years since the first high-density canine SNP (single nuclear polymorphism) chip was made available. Many of these studies used fewer than 100 dogs, yet they successfully identified associated loci and often discovered the causative mutations contributing to disease, morphology and behavioural disorders. The draw of the dog stems in large part from the unique history and population structure maintained in the species and the promise of reduced genetic complexity based on the same. While other domestic animals are also maintained as breeds, the dog has nearly 400 breeds recognized worldwide; no other species has such a vast number of highly differentiated subpopulations. This structure has been developed through centuries of selective breeding which have resulted in dramatic differences in size, shape, behaviour and even disease susceptibility. This vast diversity is found only between the breeds. Uniformity within any single breed suggests that traits, both morphological and pathological, have little heterogeneity and this leads to improved mapping power. In addition, the number of polymorphic loci involved in the formation of a complex trait is likely to be reduced within any one breed.

Given the availability of the complete genome sequence, a map of >2.5 million SNPs for analyses and medical surveillance second only to that in humans, in order to make the most of the amazing genetic system that is the domestic dog we need to understand the history of the breeds and how they were created. In this chapter, we will look at the early history and development of the breeds, examine their genetic structure and relationships to one another, and discuss the implications that the highly structured organization of breeds has for mapping simple and complex traits.

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Dog Breeds in History

We might expect the history of dog breeds to be an open book – literally – as most US and European breed clubs maintain extensive pedigree records, or stud books, chronicling the genealogy of each breed. However, such records only chronicle what has happened since the breed began the process of registration and, as such, provide little or no information about the events that led to the creation of the breed. Thus, the beginnings of most breeds are shrouded in mystery. Though there are breeds that resemble dogs from ancient art or manuscripts, it is nearly impossible to trace the individual lines through the millennia. Despite the fact that it is difficult to pinpoint the precise conception of most breeds, we can find glimpses of them throughout the ages.

As discussed in the first two chapters of this book, dogs were the first domesticated animals, and accompanied man well before the advent of agriculture or civilization (Fig. 3.1). One of the controversies associated with assigning a date to the early domestication event is that the fossils are not all one 'dog' shape and size. The earliest dog fossils are distinguished from wolves by the shape of the skull: specifically by the wide, shortened snout and increased brain stem size. Based on skull measurements, these first dogs were as big as or bigger than wolves; the earliest find, from Belgium, approaches the size of the largest modern breeds (Germonpre *et al.*, 2009). According to fossil remains, by approximately 14,000 BP the dog came in a variety of sizes. Multiple dog skeletons have been uncovered from the Levant region of the Eastern Mediterranean that date to the Nautufian era in which the dogs are much smaller in size in comparison with wolves. Tchernov and Valla (1996) surmised that one of the dogs in a grave site in Northern Israel may have been as small as 11 kg, with others at the same site ranging up to 16.7 kg (Tchernov and Valla, 1996). This reduction in overall body size may signify the change in human living conditions from the roaming hunter–gatherer lifestyle of the earliest modern human cultures to the onset of a sedentary, agricultural society. As people changed occupation, their requirements in a dog may have changed and selection for desired traits, such as small size, may have begun. It is also possible that selection for smaller body size in early dogs occurred apart from any conscious effort on the part of man; small dogs may have fitted more readily into the new niche of the human settlement.

Ancient Egyptian artwork dating as far back as 6500 BP often depicts a sighthound type of dog with long legs and a thin body. These pictures show variation in the coat colours, patterns and ear carriage of the dogs depicted. From the Urak period of Sumerian civilization, we also find depictions of a very different type of dog. In addition to the hunting scenes, there is a distinct small, shaggy, curly-tailed dog, often portrayed in jewellery. Just as humans developed many specialized occupations with the onset of city life, so too did they develop many new uses for their canine companions. They may have had different dogs to join the hunt, to guard the farm and to stay near the home either as companions or to clear vermin. First millennium BCE artwork from different continents displays a variety of dog images: the giant mastiff types in the Fertile Crescent, the heavy-headed, well-furred spitz types from the Far East, the small-framed, large-eared dogs from South America, and a variety of slender, swift breeds from all around the Mediterranean region.

While Confucius made a cryptic reference possibly referring to two different head shapes on dogs in 500 BCE, the writings of the Greek, Xenophon, in 400 BCE describe two very distinct types of dog: the fox-like 'Vulpine' which was, in his opinion, unfit for hunting, and the more desirable 'Castorian', named for a noted hunter who specialized in the chase, which comes in different varieties and specialities depending on the country of origin. Along the same lines, Flauvius Arrian in *c.* 100 CE, wrote of a breed called the Segusians, a 'shaggy, and ugly...most unsightly' type of dog that was overly excitable and barked excessively, which made them no good for the hunt. He also extolled the virtues of the Celtic 'long-dogs', an early greyhound type which may easily be a precursor to the wolfhound or deerhound. Around the turn of the common era, the poet Gratius Faliscus gave what may be the first breed description. He writes about the 'metagon', a coursing hound developed

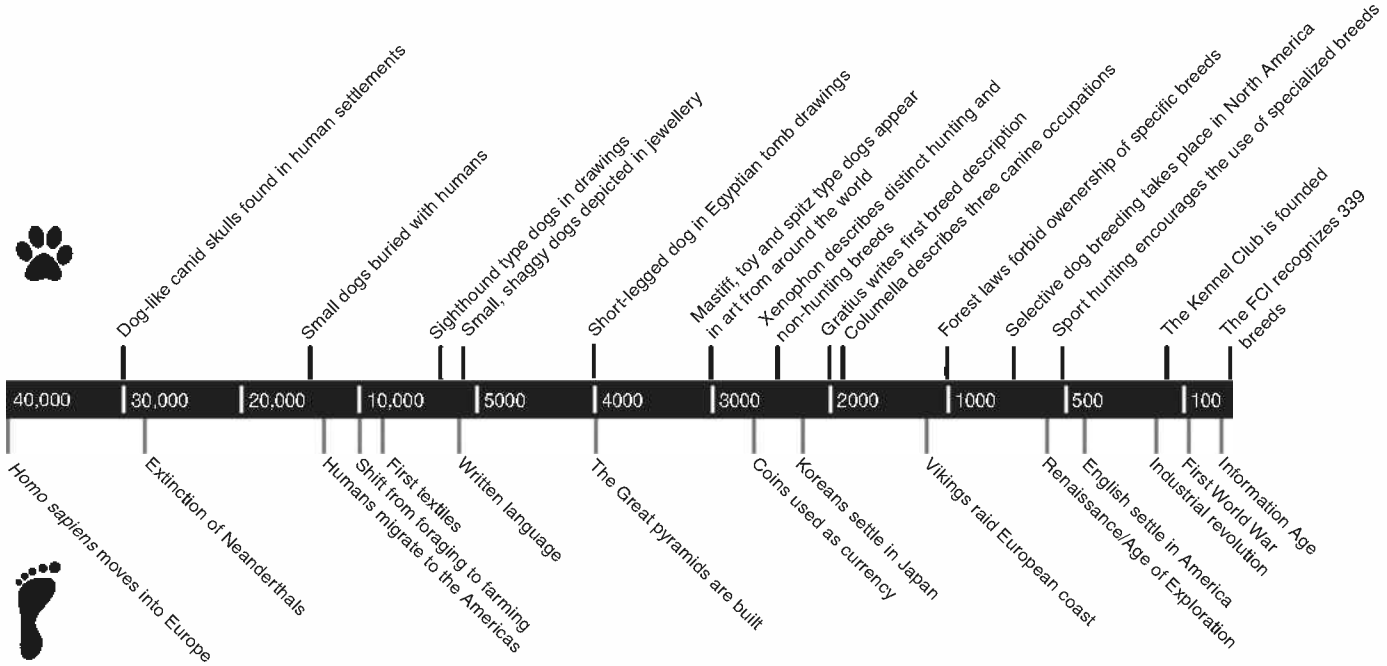

Fig. 3.1. A timeline depicting events from the last 40,000 years of human and canine history. Time points in dog breed development are shown above the line. Human events are shown below the line. FCI, Fédération Cynologique Internationale.

purposely by a man named Hagnon. This was a cross between a Cretan and a Spartan hound and reportedly retained all of the best features of each. In addition, Gratius Faliscus describes ~20 types of hunting dog, usually based on their region of origin. Though dogs of different types (or proto-breeds) were recognized, along with their individual virtues, the idea of breed isolation was not yet in effect, as we read in the agricultural text *Re Rustica c.* 70 CE in which the author describes the care of many farm animals, including the requirements for choosing a good guard dog or shepherd: 'In every kind of quadruped it is the male of fine appearance which is the object of our careful choice, because the offspring is more often like its father than like its mother.' (Columella, 70).

Skipping forward a millennium to northern Europe may provide the first case of true breed reproductive isolation, albeit forced rather than chosen. The Forest Laws enacted in the year 1087 restricted the use of forest lands solely to lords who used them for hunting. Commoners living on what was deemed forest land were not allowed to own coursing hounds or spaniels. They were, however, allowed to own mastiffs (provided they met some restrictions) to guard their homes as well as 'little dogs', as they were unable to hunt deer on their own. The creation of these laws demonstrates that it was commonplace to discriminate between different breeds of dog at this time. In addition, these edicts would have created and enforced the reproductive separation of these breeds to avoid the harsh punishment for owning a restricted dog. It is interesting to speculate that these laws may have also begun the art of crossbreeding and selection on appearance to beget a hunting dog that did not resemble any restricted breed.

Legal issues were not the only requirement for early isolation of breeds. The Salish people of North America, possibly as early as 1400 years ago, raised a breed of dog, now known as the 'woolly' dog, specifically to spin yarn to weave blankets from its coat. Recognizing the economic benefit of this breed, they were maintained in complete isolation, often on islands or in caves, to prevent any crossing with the common-coated village dogs (Crockford, 2005).

By the 1500s, we can find many treatises on dog rearing, most of them aimed at perfecting the hunt. They describe sighthounds, scent hounds, pack hounds, terriers (both short and tall, smooth and rough), mastiffs, etc., though the descriptions vacillate between dogs of different breeds and dogs displaying hunting styles gleaned from training rather than inheritance. It was not until the Victorian era that dog breeds were developed in the style that we recognize today.

With the formation of breed clubs in the late 1800s and early 1900s, dog breeding became a competition and rules were introduced. Well-documented standards were required for each recognized breed. These written standards established a specific size, shape, colour, and sometimes behaviour, from each qualifying dog (American Kennel Club, 1998). The club rules had two important impacts on breed development; they created the opportunity for new breeds – because small changes in morphology would require a new set of standards as each individual breed is supposed to breed true; further, they genetically separated the breeds by requiring the parents of each new puppy to be a registered member of the same breed in order for the puppy to be eligible for registration. This is called the 'breed barrier' rule. As a result, each modern dog breed can now be considered an isolated breeding population, defined by an assemblage of traits maintained under strong selection. As a result of this, we now have a domestic species that exists in a >50-fold range of sizes, with numerous coat types, body proportions and head shapes, which often lead the observer to question whether they are indeed all members of a single species.

Genomic Studies of Breed Relationships

The breed barrier rule has played a defining role in establishing modern dog population structure. Because of this requirement, no dog may legitimately 'immigrate' into a pure breed, and therefore gene flow between breeds is almost entirely prevented. According to the rule, each breed can merely maintain

the level of genetic diversity brought to the breed by the animals that founded it, only acquiring new diversity through the very slow accumulation of mutations within the breed. With the established breed standards comes strong selection to produce animals of uniform appearance that display similar behaviour patterns. All members of a breed are judged by conformity to the standard, and the reproductive success of any single pure-bred dog is almost wholly determined by this evaluation. This often leads to the overuse of 'popular sires' within a breed; typically, these are dogs that have performed very well in the conformation show ring and are then chosen repeatedly for matings. In many breeds today, it is common practice to store sperm from such dogs and to use that sperm to sire litters, even long after the dogs have died. Such an animal therefore plays a dominant role in determining the genetic diversity and allele frequencies in subsequent generations within the breed. In addition to the above, many breeds have experienced bottlenecks or changes in popularity that have affected the resulting population structure. Combine these factors with world events that alter the size of the population or introduce new breeds into previously uninhabited areas, and one can readily appreciate the powerful forces acting within breeds to alter allele frequencies. These forces have led to allelic distortions within each breed, making it possible for genetic drift to turn a new mutation or a formerly rare allele into a common one within that breed. The health consequences of this will be discussed in the following chapters. Here, we will show how these changes in allele frequencies help to identify each breed and give us information about their relationships.

Molecular markers have frequently been employed to examine the differences between dog breeds. Several studies have used the allele patterns from small sets of markers to examine the differences between as few as three and as many as 28 breeds. In all cases, variations in heterozygosity and allele frequency were noted and the expected deviation from Hardy-Weinberg equilibrium was also frequently observed (Zajc *et al.*, 1997; Koskinen and Bredbacka, 2000; Brouillette and Venta, 2002; Irion *et al.*, 2003).

Other studies have employed similar marker sets to address the issue of breed relatedness. Koskinen (2003) measured phylogenetic distances between five breeds and found that they were much larger than comparable distances between human subpopulations. In the same study, microsatellite allele frequencies were used to correctly assign individual dogs to their proper breed, indicating that distinctive patterns of genetic variation existed for each of the five breeds. Although the number of breeds studied in this case was very small, these data nevertheless support the expectation that there is considerably less variation within breeds than between them. Moreover, perhaps the genetic differentiation between breeds, if measured with sufficient resolution, could be used to identify nearly any dog's breed membership. This idea was put to the test in a pair of studies (the PhyDo studies) in which up to 132 breeds were compared and classified through molecular means (Parker *et al.*, 2004, 2007).

The PhyDo studies

In order to provide a genetic interpretation of modern dog breed history, five dogs from each of 85 breeds were genotyped at 96 microsatellite markers distributed throughout the 38 autosomes of the dog genome (Parker *et al.*, 2004). To assure a broad sample of the genetic patterns of each breed population, pedigrees were checked to ensure that the five dogs were not closely related and dogs were not included if they shared parents or grandparents. The heterozygosity across the breeds was examined first. Based on this data set, there was a >4-fold range in heterozygosity that corresponded loosely with the size of the breed. For instance, the Field Spaniel, a breed that averages only 125 dogs registered annually nationwide, had among the lowest heterozygosity in the sample. In contrast, the Labrador Retriever, the most popular dog for the past decade, had among the highest heterozygosity. Population size, however, does not tell the whole story when looking at a breed's genomic composition. The Boxer, one of the most popular breeds for the past decade, ranks as one of the least heterozygous, possibly owing to strong

popular sire effects or past bottlenecks that are not evident from today's demographics.

These data were then used to assign each dog to their breed of origin. Using a leave-one-out analysis implemented in the computer program Doh (Brzustowski, 2002), dogs from these 85 breeds could be correctly assigned to their originating population 99% of the time. In fact, a tremendous amount of the variation observed in the dog rests in the differences that separate breeds; 27–30% of genetic variation that exists in dogs is found between different breeds (Parker *et al.*, 2004; vonHoldt *et al.*, 2010). In comparison, only 5–10% of all human variation is found by comparing different populations or races (Cavelli-Sforza *et al.*, 1994; Rosenberg *et al.*, 2002).

In order to test the necessity of a training set to assign dogs to breeds, an untutored clustering algorithm from the program STRUCTURE was employed to group the dogs. Using this method, populations were determined solely by their genotypes rather than by any subjective classification schema, such as breed identity or morphological or functional groupings (Pritchard *et al.*, 2000). STRUCTURE was applied to the same set of genotypes as described above. The program divided the 414 individual dogs as follows. Most of the individuals, 325 dogs from 69 breeds, were assigned to 69 unique breed-specific clusters (Fig. 3.2). Twenty dogs representing four diverse breeds did not form perfect breed clusters. The last 59 dogs from 12 breeds were placed into six clusters, each composed of two historically related breeds: the Bernese Mountain Dog and Greater Swiss Mountain Dog, the Collie and Shetland Sheepdog, the Greyhound and Whippet, the Alaskan Malamute and Siberian Husky, the Mastiff and Bullmastiff, and the Belgian Sheepdog and Belgian Tervuren. Despite the close genetic relationships between these pairs of breeds, the individual breeds could nevertheless be readily distinguished for five of the pairs when the dogs from just the two breeds were analysed apart from the other dogs. The sixth pair, the Belgian Sheepdog and Belgian Tervuren, are considered to be coat variations of a single breed in Europe, and do not appear otherwise based on these data. The addition of 47 breeds in a subsequent analysis revealed another pair, the Petit Basset Griffon Vendeen and the Grand Basset Griffon Vendeen that have not yet achieved breed-specific allele patterns (Parker *et al.*, 2007). To date, only the former has been officially recognized by the American Kennel Club (AKC).

Assignment and breed-specific clustering clearly demonstrated that the dog breeds were well-isolated populations and revealed the connections between closely related breeds. However, it had yet to address how all of the breeds relate to one another. A distance-based phylogenetic approach was applied to the data set under the assumption that dogs from the same population would have genotypes more similar to each other than to dogs from different populations. Such a phylogenetic approach had been tried in previous microsatellite studies, but with only limited success, presumably due to the small numbers of breeds examined (Zajc *et al.*, 1997; Koskinen and Bredbacka, 2000; Brouillette and Venta, 2002; Irion *et al.*, 2003; Koskinen, 2003). Chord distances were calculated based on allele sharing, and neighbour-joining trees built using the data set comprising dogs from all seven AKC groupings. Significant branching (bootstrap values >75%) was observed for just nine of the breeds (Fig. 3.3a). Interestingly, these nine breeds shared one striking feature: none were of European origin. These breeds, which include the Akita, Chow Chow, Shiba Inu, Chinese Shar-pei, Alaskan Malamute, Siberian Husky, Basenji, Afghan Hound and Saluki comprise a group we labelled the 'Ancient' breeds as they group more closely to the wolf than do the other dog breeds (Fig. 3.2). In addition, 12 breeds showed significant pairing to one another and a triplet of breeds also grouped together, suggesting very recent divergence. These included the Collie and Shetland Sheepdog (which is a miniature version of the Collie), and the three Asian lapdogs: Shih Tzu, Lhasa Apso and Pekingese. Interestingly, the majority of breeds on the canine phylogenetic tree stem from a single node without significant branch structure (Fig. 3.3a). This 'hedge'-shaped topology of branches is indicative of a recent origin from a common founding pool and of hybridization between the breeds. The topology also emphasizes the fact that most of the population structure between breeds is due to individual breed-specific demographic forces rather than

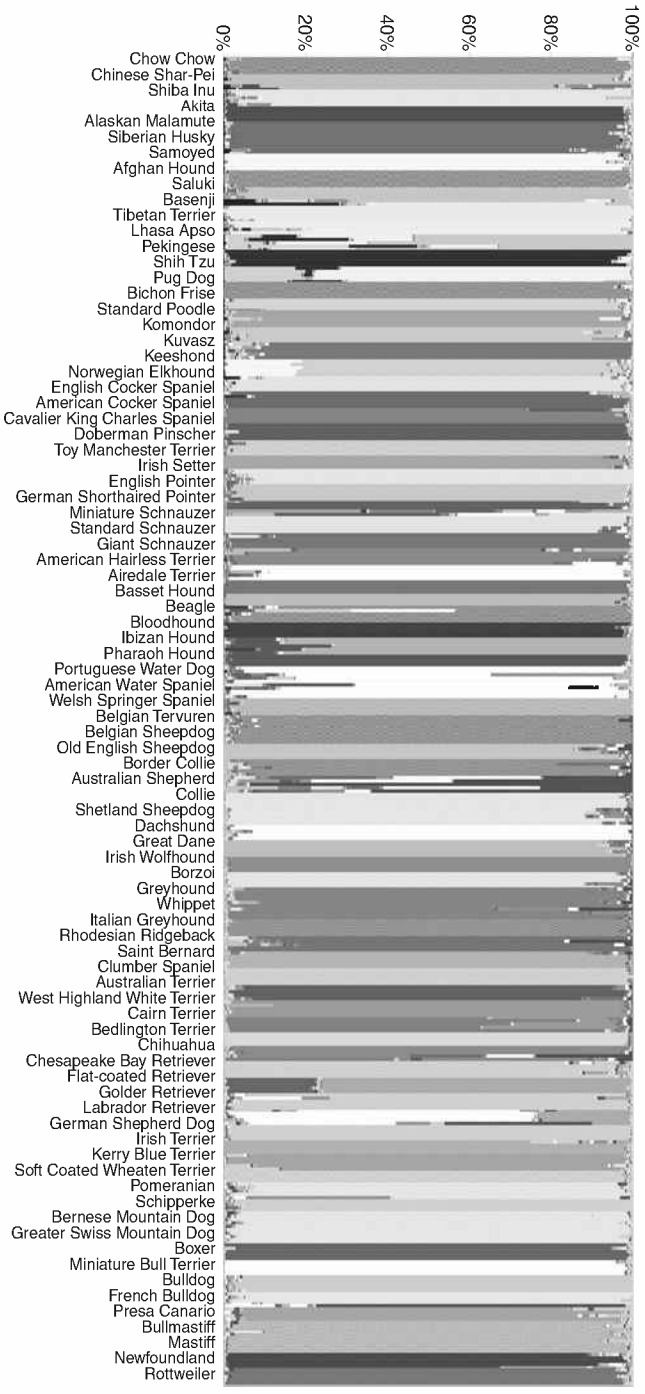

Fig. 3.2. Untutored clustering of 85 dog breeds based on genotyping 96 microsatellite markers distributed throughout the 38 autosomes of the dog genome. Each breed is represented by 4–5 dogs. Each dog is displayed as a single thin line on the graph, divided into segments (shades of grey) representing a genetic population. The majority of breeds form unique clusters distinct from all other breeds.

to an older structure based on potential ancestral lineages that each gave rise to specific groups of breeds. This hedge-shaped topology is a useful feature of the dog's population structure as each breed can be treated, to a first approximation, as an independent sample. This is an important feature of dog phylogenetics; it empowers mapping studies relying on comparison between groups of breeds with divergent phenotypes. In such studies, it is as if each breed is a sample.

Having now ascertained the relationships between the most closely related dog breeds and the most distant, the next step was to examine the population structure of the 'Modern' breeds. This was once again done with the clustering program STRUCTURE. Instead of attempting to cluster each breed into a unique population, the program was forced to group the individuals in 2–20 populations and look for repeatability in the results. The best breed groupings were initially found in 2–4 clusters. A fifth cluster was identified with an increase from 85 to 132 breeds and remained in effect with a final data set of 141 breeds (Parker *et al.*, 2004, 2007; Huson *et al.*, 2010) (Fig. 3.3b). The Ancient group was separated from the Modern breeds at two clusters. When five wolves from diverse populations were included in the clustering analysis, they also grouped with the ancient dog breeds. The second cluster (the Mastiff cluster) comprised the mastiff-like breeds, some terriers and their hybrid cousins – the bull terriers. The third cluster (the Sighthound/Herding cluster) contained a combination of herding breeds and European sighthounds distinct from the Middle Eastern sighthounds in the Ancient group. The fourth cluster (the Hunting cluster) comprised a mixture of hunting dogs from gun dogs to pack hounds, as well as many of the toy breeds and some terriers (Parker *et al.*, 2004). The fifth cluster (the Mountain cluster) included the mountain dogs or flock guards, along with a subset of spaniels and water retrievers presumably derived from similar founding stock (Parker *et al.*, 2007). Many breeds showed inclusion in more than one cluster. For instance, the Pekingese and Shih Tzu were both in cluster one, showing their original Asian origin, as well as in cluster four, displaying their relation to many modern toy breeds. The Rottweiler straddled the line between the mastiff breeds and the mountain breeds, showing its heritage as a multi-purpose working dog. Though there appeared to be additional structure within the breeds beyond the five clusters identified, no further clusters could be ascertained from this data set with any continuity. The five categories of dog breeds, which represent groupings based on geography, morphology and function, have held true under additional analyses using genome-wide SNP markers in place of the microsatellites.

The CanMap analysis

In 2007, a collaboration was developed to build a database of SNP genotypes from a large number of dogs in order to assess genome composition, map traits, infer the geographic origin(s) of domestication and further refine our understanding of breed relationships (Cadieu *et al.*, 2009; Parker *et al.*, 2009; Boyko *et al.*, 2010; vonHoldt *et al.*, 2010). More than 900 dogs were genotyped at ~48,000 SNPs from all 38 autosomes and the X chromosome using the Affymetrix v.2 Canine SNP chip. In order to assess breed structure based on this data set, the SNPs were analysed both individually and in groups of 5–15 SNPs, each covering approximately 500 kb. The alleles from each SNP in a group were assigned a chromosome of origin and the two resulting haplotypes compared across breeds and within breeds to determine the degree of sharing, and hence the level of relatedness (vonHoldt *et al.*, 2010). Phylogenetic analysis of haplotype sharing and allele sharing across 80 breeds, with >6 unrelated individuals per breed, supported the original four breed clusters, the Ancient, Mastiff, Sighthound/Herding and Modern clusters (Parker *et al.*, 2004; Parker and Ostrander, 2005). The Mountain group (Parker *et al.*, 2007) was not well represented in the set of 80 breeds, which is probably why it did not form a separate cluster. However, the few Mountain group breeds that were analysed did in fact group together within the Mastiff cluster (Fig. 3.3c). In addition, the SNP analysis divided the modern breeds into scent hound, spaniel and working dog groups, and clearly

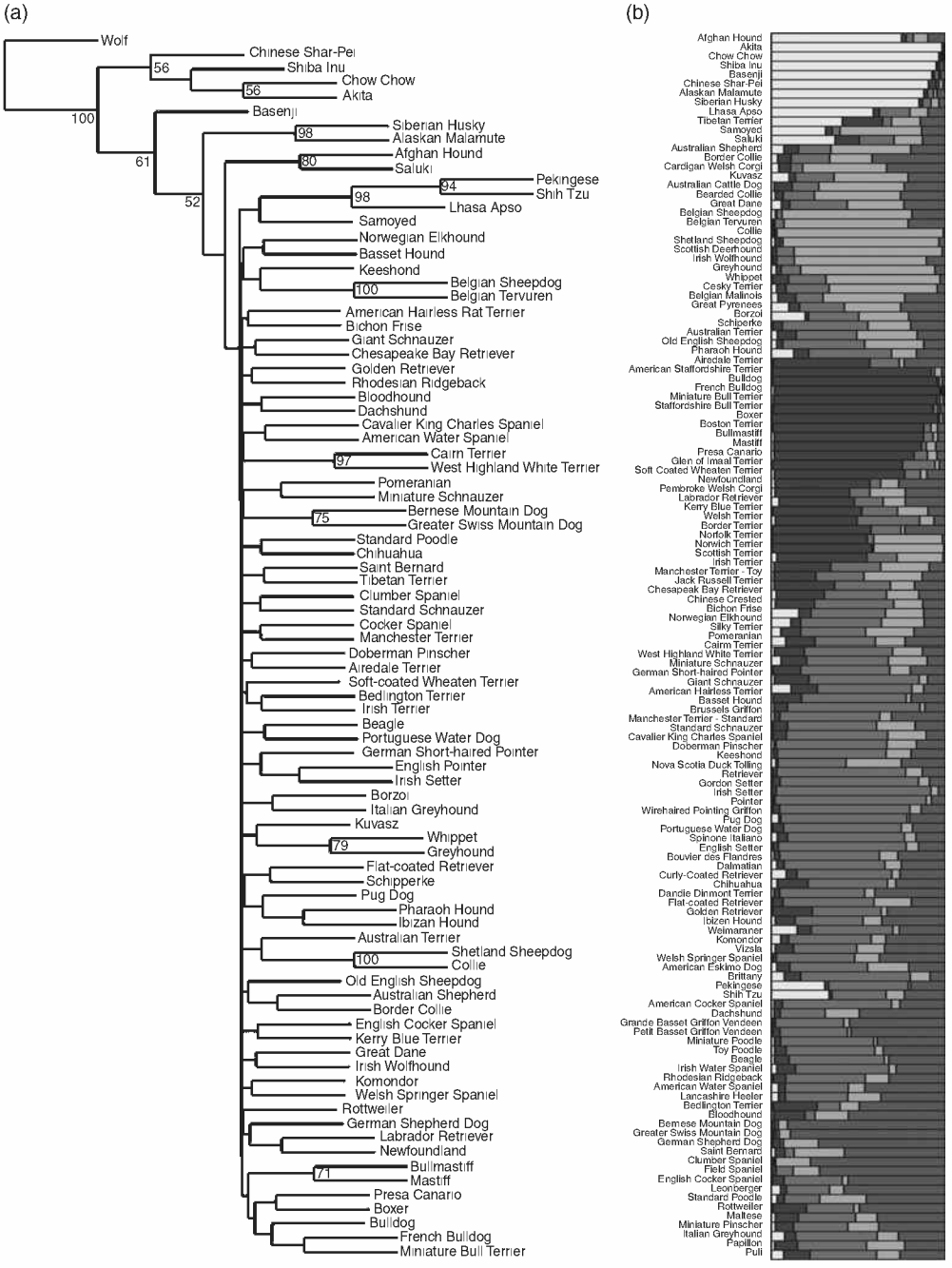

Fig. 3.3. Three genomic depictions of breed relationships. (a) A neighbour-joining tree of 85 breeds based on alleles at 96 microsatellite markers. Bootstrap values are given for branches that exceed 50%. The only values >75% are those that distinguish the 'Ancient' breeds from the modern 'hedge' breeds (those of recent origin, see text) or that group pairs of closely related breeds. Figure originally published in *Science* (Parker *et al.*, 2004). (b) Clustering analysis of 132 breeds based on alleles at 96 microsatellite markers. Four to five dogs from each breed were averaged to obtain the breed cluster assignment.

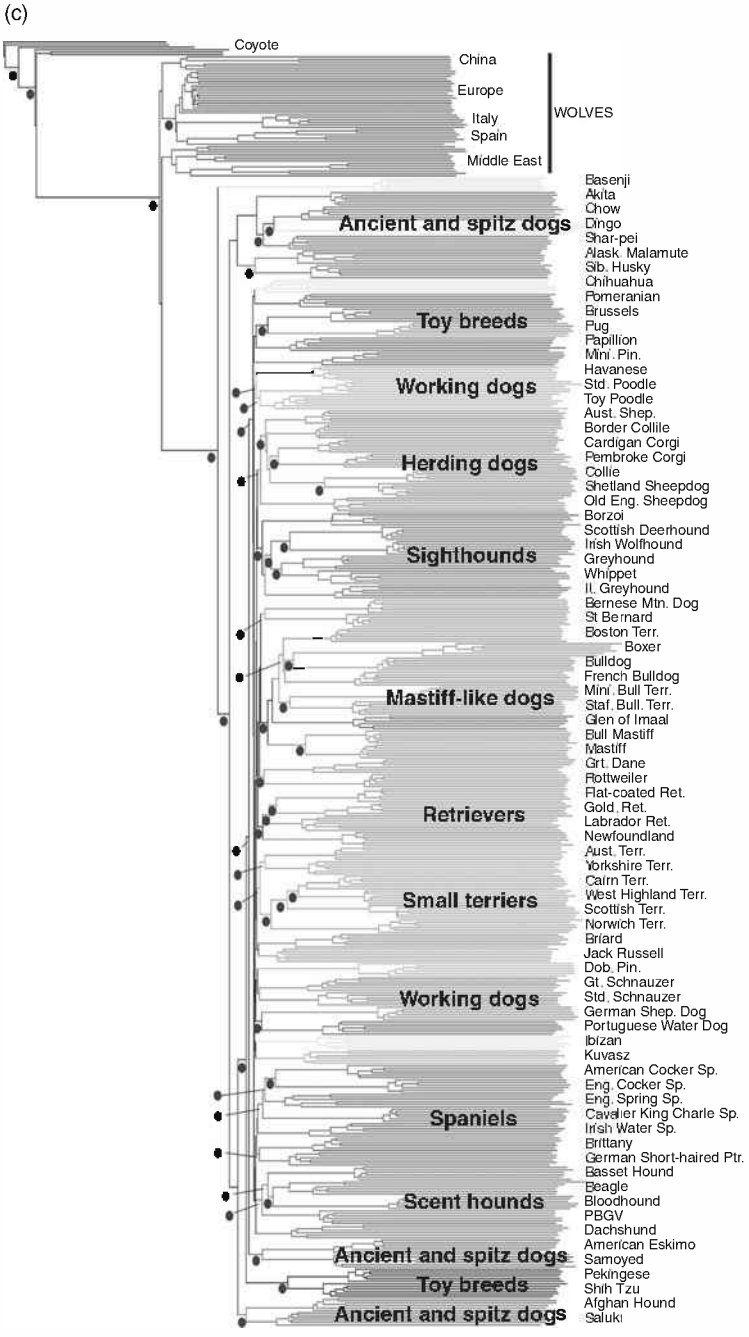

Fig. 3.3. Continued.

Five distinct clusters are obtained with many breeds showing inclusion in more than one. Figure adapted from original published in *Science* (Parker *et al.*, 2007). (c) Neighbour-joining phylogram of 80 breeds each represented by six or more individuals. Distances were measured based on haplotype sharing of 48,000 SNPs (single nucleotide polymorphisms) in five SNP windows. Bootstrap values >95% are indicated by dots. PBGV, Petit Bassett Griffon Vendeen. Figure originally published in *Nature* (vonHoldt *et al.*, 2010).

separated the sighthounds from the herding dogs within their shared cluster. The toy dogs grouped in two different positions in the analysis, one near the ancient dogs, again displaying the Asian influence on a number of toy breeds, and the other in the middle of the modern breeds. The short-legged terriers also formed a unique cluster that was only hinted at in the 2007 microsatellite analysis (Fig. 3.4).

In addition to the identification of new breed clusters, the SNP analysis also revealed that a significant amount of variation found in dogs, nearly 4%, is a result of differences between the breed clusters as opposed to breed membership or individual variation (vonHoldt *et al.*, 2010). This supports a third level of complexity in the population structure of the domestic dog, one that is already being used to develop strategies for finding mutations that cause common disorders (Parker *et al.*, 2006, 2010). For example, Modiano *et al.* (2005) sought to determine the origin of B- and T-cell lymphomas in dogs by looking at differences across the breeds. They found that, while B-cell lymphomas are most common overall, rates of T-cell lymphoma are significantly higher in breeds from the Ancient or Asian cluster. This suggests a common cause of T-cell lymphoma in the Asian dogs, while arguing against a single ancestor for B-cell lymphoma in any other group. The optimal mapping study for T-cell lymphomas would therefore focus on dogs from the ancient group. Many studies have combined similar breeds with matching phenotypes in order to improve fine mapping and mutation analysis. Some of these will be discussed in the Breeds and Mapping section.

Potential new marker sets

An area of recent interest in genome analysis is the characterization of copy number variations (CNVs) across the genome. These are regions of the genome that exist in either greater or fewer than the expected two copies. To date, two analyses have been published describing CNVs in small sets of diverse dog breeds. The study by Nicholas *et al.* (2009) describes genome-wide CNVs in 17 breeds, each represented by one individual. These researchers identified approximately 200 CNVs per dog with a range of 118–298; in addition, they noted that most of the CNVs (average 94%) were shared across breeds. It is possible that these gains and losses of genomic regions could be used to investigate the relationships

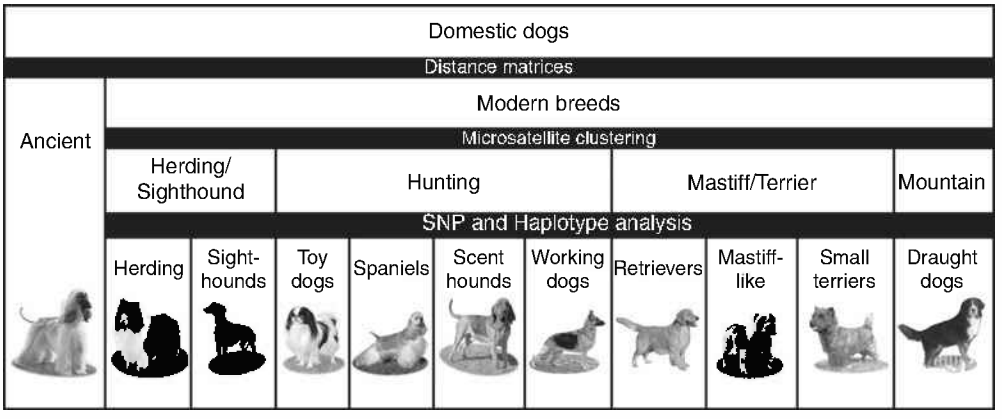

Fig. 3.4. Summary of the genetic relationships among the breeds. The first division between Ancient breeds and Modern breeds can be found using any set of markers and a simple distance measure that summarizes allele sharing. The second division of the Modern breeds into five clusters was identified using a genome-wide set of 96 dinucleotide microsatellite markers (Parker *et al.*, 2007). The final division of the modern breeds into ten occupational breed clusters was accomplished using 48,000 SNPs in haplotype groups (vonHoldt *et al.*, 2010).

between the breeds. Chen *et al.* (2009) found evidence of breed group clustering of CNVs in their analysis, although it was based on a very small sample size. These results suggest that an extensive definition of CNVs across breeds may provide support for the current breed relationship paradigm and, because the mutation process involved in CNV creation varies from site to site, may help to refine our current interpretations of breed development.

There are other markers available within the dog genome that could also prove useful for population studies. One of these is the SINE (short interspersed element) retroposons. A comparison of the two genomic dog sequences that are available, a draft Boxer genome (derived from 7.5× coverage) and a Poodle genome (derived from 1.5× coverage), has revealed >10,000 canine-specific SINEC_Cf (a major subfamily of canine-specific SINEs) repeats that are polymorphic (Wang and Kirkness, 2005). Hundreds of thousands of other copies of these transposable elements are present across the genome that have not been observed to be polymorphic. Presumably, most such elements are fixed and are present in all dogs, regardless of breed. These bimorphic elements, as Wang and Kirkness term them, in contrast to the fixed copies, contribute to sequence diversity in the dog genome. Because they are not fixed, they are likely to have been integrated relatively recently in evolutionary time. Thus, these SINEs appear to be very active in the dog genome at present, or at least were active in the relatively recent past. Even if the SINE integrations are not actively occurring in today's breed populations, the breeds should still carry distinct patterns of individual SINE integrations due to founder effect and drift. Therefore, tracing the presence and absence of SINEs within individual breeds may reveal breed relationships not yet identified by the current marker sets. Individual SINE elements provide only two allele states, presence or absence, and are, therefore, not as information dense as individual microsatellite markers. However, they do have one advantage: the ancestral state is immediately identified as the absence of the SINE, while the integration is the derived state. Furthermore, they provide better inference of identity by descent than do microsatellites.

Possibly a combination of analyses from a variety of marker types – microsatellites, SNPs, CNVs and SINEs – will ultimately provide the greatest resolution for discerning dog breed relationships. Each marker type has its own mutation rate and follows a different mutation time frame, thereby offering snapshots of breed relationships at different points in time.

Single-locus analyses

Discovery of the relationships between breeds and an investigation into the methods of creation of each has benefited mapping strategies that seek to gain mapping power by extending across breed boundaries. The mutations that cause disease or alter morphology are discussed in later chapters. However, here we will look briefly at the comparisons that have been made between the breeds based on the haplotypes carried at specific loci that control or contribute to variation in traits. Several alleles have been identified that alter the appearance of the domestic dog, and many of these are strongly selected for and fixed within individual breeds. In nearly all cases, the haplotypes, as well as the causative mutations, have been found to be identical across many or even all breeds that display the trait concerned (Clark *et al.*, 2006; Candille *et al.*, 2007; Karlsson *et al.*, 2007; Salmon Hillbertz *et al.*, 2007; Sutter *et al.*, 2007; Drögemuller *et al.*, 2008; Cadieu *et al.*, 2009; Parker *et al.*, 2009). These findings do not provide information about the development of individual breeds other than to remind us that all dogs are related and, if traced back far enough, all their ancestors come from the same population. They do emphasize the fact that crossing and selection have played a major role in the creation of the diverse breeds that we find today, as most key morphological traits are controlled by major-effect alleles that have apparently arisen only once and then been quickly selected to fixation in many diverse populations, all sharing the trait of interest. These alleles and loci under strong selection can be thought of as providing a particular view of breed relationships that is highly skewed based on the trait. So from

consideration of the *IGF1* locus (for insulin-like growth factor 1) alone one would conclude that small dog breeds are very closely related because they share a single haplotype. The same point of view applies to chondrodysplastic breeds that share the *FGF4* pseudogene (for fibroblast growth factor 4).

More information can be gleaned from the analyses of disease-related loci. These have not been consciously selected for; therefore, their sharing may be attributed to common founders of the breeds more often than single crosses made for the acquisition of a desired trait. Originally, disease mapping studies have concentrated on a single breed in which the disease is prevalent and families could be identified that carry the disease. In a few cases, multiple breeds would present with a similar disease and data would be compared for all breeds. For example, when narcolepsy was mapped to the *Hcrtr2* gene (for hypocretin receptor 2) in 1999, Lin *et al.* (1999) found that the Labrador Retriever and Doberman Pinscher carried different mutations in the same gene that caused the disease. Later, a third mutation was found in the Dachshund (Hungs *et al.*, 2001). Here were three dog breeds that are all in the modern breed cluster but lie within three different haplotype clades and show three completely different mutations in the same gene that lead to the same disease. This allelic heterogeneity mirrors what is often found in human disease loci. Because the dogs were separated into breeds before mapping, each allele could readily be identified. Similar results were found in analysis of the *Cmgb3* gene which causes cone degeneration through a deletion in Alaskan Malamutes and a point mutation in German Shorthaired Pointers (Sidjanin *et al.*, 2002). Oculoskeletal dysplasia has recently been traced to mutations in two different but related genes, *Col9a2* and *Col9a3* (which code for collagen chains), in two unrelated breeds, the Samoyed and the Labrador Retriever, respectively (Goldstein *et al.*, 2010). Allelic heterogeneity among unrelated breeds may discourage the use of multiple breeds in a mapping study, but the need to infer whether allelic heterogeneity is likely or not provides an important part of the rationale for understanding breed phylogenetics.

When dog breeds with similar origins and common ancestors are combined in a mapping study, the mutation is often shared among all the affected breeds. For instance, the multidrug resistance gene (*MDR1*) mutation plus four linked microsatellite markers were all found to segregate identical by descent (IBD) in nine related breeds. These breeds included seven herding breeds and two new variations of sighthounds with probable outcrosses to at least one of the herding breeds (Neff *et al.*, 2004). Thus, it is plausible that this group of breeds would share a haplotype by descent at some locus. In another example, a single-point mutation that alters a splice donor recognition site in the *ADAMTS17* gene (a disintegrin and metalloproteinase gene), carried within a shared 600 kb haplotype, was found in all Jack Russell Terriers, Miniature Bull Terriers and Lancashire Heelers with primary lens luxation (Farias *et al.*, 2010). In this case, none of these breeds cluster closely in genome-wide studies, although all do have terrier ancestry. Similarly, an identical *Sod1* mutation (in the superoxide dismutase gene) and 200 kb haplotype was found in a diverse set of breeds including Rhodesian Ridgebacks, German Shepherds, Pembroke Welsh Corgis and Boxers with degenerative myelopathy (Awano *et al.*, 2009). All of these breeds display the haplotype both with and without the putative causative mutation, suggesting that this is a very old ancestral mutation inherited from the source by each breed rather than from a recent crossing between them.

Progressive rod-cone degeneration has been linked to a mutation in the gene *PRCD* (Zangerl *et al.*, 2006). This mutation has been found to be identical by descent in 14 dissimilar breeds of dog. Examination of the extended haplotype in the region shows that the Labrador Retriever, Chesapeake Bay Retriever, Portuguese Water Dog, miniature and toy poodles, and English and American Cocker Spaniels share the largest haplotype, ~1.5 Mb, with the American Eskimo dog also sharing the majority of that span. The Australian Cattle Dog only shares approximately 100 kb surrounding the mutation with these breeds compared with the ~1 Mb of common haplotype shared with the Nova Scotia Duck Tolling Retriever (NSDTR) (Goldstein *et al.*, 2006). Examination of the

histories of these two breeds does not suggest that they share a lot in common, as both were only recently created on different continents. However, each of the breeds was created through mixtures of a number of existing breeds, including collie types, which may account for their common haplotypes in this region. The NSDTR was also associated with herding breeds in the mapping of collie eye anomaly. In this disease the mutation and common haplotype were found in eight breeds, most of which were herding dogs with evident shared heritage, but also included the NSDTR and Boykin Spaniel (Parker *et al.*, 2007). Whereas the NSDTR groups clearly with the other sporting breeds on whole genome analysis, by looking at individual loci we can see evidence of its herding dog origins.

Examination of additional loci has provided a mixture of information about the breeds. A common coding insertion in the *Hsf4* gene (for heat shock transcription factor 4) was found in Staffordshire Bull Terriers and Boston Terriers with cataracts, both members of the mastiff–terrier cluster (Mellersh *et al.*, 2006). However, a coding deletion was found in the same gene in similarly afflicted Australian Shepherds from the herding group (Mellersh *et al.*, 2009). Neither of these mutations was found in unrelated breeds such as the Dachshunds, Siberian Huskies, Entlebucher Mountain Dogs, English Cocker Spaniels or Komfohrlanders in subsequent studies (Engelhardt *et al.*, 2007; Muller *et al.*, 2008; Muller and Distl, 2009). Siberian Huskies and Samoyeds, both spitz-type dogs that group with the ancient breeds, share a mutation and common haplotype associated with X-linked progressive retinal atrophy, while the Akita, a spitz breed from the same cluster, does not (Zangerl *et al.*, 2007). This division of allele sharing may be the result of more recent crosses between or common ancestors among the husky and Samoyed, both Arctic working dogs, versus a much older relationship to the Akita, a hunting/fighting breed that has undergone periods of isolation and bottleneck in its native Japan.

Each region of the genome, examined independently, could be expected to provide a distinct phylogeny. The combination of all the regions will tell the complete story of dog breed creation and history. That being said, the most comprehensive look at each breed and how it shares genomic information with its neighbours will ultimately come from whole genome sequence. Examining chromosome sharing, identifying founders and their hybrid recombinants, quantifying shared segments in both total number and size, as well as placing new mutations within the common haplotypes, will not only reveal the source of each modern breed but may give each a more definite timeline for their development.

Breeds and Mapping

In any system, the intrinsic complexity of the genetic factors contributing to a trait can make the identification of the contributing loci and alleles extremely difficult. In human genetics, there is often both allelic and locus heterogeneity working together to reduce mapping power. Studies to map complex traits in humans now routinely analyse phenotypes and genotypes from tens of thousands of individuals. Even with the rapidly dropping costs for genotyping and sequencing, these studies are daunting for the most well-funded investigators. Based on the population structure described above, the dog can offer many advantages to tackling complex trait mapping on a smaller scale. First, fewer markers are required to find association in the dog. Secondly, traits can be mapped with a relatively small numbers of individuals. Thirdly, affected groups can be stratified to identify single mutations that combine to create complex phenotypes. Let us examine each of these statements in the context of the breeds.

First, the breeding rules and limited gene pools within each breed have led to lengthy stretches of linkage disequilibrium (LD) across the genome (Sutter *et al.*, 2004; Lindblad-Toh *et al.*, 2005; Boyko *et al.*, 2010). The average LD in dog breeds stretches over 1 Mb, ~50 times longer than the average LD found in humans. So completing a GWAS in a dog population will require only 20,000–40,000 SNPs spaced evenly across the genome as opposed to the million SNPs that have become standard in human studies. In addition, a range of LD

measurements have been reported in different breeds, from <50 Kb in the Labrador Retriever to >5 Mb in the Mastiff (Gray *et al.*, 2009). This would suggest that the initial association will be most easily found in the Mastiff because the LD is far reaching, and any one of many SNPs within a haplotype should be able to mark the causative mutation. Fine mapping, however, will be best performed in the Labrador as the LD blocks in the region will be divided into smaller sections, thereby better allowing for the identification of a disease-associated gene or mutation. In addition to differences in LD, haplotype structure varies across breeds. In the section on the CanMap analyses above, we discussed how haplotype sharing could be used to cluster breeds into related groups. Haplotype sharing can also be used to fine map disease-associated loci. Sutter *et al.* (2004) showed that the majority of chromosomes (80%) within a single breed are carrying only 2–3 haplotypes in any one chromosomal region. Across a set of breeds, the majority of chromosomes are carrying just 4–5 haplotypes in total. Thus, pure-bred dogs have fairly limited haplotype diversity. Furthermore, there is a great deal of haplotype sharing between dog breeds. On average, two-thirds of chromosomes within a pair of breeds carry haplotypes that the two breeds share in common. As these numbers pertain to unselected regions of the genome not associated with traits, it seems likely that breeds will share haplotypes even more frequently in regions associated with traits that they share. Careful and knowledgeable combination of breeds in studies will provide the greatest amount of information about any trait or disease.

The second statement, that mapping can be performed with fewer dogs, is also a result of breed structure. On average, less than 10% of the dogs registered in any breed contribute to the following generations. In addition to the small breeding population, there is a trend towards the use of popular sires, which can exaggerate the contribution of a single dog to the entire breed. Therefore, for any trait carried within a single breed, there is a very high likelihood that the trait is inherited from a common founder of the breed. With all of the mutant alleles coming from the same source, there is little heterogeneity at the disease locus. So a small number of cases and controls will provide sufficient power to detect an associated locus.

Finally, we come to complex trait mapping. Again, we look to the structure of the breeds for our deliverance. Other than a few exceptions, such as hairlessness in the Chinese Crested that must be maintained in a heterozygous state (Drögemuller *et al.*, 2008), all traits that are essential to a breed are homozygous in order to assure that every generation displays the trait exactly like the generation before. In order to achieve this, there must be extensive selection in the creation of the breed and fixation of a large number of alleles. This can be observed genome wide, as was reported upon completion of the dog sequence. Large stretches of homozygosity, some over 50 Mb, are scattered across all chromosomes and account for a greater fraction of the genome than do the heterozygous stretches (Lindblad-Toh *et al.*, 2005). Thus, for each multigenic attribute, each breed will probably be fixed for some subset of the genes required to create the trait or disease. This suggests that complex traits and quantitative traits will be segregating alleles at fewer loci in any one breed than in the species as a whole. By mapping the traits in separate breeds, it may be possible to find each of the alleles independently and then combine them to create a picture of the trait as a whole.

While these points may seem optimistic, in the last 5 years the field has found abundant evidence that breeds really do simplify the genetics underlying complex traits, often in striking ways. This has been found to be especially true for morphology, as is discussed in Chapter 16, but can also be seen in complex diseases such as epilepsy (Lohi *et al.*, 2005), progressive retinal atrophy (described in the section on Single-locus analyses above; and reviewed by Baehr and Frederick, 2009), neurological disorders (Chen *et al.*, 2008; Drögemuller *et al.*, 2010) and canine compulsive disorder (Dodman *et al.*, 2010). The degree to which breeds can similarly simplify complex disease susceptibility traits such as cancers will doubtless be answered through mapping projects currently underway.

Because dog breeds provide a plausible solution to many of the current problems

encountered in GWAS, it is essential to do what we can to empower these mapping studies through educated use of the available breed populations. A solid foundation of breed phylogenetic knowledge will grow even more important as the field ventures towards ever more challenging projects. The most complex disease-trait mapping efforts will require every bit of extra mapping power that we can squeeze from knowledge of breed histories and relationships.

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4 Molecular Genetics of Coat Colour, Texture and Length in the Dog

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Introduction

The genetics of coat variation is a powerful system for studying the fundamental aspects of gene action and the evolutionary mechanisms that give rise to morphological diversity. The domestic dog (*Canis familiaris*) is an ideal platform for this approach due to the variety of coat colours, textures and patterns represented among modern breeds, and a unique population history that facilitates efficient and precise gene localization.

The study of coat colour heredity in the domestic dog was among the early

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applications of Mendelian principles to mammals, and it contributed to a comparative description that demonstrated for the first time the conservation of gene action and interaction in mammals (Little, 1914; Wright, 1918; Searle, 1968). As described in an earlier edition of this chapter (Sponenberg and Rothschild, 2001), much of the early work on dog colour genetics is summarized in books by Clarence Cook Little (Little, 1957) and Ojvind Winge (Winge, 1950), and is based on a combination of segregation data and comparisons to other species, usually the laboratory mouse. Indeed, Little is perhaps best known for founding The Jackson Laboratory (an independent, non-profit organization focusing on mammalian genetics research to advance human health, first established in 1929 in Bar Harbor, Maine, and now also based in Sacramento, California) and helping to establish the first inbred strains of mice (Crow, 2002), which would serve as a critical resource for the identification of more than 100 loci that affect coat colour (Bennett and Lamoreux, 2003). The cloning and characterization of more than 50 mouse coat colour genes have greatly informed our current understanding of pigmentary biology and led to the discovery of homologous pathways participating in other biological processes, including the regulation of body weight (Barsh *et al.*, 2000) and the biogenesis of intracellular organelles such as lysosomes and platelet granules (Huizing and Gahl, 2002; Raposo and Marks, 2007).

In fact, for the most part, our current understanding of the molecular genetics of dog coat morphology has been heralded by studies in laboratory mice, because the repertoire of mouse pigmentation genes provides a useful set of candidates for identifying coat colour genes in dogs. None the less, there are important reasons to pursue studies of dog coat morphology on its own. From a practical perspective, breeders often make decisions based on coat texture or colour characteristics of potential litters, and such decisions can often have more than cosmetic ramifications, as with dogs homozygous for the *Merle* mutation, in which vision and hearing are often compromised. From a scientific perspective, analysis of multiple alleles in dogs can occasionally provide a more detailed understanding of gene function than exists in laboratory mice, as may be the case for the *Spotting* locus and the *MITF* gene. In addition, there are several examples of dog colour or hair mutations for which there is not an obvious homologue in other mammals, such as *Dominant black* (*K*), *Harlequin*, or the coat characteristic known as furnishings – facial furnishings, such as a beard, moustache or eyebrows – (*RSPO2*). These examples probably reflect the history of dog domestication and breeding, which is characterized by strong diversifying selection for many different traits; thus, studying the genetics of dog coat morphology provides important insight into evolutionary mechanisms associated with artificial selection.

Enabled by advances in genomic resources and analytical approaches, the domestic dog has recently been transformed into a tractable genetic system for efficient gene mapping. In what follows, we first describe the modern 'case-control' approach to mapping genetic traits in the dog in the context of population history. We then present an overview of coat colour genetics using the framework established by C.C. Little (Little, 1957), comparing and contrasting this with the relevant processes studied in the laboratory mouse. Although Little's opinions about allelic and locus relationships in dogs were necessarily based on limited data and speculation, much of what he described more than 50 years ago holds up very well to molecular scrutiny; in situations where this is not the case, we explain in more detail the underlying molecular basis and gene interactions. We next discuss the background for and recent advances in the genetic basis of coat structure traits, including coat length, coat texture (curly versus straight), and regional distribution of these phenotypes, e.g. furnishings. In contrast to pigmentary phenotypes for which traits often segregate within breeds, coat structure traits are commonly fixed, reflecting both the population history and the rationale for breed derivation.

Throughout the chapter, and following earlier convention in dog and animal genetics, we use the term 'locus' to refer to a phenotypic trait that segregates in a Mendelian fashion, 'gene' to refer to a fragment of coding DNA associated with and/or responsible for a specific trait, and

'allele' to refer to the different variants that are observed, sometimes at a phenotypic level (where the gene has not yet been identified, e.g. as in *Ticking*), and often at a molecular level (where the gene has been identified, e.g. as in *Spotting* or *MITF*). We note that, historically, the term 'gene' has been used to refer both to the entity responsible for Mendelian segregation (the original definition, of course) and to a fragment of DNA (a more modern, and molecular, definition). We also note that the term 'locus' was originally developed to represent the concept that genes resided in specific chromosomal locations and that, in some cases, several genes might reside at a single locus. But, for the purposes of this chapter (and with apologies to Beadle and Tatum), it will be a useful oversimplification to consider dog coat morphology in a 'one locus, one gene' framework. Summaries of coat colour and hair loci, genes, alleles and phenotypes are provided in Table 4.1, and of coat colour and hair loci, genes, alleles, mutation types, expression and functions in Table 4.2.

Our knowledge of the molecular basis of dog coat morphology has progressed dramatically in the last decade; a large number of morphology genes have been identified, and we anticipate rapid progress for understanding the remaining unknown determinants of dog coat morphological variation, including quantitative variation – such as the extent of spotting or the intensity of red coat dilution. Though the story remains incomplete, the current data set is sufficient to offer some insight into mechanisms driving the diversification of coat morphology. For example, how often does the same phenotype, occurring in multiple dog breeds, share a common origin? What proportion of phenotypic variation is due to coding versus regulatory variation? What properties are shared by genes that are targets for selection? We conclude with our perspective on these questions.

Evolutionary History of the Domestic Dog and Application to Gene Mapping

Current estimates suggest that dog domestication from the grey wolf occurred in East Asia between 15,000 and 40,000 years ago (Leonard *et al.*, 2005; see also Chapter 1), before the domestication of any other plant or animal species (Clutton-Brock, 1995). With the onset of agrarian societies, dog populations grew in number and quickly dispersed. The earliest dog breeds were probably established during this time to perform specialized tasks associated with agrarian life. Evidence for coat colour variation in these early breeds can be found in ancient Egyptian paintings dating as early as 2000 BC.

Today, as many as 1000 breeds have been described worldwide (Morris, 2001). Most of them were established in Eurasia within the past few hundred years from a well-mixed founding population. They were subsequently maintained as closed breeding lines under strong selection for desired traits, resulting in a radiation of morphological and behavioural diversity (Wayne and Ostrander, 2007). For example, the Newfoundland breed acquired a thick, water-resistant double coat and webbed feet adapted for aiding fishermen in the icy waters off the Newfoundland coast (Club, 2006). Dachshunds, which were used in Europe to hunt badgers in underground burrows, developed an elongated body, short legs and olfactory machinery honed for hunting (Club, 2006).

From a population genetics perspective, dog history is punctuated by an initial population bottleneck that occurred at least 15,000 years ago (at domestication) and a second series of bottlenecks that occurred in the past few hundred years (at breed formation). Because of these events, the dog is uniquely suited for genome-wide association mapping of genetic traits such as coat colour that segregate within breeds. The approach is conceptually similar to that being used to map human traits. Dogs are stratified into groups based on phenotype, and single nucleotide polymorphism (SNP) allele frequencies across the genome are compared. Genomic regions that harbour SNPs with large allele frequency differences are likely to signify the location of a causative mutation. When comparing dogs of different phenotypes within the same breed, the association of neighbouring SNPs extends over large genomic regions (with average haplotype blocks of 0.5 to 1 Mb)

Table 4.1. Mendelian coat colour loci in the domestic dog.

Locus	Gene	Allele[a]	Phenotype
Spotting			
S (*Spotting*)	*MITF*		
		S	Solid coat (no spotting)
		s^i	Irish spotting pattern
		s^p	Piebald spotting pattern
		s^w	Extreme white spotting
T (*Ticking*)	?		
		T	Ticking in white areas
		t	No ticking
R (*Roan*)	?		
		R	Mixture of white and coloured hairs in spots
		r	No pigmented hairs in white spots
Dilution			
B (*Brown*)	*TYRP1*		
		B	No dilution of eumelanin (black)
		b^s, b^d, b^c	Diluted eumelanin (liver, brown, chocolate)
C (*Tyrosinase*)	*TYR*		
		C	No dilution of pheomelanin (yellow, sable, fawn)
		c	Oculocutaneous albinism (in Pekingese)
D (*Dilute*)	*MLPH*		
		D	No dilution of eumelanin (black)
		d	Dilution of eumelanin (silver, blue)
G (*Progressive greying*)	?		
		G	Greying of eumelanin with age
		g	No greying
M (*Merle*)	*PMEL*		
		M	Merle pattern
		m	Non-merle
H (*Harlequin*)	*PSMB7*		
		H	Harlequin pattern (in a merle background)
		h	Merle pattern (in a merle background)
Tw (*Tweed*)	?		
		Tw^T	Large, smooth patches (in a merle background)
		tw^+	Small, jagged patched (in a merle background)
Pigment-type switching			
A (*Agouti*)	*ASIP*		
		a^y	Yellow, sable, fawn
		a^w	Agouti-banded hair, light-coloured ventrum
		a^t	Black and tan
		a	Recessive black
K (*K*)	*CBD103*		
		K^B	Black
		k^{br}	Brindle (black and yellow stripes)
		k	Wild type (allows expression of *Agouti* phenotypes)
E (*Extension*)	*MC1R*		
		E^m	Melanistic mask
		E	Extension, wild type
		e	Recessive yellow

[a]Alleles for each locus are listed in order of dominance.

Table 4.2. Types of mutations identified in dog coat colour genes.

Gene[a]	Allele	Mutation type	Expression	General function
Pleiotropic gene function				
MITF	S^i	Regulatory	Neural crest-derived cells, developing eye, osteoclasts	Eye/bone development, pigment cell development and function
MITF	S^p	Regulatory		
MITF	S^w	Regulatory		
MLPH	*d*	Regulatory	Expressed in multiple tissues	Membrane trafficking, secretory granule exocytosis
CBD103	*K*	Coding	Expressed in multiple tissues	Immune response, pigmentation
Pigment-cell specific gene function				
TYRP1	b^s	Coding	Pigment cells	Pigment synthesis
TYRP1	b^d	Coding		
TYRP1	b^c	Coding		
PMEL	*M*	Splicing	Pigment cells	Melanosome biogenesis, pigment synthesis (other?)
ASIP	a^y	Regulatory	Dermal papillae (hair follicle)	Pigment-type switching
ASIP	a^t	Regulatory		
ASIP	*a*	Coding		
MC1R	e	Coding	Pigment cells	Pigment-type switching

[a] *MITF, Microphthalmia-associated transcription factor; MLPH, Melanophilin; CBD103, Canine β-defensin 103; TYRP1, Tyrosinase-related protein 1; PMEL* (encodes a melanocyte-specific transmembrane glycoprotein); *ASIP, Agouti signalling protein; MC1R* (encodes a G-protein coupled receptor expressed on the surface of melanocytes).

because the haplotype blocks established at breed formation have not yet been broken down by recombination. In contrast, when comparing dogs of different phenotypes across breeds, haplotype blocks are much shorter (10 to 100 kb), reflecting a greater degree of recombination since the domestication bottleneck (Sutter *et al.*, 2004; Lindblad-Toh *et al.*, 2005).

Long haplotype blocks provide an advantage for genome-wide mapping because the power is higher: fewer genetic markers and fewer individuals are required to detect association for even subtle phenotypic differences. Short haplotype blocks offer higher mapping resolution. Thus, a two-step mapping approach can be employed to take advantage of both long and short haplotype structures. In the first stage, dogs of the same breed are used for genome-wide association mapping. In the second stage, dogs of different breeds are used, focusing on the genomic region identified during the first stage (Lindblad-Toh *et al.*, 2005; Karlsson *et al.*, 2007). Implementation of this strategy requires that the trait has a common genetic basis and segregates within at least two breeds. While breed standards are strict regarding morphology, they often allow for substantial variation in coat colour, making them ideal traits for genetic mapping.

In what follows, we summarize specific progress made in dog coat colour genetics, much of which involves application of the aforementioned approach. We consider three categories of coat colour variation – white-spotting, dilution and pigment-type switching – that reflect gene function in various discrete developmental and physiological aspects of the mammalian pigment cell, the melanocyte (Table 4.1).

Pigment Cell Development and Survival

During embryogenesis, melanocyte precursor cells, the melanoblasts, migrate from the neural crest to the epidermis and hair follicles in the skin (as well as to parts of the eye and inner ear). Once there, they proliferate and differentiate into pigment-producing melanocytes. A second type of pigment cell – that found in the retinal pigment epithelium (Sparrow *et al.*, 2010) – expresses many of the same melanogenic enzymes and proteins as the melanocyte, but arises directly from neurectoderm rather than from the neural crest, and has a very different shape and physiology from those of the melanocyte. White spotting on the coat usually indicates an absence of melanocytes caused by a failure of melanoblast migration, proliferation or differentiation during development. Mutations in genes that participate in one or more of these processes are responsible for coat patterns of white spots (Bennett and Lamoreux, 2003).

Little described two major loci that contribute to spotting in dogs – *Spotting* (*S*) and *Ticking* (*T*) (Little, 1957). In contrast, 25 white-spotting loci have been identified in mice and ten white-spotting genes have been cloned (Baxter *et al.*, 2004). In mice, white spotting is often associated with other congenital abnormalities including anaemia, megacolon and craniofacial malformation, indicating the importance of the neural crest in multiple developmental areas and/or a common set of signalling pathways used by multiple different cell lineages (Baxter *et al.*, 2004). In contrast, white spotting in dogs is occasionally associated with hearing deficiencies, but not with more severe problems (Strain, 2004). This difference apparently reflects the different motivations underlying trait selection: mutations in dogs are likely to have been selected and maintained by breeders only if they have little or no effect on overall health and fitness. In contrast, laboratory mice are a well-established and rich resource for collecting and studying mutations that affect a variety of disease processes.

The *Spotting* locus

Little predicted the canine *S* locus to have four alleles – *Solid* (*S*), *Irish spotting* (s^i), *Piebald spotting* (s^p) and *Extreme white spotting* (s^w) – which differ based on the degree of pigmented body surface. Alleles resulting in a more completely pigmented body surface are dominant to those with less pigmentation (Little, 1957). In some breeds, s^w/s^w animals are completely or almost completely white, e.g. the Boxer, Bull Terrier or Greyhound, while in others, s^w/s^w animals may have residual pigmentation that overlaps with the piebald spotting phenotype, e.g. the Italian Greyhound or Great Pyrenees. The substantial degree of phenotypic heterogeneity apparent among breeds fixed for a specific *Spotting* allele indicates that modifier loci contribute significantly to the phenotype.

The Irish pattern describes white markings on the face, legs and ventrum, which often extend to form a white collar around the neck. The name of the pattern was adopted from early hereditary studies of a similar pattern characterized in Irish rats by the English geneticist Leonard Doncaster (1906). In dogs, the Irish pattern can result either from homozygosity of the s^i allele or from incomplete dominance of the *S* over more severe *Spotting* alleles (S/s^p and S/s^w). This difference in the genetic basis has a practical implication: the desired Irish pattern is fixed in certain breeds, such as the Basenji (s^i/s^i), but is maintained by balancing selection (and therefore not fixed) in others, such as the Boxer (S/s^w) (Barsh, 2007; Schmutz *et al.*, 2009). Plate 1 depicts Italian Greyhound siblings, one that exhibits the Irish pattern, and one that exhibits extreme white spotting with residual pigmentation.

Microphthalmia-associated transcription factor (*MITF*) was identified as the gene responsible for white spotting in dogs using the genome-wide association mapping strategy described above (Karlsson *et al.*, 2007). The power of association-based mapping in the context of dog genetics is apparent from the strategy, which required only 19 animals – nine solid coloured (*S/S*) and ten white (s^w/s^w) – to map the spotting phenotype to a single gene.

MITF is a basic helix-loop-helix transcription factor involved in the development of several cell types, including mast cells, osteoclasts, the retinal pigment epithelium and melanocytes. In melanocytes and the retinal pigment epithelium, MITF activates the expression of many melanogenic enzymes and proteins, and has been referred to as a 'master regulator' of pigmentation (Goding, 2000; Levy *et al.*, 2006; Arnheiter, 2010). In laboratory mice, alleles that disrupt the MITF protein usually affect both melanocytes and the retinal pigment epithelium; the latter cell type is important for proper eye development, which is why many mouse alleles cause microphthalmia in addition to white spotting. Because *MITF* is expressed in many different cell types, it makes use of several alternative promoters and transcriptional initiation sites (Bismuth *et al.*, 2005; Bharti *et al.*, 2008). Work from Karlsson *et al.* (2007) demonstrated that genetic markers near the melanocyte-specific *MITF-M* promoter demonstrate the strongest association with spotting. Sequencing a 102 kb candidate region from *S* and s^w chromosomes did not uncover coding differences between the alleles (further implying a *MITF-M* regulatory mutation), but revealed 46 distinct molecular alterations, including a short interspersed nuclear element (SINE) element insertion ~3 kb upstream of the *MITF-M* promoter in s^w but not in *S* chromosomes, and a polymorphic homopolymer tract ~100 bp upstream of the *MITF-M* promoter that is longer in s^w- than in *S*-bearing chromosomes.

The situation with other *Spotting* alleles is confusing: breeds fixed for piebald spotting (s^p/s^p) carry the s^w-associated SINE element insertion, whereas breeds fixed for Irish spotting (s^i/s^i) do not. However, all three alleles associated with spotting (s^i, s^p and s^w) carry a homopolymer tract that is longer than the version found in solid (*S/S*) dogs. At present, the data suggest that a series of regulatory mutations occurred sequentially on a single *MITF* haplotype to generate increasingly severe spotting phenotypes. Comparative sequence analysis of *Spotting* alleles should help to unravel potential relationships and pinpoint causative variants.

The *Ticking* and *Roan* loci

Ticking (*T*) and *Roan* (*R*) are dominant modifiers of white spotting. Ticking causes 'ticks' of pigmented hair to emerge in regions that would otherwise be white, while roan causes a uniform mixture of white and pigmented hairs in white regions. Ticking and roan may appear anywhere but are most frequently observed on the muzzle and forelimbs, and are found in Hounds, Pointers, Spaniels, Setters and Dalmatians. (The terminology is confusing, because, in other animals, ticking is also used to describe bands or 'ticks' of pheomelanic pigment on individual hairs, better known as the *Agouti* phenotype.) Although ticking and roan are distinct phenotypes, it is unclear whether they are allelic; they are presented in Table 4.1 as distinct loci, each with two alleles: $T > t$ and $R > r$, respectively.

Ticking and roan do not manifest until 3 to 4 weeks of age (Little, 1957), and may represent a second wave of melanocyte precursor differentiation, proliferation or colonization of hair follicles. The underlying genetics of ticking is especially interesting because corresponding traits have not been recognized in laboratory mice. Little (1957) suggested that the distinctive spotting pattern of Dalmatians represents homozygosity for *T* and s^w. However, several breeds, including Cocker Spaniels, segregate the ticking phenotype (Club, 2006), providing the opportunity to map the ticking locus using genome-wide association.

Generalized Pigment Dilution

Melanocytes produce two types of pigments – black eumelanin and red pheomelanin – in specialized organelles called melanosomes. Mature melanosomes are then transferred to surrounding keratinocyte cells populating the hair and skin. Pigment dilution reflects decreased pigment production or transfer, and results from mutations in genes that function in melanosome biogenesis, pigment synthesis or melanosome transport. Eumelanin and pheomelanin differ according to amino acid content (pheomelanin is cysteine rich, eumelanin is not), solubility (eumelanin is more highly polymerized and therefore more insoluble than pheomelanin)

and structure (eumelanin exists in highly structured ovoid granules, whereas pheomelanin exists in granules that are less structured and more spherical). Many components of the pathways for eumelanin and pheomelanin synthesis are different, and so dilution phenotypes often affect either eumelanin or pheomelanin specifically (Fig. 4.1) (Searle, 1968; Hearing, 1999).

As described initially by Little (1957), five loci modify the intensity of coat colour in the domestic dog: *Brown* (*B*), *Chinchilla* (*C*), *Dilute* (*D*), *Progressive greying* (*G*) and *Merle* (*M*). In the subsections that follow, we discuss the phenotypes associated with these loci and their molecular characterization.

The *Tyrosinase* (*Chinchilla*) locus

Historical studies in laboratory mice identified the most common cause of oculocutaneous albinism as an allele of the *Chinchilla* (hence *C*) locus, later identified as *Tyrosinase* (*Tyr*). Tyrosinase is

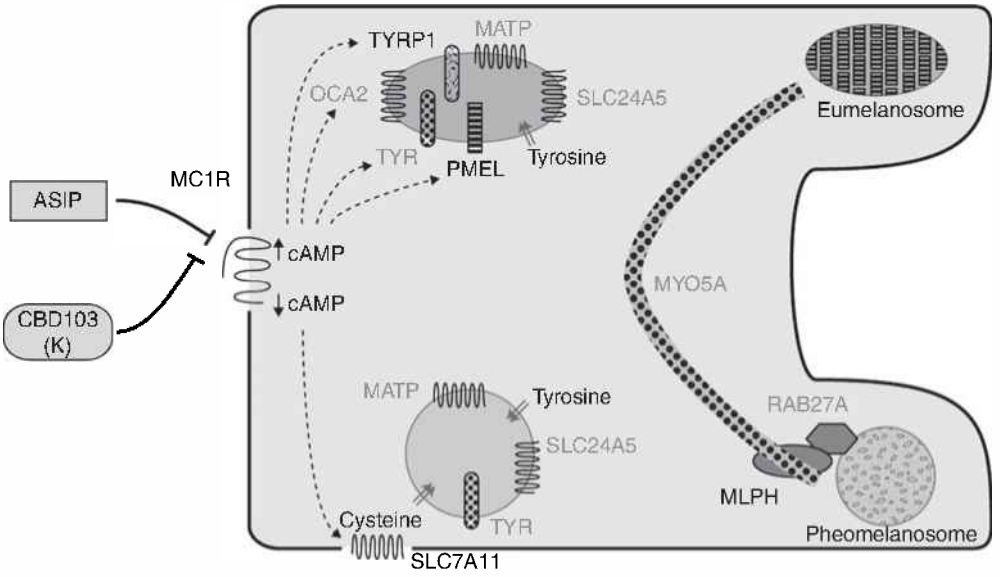

Fig. 4.1. The role of canine coat colour genes in melanocyte cell biology. The diagram shows a melanocyte, with eumelanogenesis and pheomelanogenesis depicted in the upper and lower sections, respectively. Protein names correspond to the genes discussed in the text or here. Mutations of the genes for each gene product shown here give rise to a pigmentary phenotype; those with allelic variation in dogs are shown in black, and those that have been implicated in other systems or organisms are shown in grey. The type of pigment synthesized by melanocytes is controlled by MC1R (a G-protein coupled receptor expressed on the surface of melanocytes) and its second messenger cAMP. High levels of basal MC1R signalling cause increased expression of TYR (tyrosinase), TYRP1 (tyrosinase-related protein 1), OCA2 (a membrane protein implicated in oculocutaneous albinism) and PMEL (a melanocyte-specific transmembrane glycoprotein), leading to increased eumelanin synthesis. Low levels of cAMP cause increased expression of the cysteine transporter SLC7A11, leading to increased pheomelanin synthesis. CBD103 (an MC1R ligand, encoded by the *K* locus) prevents ASIP (Agouti signalling protein) from inhibiting MC1R, thereby promoting eumelanin synthesis. The illustration is drawn to emphasize the differences between eumelanin and pheomelanin synthesis in the melanosomes; in reality, biogenesis of the different organelles involved is more complex and involves a common precursor organelle and several distinct protein trafficking steps. As melanosomes mature, they are transported to dendritic tips via a process that depends on the unconventional myosin (MYO5A), a GTP-binding protein (RAB27A), and an adapter protein (MLPH). MATP is a membrane-associated transporter protein; SLC24A5 is solute carrier family 24 member 5 (also known as sodium/potassium/calcium exchanger 5, NCKX5).

a transmembrane melanosomal protein whose intramelanosomal domain catalyses the initial and rate-limiting step of both eumelanin and pheomelanin synthesis. In mice, *Tyr* alleles, including *Albino* (*c*) and *Chinchilla* (c^{ch}), give rise to characteristic phenotypes associated with either complete albinism or the preferential dilution of pheomelanin, respectively. Tyrosinase activity is normally downregulated during pheomelanogenesis, so partial loss of function mutations, like c^{ch}, provide enough activity for eumelanin but not for pheomelanin synthesis (Ollmann *et al.*, 1998). Similar allelic series have also been described for several other species (Searle, 1968).

In dogs, oculocutaneous albinism occurs rarely in some breeds, such as the Pekingese, and is assumed to be due to a *TYR* loss-of-function allele (*c*) (Whitney, 1979). More common forms of white coat colour in dogs occur by mechanisms other than *TYR* inactivation. One common form of white is observed in spotted breeds, like the Borzoi, due to extreme white spotting (s^w/s^w) on a pale background (either a^y/a^y – the *Agouti* locus; or *e/e* – the *Extension* locus). These dogs have dark eyes with typically small amounts of residual pigmentation on the coat and skin, and produce spotted pups when mated to a solid-coloured dog. Some dark-eyed white dogs, however, are found in predominantly solid and dark coloured breeds, such as the German Shepherd, suggesting a third mechanism for producing a white coat that segregates as a monogenic trait with a recessive inheritance pattern (Carver, 1984).

Little speculated that a *Tyrosinase* allele, equivalent to c^{ch} in other animals, was responsible for variation in pheomelanin intensity. However, Schmutz and Berryere (2007) observed that *TYR* markers did not segregate with pheomelanin dilution in either Golden Retriever or Labrador Retriever pedigrees. Another candidate that has not yet been investigated is *SLC7A11*, which encodes a cysteine transporter that underlies pheomelanin dilution (the *subtle grey* mutation) in mice (Chintala *et al.*, 2005). Identification of additional components of the pheomelanin synthesis pathway is an ongoing area of investigation in pigmentation biology, and dog genetics is ideally suited to make a substantial contribution.

The *Brown* locus

In several mammals, recessive alleles of *Tyrosinase-related protein 1* (*TYRP1*) cause the dilution of black pigment to brown but do not affect the intensity of red or yellow pigment (Zdarsky *et al.*, 1990; Berryere *et al.*, 2003; Lyons *et al.*, 2005; Schmidt-Kuntzel *et al.*, 2005). *TYRP1* encodes an intramelanosomal enzyme that catalyses the oxidation of intermediates in eumelanin synthesis (Sarangarajan and Boissy, 2001). Using a candidate gene approach, Schmutz *et al.* (2002) identified three *TYRP1* coding mutations, each on a different haplotype, responsible for an indistinguishable brown coat colour in different allelic combinations. A survey of 28 breeds found the *TYRP1* alleles to be widespread, with all three alleles present in several breeds (Schmutz *et al.*, 2002). The three alleles of the *Brown* (*B*) locus are designated b^s, b^d and b^c ($B > b$) (see Table 4.1).

The domestic cat also has multiple *TYRP1* alleles that dilute black pigment, but each allele has a distinct coat colour phenotype (Lyons *et al.*, 2005; Schmidt-Kuntzel *et al.*, 2005). The identification of three molecularly distinct but functionally equivalent alleles in dogs is somewhat surprising since selection for a specific phenotype is expected to sweep a single allele to high frequency. A potential explanation is that selection for brown coat colour occurred in multiple isolated populations, each of which contributed to the formation of modern breeds.

The Dilution locus

A third colour dilution locus, *D*, has a recessive allele (*d*) that dilutes both eumelanin and pheomelanin to a metallic blue or silver (as in the Italian Greyhounds shown in Plate 1). Similar phenotypes in mice are due to disruption of the melanosome transport machinery and characterized at the cellular level by perinuclear melanosome clumping in melanocytes and abnormal melanosome distribution in the hair shaft (Searle, 1968; Silvers, 1979). Using a candidate gene approach, Philipp *et al.* (2005) revealed an association with a single haplotype

near the gene for the carrier protein melanophilin (*MLPH*) in multiple dog breeds, indicating a single causative allele of common origin. The *MLPH* coding sequence in *d/d* dogs is normal, however, which suggests an underlying regulatory mutation (Philipp *et al.*, 2005; Drögemuller *et al.*, 2007a). *MLPH* encodes a member of the exophilin subfamily of Rab effector proteins, which forms a ternary complex with a Ras-related GTPase, RAB27A, and a myosin motor protein, MYO5A, involved in the transport of melanosomes along the actin cytoskeleton (Barral and Seabra, 2004). Similar dilute phenotypes caused by *MLPH* mutations have been identified in several species, including mice, cats, chickens and humans (Matesic *et al.*, 2001; Ménasché *et al.*, 2003; Ishida *et al.*, 2006; Vaez *et al.*, 2008).

Merle and associated loci

Merle, which is referred to as dapple in some breeds, is a pattern of irregularly shaped areas of diluted pigmentation (Plate 2). The mutation (*M*) is semidominant; animals homozygous for the presumptive ancestral or wild type allele, *m*, are normally pigmented, *M/m* animals have mild-to-moderate dilution of eumelanic areas, and *M/M* animals are mainly white. Characteristically, small patches of normal colour appear within areas of diluted pigmentation in both *M/M* and *M/m* dogs. In addition, *M/M* animals occasionally exhibit deafness and ocular problems (microphthalmia, abnormal irises and/or blindness) (Sorsby and Davey, 1954; Reetz *et al.*, 1977); consequently, most guidelines recommend against interbreeding *M/M* ('double merle') dogs, and the phenotype is not fixed in any breed.

Using genome-wide association mapping of a Shetland sheepdog cohort and subsequent screening of candidates, Clark *et al.* (2006) discovered that *Silver* (*SILV*), first identified in the laboratory mouse and now referred to as *PMEL*, is likely to be responsible for *Merle*. *PMEL* encodes a melanocyte-specific transmembrane glycoprotein whose intramelanosomal domain is cleaved and localizes to the matrix of eumelanosomes, where it forms fibrillar amyloid structures that serve as substrates for the precipitation and deposition of melanin (Kobayashi *et al.*, 1994; Lee *et al.*, 1996). Mutations of *PMEL* cause pigmentation phenotypes in other species, including silvering in mice (Kwon *et al.*, 1995; Martinez-Esparza *et al.*, 1999) and horses (Brunberg *et al.*, 2006), and a series of plumage phenotypes in chickens (Kerje *et al.*, 2004). Because PMEL is localized primarily to eumelanosomes, *Merle* typically spares pheomelanin-coloured areas, as is evident in the black-and-tan, dapple Dachshund (Plate 2).

One of the most striking aspects of *Merle* is that it is genetically unstable. Matings of mutant *M/M* to 'wild-type' *m/m* dogs produce true-breeding non-merle (*m/m*) offspring at a rate of 3–4% (Sponenberg, 1984), a hallmark of so-called germline reversion, or a 'reverse mutation' of *M* to *m* (discussed further below). This observation suggests that the molecular alteration responsible for the *M* mutation is itself unstable, and can revert both in germ cells (as above) and in somatic cells, giving rise to the normal patches of colour within areas of diluted pigmentation (Plate 2, Fig. 4.2). Clark *et al.* (2006) identified, in all dogs carrying *M*, a small insertion in the *PMEL* gene for which an internal A_n (adenosine homopolymer) tract exhibited a shortened length in putative *M* to *m* revertants. The insertion itself is a SINE mobile genetic element, but Clark *et al.* (2006, 2008) suggest that reversion is not due to excision of the SINE (which happens rarely, if at all), and instead is due to shortening of the A_n tract due to errors in DNA replication during cell division. According to this suggestion, there are three groups of alleles, the ancestral allele that lacks the SINE insertion (referred to as *m*), the derivative allele with the SINE insertion that disrupts *PMEL* function (referred to as *M*) for which the A_n tract is 91–101 nt (nucleotides) long, and a revertant allele (referred to here as m^*) which carries the SINE insertion with a shorter A_n tract of 54–65 nt (Clark *et al.*, 2008). An important implication of this idea is that both the *M* and the m^* alleles would exhibit instability, the former for tract shortening to a 'normal' phenotype, and the latter for tract expansion to an abnormal phenotype, in which case the phenomenon of merle reversion might more accurately be

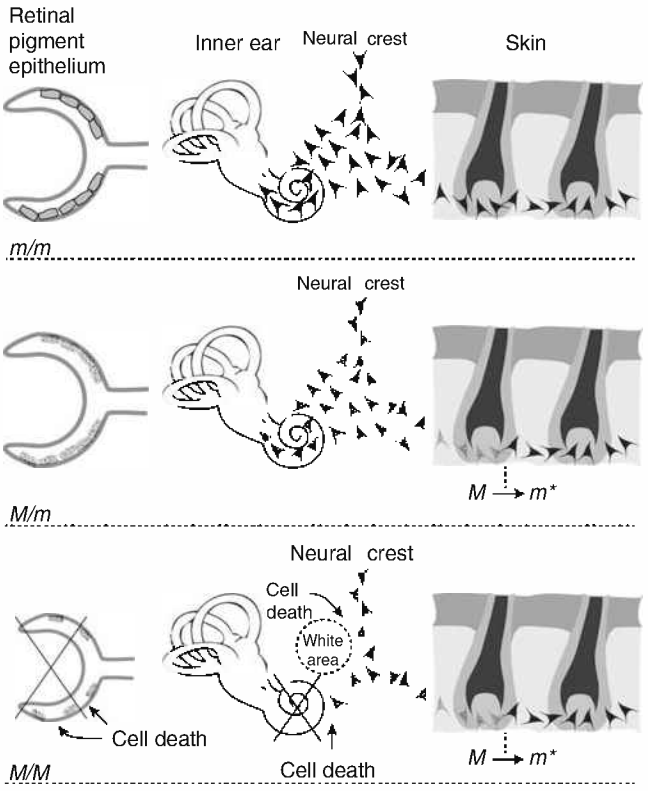

Fig. 4.2. Proposed cellular and developmental basis for *Merle*-associated (or dapple) phenotypes. The role of pigment cells in eye, ear and skin development is described in the text. In dogs heterozygous for *Merle* (*M/m*), a defective PMEL protein compromises eumelanosome formation, leading to a generalized pigmentary dilution. In dogs homozygous for *Merle* (*M/M*), increased levels of defective PMEL protein (a melanocyte-specific transmembrane glycoprotein) cause pigment cell death, which itself leads to abnormal retinal development, deafness and large white areas on the coat. As described in the text, the molecular nature of the *M* mutation is unstable, which facilitates frequent conversion to what we refer to as a pseudorevertant *m** allele. Pseudoreversion is shown here at the late stages of melanocyte development, but may also occur much earlier, giving rise to large patches of normal colour within a diluted area.

referred to as pseudoreversion. Indeed, the idea of an unstable *m** allele is likely to underlie what has been described as a cryptic or phantom merle, in which a dog with little or no pigmentary abnormalities produces typical merle offspring.

The previous discussion also provides a hypothesis for considering *Merle*-associated phenotypes from a cellular and developmental perspective (Fig. 4.2). Microphthalmia and deafness are a hallmark for death of retinal pigment cells and melanocytes in the inner ear, but pigmentary dilution is most likely caused by a structurally abnormal PMEL protein that interferes with eumelanosome formation. Death of skin melanocytes probably accounts for white areas of the coat in *M/M* animals, but is unlikely to be linked to melanogenesis itself, because the latter phenomenon yields a characteristic hair phenotype with loss of pigment at the base of the hairs. Finally, the location and size of normal colour patches that occur within diluted areas of Merle dogs probably reflects the time during melanocyte development when reversion occurs, with large and small patches signifying early and late events,

respectively. In both cases, the shape of normal colour patches should reflect the developmental history of a melanocyte clone, and is consistent with recent studies in laboratory mice (Wilkie *et al.*, 2002).

Harlequin (*H*) and *Tweed* (Tw^T) are both dominant modifiers of the merle pattern that have no apparent effect on coat colour in non-merle backgrounds. Dilute regions of merle pattern are, instead, white in a harlequin background (*H/h;M/m*) and contrast sharply with the region of full pigmentation. *H/H* genotypes cause lethality during embryogenesis (Sponenberg, 1985); Clark *et al.* (2011) showed that the phenotype is associated with a coding variant in the *20S proteasome 2 subunit* (*PSMB7*). In tweed dogs, dilute regions of the merle pattern are larger, on average, with varying shades of pigment intensity and smooth boundaries (Sponenberg and Lamoreux, 1985); there is, at present, no information on the molecular basis of *Tweed*.

The *Progressive greying* locus

A final dilution phenotype that segregates in dogs is progressive greying (the *G* locus). Common in poodles and some terrier breeds, it is dominantly inherited and causes a progressive dilution of eumelanin from black to grey (Little, 1957). Greying in dogs is similar to the progressive silvering that occurs in mice (Dunn and Thigpen, 1930) and horses (Bowling, 2000). In both cases, mutations of *PMEL* are responsible (Kwon *et al.*, 1995; Martinez-Esparza *et al.*, 1999), implicating the canine *PMEL* gene as a candidate for *G* as well as for *Merle*.

Pigment-type Switching

The synthesis of either eumelanin or pheomelanin is regulated in a time and location specific manner by an intercellular signalling pathway within the hair follicle (Silvers, 1979). Components of this pathway are encoded by the pigment-type switching gene *MC1R* and the gene for Agouti signalling protein (*ASIP*). MC1R is a G-protein coupled receptor expressed on the surface of melanocytes and its signalling activity promotes eumelanin synthesis. MC1R is primarily regulated by ASIP, a secreted ligand that antagonizes MC1R signalling and promotes pheomelanin synthesis (Fig. 4.1).

Mutations in *MC1R* or *ASIP* commonly result in phenotypes that alter the timing and/or distribution of eumelanin and pheomelanin. *MC1R* gain-of-function mutations or *ASIP* loss-of-function mutations cause exclusive production of eumelanin. Conversely, *MC1R* loss-of-function mutations or *ASIP* gain-of-function mutations cause exclusive production of pheomelanin. As a consequence, *ASIP* has a characteristic allelic hierarchy, with dominant yellow and recessive black alleles. *MC1R* has a reverse hierarchy, with dominant black and recessive yellow alleles (Searle, 1968). *MC1R* alleles are epistatic to *ASIP* alleles.

In most dog breeds (for example, the Labrador retriever in Plate 3), black coat colour is inherited as a dominant trait, consistent with a gain-of-function *MC1R* allele. However, based on segregation studies, Little (1957) postulated that dominant black coat colour was instead due to an unusual allele of *ASIP* (A^s). His observation implied that the genetics of pigment-type switching in dogs is distinct from that operating in other mammals.

The *K* locus

Enabled by genome-wide molecular markers, pedigree and linkage analysis revealed that dominant black and another unusual dog coat colour phenotype, brindle, were not alleles of either *ASIP* or *MC1R*, but instead map to a novel pigment type switching locus (*K*, so named from blacK) with three alleles – *Dominant black* (K^B), *Brindle* (k^{br}) and the ancestral allele (k^y) – listed here in order of dominance (Kerns *et al.*, 2003, 2007). A combination of pedigree- and population-based mapping approaches identified the K^B mutation as a 3bp deletion in *β-defensin 103* (*CBD103*) that predicts an in-frame glycine deletion (Candille *et al.*, 2007). Several lines of evidence suggest that CBD103 (canine β-defensin) is an MC1R ligand that promotes

eumelanin synthesis by inhibiting ASIP antagonism of MC1R (Candille *et al.*, 2007).

β-Defensins comprise a diverse and rapidly evolving family of secreted peptides (Hughes, 1999; Semple *et al.*, 2006) with a role as endogenous antibiotic agents that participate in both acquired and innate immune responses (Yang *et al.*, 1999; Biragyn *et al.*, 2002; Ganz, 2003; Soruri *et al.*, 2007). The link between *CBD103* and the melanocortin system indicates that β-defensins do more than just defend, and points to a potentially intriguing connection between pigmentation and immunity. Nevertheless, a similar pigmentary function for β-defensins in other species has yet to be uncovered.

Brindle (k^{br}), which describes the irregular pattern of black stripes on a fawn or yellow background, is common in many breeds. Little (1957) initially assigned k^{br} to the *Extension* (*E*) locus, but mapping data now verify that it is an allele of the *K* locus (Kerns *et al.*, 2003, 2007). The brindle phenotype is only apparent on pheomelanic areas of the coat and its expression requires a functional MC1R (*K* alleles are hypostatic to *E* alleles) (Kerns *et al.*, 2007). The extent of brindle striping varies considerably. Some dogs are yellow with only a few black stripes, while others are so heavily striped that they appear black. It remains unclear whether variation in the extent of brindle striping is due to stochastic or genetic mechanisms (or a combination of both).

The *Agouti* locus

The temporal and spatial regulation of *ASIP* expression has been well characterized and provides the basis for understanding common pigmentation patterns in dogs and other mammals. *ASIP* uses two alternative promoters (Vrieling *et al.*, 1994). One promoter, active on the ventral body surface, produces a light (yellow) ventrum. The second promoter is active only at a specific time during the hair cycle and produces banded hair. Variation in the activity of each promoter gives rise to a diversity of common mammalian coat patterns, in which the ventral surface is lighter than the dorsal surface, and which may contribute to countershading (Thayer, 1909; Kiltie, 1988). In dogs, four alleles of the *Agouti* series recognized by Little (1957) ($a^y > a^w > a^t > a$) reflect these aspects of *ASIP* regulation. As discussed at the beginning of this section, Little also proposed that a fifth *Agouti* allele (A^S) was responsible for dominantly inherited black coat colour, which is now known to be caused by an allele of the *K* locus (K^B).

Dogs carrying an a^y allele are uniformly yellow (commonly referred to as fawn, tan or sable), though hairs often have black tips that give a sandy appearance. The a^y allele is associated with two *ASIP* coding variants in 22 breeds, suggesting a single common origin for the allele in dogs (Berryere *et al.*, 2005). Most likely, the coding variants are not responsible for the phenotype but, rather, are in linkage disequilibrium with a regulatory mutation that causes *ASIP* expression to persist throughout most of the hair cycle. Specifically, a mutation in the hair cycle promoter could expand the timing of *ASIP* expression relative to that observed in most other animals with banded hairs. The a^y allele in dogs differs from dominant *Asip* alleles in mice, in which unusual gain-of-function regulatory alterations cause widespread *Asip* expression and non-pigmentary effects, including obesity, diabetes and increased body length (Silvers, 1979).

The a^t allele in dogs gives rise to a characteristic phenotype with a black dorsum and yellow (or tan) markings on the head, ventrum and/or legs (Plate 2). By analogy to laboratory mice, the a^t mutation is probably caused by molecular alterations that reduce or eliminate activity of the hair cycle promoter. The distribution of pheomelanin varies considerably among different breeds. Some display a minimal pattern of tan 'points' on the ventral surface. In others, such as the Airedale Terrier, the pheomelanic region extends dorsally to cover a considerable portion of the coat, limiting the expression of eumelanin to a saddle-shaped region on the back and sides (Little, 1957). Some have proposed that the 'saddle' pattern is due to modifiers of the a^t pattern, while others consider it an independent *Agouti* allele (a^s) (Little, 1957; Burns and Fraser, 1966; Willis, 1976).

The a^w allele, the presumed ancestral *ASIP* allele that produces banded hair similar

to the wolf, is present in breeds such as the German Shepherd and the Schnauzer (Little, 1957). Recessive inheritance of the *a* allele, identified as a coding variant predicted to inactivate the protein, is responsible for black coat colour in some breeds, including the German Shepherd and Australian Shepherd (Kerns *et al.*, 2004).

The *Extension* locus

The *Extension* (*E*) locus has three known alleles in dogs: $E^m > E > e$. Little (1957) also assigned a fourth allele, E^{br}, which he postulated was responsible for the brindle phenotype, but which is now recognized as an allele of the *K* locus (K^{br}) (Kerns *et al.*, 2003, 2007). The wild-type allele, *E*, encodes a functional MC1R that allows for expression of the *Agouti* and *K* locus alleles.

The dominant E^m allele is responsible for the localized distribution of eumelanin on the muzzle that resembles a darkened mask in a pheomelanic (a^y) background. The phenotype was perfectly associated with an *MC1R* coding alteration (M264V) in a survey of 12 breeds (Schmutz *et al.*, 2003), confirming it as an *MC1R* allele. The mutation occurs at a junction between a transmembrane domain and an extracellular loop that could affect ligand affinity, signalling, or stability of the receptor. Alternatively, it is possible that the coding variant is in linkage disequilibrium with a regulatory mutation that alters *MC1R* expression levels. In either case, E^m is likely to affect MC1R signalling levels rather than its regional distribution, implying that specific areas of the body have different thresholds for pigment-type switching that are revealed by perturbations in MC1R signalling efficiency (Schmutz *et al.*, 2003).

In most breeds, a uniform yellow coat colour is due to the dominant *Agouti* allele, a^y. In the Labrador Retriever, the Golden Retriever and the Irish Setter, however, uniformly yellow or red coat colour is due to recessive inheritance of the *e* allele. The molecular basis of *e* was identified as a nonsense MC1R mutation (R306ter) that truncates the final 11 amino acids of the receptor. The R306ter variant is found on two different haplotypes in the dog, implicating each as an independently occurring mutation (Newton *et al.*, 2001).

A glaring omission from the *Extension* series in dogs is a dominant black allele. Given the representation of equivalent alleles in other species and the diversity of coat colour phenotypes in dogs, it is perhaps surprising that such an allele has not been identified in the dog, but the prevalence of the K^B allele in modern dog breeds make it unlikely that a second dominant black allele would be noticed (Kerns *et al.*, 2007).

Genetics of Hair Structure in the Domestic Dog

The appearance of the canine coat is determined not only by the colour and distribution of pigment, but also by the characteristics of hair structure – its length, its texture, and regional distribution – dogs may be long haired, hairless, wire haired, curly haired, ridged or ripple coated. While colour traits often segregate within breeds, hair structure traits are more likely to be fixed. One reason for this general observation is that selection for coat structure during breed formation was in many instances a consequence of function, not form. For example, the occasional longhaired Pembroke Welsh Corgi was not desirable, because short hair, in combination with the Corgi's short stature, was most compatible with herding work. Similarly, the water-resistant, thick and curly coat of the Irish Water Spaniel was a necessary provision for retrieving game in cold water. Today, the historical motives for particular coat structures are maintained by breed standards.

The lack of hair structure variation within breeds is likely to have contributed to the paucity of classical genetic studies relative to pigmentary traits. Colour traits segregating within breeds were ripe for genetic analysis, but fixed traits were not. The early breeder and geneticist, Leon Whitney (1979), made several insightful observations about hair structure genetics from interbreed crosses, but these were necessarily limited in scope. In addition, comparative genetic studies across domestic animals have not been

done for hair structure traits to the same extent as for coat colour traits. Consequently, genetic comparisons between dogs and mice are not as straightforward. Even in mice, where anatomical and genetic studies of abnormal hair have led to substantial progress in our understanding of hair development, an integrated molecular view is incomplete, owing in part to the complexity of hair formation (Schlake, 2007).

Despite these limitations, the genes responsible for five canine hair structure traits are now known (Fig. 4.3, Table 4.3). Three of these traits – coat length, furnishings and curl – are common among many breeds. The other two – hairlessness and dorsal ridge formation – are restricted to only a few. The following section will focus on the genetic and molecular characterization of these loci, touching only briefly on aspects of hair structure where the basis and extent of genetic components remain unknown. For example, characteristics such as hair density and the presence of an undercoat are likely to have both heritable and environmental determinants (Whitney, 1979), but are not discussed here. Nor do we consider follicular dysplasias, even though the distinction between normal variation and 'disease', i.e. follicular dysplasias, is sometimes more quantitative than qualitative.

Hair Follicle Development and Biology

Variation in hair structure can represent alterations in the molecular building blocks or the underlying developmental and regenerative mechanisms of the hair follicle, and we first provide a brief review of these processes. Hair is a complex and impermanent epithelial appendage that is continually regenerated over the lifespan of an organism. The process of regeneration is commonly referred to as the hair cycle and consists of distinct stages of hair growth (anagen), regression (catagen) and rest (telogen) (Fig. 4.3) (Fuchs *et al.*, 2001). Hair cycle periodicity is influenced by genetic and non-genetic factors, including seasonal cues, breed and location on the body. The estimated hair cycle for dorsal hair on the Labrador Retriever, for example, is ~1.5 years (Diaz *et al.*, 2004), but similar estimates for other breeds have not been reported. In comparison, mouse hair cycles every ~20 days, whereas the hair on the human scalp may take 6 years to complete a single cycle. Hair length is determined by both the growth rate and the length of the growth period, and therefore is potentially affected by processes controlling the transition between hair cycle stages (Schlake, 2007).

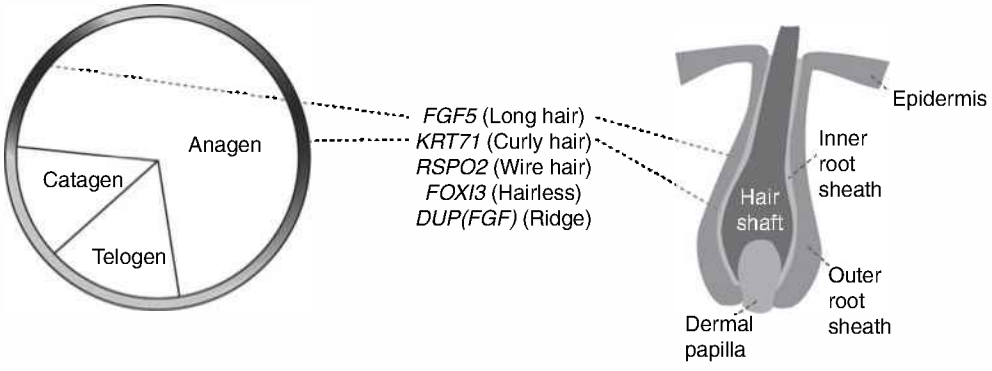

Fig. 4.3. The role of hair structure loci in dogs. *FGF5* (*Fibroblast growth factor 5*) and *KRT71* (*Keratin 71*) demonstrate restricted spatio-temporal expression during the hair growth cycle. The expression of genes affecting hair structure – *RSPO2* (*R-spondin 2*), *FOXI3* (*Forkhead Homebox Domain I3*) and the *FGF* (*Fibroblast growth factor*) mutations (*DUP(FGF)*) involved in the *Ridge* duplication – has not been reported. Hair regeneration consists of distinct stages – anagen (hair growth), catagen (hair regression) and telogen (resting phase) – collectively termed the hair cycle.

Table 4.3. Loci and alleles affecting dog hair texture.

Locus	Gene	Allele[a]	Phenotype	Breed example
L (Long hair)	*FGF5*			
		L	Shorthaired	Labrador Retriever
		l	Longhaired[b]	Golden Retriever
Hr (Hairless)	*FOXI3*			
		Hr^{hr}	Hairless[b]	Chinese Crested Dog
		hr+	Coated	
Ha (American hairless)	?			
		Ha^{+}	Coated	
		Ha^{ha}	Hairless[b]	Hairless Terrier
Wh (Wire hair)	*RSPO2*			
		Wh^{w}	Wire hair/ Furnishings[b]	Border Terrier
		wh^{+}	Smooth coat	
Cu (Curly hair)	*KRT71*			
		Cu^{c}	Curly coat[b]	Poodle
		Cu^{+}	Straight coat	German Shepherd
R (Ridge)	Duplication			
		R	Ridged[b]	Rhodesian Ridgeback
		r	Ridgeless	
Rp (Ripple coat)	?			
		Rp^{+}	Smooth coat	Weimaraner
		Rp^{r}	Ripple coat	

[a]Alleles for each locus are listed in order of dominance.
[b]Denotes the derived allele at this locus.

The hair follicle is formed during skin development by interactions between skin epithelial cells and underlying dermal cells, which transform both cell types into specialized tissues. The dermal cells condense to form a structure called the dermal papilla, which interacts with overlying epithelial cells and will also form a permanent portion of the mature hair follicle. As the follicle develops late in gestation, invagination of epithelial cells overlying the dermal papilla is accompanied by differentiation of what used to be a simple epithelial monolayer into specialized compartments of the hair. The internal compartments, the medulla and the cortex, form the hair shaft that will eventually protrude from the surface of the skin. The external compartments form layers of inner and outer root sheaths that support the growing hair shaft (Fig. 4.3). As compartments of the hair follicle differentiate, they produce specialized keratins and additional fibrous proteins that polymerize into filaments and provide integrity for the hair shaft and the root sheaths. Hair shape and texture are affected by the composition, density and distribution of these structural components in the hair follicle (Fuchs *et al.*, 2001).

The *Long hair* locus

Hair length in dogs is generally classified as either long (the Golden Retriever) or short (the Labrador Retriever), and is under the control of two alleles: $L > l$. However, within either class, there is a substantial variation both within and across breeds (Whitney, 1979). The pattern of variation suggests a major genetic determinant for hair length that can be modified by additional genetic or environmental factors. Before recent molecular genetic studies, there was a sparse but convincing literature describing coat length as a Mendelian trait, with long hair recessive to short hair. This is certainly the case in specific breeds that segregate both short and long hair varieties, such as the Saint Bernard (Plate 4) (Crawford and Loomis, 1978). The occasional occurrence of long hair variants in typically short hair breeds, like the German

Shepherd, is also consistent with low frequency segregation of a recessive long hair allele (*l*) (Housley and Venta, 2006). Also, Whitney recorded that interbred crosses between 16 different combinations of long and short hair breeds produced only short hair offspring, and that subsequent intercross or backcross matings produced ratios of short and long hair offspring indicative of recessive inheritance of long hair in all cases (Whitney, 1979).

In mice (Hebert *et al.*, 1994) and cats (Drögemuller *et al.*, 2007b; Kehler *et al.*, 2007), long hair is a recessive trait caused by loss-of-function mutations in *Fibroblast growth factor 5* (*FGF5*). FGFs comprise a large family of secreted growth factors that regulate proliferation and differentiation in a wide variety of tissues. In mice, *FGF5* is specifically expressed in the outer root sheath of the hair follicle during late anagen, where it functions as a hair growth termination signal (Fig. 4.3) (Hebert *et al.*, 1994).

Taking a candidate approach, Housley and Venta (2006) identified a coding variant in *FGF5* (C59F) that is associated with long hair in several breeds. A later survey of 106 breeds showed that $FGF5^{59F}$ was indeed fixed (or nearly fixed) within most long hair breeds (Cadieu *et al.*, 2009), consistent with a general theme that most traits shared across breeds have a common genetic basis. The Afghan Hound (Plate 5) and the Yorkshire Terrier are notable exceptions, however, for which long hair is not associated with the $FGF5^{59F}$ allele (Cadieu *et al.*, 2009), indicating that an additional *FGF5* allele or a different gene is responsible for increased hair length in these breeds. In line with this evidence, Burns and Fraser (1966) noted that some forms of long hair appear to be dominant.

The *Hairless* locus

Three breeds, the Xoloitzcuintle (Mexican hairless, Plate 6), the Peruvian Inca Orchid (Peruvian hairless) and the Chinese Crested, have hairless varieties that retain vestiges of hair on the head, ears, tail and base of the legs. Hairlessness is also associated with dental abnormalities, a syndrome regarded as ectodermal dysplasia in mice and humans (Pinheiro and Freire-Maia, 1994). In all three breeds, hairlessness is dominant to a normal coat. Selection for hairless dogs necessarily maintains both the *Hairless* (Hr^{Hr}) allele and the ancestral, wild-type (Hr^{+}) allele because Hr^{Hr}/Hr^{Hr} individuals are lethal during embryogenesis. Therefore, hairless dogs are always Hr^{Hr}/Hr^{+}, while normal-coated dogs are Hr^{+}/Hr^{+} (Anon., 1917; Robinson, 1985; Kimura et al., 1993). Using a combination of pedigree and case-control approaches in Chinese Crested dogs, Drögemuller *et al.* (2008) identified the responsible gene as *Forkhead Homebox Domain I3* (*FOXI3*), which encodes a previously uncharacterized member of the Forkhead family of helix-turn-helix transcription factors. The three breeds have an identical frameshift mutation predicted to completely disrupt protein function.

The shared genetic basis across breeds implies a common origin for the hairless trait, which is surprising in this instance because the Chinese Crested and the American hairless breeds are presumed to have distinct and ancient histories. Archaeological records suggest that American hairless dogs predated European exploration in the Americas (Drögemuller *et al.*, 2007b), and Chinese Crested dogs were believed to have originated from equatorial dogs in Asia or Africa (Plate, 1930). Thus, gene flow among the breeds may have occurred by sea trade between Asia and the Americas before Western exploration.

A distinct form of hairlessness is recognized in the American Hairless Terrier (Sponenberg *et al.*, 1988). These dogs are born with a sparse, soft coat of hair that sheds during the first hair cycle and is not replaced. Hairlessness is recessive and is not accompanied by dental abnormalities. The identity of the responsible gene, referred to as *American hairless* (*Ha*) (Sponenberg *et al.*, 1988), has not been reported, but autosomal recessive hairless phenotypes are due to mutations in the *Hairless* gene in mice (Cachon-Gonzalez *et al.*, 1994) and humans (Ahmad *et al.*, 1998, 1999) and by a *Keratin 71* (*KRT71*) mutation in cats (Gandolfi *et al.*, 2010).

The *Wire hair* locus

Wire hair describes the coarse, bristly coat especially common in terrier breeds. Little

(1934) first reported wire hair (or rough coat) as an autosomal dominant trait in Toy Griffins, and Whitney (1979) extended Little's observation about inheritance to other breeds. Although its inheritance was initially studied based on segregation of wire- and smooth-coated varieties within specific breeds, it was also appreciated that wire hair is associated with a pattern of increased hair length around the chin, muzzle and above the eyes (referred to as facial furnishings). This association later proved important for gene mapping, because facial furnishings are more distinguishable across multiple breeds than wire hair, the coarseness of which is affected by hair length and curl. For example, wire hair Dachshunds, which segregate both long (*ll*) and short (*LL* or *Ll*) hair varieties, have facial furnishings, irrespective of coat length. However, short hair wires have coarse coats, while long hair wires have soft coats and are commonly referred to as soft wires (Plate 7).

Cadieu *et al.* (2009) identified the gene responsible for wire hair and facial furnishings as *R-spondin 2* (*RSPO2*) by genome-wide association using a population of Dachshunds to map wire hair, and a multi-breed population of dogs to map furnishings. A 167 bp insertion in the 3′ untranslated region of *RSPO2* was associated with both wire hair and furnishings, and elevated *RSPO2* transcript levels in skin from Wh^W dogs suggests that the insertion stabilizes the transcript (Cadieu *et al.*, 2009).

RSPO2 is a secreted activator of the Wingless/Integrase 1 (Wnt) signalling pathway (Kazanskaya *et al.*, 2004), which has been implicated in several aspects of hair follicle biology. Wnt signalling is thought to regulate the expression of hair keratin genes and provide important cues for hair cycle initiation, but the precise functions are still not fully characterized (Fuchs *et al.*, 2001). *RSPO2* is likely to have multiple roles during the hair cycle, reflected by its association with both coat length and texture in dogs. The facial hair growth pattern is particularly interesting because it indicates that perturbation of *RSPO2* levels affects hair follicles differently in body surface locations that correspond to regions of increased and sexually dimorphic hair growth in humans.

The *Curly hair* locus

Hair shape, or curl, in dogs is a complex trait that is difficult to classify, especially when comparing phenotypes across breeds. This is partly due to a wide range of variation in hair curl and also to differences in terminology among breeds. Hair curl traits are most prominent on a long hair background, and extreme differences in phenotype are exemplified by the long, relatively straight hair of the Afghan Hound (Plate 5) and the long, tightly curled hair of the Poodle. In Table 4.3, this locus is called *Cu*, and the two alleles Cu^C and Cu^+, for curly and straight coats, respectively. Intermediate phenotypes between these two extremes, such as the loose, spiral-shaped hair characteristic of Irish Water Spaniels, are described as kinky or wavy. These terms are sometimes used interchangeably but, more specifically, kinky refers to a loose curly shape while wavy describes hair that is straight with a slight curl.

Further complicating the issue, extreme phenotypes (straight and curly hair) do not segregate within any breeds. Early genetic analysis relied on the results of cross-breed matings, which provided seemingly conflicting interpretations of inheritance. For example, Whitney (1979) postulated that wavy hair is recessive to straight hair, based on the outcomes of single-generation crosses between different breeds. Alternatively, Burns and Fraser (1966) concluded that curly hair is dominant to straight hair in crosses involving Curly Coated Retrievers and Poodles.

The Portuguese Water Dog (PWD) offered a good model for a molecular genetic characterization of hair curl because this breed has both curly-coated and wavy-coated varieties that are distinguished by the degree of curl (Plate 8). Dogs from the two varieties served as cases and controls for genome-wide mapping that identified association between a *KRT71* coding alteration, R151W, and the curly-coated PWD. Furthermore, analysis across breeds indicates that the variant allele, $KRT71^{151W}$, is uncommon in straight-coated breeds, but fixed in several curly- and wavy-coated breeds, providing another example of a widely distributed trait with a shared genetic origin (Cadieu *et al.*, 2009).

R151W occurs within the highly conserved 1A region of the keratin α-helical rod domain, which is important for keratin dimerization (Hatzfeld and Weber, 1990) and which is a frequent site for curly/wavy coat mutations in other species (Kikkawa *et al.*, 2003; Lane and Mclean, 2004; Runkel *et al.*, 2006). *KRT71* is specifically expressed in the inner root sheath (Fig. 4.3) (Aoki *et al.*, 2001; Porter *et al.*, 2001; Langbein *et al.*, 2002), the structure and integrity of which is thought to mould the shape of the growing hair shaft. (Schlake, 2007).

In mice, *KRT71* mutations responsible for wavy-coat phenotypes are dominant (Kikkawa *et al.*, 2003; Runkel *et al.*, 2006). In dogs, the genetics appears to be more complicated. Inheritance in PWDs is non-Mendelian, and the $KRT71^{151W}$ allele is not found in some curly-coated breeds, such as the Curly Coated Retrievers (Cadieu *et al.*, 2009). In fact, a recent genome scan for signatures of selection across dog breeds identified a keratin cluster, distinct from the *KRT71* cluster, under strong selection (Akey *et al.*, 2010). The apparent genetic complexity of hair curl in dogs is not surprising given that recent studies on human hair structural variation implicate multiple contributing loci with relatively small phenotypic effects (Fujimoto *et al.*, 2008a,b; Medland *et al.*, 2009), and that similar wavy coat traits in mice are due to mutations in multiple genes (Nakamura *et al.*, 2001).

The *Ridge* locus

The presence of a dorsal ridge is a fully penetrant, autosomal dominant trait (Hillbertz and Andersson, 2006) at the *R* locus (which has two alleles, $R > r$). The trait is characterized by a dorsal hair stripe that stands out from the rest of the coat because hair follicles are oriented in a lateral, instead of caudal, direction (Plate 9). The trait occurs in three related breeds – the Rhodesian Ridgeback, the Thai Ridgeback and the Vietnamese Phu Quaoc dog. The presence of the dorsal ridge is also sometimes associated with a congenital malformation called a dermoid sinus (Hillbertz, 2005), a condition similar to a neural tube defect in humans called a dermal sinus. A dermoid sinus presents as a tubular indentation of the skin with hair and keratin in the lumen. In contrast to the ridged phenotype, the dermoid sinus has variable penetrance in ridged dogs. Therefore, *R/R* and *R/r* dogs are indistinguishable with respect to the dorsal ridge trait, but the prevalence of a dermoid sinus is substantially increased in *R/R* dogs (Salmon Hillbertz *et al.*, 2007).

Taking a genome-wide association mapping approach in Rhodesian Ridgebacks, Salmon-Hillbertz *et al.* (2007) identified the genetic basis for the ridged phenotype as a 133 kb duplication that includes several genes – *FGF3, FGF4, FGF19, Oral cancer overexpressed 1*, and part of *Cyclin D1*. The authors favour the interpretation that increased expression of one or more *FGFs* is responsible for the ridged phenotype because FGFs are known to participate in skin and hair development.

The *Ripple coat* locus

In his discussion on the genetics of coat characteristics, Whitney (1979) mentions a recessively inherited, transitory striping pattern in newborn puppies of some breeds, such as the Bloodhound and the Weimaraner, which he refers to as a ripple coat (at the *Rp* locus, with two alleles, $Rp^{+} > Rp^{r}$). Puppies with the pattern have regular, laterally branching waves running along the anteroposterior axis (Plate 10). The pattern is present at birth but disappears within a week. While its aetiology and genetic basis have not been described, the trait is interesting because it has a simple genetic basis and because it resembles periodic pigmentation patterns characteristic of other mammals, such as the domestic cat.

Concluding Remarks

The development of genomic resources and powerful gene-mapping strategies has fuelled rapid progress in unravelling the molecular genetics of coat morphology in the domestic dog. Seven of the original nine pigmentation loci proposed by Little in 1957 have now been mapped to a single gene and one new locus

has been discovered. Causative mutations have been determined for eight of the 19 derived alleles at these loci, providing insight into the mechanisms of canine pigmentary diversity. Mutations have also been identified for derived alleles at five hair structure loci, three of which contribute to trait variation widely distributed across breeds.

In general, coat morphological variation in the domestic dog has a simple genetic basis, sharply contrasting with the quantitative nature of similar variation in humans, and potentially reflecting differences between artificial and natural selection. Rare mutations with large phenotypic effects and simple inheritance are ideal substrates for selection during domestication because they are easy to recognize and maintain. A small number of such variants can then interact to generate complex phenotypes, as is apparent for a range of discrete coat-texture traits that result from combinations of the *Long hair, Wire hair* and *Curly hair* alleles (Cadieu *et al.*, 2009).

Other hypotheses to explain the range of phenotypic diversity in dogs, such as accelerated rates for specific mutations (Fondon and Garner, 2004) or selection for standing variation present in ancestral wolf populations, are inconsistent with the molecular architecture of morphological traits. For example, no specific type of molecular lesion predominates among derived alleles; nucleotide substitutions, insertions and deletions, duplications and repeat element transpositions are all represented. In addition, the relatively monomorphic form of wolves suggests that derived variants with large phenotypic effects are quickly removed from natural populations by purifying selection. A notable exception is the uniformly black coat colour segregating in North American wolves and coyotes. In this case, however, the responsible molecular variant, the K^B allele of *CBD103*, was introduced into wild canids through recent hybridization with the domestic dog (Anderson *et al.*, 2009).

Across breeds, similar traits frequently have identical genetic determinants, implying strong selection and a common origin predating modern breed formation. There are, however, some notable exceptions. Three different *TYRP1* alleles cause brown dilution with no apparent phenotypic differences noted for any pairing of alleles. The $MC1R^{R306ter}$ mutation responsible for yellow coat colour is present on different haplotypes and must therefore be a recurrent mutation. Uniformly black coat colour can be caused by alleles of *CBD103* (K^B) or *ASIP* (*a*), and uniformly yellow coat colour can be caused by alleles of either *ASIP* (a^y) or *MC1R* (*e*). Hairlessness demonstrates both dominant and recessive inheritance in different breeds, and the $FGF5^{59F}$ and $KRT71^{151W}$ alleles are responsible for long hair and curly coat in some breeds but not in others.

Traits affecting coat colour were possibly early targets for selection, as they would have helped to distinguish domestic dogs from wolves. The rapid population expansion and geographical distribution of dogs after domestication would have provided sufficient opportunity for human-directed selection to act on rare mutations. Importantly, before modern breed formation, dog populations that would eventually contribute to modern breeds probably existed in geographic isolation with relatively little gene flow, permitting the occurrence of parallel variation caused by distinct genetic determinants.

Modern breed formation during the last 300 years has provided an opportunity to cherry pick, from existing populations, derived variants that are highly penetrant, highly specific and predictably inherited. Because most modern breeds were developed on the same continent at the same time, it is not surprising that variants responsible for desirable traits are shared across breeds. Given the strength of human-directed selection during breed formation, one might predict that multiple derived variants of independent origin would only persist if they conferred indistinguishable phenotypes.

The data presented in this chapter indicate that diversity in coat colour, length and texture results from both coding and regulatory variation. Coding mutations tend to occur in genes with cell-type specific functions (*TYRP1, ASIP, MC1R, FGF5* and *KRT71*), and regulatory mutations tend to occur in genes with modular regulatory units (*MITF* and *ASIP*) (see Table 4.3). In particular, the pigmentary system may be prone to

diversification in domestic species because several genes affecting pigmentation have melanocyte-specific functions, and at least some of the important pigmentation genes are known to have complex regulatory systems. From a more general standpoint, the plasticity of a trait for diversification may rely on the specificity and modularity of its underlying genetic components.

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5 Mendelian Traits in the Dog

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Introduction

The dog has been well served by cataloguers of Mendelian traits, especially inherited disorders. From the pioneering work of Hutt (1934, 1968), Patterson (Patterson and Medway, 1966; Patterson, 1977, 2000) and Robinson (1968, 1990) to the contemporary reviews by Asher *et al.* (2009), Marschall and Distl (2010), Shearin and Ostrander (2010) and Summers *et al.* (2010), there have been many detailed summaries of the state of play with Mendelian traits. Together with this wealth of published information, there are eight other chapters in the present volume that provide detailed information about certain types of Mendelian traits, namely those concerning coat colour and hair texture (Chapters 4, 16), orthopaedic disorders (Chapter 7), cancer (Chapter 8), neurological disorders (Chapter 9), eye disorders (Chapter 10), morphology (Chapter 16) and models of human disorders (Chapter 21).

From 1978, the author of the present chapter has been compiling a catalogue of inherited disorders and traits in a wide range of animals, including dogs. This catalogue, which is available on the Internet as Online Mendelian Inheritance in Animals (OMIA) (http://omia.angis.org.au) is modelled on, complementary to, and reciprocally hyperlinked to the definitive catalogue of inherited disorders in humans, namely McKusick's Online Mendelian Inheritance in Man (OMIM) (http://omim.org).

OMIA includes entries for all Mendelian traits in dogs, together with other traits in dogs for which single-locus inheritance has been claimed, however dubiously. Each entry comprises a list of references arranged chronologically, so as to present a convenient history of the advance of knowledge about each disorder/trait. For some entries, there is additional information on inheritance and/or molecular genetics. If the trait has a human homologue, this is indicated by including the relevant MIM number(s) from the McKusick human catalogue. These MIM numbers provide a direct hyperlink to the relevant entry in OMIM. Finally, by searching on the word 'review' in OMIA, readers can access a full list of reviews of Mendelian traits in dogs.

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The Present Chapter

Given the comprehensive nature of the other chapters of this book, as outlined above, there is no need to produce a full list of Mendelian entries from OMIA in the present chapter. It is, however, useful to produce a list that complements the discussion in those other chapters. With the recent publication of the first assembly of the canine genome (Lindblad-Toh *et al.*, 2005), it is now possible to produce the canine equivalent of Victor McKusick's morbid map of the human genome (McKusick, 1986). A regularly updated version of the human morbid map, presented in tabular form, is obtainable from http://omim.org/downloads. As not all canine Mendelian traits are disorders, the canine map is better called a mostly-morbid map. Following the convention of McKusick (1986), and the general philosophy of Morton (1991), the canine mostly-morbid map is presented in Table 5.1. This map lists all 150 canine Mendelian traits that are known to have been characterized at the DNA level, in the order in which they are located in the canine genome, starting at the top of chromosome 1. Included in the table are the name of the trait, the gene symbol, the OMIA number (which has the format xxxxx-9615, where xxxxx is the 6-digit OMIA phene ID and 9615 is the species ID for dogs), the chromosome number (or other designation) and the location within the chromosome (as nucleotide range). Occasionally, a disorder is characterized at the DNA level but the results are not published, often to protect intellectual property. Table 5.1 includes nine such disorders. Further information on each trait, including a comprehensive list of references arranged in chronological order, is available by searching http://omia.angis.org.au with the relevant OMIA phene ID. With a total of 150 Mendelian traits that have been characterized at the DNA level, dogs account for roughly one third of Mendelian traits that have been characterized at this level across all non-laboratory animals. Furthermore, DNA-level knowledge of Mendelian traits in the dog is almost twice as advanced as that of its nearest domestic-animal rival, namely cattle, for which 71 Mendelian traits have been characterized at the DNA level.

The genomics revolution has greatly increased the ability of researchers to discover the DNA basis of Mendelian traits (Parker *et al.*, 2010). This is clearly illustrated in Fig. 5.1, which shows a dramatic increase in the number of Mendelian traits characterized at the DNA level in recent years. This increasing rate of discovery is likely to continue in the future, and raises the question of how many Mendelian traits there are likely to be in the dog. From our current understanding of mutation, the answer to this question must be 'a very large number'. In principle, a Mendelian trait could result from mutations in any one of the 19,300 canine protein-coding genes documented by Lindblad-Toh *et al.* (2005), although obviously not all mutations in such genes are likely to cause disorders. Add to this the unknown number of potential mutations in non-coding regions that could also give rise to Mendelian traits, and we are looking at a very large number of potential traits, even after allowing for the sizeable proportion that are likely to be embryonic lethals. It seems reasonable to conclude that, despite the truly impressive progress in recent years, we are still a long way from having a complete mostly-morbid map of Mendelian traits in dogs.

Table 5.1. A mostly-morbid map of the canine genome, incorporating all Mendelian traits that have been characterized at the DNA level, as at 29 September 2011.

			Location in canine genome assembly Dog2.0 (as gleaned from NCBI's Gene database[b], unless otherwise indicated)		
Name of trait	Gene	OMIA[a] no.	Chromosome no. (CFA[c].no.)	Nucleotide range	
Neuronal ceroid lipofuscinosis, adult-onset, Tibetan Terrier	*ATP13A2*	001552-9615	1	17338422	17312452
Ataxia, cerebellar	*GRM1*	000078-9615	1	40249115	40640635
Tail, short	*T*	000975-9615	1	57237727	57228891
Polycythaemia	*JAK2*	000809-9615	1	96433874	96502703
Malignant hyperthermia	*RYR1*	000621-9615	1[d]	117371505[d]	117483397[d]
Myopathy, centronuclear	*PTPLA*	001374-9615	2	22195369	22195369
Pancreatitis, hereditary	*SPINK1*	001403-9615	2	44969159	44960461
Fucosidosis, alpha	*FUCA1*	000396-9615	2	78558119	78569733
Neuroaxonal dystrophy	*MFN2*	000715-9615	2	87192427	87166391
Mucopolysaccharidosis VI	*ARSB*	000666-9615	3	30752753	30917512
Neutropenia, cyclic	*AP3B1*	000248-9615	3	31369512	31635610
Lens luxation	*ADAMTS17*	000588-9615	3	43471215	43794100
Urolithiasis	*SLC2A9*	001033-9615	3	72227604	72416756
Epilepsy, benign familial juvenile	*LGI2*	001596-9615	3	87982045	88033576
Mucopolysaccharidosis I	*IDUA*	000664-9615	3	94353697	94332044
PRA[e]-rod–cone dystrophy type 1	*PDE6B*	000882-9615	3	94601411	94573131
PRA-rod–cone dystrophy type 3	*PDE6A*	001314-9615	4	62307500	62307500
Renal cystadenocarcinoma and nodular dermatofibrosis	*FLCN [BHD]*	001335-9615	5	45177319	45196433
Cone–rod dystrophy 2	*NPHP4*	001455-9615	5	62935637	62819749
Coat colour, extension	*MC1R*	001199-9615	5	66693348	66692397
Coat colour, grizzle	*MC1R*	001495-9615	5	66693348	66692397
Coat colour, melanistic mask	*MC1R*	001590-9615	5	66693348	66692397
Cataract	*HSF4*	000168-9615	5	85208678	85204147
Mucopolysaccharidosis VII	*GUSB*	000667-9615	6	3745878	3732316
Polycystic kidney disease	*PKD1*	000807-9615	6	41852432	41906884
Glycogen storage disease IIIa	*AGL*	001577-9615	6	53165746	53095414
Retinal pigment epithelial dystrophy	*RPE65*	001222-9615	6	79953980	79977714
PRA-rod–cone dystrophy type 2	*RD3*	001260-9615	7	12835762	12835762
Episodic falling	*BCAN*	001592-9615	7	44295188	44289269
Pyruvate kinase deficiency of erythrocyte	*PKLR*	000844-9615	7[d]	45236842[d]	45244521[d]
Epidermolysis bullosa, junctionalis	*LAMA3*	000342-9615	7	67691833	67442657
Ichthyosis	*TGM1*	000546-9615	8	7247395	7235365
L-2-hydroxyglutaricacidaemia	*L2HGDH*	001371-9615	8	29766747	29729528

Continued

Table 5.1. Continued.

Name of trait	Gene	OMIA[a] no.	Location in canine genome assembly Dog2.0 (as gleaned from NCBI's Gene database[b], unless otherwise indicated)		
			Chromosome no. (CFA[c].no.)	Nucleotide range	
Elliptocytosis	*SPTB*	001318-9615	8	42251322	42199601
Krabbe disease	*GALC*	000578-9615	8	62363861	62306852
Intestinal cobalamin malabsorption	*AMN*	000565-9615	8	73843067	73850598
Mucopolysaccharidosis IIIA	*SGSH*	001309-9615	9	4530025	4536121
Coat colour, harlequin	*PSMB7*	001454-9615	9	61870594	61933886
Rod–cone degeneration, progressive	*PRCD*	001298-9615	9	7186823	7183512
Neuronal ceroid lipofuscinosis, 4A	*ARSG*	001503-9615	9	18177870	18243127
Thrombasthenia	*ITGA2B*	001000-9615	9	22368164	22382024
Glycogen storage disease I	*G6PC*	000418-9615	9	23461490	23450826
Mucopolysaccharidosis IIIB	*NAGLU*	001342-9615	9	23731475	23724623
Hyperkeratosis, epidermolytic	*KRT10*	001415-9615	9	25192205	25196077
Musladin-Lueke syndrome	*ADAMTSL2*	001509-9615	9	53263401	53263401
Exercise-induced collapse	*DNM1*	001466-9615	9	58642322	58596880
Coat colour, merle	*PMEL [SILV]*	000211-9615	10	3281379	3273878
May-Hegglin anomaly	MYH9	001608-9615	10	31135178	31194505
Cystinuria	*SLC3A1*	000256-9615	10	49826796	49862067
Wilson disease	*COMMD1 [MURR1]*	001071-9615	10	65037468	65210788
Coat colour, brown	*TYRP1*	001249-9615	11	36344711	36362564
Histiocytosis, malignant	*CDKN2A*	000620-9615	11	44256009	44255629
Histiocytosis, malignant	*CDKN2B*	000620-9615	11	44294327	44291166
Thrombocytopenia	*TUBB*	001001-9615	12	3514113	3518491
Narcolepsy	*HCRTR2*	000703-9615	12	25519536	25519536
Trapped Neutrophil Syndrome	*VPS13B*	001428-9615	13	4101618	4834310
Furnishings (moustache and eyebrows)	*RSPO2*	001531-9615	13	11792457	11636298
Improper coat	*RSPO2*	001498-9615	13	11792457	11636298
Periodic Fever Syndrome	*HAS2*	001561-9615	13	23378569	23348486
Polyneuropathy	*NDRG1*	001292-9615	13	32773306	32727266
Gastrointestinal stromal tumour	*KIT*	001516-9615	13	50040810	50122335
Gallbladder mucoceles	*ABCB4*	001524-9615	14	16564269	16492725
Multidrug resistance 1	*ABCB1*	001402-9615	14	16692598	16594869
Osteogenesis imperfecta	*COL1A2*	000754-9615	14	22830976	22866772
Oculoskeletal dysplasia, 2	*COL9A2*	001523-9615	15	5619318	5665247
Neuronal ceroid lipofuscinosis, 1	*PPT1*	001504-9615	15	5864001	5889887
Cone–rod dystrophy 1	*RPGRIP1*	001432-9615	15	21323664	21394685
Myotonia	*CLCN1*	000698-9615	16	9353532	9353532
Cone–rod dystrophy 3	*ADAM9*	001520-9615	16	29504644	29368233
Factor XI deficiency	*F11*	000363-9615	16	47447574	47431143
Prekallikrein deficiency	*KLKB1*	000819-9615	16	47477798	47454293
Coat colour, dominant black	*CBD103*	001416-9615	16	61904105	61902548
Hypothyroidism	*TPO*	000536-9615	17	3751488	3787571

Continued

Table 5.1. Continued.

Name of trait	Gene	OMIA[a] no.	Location in canine genome assembly Dog2.0 (as gleaned from NCBI's Gene database[b], unless otherwise indicated)		
			Chromosome no. (CFA[c].no.)	Nucleotide range	
Right ventricular cardiomyopathy	*STRN*	000878-9615	17	32454489	32376980
Ectodermal dysplasia	*FOXI3*	000323-9615	17	41097328	41101273
Severe combined immunodeficiency disease, autosomal, T cell negative, B cell negative, NK cell positive	*RAG1*	001574-9615	18	34654727	34651596
Hypocatalasia	*CAT*	001138-9615	18	36442060	36402617
Neuronal ceroid lipofuscinosis, 10	*CTSD*	001505-9615	18	49046651	49046651
Dermoid sinus	*FGF3*	000272-9615	18[d]	51409289[d]	51414255[d]
	FGF4		18	51439419	51441145
	FGF19		18	51490870	51494721
Chondrodysplasia	*FGF4*	000187-9615	18	51439419	51441145
Leucocyte adhesion deficiency, type III	*FERMT3*	001525-9615	18	55877772	55859993
Retinopathy, multifocal, 1	*BEST1 [VMD2]*	001444-9615	18	57509682	57498215
Retinopathy, multifocal, 2	*BEST1 [VMD2]*	001553-9615	18	57509682	57498215
Retinopathy, multifocal, 3	*BEST1 [VMD2]*	001554-9615	18	57509682	57498215
PRA-autosomal dominant	*RHO*	001346-9615	20	8641465	8636211
Coat colour, white spotting	*MITF*	000214-9615	20	24884774	24853656
Progressive retinal atrophy, Schapendoes	*CCDC66*	001521-9615	20	36756150	36711650
Epidermolysis bullosa, dystrophic	*COL7A1*	000341-9615	20	43518605	43549101
C3 deficiency	*C3*	000155-9615	20	56511783	56545477
Osteogenesis imperfecta, Dachshund	*SERPINH1*	001483-9615	21	26046669	26037811
Neuronal ceroid lipofuscinosis, 2	*TPP1*	001472-9615	21	33114262	33109052
Hyperekplexia	*SLC6A5*	001594-9615	21	45773835	45825874
Histiocytosis, malignant	*RB1*	000620-9615	22	6146196	6004672
Neuronal ceroid lipofuscinosis, 5	*CLN5*	001482-9615	22	33515050	33515050
Factor VII deficiency	*F7*	000361-9615	22	63531342	63541574
Gangliosidosis, GM1	*GLB1*	000402-9615	23	6743034	6824922
Bleeding disorder due to P2RY12 defect	*P2RY12*	001564-9615	23	48963606	48962565
Coat colour, agouti	*ASIP*	000201-9615	24	26327359	26366321
Oculoskeletal dysplasia	*COL9A3*	001522-9615	24	49699827	49715610
Nephropathy	*COL4A4*	000710-9615	25	42958216	42853856
Alopecia, colour mutant	*MLPH*	000031-9615	25	51144487	51190204
Leukaemia, chronic monocytic	*BCR*	001573-9615	26	30921293	31043453
Colorectal hamartomatous polyposis and ganglioneuromatosis	*PTEN*	001515-9615	26	40921801	40981820
Histiocytosis, malignant	*PTEN*	000620-9615	26	40921801	40981820

Continued

Table 5.1. Continued.

Name of trait	Gene	OMIA[a] no.	Location in canine genome assembly Dog2.0 (as gleaned from NCBI's Gene database[b], unless otherwise indicated)		
			Chromosome no. (CFA[c].no.)	Nucleotide range	
Persistent Müllerian duct syndrome	*AMHR2 [MISRII]*	000791-9615	27	4796698	4790859
Curly coat	*KRT71*	000245-9615	27	5540425	5548697
Glycogen storage disease VII	*PFKM*	000421-9615	27	9652207	9652207
Vitamin D-deficiency rickets, type II	*VDR*	001431-9615	27	9884542	9884542
Retinal degeneration, early	*STK38L*	001297-9615	27	23521784	23441254
Von Willebrand disease II	*VWF*	001339-9615	27	41865032	42002563
Von Willebrand disease III	*VWF*	001058-9615	27	41865032	42002563
Myasthenic syndrome, congenital	*CHAT*	000685-9615	28	4477288	4477288
Severe combined immunodeficiency disease, autosomal	*PRKDC*	000220-9615	29	3245933	3026452
Achromatopsia-3	*CNGB3*	001365-9615	29	35896147	35752878
Pyruvate dehydrogenase deficiency	*PDP1*	001406-9615	29	41759830	41774139
Neuronal ceroid lipofuscinosis, 6	*CLN6*	001443-9615	30	35257097	35239268
Metabolizer of a cognitive enhancer	*CYP1A2*	001405-9615	30	40815980	40815980
Thrombopathia	*RASGRP1*	001003-9615	30	8905228	8832112
Degenerative myelopathy	*SOD1*	000263-9615	31	29559414	29563335
Leucocyte adhesion deficiency, type I	*ITGB2*	000595-9615	31	40874865	40863039
Hair length	*FGF5*	000439-9615	32	7470760	7490740
Ciliary dyskinesia, primary	*CCDC39*	001540-9615	34	16967222	16925359
Myoclonus epilepsy of Lafora	*NHLRC1 [EPM2B]*	000690-9615	35	19940840	19939698
Neonatal encephalopathy with seizures	*ATF2*	001471-9615	36	22127418	22127418
Muscular hypertrophy	*MSTN*	000683-9615	37	3734285	3729202
Collie eye anomaly	*NHEJ1*	000218-9615	37	28714885	28636728
Progressive retinal atrophy, SLC4A3	*SLC4A3*	001572-9615	37	29136298	29151030
Neuronal ceroid lipofuscinosis, 8	*CLN8*	001506-9615	37	33879437	33879437
Muscular dystrophy, Duchenne type	*DMD*	001081-9615	X	28288322	26243436
Progressive retinal atrophy, X-linked	*RPGR*	000831-9615	X	33055106	33006439
Progressive retinal atrophy, X-linked, 2	*RPGR*	001518-9615	X	33055106	33006439
Anhidrotic ectodermal dysplasia	*EDA*	000543-9615	X	57008109	57439950
Severe combined immunodeficiency disease, X-linked	*IL2RG*	000899-9615	X	58410933	58407336

Continued

Table 5.1. Continued.

Name of trait	Gene	OMIA[a] no.	Location in canine genome assembly Dog2.0 (as gleaned from NCBI's Gene database[b], unless otherwise indicated)		
			Chromosome no. (CFA[c].no.)	Nucleotide range	
Tremor, X-linked	*PLP1*	000770-9615	X	80267541	80272546
Nephritis, X-linked	*COL4A5*	001112-9615	X	85051677	85051677
Haemophilia B	*F9*	000438-9615	X	112569285	112601742
Myotubular myopathy 1	*MTM1*	001508-9615	X	121864050	121958948
Haemophilia A	*F8*	000437-9615	X	126063524	125917393
Sensory ataxic neuropathy	*mitochondrial tRNATyr*	001467-9615	MT[g]	5347	5280
Leucodystrophy	*CYTB*	001130-9615	MT	14182	15321
Black hair follicle dysplasia	*NA*[h]	000110-9615	NA[h]	NA	NA
Dry eye curly coat syndrome	*NA*	001591-9615	NA	NA	NA
Fanconi syndrome	*NA*	000366-9615	NA	NA	NA
Gangliosidosis, GM2	*NA*	000403-9615	NA	NA	NA
Hyperparathyroidism	*NA*	000508-9615	NA	NA	NA
Ichthyosis, Golden Retriever	*NA*	001588-9615	NA	NA	NA
PRA-rod–cone dystrophy type 4	*NA*	001575-9615	NA	NA	NA
Renal dysplasia	*NA*	001135-9615	NA	NA	NA
Von Willebrand disease I	*NA*	001957-9615	NA	NA	NA
Deficiency of cytosolic arylamine N-acetylation	*NAT1, NAT2*	001587-9615	These two genes are lacking in all dogs		

[a] OMIA, *Online Mendelian Inheritance in Animals* (available at: http://omia.angis.org.au).
[b] http://www.ncbi.nlm.nih.gov/gene.
[c] CFA, canine (*Canis familiaris*) chromosome.
[d] Information taken from Ensembl (http://www.ensembl.org) because this gene is not annotated in Homologene.
[e] PRA, progressive retinal atrophy.
[f] MT, mitochondrial.
[g] NA, not available.

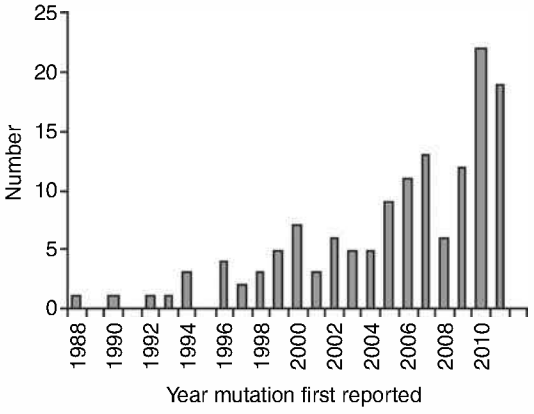

Fig. 5.1. The timescale of discovery of the molecular basis of Mendelian traits in dogs, since the first such discovery in 1988, as at 19 September 2011.

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6 Canine Immunogenetics

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Introduction

Both innate and acquired immunity represent dynamic complex regulatory biological networks that protect the host organism from a wide range of harmful situations, including viral and bacterial infection, and intracellular and ectoparasites. Both systems allow the discrimination of foreign and novel proteins and, to some degree, of other biological molecules. Innate immunity represents a more primitive system that provides an immediate and formidable protective barrier; it is largely non-specific and directed equally at all infectious insults. In contrast, the acquired immune response is only found in higher animal orders and is sufficiently sophisticated to develop specific responses and recognize non-self from self-proteins (Klein, 1982). While this mechanism provides highly selective immunity to pathogens, it is also effective in providing immune surveillance against the development of malignant cells.

Genes involved in the acquired immune response contribute in two broad areas: (i) in the specific recognition of foreign antigen; and (ii) in the regulation and intensity of the response that ensues. The specificity of antibody (represented on B cells) and cell-mediated (represented on T cells) immune recognition of non-self peptide antigens is encoded by immunoglobulin genes and T cell receptor genes, respectively. By rearranging different combinations of gene segments, it is possible to generate vast numbers of unique antigen recognition receptors. Specific processes that occur during the differentiation of lymphocytes are capable of creating an enormous repertoire of B cell receptor (BCR) and T cell receptor (TCR) phenotypes. This includes a combination of genetic mechanisms operating at a somatic cells level: recombination-activating gene (RAG) protein-mediated somatic recombination, gene segment rearrangement, junctional diversity, and somatic mutation mechanisms, to generate a vast repertoire of TCR and BCR phenotypes with which to sample the antigenic environment (Table 6.1) (Paul, 2008; Barreiro and Quintana-Murci, 2010; Lunney *et al.*, 2011). In addition to these lymphocyte-specific events there is somatic hypermutation, which generates large numbers of new mutations in the newly reconstructed BCR and TCR genes. The enormous potential variety of the proteins produced by the lymphocytes is the basis for a highly specific ability to recognize and eventually to destroy non-self antigens.

A large proportion of the newly generated BCRs and TCRs may have specificity for recognizing self-antigen. Additional mechanisms regulating immune tolerance to self-antigens have evolved to overcome the danger of making an immune response to self-protein. Major histocompatibility complex (MHC) genes are critical for this process, as an immune response can only develop if peptide antigen is presented by an MHC cell surface molecule to a TCR. The vast majority of T lymphocytes bearing a TCR are eliminated in the thymus, and only T cells bearing receptors capable of recognizing non-self antigen, in the context of being presented by self-MHC, survive and move into the periphery. This process is also responsible for restricting antibody production to non-self antigens, as B cells will only go into clonal

Table 6.1. The genetic diversity of the antigen receptors of the adaptive immune system (adapted from Lunney *et al.*, 2011, with the author's permission).

	Origin of specificity			Origin of genetic diversity	
Receptor[a]	Germ line	Somatic	Rearrangement	SHM[b]	Polygenic
BCR	−	+	+	+	+
TCR	−	+	+	−	+
MHC I	+	−	−	−	+
MHC II	+	−	−	−	+
Non-classical MHC	+	−	−	−	±

[a] BCR, B cell receptor; TCR, T cell receptor; MHC, major histocompatibility complex.
[b] SHM, somatic hypermutation.

expansion and develop into antibody-secreting plasma cells if: (i) the surface immunoglobulin receptor recognizes its specific antigen; and (ii) the B cell receives an appropriate signal from T cells that also recognize the antigen. Thus, although MHC molecules do not determine specific antigen recognition, they are critical in presenting antigen to T cells and regulating a repertoire of T cell receptors that do not recognize self. TCR, immunoglobulin (Ig) and MHC genes and their genomic organization/polymorphism are central to immunogenetic studies. Unlike Ig and TCR, MHC is highly variable. This is not due to somatic recombination and ongoing somatic mutation events, but results from high levels of allelic polymorphism in many MHC genes (Table 6.1; Klein, 1986). There are three classes of MHC antigens, which are considered further later in this chapter.

The genes encoding BCR, TCR and MHC class I and class II molecules exhibit a precise correlation between exons and the protein domains that they encode. The DNA sequences of the MHC class I alpha 3 domain, the class II alpha 2 domain and the CH3 domain of immunoglobin (BCR) constant regions as well as parts of the TCR are homologous. These similarities indicate that the DNA sequences have descended from a common ancestral gene and are members of one superfamily. Indeed, the proteins supporting the immune response (in BCRs, TCRs and the MHC) are members of the immunoglobulin superfamily, a large group of soluble and cell-surface proteins that are essential for protein recognition and binding. The criterion for the inclusion in this superfamily is based on the presence of a so-called immunoglobulin domain initially discovered in (BCR) immunoglobulins (Figs 6.1 and 6.2).

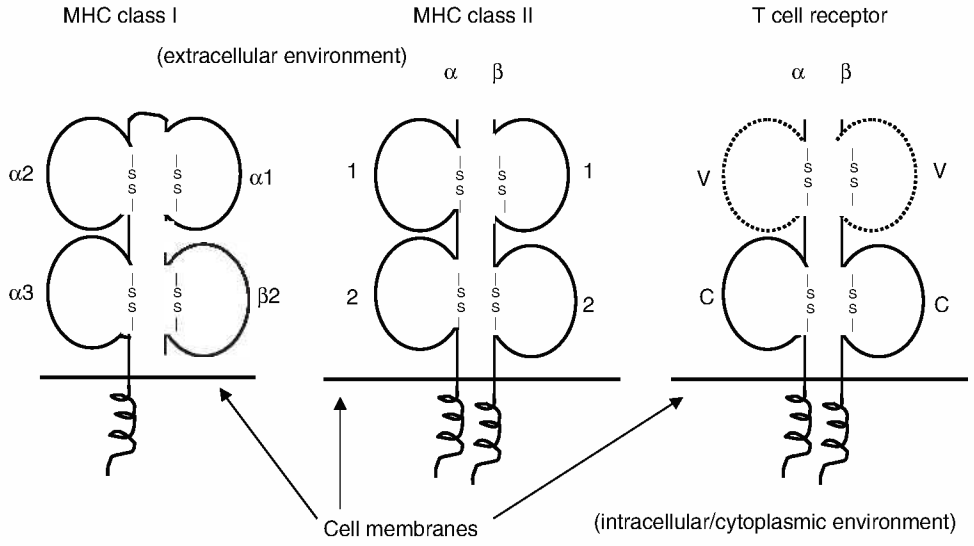

Fig. 6.1. Schematic representations of the prototypical structures of MHC (major histocompatibility complex) classes I and II, and T cell receptor molecules. Each of the circular domains represents approximately 200–220 amino acid residues and ' –S S– ' represents the placement of disulfide bonds. The most cytoplasmic domains exhibit the greatest genetic variability. The MHC class I gene encodes a single molecule comprising three domains (α1, α2, α3). It is functionally complete in association with the beta-2 microglobulin molecule (β2), a protein unrelated to this gene family. The MHC class II molecule results by association of two MHC gene products, the α chain and the β chain, each comprising two domains. The T cell receptor has a similar structure, either with an α and β chain, as shown here, or with a γ and δ chain. The dotted lines for the T cell receptor indicate the 'V' region, which has variation as the result of somatic recombination or other mutational events; the C region is the constant region (adapted from Bailey *et al.*, 2001, with permission).

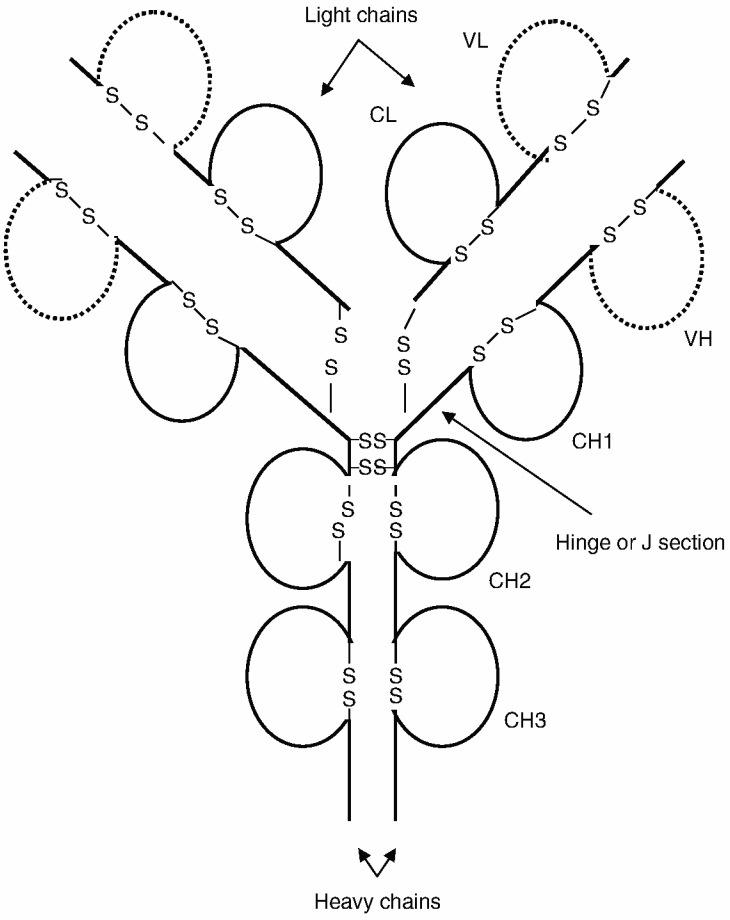

Fig. 6.2. Schematic representation of the structure of an immunoglobulin (Ig) molecule. Like MHC molecules of the MHC (major histocompatibility complex; see Fig. 6.1), this is also composed of a series of 220 amino acid residues. The heavy chain determines the function of the molecule. The variable region (V), denoted by dotted lines, determines the antigenic specificity. The variable region is the product of somatic recombination and other mutational events. The heavy chain constant domains are denoted CH1, CH2 and CH3 while the variable domain is identified as VH; likewise, the constant region of the light chain is denoted as CL and the variable region as VL; ' –S S– ' represents the placement of disulfide bonds (adapted from Bailey *et al.*, 2001, with permission).

A separate system of molecules has evolved to regulate the immune response. These are referred to as cytokines and chemokines, and they broadly control immune kinetics and immune chemotaxis/cell trafficking, respectively (Thomas and Lotze, 2003). The molecules are soluble peptides released into the circulation by a variety of cell types as immune- or inflammatory-driven processes develop. They are often referred to as 'immune hormones'. A large number of cytokines, chemokines and growth factors have now been characterized and they exhibit a wide range of immunological properties. The genes that encode these factors, and the cell-surface receptors that bind them, can exhibit polymorphism. So their contribution to health and disease is also a key area of study within immunogenetics. Similarly, the scope of immunogenetics can also be extended to include

cell-surface accessory molecules involved in immune cell–cell interactions. Furthermore, a case can also be made for including the study of genes involved in antigen processing and secondary signalling pathways mediating immune receptor binding.

Canine diseases that exhibit a major immune component represent some of the most prevalent conditions observed in the dog. These include cancers, autoimmunity, infections, atopy and parasitic diseases. In addition, vaccine failure and vaccine-associated adverse hypersensitivity reactions are also clinically important. Consequently, canine immunogenetics is now recognized as being an important area for study. The dog exhibits many immune-related conditions that represent excellent comparative models for human counterpart diseases such as autoimmunity. The dog also serves as an important model for drug toxicity trials and for a variety of human diseases, such as cyclic neutropenia (Weiden *et al.*, 1974), X-linked severe combined immunodeficiency syndrome (SCID) (reviewed in Felsburg *et al.*, 1999), von Willebrand's disease (reviewed in Thomas, 1996), severe hereditary haemolytic anaemia (Weiden *et al.*, 1976), haemophilia (reviewed in Fogh *et al.*, 1984), gluten-sensitive enteropathy (Hall and Batt, 1990), rheumatoid arthritis (Halliwell *et al.*, 1972), systemic lupus erythematosus (Lewis and Schwartz, 1971) and narcolepsy (Baker *et al.*, 1982). Dogs have high rates of spontaneous malignancies and have therefore served as models for a variety of cancers, including breast cancer (Mol *et al.*, 1999), non-Hodgkin's lymphoma (Weiden *et al.*, 1979) and prostate cancer (Navone *et al.*, 1998). For nearly 50 years the dog has also served as a valuable model for haematopoietic stem cell transplants (reviewed in Thomas and Storb, 1999).

Overview of the major antigen receptors of the immune system

Innate immunity and its genetic diversity

As mentioned earlier, the immune response in vertebrates has two principally different strategies: innate and adaptive. The first is available immediately but exhibits broad antigen specificity, mainly to common antigens. This system is the first line of defence against bacterial and viral infections. Toll-like receptor (TLR) loci are the prominent components of the innate immune system. Molecular phylogenetic analysis reveals that TLRs existed at the early stages of vertebrate evolution (Roach *et al.*, 2005). Due to strong selective pressures, the set of TLR genes and their structure remains pretty stable in vertebrates. There are six major families of vertebrate TLRs (TLR 1, 3, 4, 5, 7, 11); members of the same family recognize a general class of pathogen-associated molecular patterns.

Single nucleotide polymorphisms (SNPs) of TLR genes have been reported in several mammalian species and may reflect differences between individual animals, particularly during the first hours after contact with viral and bacterial infections. TLRs and other systems of innate immunity, as well as MHC antigens – which, in contrast, interact with components of adaptive immunity – fully correspond to the notions of classical Mendelian genetics: they behave as co-dominantly inherited genes, exclusively determined by the germ line, and both alleles are not expressed on any one cell.

A great deal of progress has been achieved in recent years in understanding TLR structure and function. Canine investigations have also contributed to this research (Table 6.2). However, this is only the beginning, and further studies are needed for a more comprehensive understanding of the role of TLRs, which is probably not limited to external microbial pathogens but is also related to the recognition of non-self antigens and, hence, to autoimmune conditions and possibly cancer.

Adaptive immunity

The adaptive immunity represented in mammals by B and T cells is very different from the innate system. Neither the BCRs nor the TCRs are determined by the germ line alone, but are the result of complex somatic gene rearrangements that occur during the development of lymphocyte cell lines. The somatic processes that control their generation are controlled by nuclear enzymes such as the RAG complex, Tdt (terminal deoxynucleotidyl transferase), AID (a B cell specific cytidine deaminase: activation-induced deaminase) and various constitutive DNA repair enzymes. As a result, an

Table 6.2. The Toll-like receptor genes (TLRs) and the Nod-like receptor genes (NLRs)[a] involved in innate response in dogs. (The map data were obtained from the *Ensembl* genome browser[b] on 5 March 2011.)

Gene	Map location (CFA[c]: position in Mb)	Some effects on immune response	Reference
TLR1 *B2CW35*[d]	3: 76.3	Whole blood hyper-inflammatory responses to pathogen-associated molecules; sepsis-associated multi-organ dysfunction and acute lung injury in vertebrates	Pino-Yanes *et al.* (2010)
TLR10 *TLR2*	15: 54.5	Responses towards bacterial flora of the gut; the pathogenesis of canine inflammatory bowel disease (IBD)	McMahon *et al.* (2010)
TLR3	16: 47.6	Antiviral responses in virus-infected animals; type I interferon (IFN) and cytokine production, secondary natural killer (NK) cell responses, and final cytotoxic T lymphocyte (CTL) responses and antibody production	Matsumoto *et al.* (2011)
		Protective role for TLR signalling in the vessel wall	Cole *et al.* (2011)
TLR4 *NP_001002950*[d]	11: 74.4	Role in the pathophysiology of stifle joints of dogs with or without osteoarthritis	Kuroki *et al.* (2010)
TLR5 *B2CW36*[d]	38: 26.7	Association with IBD	Kathrani *et al.* (2010)
TLR6	Unknown	Pathogen recognition in some mammals; structurally similar to TLR1	Pino-Yanes *et al.* (2010)
TLR7	X: 9.3	Induces interferon production; expression in dogs mainly in large intestine, lung, pancreas, small intestine and skin	Okui *et al.* (2008)
TLR8	X: 9.3	Sensing foreign RNA, including viral, likely cooperative connection with the RIG-I like helicases (cytosolic RNA helicases), similarity with TLR7	Bauernfeind *et al.* (2010)
TLR9	20: 40.5	Activated by bacterial DNA and induces production of inflammatory cytokines, one of the expression focuses is the lungs	Hashimoto *et al.* (2005) Schneberger *et al.* (2011)

Continued

Table 6.2. Continued.

Gene	Map location (CFA[c]: position in Mb)	Some effects on immune response	Reference
NOD1	14: 46.1	Initiate or regulate host defence pathways through formation of signalling platforms that subsequently trigger the activation of inflammatory caspases and NF-κB (nuclear factor kappa B)	Proell *et al.* (2008)
NOD2	2: 67.5		

[a] Nucleotide oligomerization domain receptors (cytoplasmic proteins that may have a variety of functions in regulation of inflammatory and apoptotic responses).
[b] http://www.ensembl.org (accessed 5 March 2011).
[c] CFA, canine (*Canis familiaris*) chromosome.
[d] Current *Ensembl* symbols.

individual can potentially produce an enormous variety ($\sim 10^{10}$) of protein receptor molecules, which strictly speaking are not directly encoded by the germ line genes, but rather are an outcome of random rearrangements of DNA segments existing in the germ line. This sharply differs from classical Mendelian inheritance.

Another unusual feature common to both TCRs and BCRs is that the expression of these receptors on lymphocytes is controlled by allelic exclusion. This phenomenon is based on the expression of only one receptor on any one B or T cell; thus, lymphocytes are monospecific. The latter can be understood in terms of dual receptor avoidance on a B or T cell, because such a lymphocyte might recognize both a dangerous pathogen and an own self-antigen. As follows from the above, the differences between the innate and adaptive systems are significant, but the two systems complement one other during the immune response.

The Major Histocompatibility Complex (MHC)

Overview of the canine major histocompatibility complex

An understanding of the general structure and function of the MHC is helpful in order to comprehend its importance in transplantation and disease. The canine MHC is also known as the dog leucocyte antigen (DLA) and is located on chromosome 12 (Fig. 6.3). Each of the three classes of DLA genes are located in clusters: DLA class I genes are located proximally, DLA class II genes have the most distal location and DLA class III genes are in an intermediate position. The MHC is one of the most extensively studied regions of the genome in mammalian species owing to its biological role and very high level of allelic polymorphism.

The products of MHC genes interact with bound peptide ligands and with products of rearranged TCR genes in the thymus, which results in positive and negative selection of the peripheral T cell repertoire. This region of tightly linked genes is responsible for the presentation of self and non-self antigens to the immune system and is, therefore, fundamental to the recognition and regulation of the immune response. MHC molecules perform these roles by binding and presenting peptide antigens to T cells. This antigen presentation can lead to several events, including the elimination of infected cells or cellular rejection of transplanted organs.

DLA class I and class II molecules are cell surface glycoproteins of similar structure that are involved in antigen presentation to T cells. Allelic variation occurs typically because of polymorphism in or around the peptide-binding site, and there may be more than 100 alleles for a given gene (Table 6.5). Class III molecules

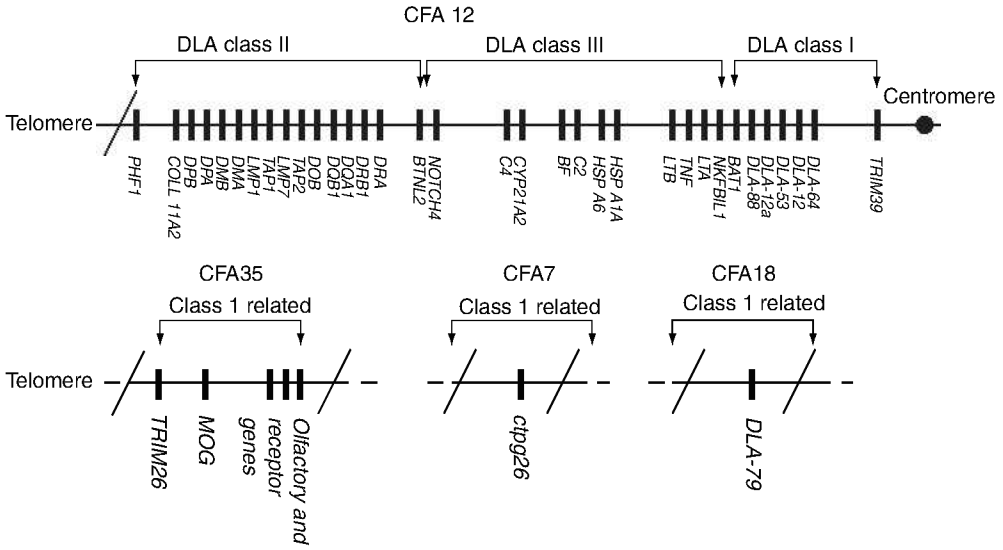

Fig. 6.3. Chromosome location and orientation of some canine MHC (major histocompatibility complex) class I, II and III region genes. Note that: only selected genes are shown in the diagram; the distances between genes are not accurately represented; and the number of class II genes may vary on different ancestral DLA (dog leucocyte antigen) haplotypes seen in some breeds. Information on normal functional genes and on pseudogenes can be found in Debenham (2005) and Yuhki *et al.* (2007). CFA, canine (*Canis familiaris*) chromosome.

are structurally unrelated to class I and class II molecules, and are not relevant to antigen presentation, but can be important in other aspects of the immune system, such as complement activation. Class I antigens are expressed on all somatic cells. Interestingly, in contrast to mice and humans, canine class II gene products are present on almost all lymphocytes (Doxiadis *et al.*, 1989).

Other genes located within this complex are critical for how antigens are processed, transported within the cell and loaded into MHC molecules, which then act as cell-surface moieties capable of presenting antigens to TLRs. It is, therefore, not surprising that key immune-driven mechanisms, such as graft rejection and levels of immune response to viral, bacterial and other exogenous and endogenous antigens, are regulated by genes in this region. Given the need to maintain population immuno-diversity to deal with new and changing antigen challenges in the environment, it is also understandable why this genetic system has evolved with such extreme polymorphism. A major consequence of the biological role that MHC genes play, and of the level of polymorphism seen between individuals within a population, is that some individuals will be more able to deal with particular infections and immunological insults than others; i.e. some will be resistant while others are more susceptible to disease. Thus, susceptibility towards many diseases such as infections, cancers and autoimmune conditions are related to particular MHC polymorphisms. The domestic dog represents an ideal species for elucidating the relationship between MHC genes and immune-biological function. The extreme phenotypic diversity generated and maintained through selective dog breeding has resulted in over 400 distinct breeds. Such breeds are likely to have characteristic distributions of MHC gene polymorphism and, as a consequence, breed-related predisposition to particular immune-related conditions. Through the study of the dog MHC, both veterinary and human medical sciences are likely to benefit. Fixed dog breeds share many

genetic parallels seen in highly inbred human founder populations. Comparative studies of MHC-related conditions in predisposed dog breeds represent a powerful approach to understanding the aetio-pathology of diseases common to both species (see the European LUPA project for the study for animal models – specifically dog models – of human diseases at www.eurolupa.org).

Discovery of the dog leucocyte antigen (DLA) system

The characterization of the dog gene complex of the MHC began in the early 1960s when allo-antisera were produced that were capable of serologically defining discrete cell-surface antigens on canine white blood cells (Puza *et al.*, 1964; Rubinstein and Ferrebee, 1964). A major biological role for these dog antigens was established, as they determined the rejection/survival outcome of allogeneic tissue transplants (Epstein *et al.*, 1968). Interest in using the dog as a model for transplantation drove much of the early research into the canine MHC, and the results of these investigations were summarized in the first edition of *The Genetics of the Dog* (Wagner *et al.*, 2001). The genetic control of dog histocompatibility antigens and their influence on graft survival were co-located to the same chromosomal region (Vriesendorp *et al.*, 1971). This chromosomal region was also found to regulate the level of recognition and cell proliferation between the lymphocytes of two dogs when they were cultured together within a mixed lymphocyte culture (Templeton and Thomas, 1971). In keeping with the terminology used for the human HLA (human leucocyte antigen) system, the abbreviation DLA (dog leucocyte antigen) was introduced to describe these antigens in the dog.

The serological (using allo-antisera) and cellular (using mixed lymphocyte culture) definition of the DLA system was significantly taken forward through several international collaborations. This was largely coordinated and reported through international histocompatibility workshops held in 1972, 1974 and 1981 (Vriesendorp *et al.*, 1973, 1976; Deeg *et al.*, 1986; Bull *et al.*, 1987). Following a hiatus of activity through the early 1980s, a resurgence of interest in characterizing the DLA system began again in the 1990s as new molecular methods for the definition of alleles were introduced and the great potential of using spontaneous dog diseases as homologous disease models for human conditions was increasingly recognized. A DLA component was reported at the 14th International Histocompatibility Workshop (Australia) (Kennedy, 2007; Kennedy *et al.*, 2007b,c,d) and a major DLA component was included in the European Union 7th Research Framework Programme grant that funds the LUPA project. Our increasing recognition of the complexity of the DLA system and the diversity of DLA polymorphism observed between different domestic dog breeds are now bringing insight into and increasing our understanding of the relationship that the dog has to other Canidae, and of how DLA contributes to disease susceptibility.

Relationship between early immunological studies of the dog MHC and the current view of DLA genes

Early studies using serological and cellular/functional methodologies demonstrated the existence of a highly polymorphic series of alleles which were originally divided into two loci (Vriesendorp *et al.*, 1972). These were subsequently further resolved into four serologically defined loci: DLA-A, DLA-B, DLA-C and DLA-D. Although DLA-A, -B and -C were all first considered to be class I molecules as they were constitutively expressed on all lymphocytes, immuno-precipitation studies demonstrated that DLA-B was not co-associated with the beta-2 microglobulin invariant chain, which is a standard criterion for the designation of MHC class I status (Krumbacher *et al.*, 1986). Further studies using two-dimensional gel electrophoresis confirmed that DLA-B molecules were heterodimers made up of alpha and beta chains (Fig. 6.1), thus confirming DLA-B as being a class II molecule (Doxiadis *et al.*, 1989). As seen in humans, this dog alpha chain was invariant, suggesting

that DLA-B corresponded to a DRB/A heterodimer.

The DLA-D locus, previously identified by both serology and cellular assays, was later established as being a class II locus by molecular genetic techniques using restriction fragment length polymorphism (RFLP) analysis and cross-hybridizing human HLA cDNA probes (Sarmiento and Storb, 1988a,b). Using probes corresponding to class II alpha and beta genes, it was possible to detect the presence of DR, DQ, DP and DO genes within the dog MHC. The alpha probes identified a *DRA* gene with limited polymorphism and two *DQA* genes, one being highly polymorphic and the other non-polymorphic. At least one non-polymorphic *DPA* gene was also identified. The molecular (gene) equivalents for DLA-A, -C and –D have never been established, but it seems likely that DLA-A is *DLA-88*, DLA-C may be *DLA-64* or *DLA-79*, and DLA-D is probably *DLA-DQ*.

The genes for DLA class II beta were also analysed using RFLP patterns detected with human class II beta probes. This identified two polymorphic *DRB* genes, two *DQB* genes (one polymorphic and the other non-polymorphic), two polymorphic *DPB* genes, and one *DOB* gene with only limited polymorphism. The high level of cross-reactivity observed between these loci and all of the human class II probes used confirmed the large amount of similarity between all dog class II loci and between class II genes in both the human and the dog. This was further confirmed by a study which identified 87% nucleotide identity between *DLA-DRB1* and *HLA-DRB1* (Sarmiento and Storb, 1990a).

Full characterization of the DLA class II region was determined when 711,521 base pairs of sequence was generated from six overlapping BAC (bacterial artificial chromosome) clones covering the classical and extended class II region (Debenham, 2005; Debenham *et al.*, 2005). Analysis and annotation of this sequence revealed the presence of 45 loci of which 29 were predicted to be functionally expressed, five were unprocessed pseudogenes, ten were processed pseudogenes and there was one novel transcript. Key DLA class II genes and their chromosomal order are summarized in Fig. 6.3 and a full list with annotations is provided by Debenham (2005). This study confirmed the presence of many DLA class II genes as being homologues of human counterparts and that the dog has three DP genes, all of which are non-transcribed pseudogenes. Furthermore, this study identified 23 useful class II region microsatellite markers.

Dog class I genes have also been resolved using RFLP analysis and human cDNA probes for HLA-A, -B and -E sequences. This revealed the presence of canine homologues to these genes and at least five additional class I-like loci (Sarmiento and Storb, 1989). The classical DLA class I locus corresponding to the serologically defined DLA-A locus was characterized by sequencing a canine cDNA clone (Sarmiento and Storb, 1990b). This was subsequently renamed as *DLA-88* (Burnett *et al.*, 1997). A further non-classical class I gene with limited polymorphism, named *DLA-79*, was characterized by clones pulled from a dog genomic library using an HLA-E probe (Burnett and Geraghty, 1995). This gene has been located to a region on dog chromosome 18 (Wagner, 2003). Four additional DLA class I genes were subsequently characterized by sequencing clones from canine DNA Libraries (Burnett *et al.*, 1997). Two were classified as being functional class I genes (*DLA-12* and *DLA-64*) and two others as being pseudogenes (*DLA-12a* and *DLA-53*), the latter lacking complete homology to class I genes. A fifth class I-like sequence, *ctpg-26*, has been identified that appears to be a processed pseudogene. RFLP analysis indicated that this gene was linked to the DLA region (Burnett *et al.*, 1997). *In silico* analysis of the Boxer full genome sequence revealed that it is located on dog chromosome 7 (Yuhki *et al.*, 2007). The designation of class I loci in the dog has not followed that already given to human class I loci as they do not appear to have maintained any significant orthology. Moreover, there has been considerable species-specific evolution in the DLA class I region (Yuhki *et al.*, 2007). This is important to bear in mind when any biological interpretation is attempted, as extrapolation from human and other non-carnivore mammal data is likely to be incorrect; it has also been demonstrated by the fact that the dog does not have any MIC-like genes in the class I region compared with those seen in the corresponding human HLA-

B/C region (Yuhki *et al.*, 2007). As the MIC genes are important in the natural killer cell control seen in man, it suggests that dogs have evolved different regulatory mechanisms.

Earlier studies also identified that a class III region was present within the DLA complex. Class III genes identified within this region include, *TNF* (Zucker *et al.*, 1994), *CYP21* (Takada *et al.*, 2002) and *C4* (Grosse-Wilde *et al.*, 1983). There is some evidence that the canine *C4* gene may be duplicated (Doxiadis *et al.*, 1985). DNA sequencing studies have now resolved the DLA class I–III boundary more precisely, and this has confirmed that it has similar structure and organization to those seen in the human MHC (Wagner, 2003; Wagner *et al.*, 2005). A number of class I and III genes were identified in this region, including: *DLA-12, DLA-12a, DLA-53, DLA-64, TNF, HSPA1A* (resembling *HSP70-1*), *BAT-1, NFKBiII, ATP6G* and *LTA* (lymphotoxin).

Extensive studies to determine the tissue distribution and expression of DLA antigens have not been conducted. Early studies demonstrated that they are present on peripheral blood lymphocytes and thrombocytes but are not found on erythrocytes (Vriesendorp *et al.*, 1977). Some circumstantial evidence has been reported for DLA molecules being expressed on skin and in the small bowel, pancreas and heart tissues, as allograft recipients produce high titre anti-DLA antibodies and reject grafts (Vriesendorp *et al.*, 1971; Westbroek *et al.*, 1972). It is unclear whether the DLA antigens expressed on these tissues are class I or class II or both. In both primates and rodents, most class I MHC molecules are expressed on all somatic cells, whereas class II antigens are only constitutively expressed on 'professional' antigen-presenting cells such as macrophages and dendritic cells and also on B lymphocytes. In contrast, dogs and presumably other canids appear to constitutively express DLA class II molecules on the majority of peripheral blood lymphocytes (Deeg *et al.*, 1982; Doxiadis *et al.*, 1989). This is not unique, and the same wide pattern of class II expression on lymphocytes has also been observed in both the cat (Neefjes *et al.*, 1986) and the horse (Crepaldi *et al.*, 1986). More recently, from molecular analysis of the dog class II region, it has been determined that the *DLA-DP* genes are not capable of expression (Debenham *et al.*, 2005).

Genomic organization of the canine MHC

Early studies recognized that a series of tightly linked genes within the canine MHC (to which a range of immunologically defined phenotypes could be attributed) segregated in a Mendelian fashion and was located in a discrete position (Vriesendorp *et al.*, 1977). It was also recognized that some histocompatibility-attributed biological effects were potentially encoded on another chromosome (Vriesendorp *et al.*, 1975). Eventually, the DLA region was localized to the dog chromosome CFA12q (canine chromosome 12q) by fluorescence *in situ* hybridization using two canine clones containing the *DLA-64* and *DLA-88* genes, respectively (Dutra *et al.*, 1996). This canine region was confirmed as being syntenic to the HLA region on human chromosome 6p22.1 using chromosome painting (Breen *et al.*, 1999).

The sequencing of the dog genome has enabled a more precise chromosomal location of canine MHC genes (Yuhki *et al.*, 2007). The genes of the dog MHC that have so far been investigated and their locations within the DLA regions are summarized in Fig. 6.3. It is important to recognize that this summary is based on limited information. Several genes of DLA class I (*DLA-64, DLA-12, DLA-53, DLA-12a* and *DLA-88*) are located in the proximal region of chromosome 12 and only three genes, *DLA-64, DLA-12* and *DLA-88*, are fully functional. The class III region and the following class II region are located distally from the class I region. The class II region also contains four fully functional genes: *DLA-DRA, DLA-DRB1, DLA-DQA1* and *DLA-DQB1*. In all, the DLA region on chromosome 12 covers approximately 3 Mb of sequence. It is also clear now that the dog class I region differs from that in the human and in the mouse in that approximately 0.5 Mb resides on a different chromosome (the telomeric region of chromosome 35). The chromosomal location for this break point resides within the TRIM gene family region in both dogs and cats, and is thought to have occurred some 55 million years ago

before the split between canids and felids (Yuhki *et al.*, 2007). Another study has indicated that two other class I genes, *ctpg26* and *DLA-79*, are located on dog chromosomes CFA7 and CFA18 respectively (Mellersh *et al.*, 2000; Wagner, 2003). The *ctpg26* gene is considered to be a processed pseudogene, while *DLA-79* is a potentially functional gene. The *TNF* (tissue necrosis factor) gene also maps to CFA12 within the DLA class III cluster (Mellersh *et al.*, 2000). While many genes within the canine MHC have been sequenced and annotated, the order of the genes on chromosome 12 and the genetic distances between them require further investigation. A significant amount of information regarding the DLA can be inferred from other mammalian species as the MHC was highly conserved throughout mammalian evolution.

DLA nomenclature and the immuno-polymorphism MHC database

In 1998, a new DLA nomenclature committee was established under the auspices of the International Society for Animal Genetics for recognizing and naming DLA genes and alleles. Several reports have now been published using a revised nomenclature system (Kennedy *et al.*, 1999b, 2000a, 2001a,b). The system used for naming dog MHC alleles has continued to use DLA. In 2010, the HLA nomenclature committee (Marsh *et al.*, 2010) established a new nomenclature for HLA, which also includes the use of italics for alleles and genes. Previously, italics have not been used for MHC nomenclature. In 2011, DLA nomenclature also changed in line with the new HLA nomenclature (L.J. Kennedy, paper in preparation).

The Immuno-Polymorphism Database (IPD-MHC) website (www.ebi.ac.uk/ipd/mhc/dla/index.html) contains the allele sequences for all officially recognized DLA alleles, plus tables indicating in which canid species each allele has been found. A complete list of DLA class II (and class I) haplotypes is available on this website. There are also details regarding the DLA haplotypes found in dog breeds.

DLA class I

Molecular characterization of DLA class I

Annotation of the DLA class I genes is ongoing at the moment. The description of at least one gene, *DLA-88*, can be found in *Ensembl* (http://www.ensembl.org) and some other genomic browsers; this gene contains eight exons (encoding 361 amino acids). DLA class I genes encode highly polymorphic proteins that have structural similarity to DLA class II proteins and immunoglobulins (Fig. 6.1). These proteins consist of one α chain with three domains, α1, α2 and α3, interacting with a separate β2 microglobulin. These molecules are expressed on the surface of all nucleated cells and platelets, and present cytosolic peptides to the $CD8^+$ receptor expressed on T lymphocytes. These proteins are also able to bind inhibitory receptors on natural killer cells. The molecular characterization of DLA class I alleles has lagged behind that for class II for several technical reasons. First, both exons 2 and 3 are polymorphic, requiring a longer sequence to be characterized (~1000 bp), but, secondly, the several class I genes known are very similar, which creates obstacles in designing specific primers. Various methods have been tried, including RFLP (Sarmiento and Storb, 1989), DNA cloning and sequencing (Graumann *et al.*, 1998), PCR-SSCP (PCR-single strand conformational polymorphism) (Wagner *et al.*, 2000), a modified PCR-SSCP (Venkataraman *et al.*, 2007) and sequence-based typing (SBT) (Hardt *et al.*, 2006). Unfortunately, none of these methods has proved easy for large-scale DLA class I typing. SBT is possible in some cases, but there are obstacles, including the fact that many DLA haplotypes carry two copies of the *DLA-88* gene, so that an individual dog can have between one and four *DLA-88* alleles. Therefore, DNA cloning and sequencing is the current approach for identifying all *DLA-88* alleles in all dogs.

Polymorphism: alleles, haplotypes and duplicated genes

To date, there are 71 recognized *DLA-88* alleles plus another 11 provisional alleles. Currently, names are based on the exon

2 and 3 sequences, but collecting sequence data for the introns as well as the exons continues. There is an expectation that alleles with identical exon sequences and different intron sequences, as in humans, might be found (Marsh *et al.*, 2010). None of the other class I genes have been investigated for polymorphism beyond the original reported study (Graumann *et al.*, 1998).

Typing *DLA-88* in 428 dogs revealed that 129 were homozygous for DLA class II genes. The dogs have been characterized for *DLA-88* using several methods. Some dogs have more than two *DLA-88* alleles; Table 6.3 gives a summary of the number of *DLA-88* alleles found in each dog. The majority of dogs, 351/428 (82%) appear to have just one *DLA-88* allele on each of their haplotypes. However, 77/428 (18%) of dogs show evidence of two *DLA-88* alleles on at least one of their haplotypes. Whether both alleles on a haplotype are expressed is currently unknown, and it is not yet possible to assign alleles to specific loci. There is some evidence that the same *DLA-88* allele can occur in combination with several different alleles, on different haplotypes.

There were 28 dogs that were homozygous for DLA class II but had two different *DLA-88* alleles present. To establish whether these dogs carried both of these *DLA-88* alleles on each haplotype, or whether they carried a single, different *DLA-88* allele on each haplotype, all the homozygous and heterozygous dogs were analysed. By pattern analysis we could show that 12 of these dogs had haplotypes that carried two alleles and that the other 14 had two identical class II haplotypes that carried single different class I alleles. Table 6.4 shows some examples of the same class II haplotypes carrying different class I alleles.

One explanation for the duplicated *DLA-88* genes is that the primers are amplifying some alleles from another class I locus, such as *DLA-64*, and, because the primers are not an exact match for *DLA-64*, not all alleles are amplified and so some dogs appear to have no alleles from that locus. This is unlikely, as we have examined genomic sequences from many dogs and showed that all the alleles we have defined as *DLA-88* are much more similar to *DLA-88* than to *DLA-64* or any of the other class I genes. While the class I genes are more similar in their exons, the introns are quite different and appear to have clear distinguishing features (Graumann *et al.*, 1998).

Table 6.3. Number of *DLA-88* alleles found on each haplotype in 299 different dogs that have been characterized for *DLA-88*.

DLA[a] class II genotype	No. of *DLA-88* alleles identified	Haplotypes present with duplicated *DLA-88* genes?	No. of *DLA-88* alleles on each haplotype	No. of dogs studied
Homozygous	1	No	1/1	100
Homozygous	2: a different one on each haplotype	No	1/1	14
Homozygous	2: the same two on each haplotype	Yes	2/2	12
Homozygous	3	Yes	1/2	2
Homozygous	4	Yes	2/2	1
Total				129
Heterozygous	1	No	1/1	38
Heterozygous	2	No	1/1	199
Heterozygous	3	Yes	1/2	57
Heterozygous	4	Yes	2/2	5
Total				299

[a]DLA, dog leucocyte antigen.

Table 6.4. DLA[a] class II haplotypes carrying different *DLA-88* alleles, identified in dogs that are homozygous for class II.

Haplotype	*DRB1**[b]	*DQA1**[b]	*DQB1**[b]	*DLA-88**[b]	*DLA-88**[b]
a	*001:01*	*001:01*	*002:01*	*002:01*	
b	*001:01*	*001:01*	*002:01*	*004:02*	
c	*001:01*	*001:01*	*002:01*	*006:01*	*046:01*
d	*001:01*	*001:01*	*002:01*	*501:01*	
e	*001:01*	*001:01*	*002:01*	*508:01*	
f	*006:01*	*005:01:1*	*007:01*	*004:02*	
g	*006:01*	*005:01:1*	*007:01*	*005:01*	
h	*006:01*	*005:01:1*	*007:01*	*006:01*	
i	*006:01*	*005:01:1*	*007:01*	*035:01*	
j	*006:01*	*005:01:1*	*007:01*	*501:01*	
k	*006:01*	*005:01:1*	*007:01*	*508:01*	

[a] DLA, dog leucocyte antigen.
[b] *DLA* allele names follow similar nomenclature to HLA (human leucocyte antigen) alleles. A typical example would be *DRB1*001:01*.

Inter- and intra-breed diversity

The existing data are not sufficient for describing *DLA-88* allele and haplotype frequencies for different breeds of dogs. However, preliminary observations indicate that within a breed will be two to four major haplotypes. For the relationship between class I and class II, we can show that one particular class II haplotype can carry different *DLA-88* alleles, and that within a breed there will tend to be only one of the possible combinations. Thus, when we consider the class II haplotye *DLA-DRB1*006:01–DQA1*005:01:1–DQB1*007:01* in 20 Beagles, seven had haplotype d and two had haplotype e (see Table 6.4). Conversely, in Cocker Spaniels, four different *DLA-88* alleles were found. Fourteen dogs had haplotype f, one had haplotype g, six had haplotype h and six had haplotype j (see Table 6.4). Obviously further work is necessary to characterize dog breeds.

DLA class II

Molecular characterization of DLA class II

Three DLA class II genes have been annotated and details of their structures can be found in *Ensembl* (http://www.ensembl.org) and some other genomic browsers. The *DLA-DRA* gene contains 5 exons (encoding 244 amino acids), *DLA-DQA1* also consists of 5 exons (encoding 269 amino acids) and *DLA-DRB1* – 8 exons (encoding 296 amino acids). Sequence variation in exon 2 is the major source of polymorphism in *DLA-DRB1* gene. These genes code for glucoproteins which are formed by two chains (α and β). Each chain is linked to the cellular membrane through a transmembrane region and expressed on a membrane of the so-called antigen-presenting cells capable of interacting with the helper T lymphocytes ($CD4^+$).

Many different molecular typing methods have been used to assess the polymorphism of DLA class II genes which were later superseded by DNA cloning and sequencing (Sarmiento *et al.*, 1990, 1992, 1993; Wagner *et al.*, 1996a,b, 1998, 2000; Kennedy *et al.*, 1999a, 2000b, 2005). The current method of choice is SBT, which was established years ago (Kennedy *et al.*, 1998).

A transcription control of DLA genes is necessary for a functional immune system, otherwise immunodeficiency and autoimmune syndromes seem to be inevitable. The polymorphism of the promoter regions of the *DLA-DRB1*, *DLA-DQA1* and *DLA-DQB1* loci was studied in wolves and dogs (Berggren and Seddon, 2005). The level of polymorphism was high in the *DLA-DQB1* promoters, including binding sites for transcription factors. Associations between *DLA-DQB1* promoters

and exon 2 alleles were noted in wolves, indicating strong linkage disequilibrium in this region. The *DLA-DRB1* and *DLA-DQA1* promoter regions have a low level of polymorphism. Also, a variable site was identified within a TNF-alpha response element of the *DLA-DQA1* promoter, as well as a previously unknown 18-base pair deletion within exon 1 of the *DLA-DQB1* locus (Berggren and Seddon, 2005).

Deviations from normal MHC expression patterns have been associated with autoimmune diseases, which occur frequently in several dog breeds. Further knowledge about these deviations may be helpful for understanding the aetiology of such diseases (Berggren and Seddon, 2008).

Polymorphism: alleles, haplotypes and linkage disequilibrium

The causes of polymorphism in molecules encoded by the class II genes are similar to those in class I, but the polymorphism is even more complex and generates huge variation. This is happening despite the fact that the *DLA-DRA* gene appears to be monomorphic in the domestic dog, and in other closely related canids. Sequence variation in exon 2 is the major source of polymorphism in the *DLA-DRB1* gene.

Numerous alleles have been discovered so far in the three highly variable DLA class II genes. All of these alleles have been officially named. Some of them are unique to particular species, but many are shared between different canids. In addition, there are many more unconfirmed (provisional) alleles that have only been found in a small number of dogs, which are awaiting confirmation (Table 6.5). The three class II genes, *DLA-DRB1, -DQA1* and *-DQB1*, are located in close proximity, and are subject to strong linkage disequilibrium. Overall, about 35% of dogs are homozygous for their class II alleles and it is, therefore, easy to identify three class II loci haplotypes (*DLA-DRB1–DQA1–DQB1*). Of the 176 official *DLA-DRB1* alleles, 107 are found in the dog only, 53 are found in one or more canid species, 11 are found in both the dog and other canids, and five have not been found since their original succession to GenBank. For the 31 *DLA-DQA1* alleles, there are six found only in the dog, 11 in other canids only, 11 are shared by the dog and other canids, and three have not been found again. For the 76 official *DLA-DQB1* alleles, 46 are found in the dog only, 14 are in other canids only, 12 are shared by the dog and other canids and four have not been found again (L.J. Kennedy, unpublished data).

Each *DLA-DRB1* allele is found in at least one haplotype, and many are found in several different haplotypes. Based on data from over 10,000 domestic dogs from 204 different dog breeds, there are 157 different haplotypes, each of which has been found in more than one homozygous dog, or at least three heterozygous dogs. There are 22 DLA class II haplotypes with a frequency greater than 1% in these 10,253 dogs. These are listed in Table 6.6, together with the number of breeds (n = 204) in which each haplotype has been found, and breeds with a high frequency (>40%) of that haplotype. Interestingly there are only three DLA class II haplotypes that are shared between the dog and the grey wolf (Kennedy *et al.*, 2007a), and two of these are in this group of 22 most frequent haplotypes in the domestic dog. These haplotypes are highlighted in bold in Table 6.6.

Table 6.5. Number of alleles at DLA[a] class II genes.

Locus	Official alleles: dog and other canids	Unconfirmed alleles: dog and other canids	Total
DLA-DRB1	176	65	241
DLA-DQA1	31	8	39
DLA-DQB1	76	41	117

[a] DLA, dog leucocyte antigen.

Table 6.6. Common class II DLA[a] haplotypes found in the domestic dog.

*DRB1** allele[b]	*DQA1** allele[b]	*DQB1** allele[b]	No. of haplotypes (10253)	Haplotype frequency (%)	No. of breeds	Breeds with high frequency (>40%) of this haplotype
001:01	*001:01*	*002:01*	1770	8.65	92	10 breeds
002:01	*009:01*	*001:01*	818	4.00	73	Shetland Sheepdog, Border Terrier
004:01	*002:01*	*015:01*	571	2.79	21	Boxer
006:01	*004:01*	*013:03*	548	2.68	20	Dobermann
006:01	***005:01:1***	***007:01***	**2067**	**10.10**	**104**	**Cocker spaniel + 5 other breeds; also found in grey wolf**
006:01	*005:01:1*	*020:01*	417	2.04	23	
009:01	***001:01***	***008:01:1***	**775**	**3.79**	**84**	**Miniature Schnauzer + 3 other breeds; also found in grey wolf**
011:01	*002:01*	*013:02*	389	1.90	28	German Shepherd Dog
012:01	*001:01*	*002:01* *013:03*	235	1.15	17	
012:01	*004:01*	*017:01*	1373	6.71	49	Hovawart
012:01	*004:01*	*013:03*	297	1.45	12	
013:01	*001:01*	*002:01*	564	2.76	52	
015:01	*006:01*	*019:01* *054:02*	262	1.28	4	Samoyed
015:01	*006:01*	*003:01*	750	3.66	57	Clumber Spaniel, Norwegian Elkhound
015:01	*006:01*	*020:02*	342	1.67	50	Weimaraner
015:01	*006:01*	*023:01*	1173	5.73	82	Poodle, Bichon Frise + 3 other breeds
015:02	*006:01*	*023:01*	1065	5.20	78	Norwich Terrier, Bulldog
018:01	*001:01*	*002:01*	238	1.16	20	Bearded Collie
018:01	*001:01*	*008:02*	640	3.13	40	Whippet, Greyhound, Bullmastiff
020:01	*004:01*	*013:03*	712	3.48	77	Norfolk Terrier, Saint Bernard
023:01	*003:01*	*005:01*	250	1.22	32	
025:01	*012:01:2*	*035:01*	242	1.18	8	Shih Tzu

[a] DLA, dog leucocyte antigen.
[b] DLA allele names follow similar nomenclature to HLA (human leucocyte antigen). A typical example would be *DRB1*001:01*.

A complete list of DLA class II (and class I) haplotypes is available on the Immuno-Polymorphism Database (IPD) website (www.ebi.ac.uk/ipd/mhc).

Analysis of the *DLA-DQ* haplotypes in the data set of the 10,253 dogs mentioned above shows that only certain *DLA-DQA1–DQB1* combinations occur, suggesting that the polymorphism of both chains (α and β, Fig. 6.1) affects the conformation of the molecules and only allows certain combinations to form viable heterodimers, as has been found in humans (Lotteau *et al.*, 1987; Kwok *et al.*, 1993). If a table is constructed of *DLA-DQA1* alleles against *DLA-DQB1* alleles, and the *DLA-DRB1* alleles are written in the appropriate intersecting box representing the three-locus haplotype (i.e. insert *DRB1*00101*, in the box where *DQA1*00101* and *DQB1*00201* intersect, and so on, for each three-locus haplotype), the most striking thing about the table is the large number of empty boxes (Table 6.7). This table includes 157 haplotypes which occur at a frequency of >0.01% in the total population, and excludes 143 haplotypes that had a frequency of 0.01%. Rather than list the *DLA-DRB1* alleles found with each *DLA-DQA1–DQB1* combination, we have indicated the number of different *DLA-DRB1* alleles that have been found with each *DQA1–DQB1* combination. The *DLA-DQA1–DQB1* combinations that intersect at an empty box space have not been seen to date in a three-locus haplotype. Full details of all these three locus haplotypes can be found on the IPD website.

Inter- and intra-breed diversity

It is clear from the previous sections of this chapter that there is a large amount of diversity in domestic dog breeds as a whole. Whenever a previously uncharacterized dog breed is tested for DLA, almost invariably new alleles are found (Kennedy *et al.*, 2002b, 2008b). There is clearly much diversity to be found in semi-tame and feral street dogs, as has already been demonstrated in Bali street dogs (Runstadler *et al.*, 2006). Similarly, according to preliminary data, indigenous dogs from other locations are very diverse; the trend is supported by observations of Australian dingoes and mongrels from Brazil (Kennedy *et al.*, 2002b). These dog populations are more outbred than most domestic dog breeds, and this is demonstrated when we assess the number of different haplotypes found in each group (where n = 50–100 dogs) and compare the frequency of the most common haplotypes. The highest haplotype frequency is around 12%, and there are, on average, 15 haplotypes with frequencies of 2–10%, with a further 30 or more haplotypes at lower frequencies. Dingoes appear to be an exception, as only five haplotypes were found.

Pure-bred dog breeds have quite different haplotype profiles (Kennedy *et al.*, 2002a). We analysed 42 breeds where n > 50 (the range was 50–1080) and found that, on average, each breed had seven haplotypes: one at a frequency of >20%, two with frequencies of 10–20% and four with frequencies of 2–10%. The frequency of the commonest haplotypes varied from 12–72% (Pestka *et al.*, 2004), with 15 breeds having a frequency for one haplotype of >40%. Most breeds have a 'tail' of haplotypes at frequencies of less than 2%, which in part might be explained by some inaccuracy in breed identification and possible crossbreeding. Groups of dogs that have been more thoroughly collected through breed clubs have much 'tighter' haplotype profiles and generally lack a tail of low-frequency haplotypes. At one extreme of the spectrum are Rottweilers, which have only two haplotypes at frequencies of 58.2% and 41.8%; at the other end of the spectrum there are breeds like the Husky, which has ten haplotypes with frequencies of >2%, although this is not considered to be a homogeneous breed in the strict sense, as they are dogs that are selected for endurance at pulling dog sleds (Huson *et al.*, 2010).

The Saluki is another extreme case; 228 dogs were analysed plus a further 26 closely related dogs. There was one major haplotype at a frequency of 37.9%, plus 12 haplotypes with frequencies of 2–10%, and a further 15 with frequencies of <2%. The majority of atypical haplotypes occur in at least two dogs, and often in 4–7 dogs. Many haplotypes represent new combinations of *DLA-DRB1* alleles with *DQA1–DQB1*, so that we see

Table 6.7. Three-locus DLA[a] class II haplotypes in domestic dog breeds: the numbers in the boxes represent the number of different *DRB1* alleles that are found with combination of *DQA1–DQB1.*

	*DQA1**														
*DQB1**	*001:01*	*002:01*	*003:01*	*004:01*	*004:02*	*005:01:1*	*006:01*	*009:01*	*010:01*	*012:01:1*	*012:01:2*	*015:01*	*018:01*	*021:01*	*New*
001:01								9							
002:01	14						1								
003:01							4								
004:01			5												
005:01			6												
005:02			1												
005:03			1												
007:01						5									
008:01:1	4														
008:01:2	4														
008:02	13											1			
011:01							1								
013:01				2											
013:02		2		1											
013:03		11		5											
013:03+017:01				2											
013:04		1													
013:05							1								
013:06				1											
015:01		3													
019:01									1						
019:01+054:02							1								
020:01						1	1								
020:02	1						6								
020:03							1								
022:01							3								
023:01				1	4		8								
026:01							1								
028:01						1									
031:01					1										
035:01										2	1				
036:01	1	1													
036:03	1														
037:01	1														
038:01			4												
044:01															1
048:01							1								
049:01							3								
050:01													1		
053:01														1	
054:01			1				4								
054:02					1										
057:01							1								
058:01							1								
060:01								1							
None					1										

[a]DLA, dog leucocyte antigen.

*DLA-DRB1*015:01* with *DQA1*004:01–DQB1*013:03–DQB1*017:01*, rather than *DLA-DRB1*012:01*. The Saluki is an ancient breed, but does not have a large population base, so why does this breed have so much more variation than any other breed? Perhaps it is not subject to such strict inbreeding as other breeds.

DLA class III gene polymorphisms

Proteins encoded by this group of genes are very different from DLA class I or class II proteins. However, this cluster of genes located between class I and II genes (Fig. 6.3) is traditionally designated as DLA class III. The genes comprising this group encode several proteins with immune functions like components of the complement system (such as C2, C4 and B factor) and cytokines such as TNFα, LTA (lymphotoxin alpha) and LTB (lymphotoxin beta), which are related to the inflammation process.

It is now becoming increasingly recognized that class III region genes can also be highly polymorphic in dogs, which is also the case in the human HLA system. This has been described for the canine *TNF*α gene, in which a considerable number of SNPs have been identified (Short, 2006; Barnes *et al.*, 2009). These polymorphisms, which exhibit linkage disequilibrium across the *TNF* SNP haplotypes, were described in several dog breeds (Short, 2006). Furthermore, these *TNF* haplotypes are found to be associated with distinct and particular class II haplotypes (Short, 2006; Short, personal communication). *TNF* haplotypes based on ten SNPS spanning the gene were observed in the German Shepherd Dog (Barnes *et al.*, 2009). Of these, four haplotypes were found to be common in this breed, with frequencies 8–39%. It is likely that *TNF* alpha haplotypes may differ in the production of cytokine following stimulation. This is the case in humans, where previous studies using human monocytes have demonstrated that the level of *in vitro* TNF production is dependent on the HLA class II background that the gene resides on (Jacob *et al.*, 1990). Similar functional studies are yet to be done in the dog. Another study has confirmed that polymorphism also exists within the canine 21-hydroxylase gene in the DLA class III region (Takada *et al.*, 2002), and it is likely that this will also be the case for other genes of the region.

Canine MHC associations with disease susceptibility and immune function

The domestic dog represents an ideal species for characterizing the genetic and environmental factors underlying the aetio-pathology of a wide range of diseases owing to its high levels of genetic homogeneity and consequent significant risk of developing certain diseases. Although there is a paucity of systematic epidemiological prevalence studies, anecdotally it is widely recognized by both the veterinary profession and by dog breed clubs that some diseases constitute a significant clinical problem in particular breeds. These include autoimmunity, hypersensitivity (including atopy), susceptibility to bacterial, viral, fungal, protozoan, endoparasitic and ectoparasitic infections, vaccination failures and adverse reactions, and the development of malignancies. Many such diseases are influenced by multiple genetic factors and environmental conditions. It has long been recognized that MHC genes in particular constitute a major genetic risk for many immune-mediated diseases. However, the precise mechanisms by which the MHC causes different diseases remains in many instances unknown. Studies of immune-mediated diseases in dogs are now providing an important comparative genetic approach for investigating homologous human diseases.

DLA and autoimmunity

Dogs can spontaneously develop a wide range of autoimmune conditions, most of which display great clinical similarity to conditions seen in man. Examples include Addison's disease, autoimmune lymphocytic thyroiditis (Hashimoto's thyroiditis), rheumatoid factor positive symmetrical polyarthritis (rheumatoid arthritis), dermatomyositis, systemic lupus erythematosus (SLE),

immune-mediated haemolytic anaemia, pemphigus, myasthenia gravis and immune-mediated diabetes mellitus. While most of these autoimmune conditions can occur in most dog breeds, many conditions appear to have a much higher prevalence in certain breeds or, alternatively, are extremely rare. A good example is the non-gestational form of diabetes mellitus in dogs; breeds such as the Samoyed are at high risk for the condition whereas, in stark contrast, diabetes is rarely, if ever, observed in the Boxer (Catchpole *et al.*, 2008). Some canine autoimmune conditions could prove to be important models for human autoimmune disease. For example, canine anal furunculosis, an immune-mediated condition largely found in German Shepherd Dogs, may represent an excellent model for a subset of human Crohn's disease patients who develop peri-anal complications (Galandiuk *et al.*, 2005).

The earliest investigations into DLA associations with canine autoimmune disease began with serology-based antigen definition. In 1990, an association between DLA-A7 and SLE was reported in German Shepherd Dogs (Teichner *et al.*, 1990). Modern methods have revitalized these investigations. A range of studies have now been conducted and the majority are summarized in Table 6.8. These studies reveal a number of important findings. First, a number of disease associations are being replicated and confirmed both in the same breeds and, in some cases, across different breeds. This is the case for autoimmune lymphocytic thyroiditis, in which the *DLA-DQA1*00101* allele is a risk allele across multiple breeds, and the *DLA-DRB1*012:01–DQA1*001:01–DQB1*002:01* haplotype was found to be associated with disease in both Dobermans and Giant Schnauzers (Kennedy *et al.*, 2006a; Wilbe *et al.*, 2010a). A similar situation has been reported for symmetrical lupoid onychodystrophy, in which the same class II risk haplotype was seen in both Gordon Setters and Bearded Collies (Wilbe *et al.*, 2010b). Although it is likely that some studies may reveal different DLA types associated with the same disease in different breeds (as has often been described in human HLA-disease studies), studies comparing across a number of breeds may help us to focus in on which particular locus represents the primary disease susceptibility factor.

Secondly, some DLA alleles and haplotypes are associated with multiple autoimmune diseases. This has also been reported for human HLA disease studies. For example, the *DLA-DRB1*015–DQA1*006–DQB1*023* haplotype was observed in both diabetes and Addison's disease, and *DLA-DRB1*015–DQA1*006* was observed in immune-mediated haemolytic anaemia (see Table 6.8). Similarly the *DLA-DRB1*006:01–DQA1*005:01:1* haplotype was seen in both immune-mediated haemolytic anaemia and SLE-related rheumatic disease. Of further possible interest is the observation that both *DLA-DRB1*002* and *DLA-DRB1*009* are associated with both polyarthritis and diabetes, while *DLA-DRB1*018* is seen in both polyarthritis and symmetrical lupoid onychodystrophy. In humans, it is known that some autoimmune diseases are more often found together in the same patients or their family members, and in some cases these have a common HLA association. We also know that some breeds of dog such as the Bearded Collie and Nova Scotia Duck Tolling Retriever appear to be high-risk breeds for a range of autoimmune conditions. The observations that we are now generating appear to reveal a similar situation in the dog. Similarly, in humans of Caucasian origin there appears to be one particular HLA haplotype (*HLA-A1, -B8, DRB1*03*), which is associated with a wide number of autoimmune conditions. From the dog data summarized in Table 6.8 it appears that the *DLA-DQA1*001:01* allele is associated with symmetrical lupoid onychodystrophy, autoimmune lymphocytic thyroiditis and anal furunculosis, suggesting that some alleles and haplotypes may carry a wide range of risk for autoimmunity in dogs.

Thirdly, the level of risk that DLA alleles and haplotypes appear to convey is more or less in line with the risk levels seen in human HLA-associated diseases, although these levels of risk are considerable compared with most other risk factors identified for complex conditions. As autoimmune diseases are recognized as being complex phenotypes, it is highly likely that the majority of genetic pre-

Table 6.8. DLA[a] associations with canine autoimmune condition.

Condition	DLA association			Comment	Breeds	Level of risk (odds ratio)	References
	*DRB1**	*DQA1**	*DQB1**				
Canine diabetes mellitus	*009:01*	*001:01*	*008:02*		Range of breeds;	2.1	Catchpole *et al.* (2005)
	015:01	*006:01*	*023:01*		Samoyed, Cairn and	1.5	Kennedy *et al.* (2006c)
	002:01	*009:01*	*001:01*		Tibetan Terriers	1.5	Kennedy *et al.* (2007d)
Addison's disease	*015:02*	*006:01*	*023:01*		Nova Scotia Duck Tolling Retriever	6.7 for homozygotes	Hughes *et al.* (2010)
Primary immune-mediated haemolytic anaemia	*006:01*	*005:01:1*	*007:01*		Range of breeds	1.8	Kennedy *et al.* (2006d)
	015:01	*006:01*	*003:01*			2.6	
	001:01						
SLE[b]-related immune-mediated rheumatic disease	*006:01*	*005:01:1*	*007:01*	Rheumatoid arthritis (RA) shared epitope (RARAA)	Nova Scotia Duck Tolling Retriever	4.9 for IMRD; 7.2 for ANA positive	Wilbe *et al.* (2009)
Chronic inflammatory hepatitis	*006:01*	*004:01*	*013:03*		Dobermann	14.9 for homozygotes	Dyggve *et al.* (2011)
Canine polyarthritis	*002:01*			RA-shared epitope (QRRAA, RKRAA)	Range of breeds	2.4	Ollier *et al.* (2001)
	009:01					4.3	
	018:01					3.5	
Symmetrical lupoid onychodystrophy	*018:01*	*001:01*	*008:02*		Gordon Setter	2.1;	Wilbe *et al.* (2010b)
	018:01	*001:01*	*008:02*		Bearded Collie	5.4 for homozygotes	
	018:01	*001:01*	*002:01*		Bearded Collie		
	001:01	*001:01*	*002:01*		Giant Schnauzer		
Autoimmune lymphocytic thyroiditis		*001:01*			Range of breeds	2.3	Kennedy *et al.* (2006b)
		001:01			Range of breeds	1.7	Kennedy *et al.* (2007d)

Continued

Table 6.8. Continued.

Condition	DLA association			Comment	Breeds	Level of risk (odds ratio)	References
	*DRB1**	*DQA1**	*DQB1**				
	012:01	*001:01*	*002:01*		Doberman	2.4	Kennedy *et al.* (2006a)
	012:01	*001:01*	*002:01*		Giant Schnauzer	6.5	Wilbe *et al.* (2010a)
Anal furunculosis	*001:01*	*001:01*	*002:01*		German Shepherd Dog	5.1	Kennedy *et al.* (2008a), Barnes *et al.* (2009)
Necrotizing (MS[c]-like) meningoencephalitis	*010:01:1*	*002:01*	*015:01*		Pug Dog	12.7 for homozygotes	Greer *et al.* (2010)
Uveodermatologic (VKH[d]-like) syndrome		*002:01*			Akita	15.9	Angles *et al.* (2005)
Canine chronic superficial keratitis	*015:01*	*006:01*	*003:01*		German Shepherd Dog	8.5 for homozygotes	Jokinen *et al.* (2011)
Meningoencephalitis	*018:02*	*001:01*	*008:02*		Greyhound	4.7	R. Shiel, personal communication

[a] DLA, dog leucocyte antigen.
[b] SLE, systemic lupus erythematosus.
[c] MS, multiple sclerosis.
[d] VKH-like, Vogt–Koyanagi–Harada-like.

disposition will lie with genes outside the DLA complex. Whole-genome association studies of human autoimmune diseases are now beginning to identify a substantial number of susceptibility genes, some of which appear to be common to a range of conditions. International collaborative studies of dog autoimmunity in Europe will hopefully reveal similar effects.

DLA studies of dog autoimmunity are also showing that some DLA alleles and haplotypes confer protection against disease rather than causing susceptibility. Examples of this are reported in a number of studies listed in Table 6.8. A strong protective effect was observed for *DLA-DRB1*020:01–DQA1*004:01–DQB1*013:03* in canine symmetrical lupoid onychodystrophy (Wilbe *et al.*, 2010b) and for *DLA-DQA1*009:01–DQB1*001:01* in canine hepatitis (Dyggve *et al.*, 2011). It is important to interpret resistance effects with caution; if a disease susceptibility allele is at very high frequency in some cases then, by default, this will mean that frequencies of some or all other alleles will be reduced compared with controls. However, protective alleles and haplotypes are likely to be real in many cases and such effects have been confirmed in human studies. It is possible that the highly protective DLA haplotypes seen in some breeds may go some way to explaining why such breeds are highly resistant to some conditions. It is clear that the Boxer breed is almost completely resistant to diabetes mellitus, and future studies may attribute at least some of this protection to the DLA profile of the breed. A recent study has used SNP and linkage disequilibrium analysis to examine the relationship between DLA class II haplotypes and their ability to confer either susceptibility or resistance to diabetes mellitus (Seddon *et al.*, 2010). This study revealed that exon 2 haplotypes have arisen through both recombination and convergence events. A region of fixed differences in SNPs across the DQ region was observed for exon 2 haplotypes associated with diabetes susceptibility and resistance, suggesting that a region outside exon 2 may be implicated in this condition. This study went on to identify four *DLA-DQB1* promoter alleles that were restricted to diabetic dogs.

As recombination/gene conversion is a relatively common event leading to diversity, this may present a better explanation for DLA-encoded disease susceptibility rather than fixation on a novel allele.

DLA associations with other conditions

There is now increasing evidence to demonstrate that genes within the DLA region are associated with a range of other immune-mediated conditions and functions. One such area is in the field of canine cancers, where clear associations are becoming apparent. A recent study has reported an association between the *DLA-DQB1*007:01* allele and anal sac gland carcinoma in the Cocker Spaniel (Aguirre-Hernandez *et al.*, 2010). Canine infections and parasitic conditions are likely to be other areas in which a DLA contribution to susceptibility/resistance will be seen. A previously reported study has demonstrated that susceptibility to visceral leishmaniasis in the domestic dog is associated with a particular class II polymorphism (Quinnell *et al.*, 2003). This study examined the relationship between DLA and the natural course of infection in Brazilian mongrel dogs exposed to *Leishmania infantum*. Dogs carrying the *DLA-DRB1*015:02* allele were significantly associated with higher levels of anti-leishmania IgG and an increased risk of being parasite positive compared with animals without this allele. A recent study has also reported an involvement of DLA polymorphisms in canine juvenile generalized demodicosis (It *et al.*, 2010); using DLA class II microsatellite markers, highly significant associations were seen between microsatellite alleles and demodicosis in the Boxer, Argentinean Mastiff and mixed breeds.

An important clinical area which remains poorly researched relates to DLA in relation to vaccine response. Appropriate vaccination is vital for maintaining good health in dogs and for reducing the development of infections, some of which can lead to zoonotic infections in man. Dogs are often vaccinated regularly and concerns exist both with regard to whether the adverse

reactions seen in some animals are related to hyper-responsiveness/hypersensitivity and also to whether other animals might be hypo-responders and constitute vaccine failures. Given what is already known from other species about the role of MHC genes in determining immune response level, we should expect a similar relationship for DLA genes in the dog. Furthermore, given the major differences that we have documented in terms of frequencies of DLA allele and haplotypes across different breeds, it would be unusual if the risk of hyperimmune or hypo-immune responsiveness to particular vaccine formulations was not a reality. A possible role for DLA in the lower responses seen in Dobermans and Rottweilers to rabies vaccination has been speculated upon (Kennedy *et al.*, 2007e).

T Cell Receptors (TCRs) and Other T Cell Surface Proteins

T cells and TCR receptors

Thymus-derived (T) lymphocytes play an important role in the immune system. T cells not only control antibody production by B cells but can also regulate cellular immune responses. T cells can recognize foreign antigens (e.g. from virus-infected or tumour cells) in the context of self-MHC molecules and are capable of killing infected cells (Janeway and Travers, 1997).

In the vertebrate immune system, there are three major types of T cells: (i) T-helper cells; (T_H); (ii) cytotoxic T lymphocytes; and (iii) $\gamma\delta$ cells (Janeway and Travers, 1997). The T cells interact with antigen-presenting cells via their TCR protein molecules, which are found on the surfaces of these cells. These molecules are responsible for recognizing antigens, which are bound to DLA molecules.

The TCR of $CD4^+$ T cells (MHC class II restricted) and the TCR of $CD8^+$ T cells (MHC class I restricted) consist of an $\alpha\beta$ heterodimer (Fig. 6.1) encoded by two separate genes. The vast majority of T cells carry TCRs composed of α and β chains, and only a small minority of these cells have TCR molecules with γ/δ chains. The TCR γ and δ chains are also encoded by separate genes.

The variety of TCR molecules produced by an individual is huge. As follows from Table 6.1, the polymorphism is generated in somatic cells undergoing differentiation and is caused by the gene rearrangements. This process has many similarities with the one occurring in the B cells that produce immunoglobulins (see the following section on immunoglobulins, including Fig. 6.5).

In mammals, the α, β, γ and δ chains are composed of variable (V) and constant regions (C) separated by a joining (J) region, just like immunoglobulin molecules (Janeway and Travers, 1997). While a great deal of progress has been achieved in studying canine TCRs, further investigations are desirable. The C regions of the α and the β chains were cloned and sequenced long ago (Ito *et al.*, 1993; Takano *et al.*, 1994). These C regions have 46% and 84% amino acid homology, respectively, with the corresponding human sequences. The V regions of the TCR chain have also been cloned and sequenced, and seven distinct genes have been identified (Dreitz *et al.*, 1999). The partial mRNA sequences of the TCR V and J regions of the chains were also cloned long ago and were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) in 1999 by Avery and Burnett (unpublished). As far as the annotation of the dog genome goes, all relevant genes have become available for comparisons and further analysis. The TCR genes encoding the V region of the chain (*TRAV*) are located on chromosome 8.

As in other mammals, recombination between the V, D (diverse) and J gene segments (V(D)J recombination) is a key process generating an enormous variability in TCR and Ig molecules during the differentiation of T and B lymphocytes (see the following section on immunoglobulins). Comparison of information from the Ensembl and GenBank databases has revealed major differences in the dog TCR beta (TRB) region annotations (Matiasovic *et al.*, 2009). The TRB genes encoding the V region of the chain (*TRBV*)

are located on chromosome 16. While the human–dog TRB sequence comparison shows a significant similarity, there is only one cluster of DJC segments in dogs. The 38 canine V segments are followed by one D segment, six J segments and one C segment (Matiasovic *et al.*, 2009). As in the human and murine clusters, the dog also has an additional V segment in opposite orientation downstream of the C segment.

The canine genome sequence was used for deducing the structure and the putative origin of the TCR gamma (TRG) locus (Massari *et al.*, 2009). Forty variable (*TRGV*), joining (*TRGJ*) and constant (*TRGC*) genes are organized into eight cassettes aligned in tandem in the same transcriptional orientation. Each cassette contains the basic recombinational unit V-J-J-C, except for a J-J-C cassette, which lacks the V gene and occupies the 3′ end of the locus. The canine TRG locus is located on chromosome 18 and spans about 460 kb. Eight of the 16 *TRGV* genes are functional and they belong to four different subgroups. Each cassette has two *TRGJ* genes and some of them are functional. The germ-line configuration and the exon–intron organization of the eight *TRGC* genes have been determined, and six of them are functional (Massari *et al.*, 2009). The low ratio of functional genes to the total number of canine *TRG* genes (21/40) is an interesting and surprising feature.

Rearranged TCR genes can be used as markers for malignant T cells (Dreitz *et al.*, 1999).

Other T cell surface proteins

There are other cell-surface proteins on T cells beside the TCRs that are of immunological importance and have been cloned. CD4, which is an accessory molecule for TCR–MHC–antigen recognition, has been cloned and sequenced in the dog (Gorman *et al.*, 1994). Unlike the situation in humans, the CD4 antigen is expressed not only on T_H cells but also on canine neutrophils (Williams, 1997). The CD8 antigen, which has the same function as the CD4 antigen, but is expressed on cytotoxic or suppressor T cells, has also been cloned in the dog (Gorman *et al.*, 1994). Other canine T cell surface proteins that have been cloned include CD28 (Pastori *et al.*, 1994) that binds CD80, CD38 (Uribe *et al.*, 1995) and CD44 (Milde *et al.*, 1994).

Monoclonal antibodies are useful for studying the distribution and expression of these receptors. The CA15.8G7 monoclonal antibody (Moore and Rossitto, 1993) recognizes TCRαβ, and CA20.8H1 recognizes TCRγ (Moore *et al.*, 1994). Analysis of T cell receptor proteins, as well as other proteins, can aid in the study and diagnosis of canine leukaemias and lymphomas (Vernau and Moore, 1999). Monoclonal antibodies that recognize several other canine T-cell surface proteins are available and have been summarized in various workshops (Cobbold and Metcalf, 1994; Williams, 1997). Many antibodies have been found by screening monoclonal antibodies that recognize human proteins for cross-reactivity with canine proteins (Chabane *et al.*, 1994).

Biller *et al.* (2007) investigated the expression of the X chromosome-linked *FOXP3* gene in healthy and cancer-affected dogs. Attention was given to regulatory T cells (Treg), which are a distinct group of T lymphocytes with immunosuppressive properties that normally prevent harmful autoimmune responses. However, Tregs can also interfere with beneficial anti-tumour and antiviral immune responses. FoxP3, a transcription factor, can be used for the identification of these cells in dogs. A cross-reactive FoxP3 antibody identified a subset of CD4(+) T cells in the blood and lymph nodes of dogs. The mean percentage of FoxP3(+)–CD4(+) T cells in normal dogs was 4.3% in blood and 9.8% in the lymph nodes. In dogs with cancer, the numbers of Treg cells were higher both in the blood (7.5%) and in tumour-draining lymph nodes (17.1%). TCR activation, together with addition of TGF (transforming growth factor) beta and IL-2 (interleukin 2) had an activation effect on FoxP3(+)–CD4(+) T cells in dogs.

Immunoglobulins

Immunoglobulins – an overview

Immunoglobulins (Ig), also called antibodies, are glycoproteins that mediate humoral immunity and are produced by B lymphocytes. Activated B cells differentiate into immunoglobulin-producing plasma cells. Immunoglobulins produced by one plasma cell are normally specific for a single antigen. The basic structure of all immunoglobulin molecules is a Y-like unit consisting of two identical light chains and of two identical heavy chains linked together by disulfide bonds (Figs 6.2, 6.4a–c). Heavy and light chains have N-terminal variable (V) and C-terminal constant (C) regions. Each immunoglobulin molecule is bifunctional: the V region of the molecule binds to the antigen while the C region mediates binding of the immunoglobulin to host tissues, including various cells of the immune system and the first component of the classical complement system. The class and subclass (also called isotype) of an immunoglobulin molecule is determined by its heavy chain type. The different isotypes are associated with different immunoglobulin functions. Mammals express some or all of the five known immunoglobulin classes: IgM, IgD, IgG, IgA and IgE. Furthermore, different subclasses of immunoglobulins are often found, as, for example, IgG1, IgG2, IgG3 and IgG4 in humans.

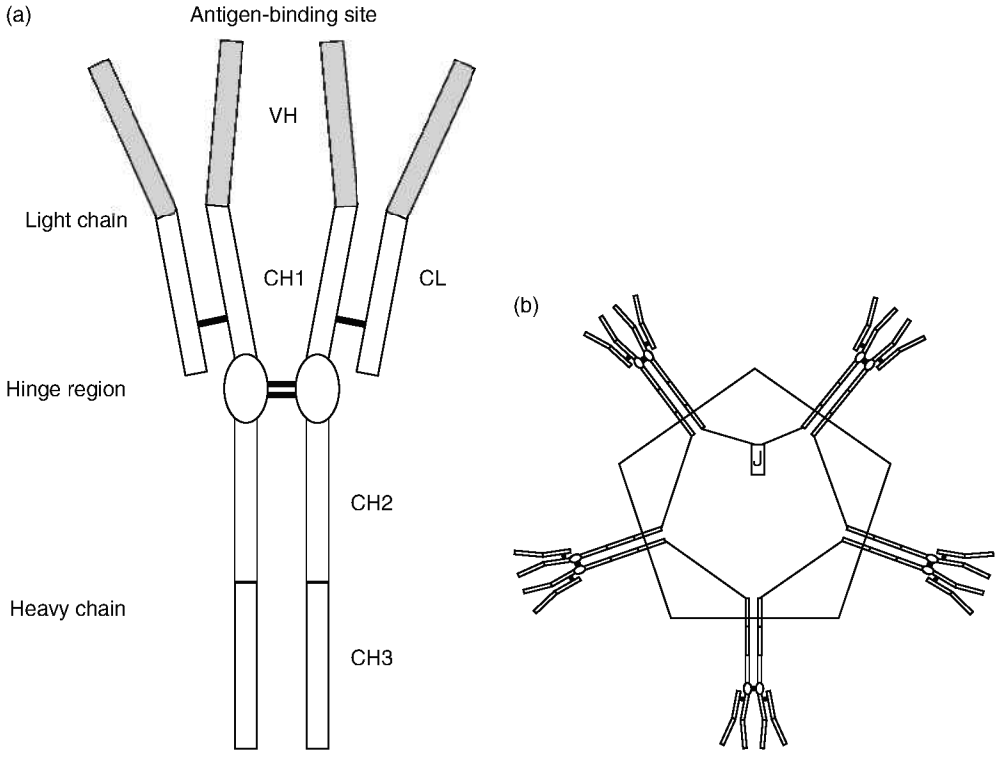

Fig. 6.4. Immunoglobulin structure: (a) IgG, (b) IgM and (c) IgE. Immunglobulins consist of two identical light chains and two identical heavy chains linked together by disulfide bonds. The heavy (H) and light (L) chains both have N-terminal variable (V) and C-terminal constant (C) regions. The number of constant region (CH) domains varies between immunoglobulin classes. IgG has three CH domains (CH1–CH3), and IgE and IgM each has four CH domains (CH1–CH4). The secreted form of IgM is a pentamer linked by disulfide bonds into a circle, which is completed by a small peptide (the J chain) that binds two of the units together, as shown in (b).

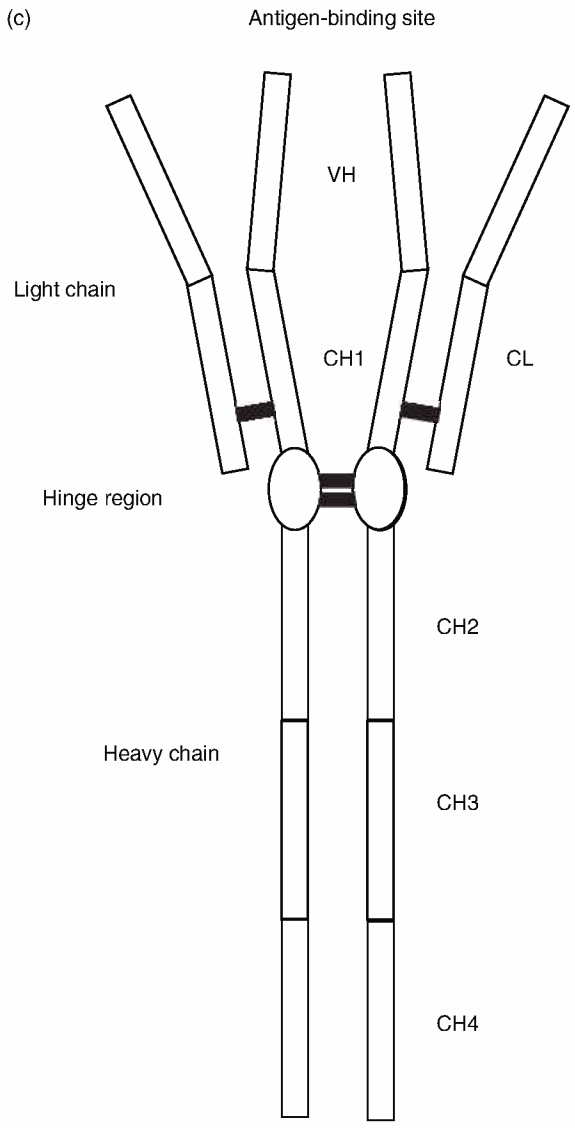

Fig. 6.4. Continued.

The immunoglobulin subclasses can have various biological activities. The differences between the various subclasses within an immunoglobulin class are less than the differences between the different classes. Unlike the immunoglobulin classes, the number and properties of the subclasses vary greatly between species.

IgG (which has a gamma (γ) heavy chain) is the major immunoglobulin in serum, and accounts for about 70–75% of the total immunoglobulin pool in humans. IgG has a molecular weight of approximately 180 kDa and consists of three heavy chain constant region domains (Fig. 6.4a). In the dog, four IgG subclasses, IgG1, IgG2, IgG3 and IgG4, have been defined based on their electrophoretic mobilities and on data from chromatography (Mazza and Whiting, 1994).

IgM (which has a mu (μ) heavy chain) accounts for about 10% of the immunoglobulin

pool in humans and is the class that predominates in a primary immune response, i.e. after the first contact of the immune system with an antigen. While it is on the B cell surface, IgM is a single 180 kDa monomer. However, the secreted form of IgM is a polymer consisting of five 180 kDa subunits linked by disulfide bonds in a circle. A small peptide called the J chain binds two of the units to complete the circle (Fig. 6.4b). The molecular weight of IgM is 900 kDa, and it is the major immunoglobulin produced during the primary immune response. Compared with IgG, IgM has a fourth constant domain but does not contain a hinge region.

IgA (which has an alpha (α) heavy chain) is the predominant immunoglobulin in seromucous secretions such as saliva, tracheobronchial secretions, milk and genito-urinary secretions. Mucosal IgA mainly consists of dimers and some larger polymers. This is the major source of IgA present in the sera of most animals, including dogs, while the serum IgA of humans and other primates is chiefly monomeric (reviewed in Snoeck *et al.*, 2006). IgA represents 15–20% of the human serum immunoglobulin pool. While two IgA subclasses are present in humans, only one single gene has been described for the alpha heavy chain constant region (*Cα*) in dogs (Patel *et al.*, 1995). However, four allelic variants of canine IgA have been identified that differ in the length of the hinge region (Peters *et al.*., 2004). A selective IgA deficiency has been described in the dog (Felsburg *et al.*, 1987; Littler *et al.* 2006).

IgE (which has an epsilon (ε) heavy chain) is only present in traces in serum and is mainly bound on the surface of basophils and mast cells. It has a molecular weight of 190 kDa and consists of four heavy chain constant domains (Fig. 6.4c). IgE plays an important role in defence against endoparasites and in the pathogenesis of allergic diseases, which are rather frequent in the dog; this explains the comparatively good knowledge of canine IgE. A study by Peng *et al.* (1997) showed the functional and physical heterogeneity of canine IgE, suggesting that dogs may have two IgE subclasses, IgE1 and IgE2. A canine IgE monoclonal antibody specific for a filarial antigen has been produced (Gebhard *et al.*, 1995), as well as recombinant fragments of the constant region of the IgE heavy chain (Griot-Wenk *et al.*, 1998; Ledin *et al.*, 2006). These reagents will facilitate the study of allergic diseases in the dog.

IgD (which has a delta (δ) heavy chain) is expressed as an antigen receptor on naive B cells. It accounts for less than 1% of total plasma immunoglobulins in humans, but is present in large quantities on the membrane of many circulating B lymphocytes. It plays a role in the antigen-triggered lymphocyte differentiation. In contrast to reports in the earlier literature, there is growing evidence that IgD is present in most mammals and even in fish, and is probably functionally important (Rogers *et al.*, 2006). An immunoglobulin IgD-like molecule was first identified in the dog by Western blot analyses by Yang *et al.* (1995). Canine IgD was then characterized at the molecular level by Rogers *et al.*, (2006). Canine, like human, IgD consists of three heavy chain constant region domains and a long hinge. It is not known yet whether the protein detected by Yang *et al.* (1995) corresponds to the one that would be produced by the canine heavy chain constant delta gene (*IGHD*) described by Rogers *et al.* (2006).

All the immunoglobulin classes that can be detected in the dog are summarized in Table 6.9. The genetic organization and regulation of immunoglobulins are among the most complex systems yet known. Antibodies have to be so diverse that they can recognize millions of antigens. What is more, the class of antibody changes during the course of an antibody response (a class switch), although the antigen-binding ability does not. Thus, a B cell will first make IgM and/or IgD. Eventually, the responding B cell switches to synthesizing either IgG or IgA or IgE. The unwanted heavy chain constant region (C_H) genes are excised, and the required C_H gene is spliced directly to the *V* genes (Esser and Radbruch, 1990).

Immunoglobulin heavy chain genes

The immunoglobulin heavy chain results from the expression of different variable and constant region genes on the immunoglobulin heavy chain locus (the *IgH* locus) after recombination has occurred. The genes coding for the variable region consist of *V* (variability), *D* (diversity) and

J (joining) genes. About 100 different V_H genes have been characterized in humans. They are located at the 5′ end of the *IgH* locus. They are followed in the 3′ direction by *D* genes (four in humans) and by *J* genes (nine in humans). As far as is known today, the organization of the *VDJ* genes is similar between species, but the number of *V, D* and *J* genes at the *IgH* locus varies from species to species. The genes coding for the constant region of the immunoglobulin heavy chain (also called C_H genes, as above) follow the *VDJ* genes in the 3′ direction. The heavy chain from each immunoglobulin isotype is coded by its particular C_H gene (Esser and Radbruch, 1990).

A recent analysis of the canine genome sequence of the *VH* gene segments showed the presence of eighty *VH*, six *DH* and three *JH* genes, mapping to a 1.28 Mb region of canine chromosome 8 (Bao *et al.*, 2010). Thirty nine of the *VH* genes were identified as pseudogenes and 41 as potentially functional. Analysis of the sequence similarities suggest that they belong to three VH gene families, but that the majority belongs to the VH1 family. This was confirmed by the sequencing of over 100 randomly selected cDNA clones containing almost full-length VH, DH and JH segments, and C_H genes representative of all five immunoglobulin classes. All of the sequences except one (the VH2 family) originated from the canine VH1 family. Furthermore, the generation and validation of canine single-chain variable fragment phage display libraries confirmed that VH sequences belonging to the VH1 family predominate (Braganza *et al.*, 2011). This suggests that the canine VH repertoire seems to be derived from limited germ-line gene families, and its diversity may be achieved through junctional diversity and somatic hypermutation (SHM).

The canine IgA and IgE C_H genes have been cloned and sequenced (Patel *et al.*, 1995) and mapped to chromosome 8 (Priat *et al.*, 1998; Mellersh *et al.*, 2000). The canine IgA C_H gene codes for a protein of 343 amino acids which displays 57–82% identity with the corresponding human sequence (depending on the subclass and allotype of the human IgA), 72% identity with the bovine sequence, 70% with the ovine sequence, 69% with the pig sequence, 61% with the mouse sequence and 57% with the rabbit sequence.

The constant region of the canine IgE heavy chain is 426 amino acids long. Comparison with the corresponding amino acid sequences of other species shows that dog IgE C_H has the highest identity with cat IgE C_H (76%) followed by horse (64%), pig (60%), human (55%), sheep (54%), bovine (53%) and mouse (48%). The canine high-affinity receptor for IgE has been cloned and sequenced (Goitsuka *et al.*, 1999). Knowledge of the DNA sequence of the canine IgE high-affinity receptor gene may be useful in searching for genetic markers associated with genetic predisposition to IgE-mediated allergic diseases (atopy) in the dog (de Weck *et al.*, 1997). In humans, genetic linkage has been demonstrated between atopy and the beta subunit of the high-affinity IgE receptor (Sandford *et al.*, 1993).

Canine IgD consists of three CH domains and a long hinge. Its structure is thus similar to those of primates, horses, cows and sheep, but not to those of mice and rats, in which IGHD has no CH2 domain (Rogers *et al.*, 2006). The canine IGHD CH1, hinge, CH2 and CH3 domains are most similar to the corresponding domains of the horse, with percentage identities of 40, 37, 58 and 67%, respectively.

While papers analysing the genetic organization of the canine IgG and IgM C_H genes have not been published to date, the amino acid sequence of the constant heavy chain region of canine IgM was published long ago (Wasserman and Capra, 1978; McCumber and Capra, 1979).

Immunoglobulin light chain genes

Immunoglobulin light chains are common to all classes of immunoglobulins. They also consist of a variable region, coded by *V* and *J* genes, and a constant region, coded by *kappa* or *lambda* genes. All species possess two classes of light chains, kappa and lambda. DNA sequences from the kappa and lambda chains of the dog have been determined. As shown immunohistochemically, tissue from the tonsils, spleen and cervical lymph nodes from normal dogs express mainly lambda (>91%) and rarely kappa light chains (9%) (Arun *et al.*, 1996), and this seems to have been confirmed

in a recent study (Braganza *et al.*, 2011). Conversely, in pigs and humans, the kappa/lambda ratio is more or less balanced (Arun *et al.*, 1996).

In humans and many other species, immunoglobulin light chains are encoded on other chromosomes than the heavy chain. The dog *Ig kappa* gene is on chromosome 17 and the *Ig lambda* gene is on chromosome 26.

V(D)J recombination

In humans and mice, and to a lesser extent in the dog, recombination of the variable (V), diversity (D) and joining (J) gene segments at the heavy chain locus and of the V and J gene segments of the light chain locus are used to generate the extremely high diversity at the antigen-binding site. The first recombination of Ig genes occurs at the heavy chain locus, followed by recombination at the light chain locus. Recombination at the heavy chain locus of an Igμ heavy chain is depicted in Fig. 6.5, and is briefly summarized here. During V(D)J recombination, gene segments encoding V, D and J proteins must be rearranged and brought into proximity for transcription and translation. This recombination brings together one D and one J segment, with deletion of the intervening DNA. The D segments that are 5′ of the rearranged D, and the J segments that are 3′ of the rearranged J segment, are not affected by this recombination. After the D–J recombination event, one of the many 5′ *V* genes is joined to the DJ unit, resulting in a rearranged VDJ exon. All V and D segments between the rearranged *V* and *D* genes are deleted. The heavy chain C region exons remain separated from the VDJ complex by DNA containing the distal J segments and the J-C intron. The rearranged Ig heavy chain gene is transcribed to produce a primary transcript that includes the rearranged VDJ complex and the Cμ exons. A poly-A tail is added to the 3′ end of the Cμ RNA. The nuclear RNA undergoes splicing, i.e. the introns are removed and the exons joined together. In the example shown in Fig. 6.5, introns between the VDJ exon and the first exon of the Cμ locus, and between each of the following constant region exons of the Cμ, are removed, giving rise to a spliced mRNA of the μ heavy chain. Translation of the rearranged μ heavy chain mRNA will then lead to synthesis of the μ protein. At the next stage, a kappa or lambda light chain is rearranged and a light chain is produced, which will associate with the previously synthesized μ chain to produce a complete IgM protein. DNA recombination at the light chain locus occurs in a similar manner. As there are no D segments in the light chain loci, recombination only involves the joining of one V to one J segment, forming a VJ exon.

Two groups of enzyme genes are critical for these gene rearrangement events: recombinase-activating genes 1 and 2 (*RAG1* and *RAG2*) and the deoxyribonucleic acid (DNA)-dependent protein kinase (DNA-PK) gene (*PRKDC*). V(D)J recombination is initiated by the binding of RAG-1 and RAG-2 to recombination signal sequences that flank these gene segments. DNA-PK acts after RAG-1 and RAG-2 and anneals severed DNA to produce genes that encode the antigen-specific TCR and surface immunoglobulin receptors of mature lymphocytes. T and B lymphocyte precursors that successfully complete V(D)J recombination and migrate to peripheral lymphoid tissue communicate through the release and binding of various interleukins through specific interleukin receptors. The receptors for some of these interleukins contain a common γ chain (γc). This γc is essential for lymphokine-dependent signal transduction. Mutations in *RAG, DNA-PK* or γc genes result in severe combined immunodeficiency (SCID), which has been described in mice, humans, horses and dogs. Three distinct molecular mechanisms resulting in canine SCID have now been described. A *RAG1* mutation has recently been identified as causing SCID in Frisian Water Dogs (Verfuurden *et al.*, 2011). SCID in Jack Russell Terriers is due to near absence of DNA-PK activity and is caused by a point mutation resulting in a premature stop codon in the DNA-PKcs (DNA-PK catalytic subunit) gene (Meek *et al.*, 2001; Bell *et al.*, 2002); mutations within this gene also cause murine and equine SCID (Blunt *et al.*, 1995; Shin *et al.*, 1997). Finally, two different mutations in the gene encoding the γ chain (γc) of the IL-2 receptor are responsible for X-linked SCID in Basset Hounds and Cardigan Welsh Corgis (Henthorn *et al.*, 1994; Somberg *et al.*, 1995; Pullen *et al.*, 1997) and are responsible for most X-linked SCID in children (Puck, 1999).

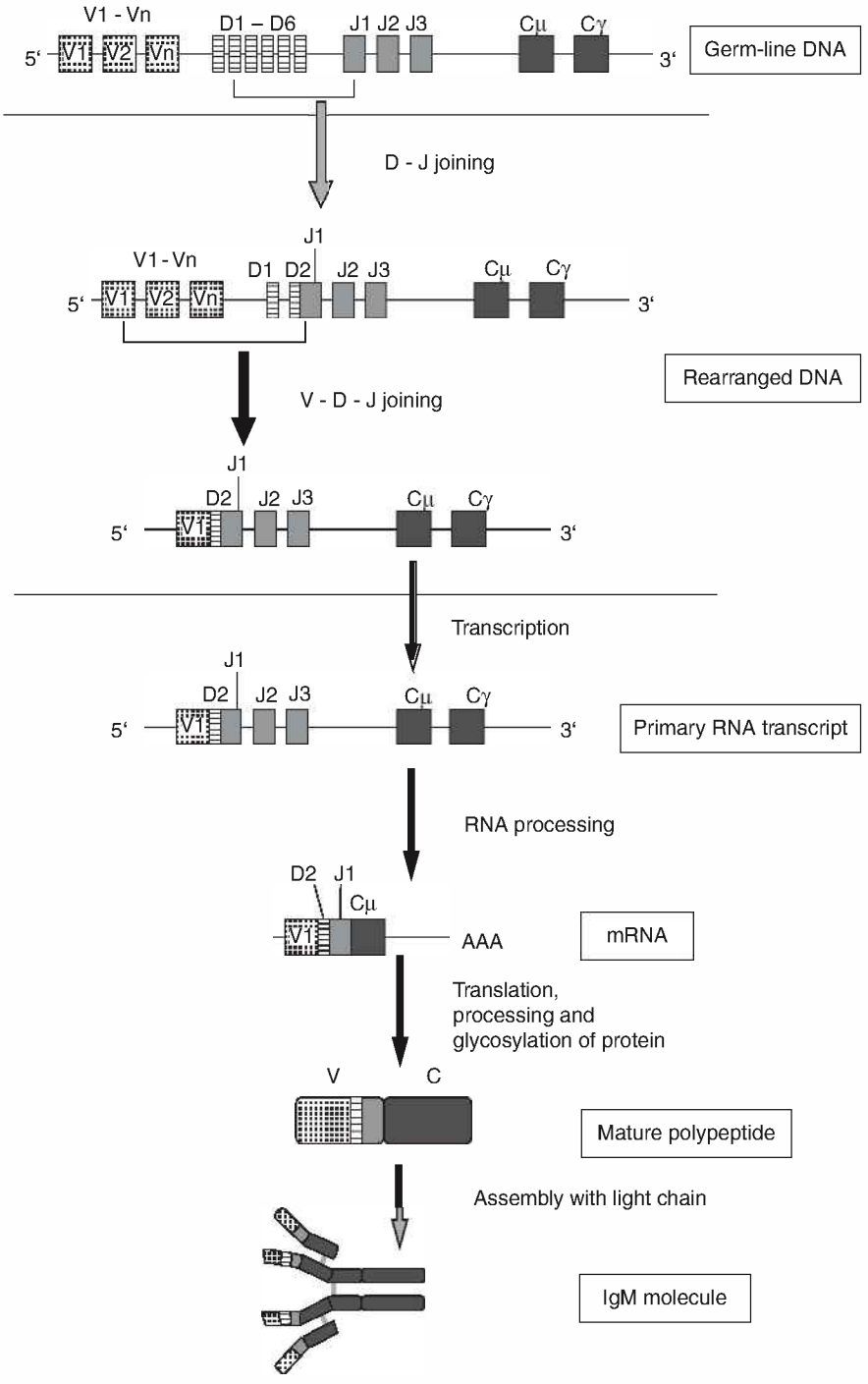

Fig. 6.5. Gene recombination and expression events are shown for an Igμ (Ig mu) heavy chain. In this example, the variable region is encoded by the exons V1, D2 and J1. First, the D2 and the J1 segments are brought together, with deletion of the intervening DNA. The D segments that are 5′ of the rearranged D and

Dog Cytokine and Chemokine Immunogenetics

Overview

Cytokines and chemokines are proteins or glycoproteins produced and released by a wide range of cell types. The regulatory role of cytokines includes cellular differentiation, tolerance, immunity and memory. The term chemokine has been attributed to immunologically active proteins that attract leucocytes to sites of inflammation. There is also evidence that some chemokines contribute to T-cell activation, B-cell antibody class switching and dendritic cell maturation, in addition their chemotactic functions (Balkwill, 2003). Chemokines are grouped based on their protein structure. The CC chemokines contain four cysteine residues, while the CXC chemokines have four cysteines with an intercalating amino acid. The four cysteines form two disulfide bonds (Renmick, 2005).

Cytokines and chemokines are often highly effective at low concentration. They are produced transiently and, in some instances, they can act systemically to generate immune and widespread physiological effects, e.g. the role of IL-6 (interleukin 6) cytokine in the generation of the acute-phase response. Other cytokines act locally on the tissues in which they are produced, and some have a relatively short half-life. Many cytokines are self-regulatory and are capable of inducing their own expression via autocrine or paracrine methods. Cytokines and chemokines manifest their effects on cells through their ability to indirectly precipitate upregulation or downregulation of specific genes. They initiate intracellular secondary signalling messengers by binding to specific cell-surface receptors on their target cells. In so doing, they initiate phenotypic changes within the cell by altering gene regulation. Many cytokines function as part of a cascade system involving the innate and adaptive immune system. A significant level of functional redundancy is seen between some cytokines in which there is a level of overlap or duplication of action. Thus, cytokines can be thought of as belonging to an overarching regulatory network which has evolved in an immuno-homeostatic fashion to regulate the amplitude of the immune response in a dynamic and self-regulating way.

This is clearly demonstrated in the way that the Th1 and Th2 cytokines act antagonistically to direct either a cell-mediated or an antibody type of response. T cells can be divided by their cytokine expression profiles into Th1 and Th2 cells. Cytokines generated by Th1 cells, such as interferon gamma and IL-2, mediate functions associated with cytotoxicity and local inflammation, and so are more effective against intracellular pathogens such as viruses and parasites. In contrast, Th2 cell derived cytokines such as IL-4 and IL-10 stimulate the proliferation of B cells and increase antibody production, protecting the host against free-living pathogens such as bacteria.

In addition to the cytokines and chemokines, the regulation of the immune response can also be potentially influenced by structural variation in cytokine/chemokine receptors. Cytokine receptors fall into two groups. Class I receptors include those that recognize most of the interleukins, erythropoietin, granulocyte

Fig. 6.5. Continued.
the J segments that are 3′ of the rearranged J segment are not affected by this recombination. After the D–J recombination event, the *V1* gene is joined to the DJ unit, resulting in a rearranged VDJ exon. All V and D segments between the rearranged V and D genes are deleted. The heavy chain C region exons remain separated from the VDJ complex by DNA containing the distal J segments and the J-C intron. The rearranged Ig heavy chain gene is transcribed to produce a primary transcript that includes the rearranged VDJ complex and the Cμ (constant region mu) exons. A poly-A tail is added to the 3′ end of the Cμ RNA. The introns between the VDJ exon and the first exon of the Cμ locus, and between each of the following constant region exons of the Cμ, are now removed, giving rise to a spliced mRNA of the μ heavy chain. Translation of the rearranged μ heavy chain mRNA then leads to synthesis of the μ protein. To produce a complete IgM monomer, the synthesized μ chain is now associated with a light chain which has been rearranged in a similar manner (not shown).

colony stimulating factor and granulocyte macrophage stimulating factor. These receptors share a cytokine-binding domain with a conserved cysteine motif (four conserved cysteines and one tryptophan) and a conserved membrane region (Trp-Ser-X-Trp-Ser). Class II receptors include those for interferon gamma, interferon alpha and IL-10. They are much more divergent than the class I receptors, sharing only one tryptophan and one pair of conserved cysteines; they do, however, have an additional conserved cysteine pair in addition to several conserved tyrosines and prolines (Pestka *et al.*, 2004). Both the class I and II cytokine receptor families exhibit multiple ligand binding which could explain some of the functional redundancy observed among cytokines. Similarly, there are discrete cell-surface receptors for chemokines that can bind either CC chemokines or CXC chemokines.

The identification and characterization of cytokines and chemokines have largely been driven through research into human disease. However, the veterinary canine research community has also significantly contributed to this field, especially in clinical areas such as haematopoiesis, where the dog has provided an important model for haematopoietic stem cell transplantation (Thomas and Storb, 1999). Much of the functional research to date on canine cytokines and chemokines shows that they display identical or highly similar immunoregulatory and physiological properties to those observed for their human counterparts. Given the high level of amino acid sequence homology seen between dog and human cytokines, this is not surprising (Wagner *et al.*, 2001).

Until recently, the identification and characterization of canine cytokines and chemokines was a laborious process involving the interrogation of molecular libraries, followed by cloning and sequencing. This was the methodology available when the previous edition of this book was published. In the chapter describing canine immunogenetics, 23 discrete canine cytokines were described (Wagner *et al.*, 2001). Since the completion and publication of the dog genome sequence (Lindblad-Toh *et al.*, 2005), the study of dog cytokines and chemokines has been revolutionized. Their identification and molecular characterization are now a relatively straightforward process of interrogating an online annotated canine genome sequence or, in some circumstances, of performing a comparative homology scan between human and canine sequences.

Genetic polymorphism in cytokines, chemokines and their receptors

Genetic variation may result in a quantitative difference in the level of production of cytokines or chemokines, or in qualitative differences in their structure and function due to amino acid changes. Both types of variation can have a major impact on immune physiology and regulation, and can be related to disease aetiology and/or pathology. Differences in the level of production can be due to polymorphism in the promoter region of the gene, which affects the binding of transcription factors and, thereby, downstream gene expression. Such functional variation in human cytokine production is well documented. The inappropriate introduction of a stop codon mutation in a range of positions within a gene can result in total failure of production, but this would have to be present as a homozygous mutation in both gene copies. Variation in the functional levels of cytokines can also be caused by 3′ encoded polymorphisms that result in a decreased half-life of mRNA, and consequential reduction in levels of translation. Any differences resulting in an increased mRNA half-life would have the opposite effect. In some individuals, the inheritance of duplicated genes can result in higher levels of cytokine production.

SNPs that reside within the exons of cytokine and chemokine genes can result in either synonymous or non-synonymous amino acid changes which may or may not have functional consequences. Gene insertions or deletions are also likely to cause a change in function. Polymorphisms that reside in gene introns are less likely to result in functional differences. However, they can cause the production of splice variants which result in a reduced, or loss of, function. Thus, a wide range of possibilities exist for how genetic polymorphism can alter the level of function of cytokine/chemokine gene products. Any changes may result in a disease-associated consequence, but in many cases these are unlikely to be dramatic

owing to the considerable built-in redundancy of the cytokine network.

In contrast, any polymorphisms within cytokine or chemokine receptors that results in a loss of function or cell-surface expression can have severe or even fatal consequences, if inherited in the homozygous state. Polymorphisms causing a loss of function in the human receptor for interferon gamma result in an inability to mount an effective immune response to tuberculosis and can be fatal (Jouanguy *et al.*, 1996). Similarly, polymorphisms in the TNF receptor can result in severe periodic fevers (Simon *et al.*, 2010). Cytokine receptor polymorphisms in the dog that lead to a loss of function are likely to have a dramatic impact on health. The canine *IL-2* receptor gamma chain gene has been cloned and sequenced (Henthorn *et al.*, 1994). Different mutations in this gene, either a frameshift mutation that results in a premature stop codon (Henthorn *et al.*, 1994), or a single nucleotide insertion (Somberg *et al.*, 1995), can cause X-linked SCID. This condition in dogs is highly similar to that seen in humans.

Given the recent origin of most dog breeds and the high level of genetic homogeneity within them, a minimal level of intra-breed variation in the distribution of cytokine gene polymorphisms is expected. In contrast, it is likely that major differences in cytokine/chemokine gene polymorphism will exist between different breeds. If such interbreed differences in cytokine/chemokine genes are related to functional variation, it is highly likely that the result will be immuno-regulatory consequences and that it will be significantly related to the predisposition of certain breeds to specific diseases. This would clearly apply to conditions where hyper- or hypo-immunoresponsiveness is a key factor, e.g. autoimmunity, infection, atopy and cancer.

Although it is possible to identify SNPs through *in silico* data mining of existing dog genome sequence (and this has been effectively used to identify SNPs for the construction of canine high-density SNP microarrays for genome-wide association studies), it should be appreciated that this is largely based on the Boxer and Standard Poodle genomes and will not fully capture the great diversity that exists across other dog breeds. To date, only limited studies have been performed to investigate the extent of cytokine/chemokine gene polymorphism across breeds, and even less effort has been addressed to whether such polymorphisms relate to variation in functional consequence.

A series of studies by Short (Short, 2006, Short *et al.*, 2007, 2009) have determined inter-breed cytokine/chemokine gene variation for a range of breeds. The approach taken was to PCR amplify gene fragments for a wide range of cytokine genes using DNA samples from different breeds. These fragments were subjected to denaturing high-performance liquid chromatography (dHPLC) mutation screening, and, where appropriate, DNA sequencing. These analyses identified a large number of SNPs not previously found in the Boxer and Standard Poodle genomes. Subsequent analysis of these SNPs in larger panels of different breeds revealed major differences in alleles and haplotype frequencies between breeds, and specific haplotype profiles for each breed. A limited example is shown in Table 6.9. This demonstrates that for the seven most common *IL-4* gene haplotypes (based on eight different SNPs): (i) some haplotypes appear to be present in nearly all breeds, and probably represent 'wild' haplotypes that were common in the original dog/wolf populations before breed development; (ii) some breeds have limited gene polymorphism, e.g. the Boxer; and (iii) some haplotypes are relatively uncommon across breeds, but can be at very high frequency in a small number of breeds, This presumably reflects founder effects within the creation of some breeds.

Canine cytokine/chemokine polymorphism and disease predisposition

A primary or secondary involvement of cytokines or chemokines in the pathology of a wide range of canine diseases has been clearly demonstrated. Such studies have used gene expression microarrays or quantitative PCR analysis to measure levels of cytokine/chemokine gene transcript levels in affected tissues in comparison with control material (Nuttall *et al.*, 2002; Maeda *et al.*, 2009). Other approaches have focused on either visualizing or measuring

Table 6.9. Immunoglobulin isotypes in the dog and reagents currently available for their detection.

Class	Subclasses	Detected with	Reference
IgG	IgG1, IgG2, IgG3, IgG4	Polyclonal Ab Monoclonal Ab	German *et al.* (1998) Pérez *et al.* (1998) Mazza and Whiting (1994)
IgA	–	Polyclonal Ab Monoclonal Ab	German *et al.* (1998) Pérez *et al.* (1998)
IgM	–	Polyclonal Ab	German *et al.* (1998) Pérez *et al.* (1998)
IgE	IgE1, IgE2?	Polyclonal Ab Monoclonal Ab α-Chain of IgE receptor (human)	Halliwell and Longino (1985) DeBoer *et al.* (1993) Hammerberg *et al.* (1997) Wassom and Grieve (1998)
IgD-like molecule	–	Monoclonal Ab	Yang *et al.* (1995)

[a] Ab, antibody.

cytokine/chemokine proteins using immunohistology or ELISA bioassays (Quinnell *et al.*, 2001). Documenting such studies falls outside the scope of this chapter, although the underlying genetic basis for how cytokine production contributes to disease risk is relevant.

To date, only limited studies on the role of cytokine gene polymorphism in disease have been performed, although this is now an area receiving considerable attention. The potential contribution of Th1 and Th2 cytokine polymorphism and other candidate cytokines and chemokines to canine diabetes has been studied in detail (Short, 2006; Short *et al.*, 2007, 2009). A previous study has identified exonic SNPs in canine *TNF* alpha, *IL-1* alpha and *IL-1* beta genes within Bernese Mountain Dogs, Collies and West Highland White Terriers to determine a possible involvement in canine malignant histiocytosis (Soller *et al.*, 2006). A previous study has also identified a significant association of a particular *TNF* alpha haplotype with canine anal furunculosis in German Shepherd Dogs (Barnes *et al.*, 2009). Further analysis identified that this *TNF* alpha association was secondary to the DLA class II haplotype due to underlying linkage disequilibrium.

Summary

The sequencing of the dog genome has greatly facilitated research into the field of dog genetics and how genotype relates to normal structural and aetio-pathological phenotype, and also to disease susceptibility and aetio-pathogenesis. Easy access to knowledge of the genome sequence and the annotation of gene structure has had a major impact in the field of canine immunogenetics. This has facilitated the molecular investigations of genes involved in immune recognition and response, and their regulation. It has also allowed us to begin the task of documenting the significant levels of gene polymorphism seen across different dog breeds and other members of the order Canidae. This will ultimately enable us to fully document and quantitate the risk contribution that such immune-related genes make to disease.

In the 10 years since the first edition of this book was published, major progress has been made in determining the extent of DLA gene polymorphism. A staggering feature of the DLA allele and haplotype data emerging from the studies is the extensive level of polymorphism seen across the hundreds of domestic dog breeds that have been developed. This begs the question of whether this has recently been generated or reflects polymorphic signatures that were present in founder wolf populations. Of major significance is the finding that some of the most frequent DLA haplotypes present across the total domestic dog population are also present in wolves.

The increasing characterization of the DLA region and the genomic organization seen also poses some interesting questions for immunologists who have largely viewed the relationship between MHC and immune response/function through the lens of human or mouse species. The dog MHC may well provide interesting insights into how DRB1 and DQ molecules could both interact to regulate immune response, and why levels of *DP* polymorphism have been maintained even though the genes are not expressed. A considerable number of DLA associations with canine diseases are now rapidly emerging in the literature. The relationship between DLA and disease susceptibility/resistance offers great opportunity and clinical potential for veterinary medicine. In addition to taking forward our understanding of disease aetiology, it has the potential to help inform selective breeding programmes within high-risk breeds. The application of the DLA knowledge now being gained will hopefully also help in the design of better vaccines and resolve phenotypes seen in a wide range of immune-mediated conditions.

Access to genome sequences has rapidly driven studies into canine cytokines/chemokines and their receptors. An extensive number of canine cytokine/chemokine genes have now been characterized, and the level of polymorphism seen across breeds is now being established. It is likely that such studies will have a significant impact on understanding breed predisposition to and development of a number of immune-related diseases.

One ambition should now be to translate this rapidly emerging knowledge into health benefits for both dogs and, ironically, their owners. The screening and diagnostic potentials of genetic testing for improving the health of existing dog breeds are increasingly obvious. The benefit of using immunogenetic studies in dog diseases as a comparative inroad into human conditions is now being increasingly recognized.

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7 The Genetics of Canine Orthopaedic Traits

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Introduction

The sequencing and annotation of the canine genome has resulted in a renewed interest in the genetics of canine disorders with orthopaedic manifestations. In addition, the high incidence of debilitating orthopaedic disease in popular breeds of dog is driving the need for a better understanding of the aetiological basis of these conditions. Although a genetic aetiology and/or other contributions to the aetiology (multifactorial aetiology) of many orthopaedic diseases has been suggested, molecular confirmation is still lacking for a lot of them. In general, a genetic aetiology of a trait is suspected if a breed or familial predisposition has been identified (Patterson *et al.*, 1989). A genetic aetiology may be considered confirmed if a mode of inheritance has been demonstrated, or if the molecular basis of the condition has been established. Based on these criteria, many orthopaedic conditions have a suspected or confirmed genetic aetiology. Much progress has been made in confirming the genetic aetiology of orthopaedic manifestations since the first edition of this chapter (Breur *et al.*, 2001). Here, we will give an overview of the genetic basis of canine orthopaedic conditions, as well as an update on the molecular genetics of selected canine orthopaedic traits.

Genetic Basis of Canine Disorders with Orthopaedic Manifestations

It has been reported that at least 30% of all canine patients presented with a musculoskeletal problem were diagnosed with a disease with a suspected or confirmed genetic aetiology (Johnson *et al.*, 1994). Thus, many canine disorders with orthopaedic manifestations may have a genetic basis. These conditions are not restricted to the young dog and may be seen in older dogs as well. In this section, orthopaedic diseases with a proven or presumed genetic aetiology will be

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classified as *congenital, paediatric* or *adult* bone and cartilage diseases. Orthopaedic conditions will be discussed, with an emphasis on aetiology, known breed predispositions, heritability, mode of inheritance, and the biochemical and molecular defects causing the disease, if they are known.

Congenital bone and joint diseases

Congenital bone and cartilage diseases, referred to as skeletal dysplasias, bone dysplasias or osteochondrodysplasias, can be identified at or shortly after birth. They are a heterogeneous group of diseases directly affecting skeletal development and growth. The classification of these dysplasias in children has evolved over time and, presently, they may be further classified as dysostoses, chondrodysplasias and osteodysplasias (Horton, 2003a; Rimoin *et al.*, 2007; Krakow and Rimoin, 2010). Dysostoses are malformations of individual bones or groups of bones, caused by a failure of bone model formation or failure of the transformation of the bone model into cartilage or bone (Spranger *et al.*, 1982; Noden and Delahunta, 1985; Towle and Breur, 2004). Congenital abnormalities affecting growth cartilage and resulting in retarded growth and a disproportionately small stature are classified as chondrodysplasias (Horton, 2003a; Horton and Hecht, 2007; Rimoin *et al.*, 2007). Osteodysplasias are caused primarily by abnormalities in bone, leading to abnormal bone mineralization, bone density and bone mechanics (Horton and Hecht, 2007; Rimoin *et al.*, 2007). The division between the last two groups is indistinct as mutations can cause abnormalities in both bone and cartilage, making the nomenclature somewhat misleading. Congenital bone and cartilage diseases may also be caused by conditions that indirectly affect skeletal development. These will not be discussed here, but may include conditions such as spinal stenosis, Chiari-like malformations and endocrinopathies for example, as well as congenital hypothyroidism and growth hormone deficiency.

Dysostoses

There are many reports on dysostoses in domesticated animals, but these studies are usually case reports or short case series. It is difficult to determine the exact incidence of dysostoses in dogs, but it is clear that the incidence is low (Towle and Breur, 2004). It appears that most canine dysostoses do not have a genetic aetiology, but occur as a result of an adverse *in utero* event (Giger *et al.*, 2006). The assumption of a genetic aetiology is usually based on the general, litter and gestational history, and on clinical examination and radiography (Towle and Breur, 2004). Dysostoses may be classified as those with: (i) cranial and facial involvement (not discussed here); (ii) predominant axial involvement; and (iii) predominant involvement of extremities (International Working Group on Constitutional Diseases of Bone, 1983). Axial dysostoses include hemivertebrae, spina bifida, axial malformation and anury and brachyury. Dysostoses with predominant involvement of extremities (appendicular dysosytoses) include amelia, hemimelia, dimelia, polydactyly, syndactyly and ectodactyly (Towle and Breur, 2004). We refer elsewhere for detailed information on the diagnosis and treatment of these dysostoses (Towle and Breur, 2004; Breur *et al.*, 2010). Dysostoses with a proven or presumed genetic basis are listed in Table 7.1.

Chondrodysplasias and osteodysplasias

Puppies with retarded growth may only attain small stature (dwarfism) when they may only be seen to attain skeletal maturity. The distribution of retarded growth about the body can be proportionate or disproportionate. Animals with proportionate dwarfism are usually not suffering from a primary bone or cartilage disease, but from another disease affecting skeletal development. Examples of such diseases are endocrinopathies, nutritional deficiencies, chronic inflammation, and congenital or acquired major organ failure or insufficiency. Dwarfs are called disproportionate if their legs or trunk are relatively short (Breur *et al.*, 2010). Dogs with disproportionate dwarfism are usually suffering from a chondrodysplasia or an osteodysplasia.

The most recent classification (the revision of 2006) of human chondrodysplasias and osteodysplasias is based on clinical, radiographic, biochemical, molecular and other

Table 7.1. Canine dysostoses with a proven or presumed genetic basis. The conditions in bold are discussed under the section on 'Molecular Genetics of Canine Disorders with Orthopaedic Manifestations' (after Breur *et al.*, 2001, 2010).

Trait	Breed	Mode of inheritance	Comments
Hemivertebra	Bulldog, Pekingese, Pug, Boston Terrier (Beaver *et al.*, 2000)	Unknown	
	German Short-haired Pointer (Kramer *et al.*, 1982)	Autosomal recessive	
Transitional vertebrae	German Shepherd Dogs (Morgan *et al.*, 1993; 2000)	Unknown	Mainly the last lumbar or first sacral vertebrae
Brachyury (Short or bobtail)	Beagle (Hall *et al.*, 1987)	Autosomal dominant with reduced penetrance	
	Pembroke Welsh Corgi (Haworth *et al.*, 2001)	**Dominant mode of inheritance**	**Mutation in *T*-box gene**
Anury	Cairn Terrier, Cocker Spaniel, Dachshund, Doberman Pinscher, Rottweiler, Schipperke (Fritsch and Ost, 1983; Hall *et al.*, 1987; Hytönen *et al.*, 2009)	Unknown	
	Cocker Spaniel (Pullig, 1953)	Autosomal recessive	
	Pembroke Welsh Corgi (Haworth *et al.*, 2001, 2007; Hytönen *et al.*, 2009; Indrebø *et al.*, 2008)	**Dominant mode of inheritance**	**Mutation in *T*-box gene Homozygous phenotype is embryonic lethal**
Hemimelia	Chihuahua (Alonso *et al.*, 1982)	Unknown	
Polydactyly	Saint Bernard (Villagómez and Alonso, 1998)	Autosomal recessive. X-linked lethal or sex-influenced autosomal	
	Australian Shepherd (Sponenberg and Bowling, 1985, Freeman *et al.*, 1988)		
	Great Pyrenees (Jezyk, 1985)	Autosomal dominant	
	Sapsaree (Park *et al.*, 2004, 2008)	**Dominant mode of inheritance**	**Mutation in *LMBR1***

descriptors (Superti-Furga and Unger, with the Nosology Group of the International Skeletal Dysplasia Society, 2007). This 'Nosology and Classification of the Osteochondrodysplasias' includes 372 different conditions divided into 37 groups. A molecular–pathogenetic classification, in which the classification of genetic disorders is based on the structure and function of genes and proteins causing the dysplasia, complements the nosology (Superti-Furga *et al.*, 2001). Use of these classifications for the clinical diagnosis of canine skeletal dysplasias may be perilous, as the molecular basis of canine dwarfism is rarely known, and dwarfisms with similar morphology can have a different molecular basis. In addition, limb-loading differences between bipeds and

quadrupeds also may lead to different secondary deformities for the same trait. For instance, Scottish Deerhounds suffering from pseudoachondroplasia have secondary carpal hyperextension, while in people with the same condition this is not a significant clinical feature (Breur *et al.*, 1989). However, the molecular–pathogenetic classification may be beneficial when determining the molecular basis of a canine chondrodysplasia or osteodysplasia and also for our understanding of mechanism of skeletal development (Superti-Furga *et al.*, 2001; Horton, 2003b; Baldridge *et al.*, 2010).

A presumptive diagnosis of canine osteodysplasia or chondrodysplasia is based on breed, family and gestational history, and on phenotypic criteria such as clinical presentation, radiographic and/or histopathological evidence of abnormal endochondral ossification of the appendicular and/or axial skeleton. Once the presumptive diagnosis is made, the condition may be further characterized. Laboratory tests are only available for mucopolysaccharidosis and *osteogenesis imperfecta* (Breur *et al.*, 2010). As the incidence of skeletal dysplasias is low and most chondrodysplasias are restricted to only one breed, it is most useful to determine whether dwarfism in the breed of interest has been reported previously. Unfortunately, reports of these dysplasias have not been well collated and exist in multiple disparate publications. Also, the veracity of many of these reports has not been vigorously established. Chondrodysplasias and osteodysplasias reported in detail in the veterinary literature are listed in Table 7.2. Canine chondrodysplasias and osteodysplasias reported in other sources are listed in various references. Websites include: the Canine and Feline Genetic Musculoskeletal Diseases site of Purdue University, Indiana (Breur, 2011); the Canine Inherited Disorders Database (Crook *et al.*, 2011); The Orthopedic Foundation for Animals (OFA) at Columbia, Missouri (OFA, 2011); and the Online Mendelian Inheritance in Animals (OMIA) database (OMIA, 2011). Book chapters include: Sande and Bingel (1983); Jezyk (1985); Breur *et al.* (2001); and Johnson (2010). Lay books include that of Clark and Stainer (1994). The health committee of breed clubs may be another good resource of information on known disorders in the breed of interest (a list is provided by the American Kennel Club (2011)).

Paediatric bone and joint diseases

Paediatric bone and joint diseases are a group of orthopaedic diseases not identifiable at birth, with clinical signs developing during growth and adolescence (Breur *et al.*, 2010). They are also known as developmental orthopaedic diseases. Diseases in this group are often breed related, and have a consistent age of onset and a consistent clinical course. Many are multifactorial or complex in origin, and genetic, nutritional and environmental factors have all been implicated in their aetiology. Paediatric bone and joint diseases usually affect either bones (hypertrophic osteodystrophy, craniomandibular osteopathy, panosteitis, tibia varus) or joints (hip dysplasia, elbow dysplasia, osteochondrosis, Legg–Calvé–Perthes disease and patella luxation). More detailed discussion regarding their aetiology, pathophysiology, diagnosis and treatment can be found elsewhere (Breur *et al.*, 2001, 2010). Paediatric bone and joint diseases with a proven or presumed genetic aetiology, based on epidemiological or genetic studies, are listed in Table 7.3. Breed predispositions suggest that paediatric bone and joint diseases occur within all breed clusters (Parker and Ostrander, 2005; Parker *et al.*, 2007). Only craniomandibular osteopathy, Legg–Calvé–Perthes disease and osteochondritis dissecans (OCD) of the talocrural joint are restricted to just two or three breed clusters. The affected breed clusters are also listed in Table 7.3.

Adult bone and joint diseases

Several adult bone and joint diseases that develop after skeletal maturation have strong breed predispositions and may have a genetic aetiology. These conditions include cranial cruciate ligament disease (CCLD, which involves degeneration and rupture), fractures, neoplasia, intervertebral disc disease (IVDD), spondylosis deformans and diffuse idiopathic skeletal hyperostosis (DISH). Conditions and breed predispositions are listed in Table 7.4.

Table 7.2. Canine chondro- and osteodysplasias. The conditions in bold are further discussed in the section on 'Molecular Genetics of Canine Disorders with Orthopaedic Manifestations' (after Breur *et al.,* 2001, 2010).

Breed	Trait	Mode of inheritance	Laboratory test	Demonstrated biochemical and/or molecular defects
Akita (Sande *et al.*, 1994)	Achondrogenesis	Unknown	No	
Alaskan Malamute (Fletch *et al.*, 1973, 1975; Bingel *et al.*, 1980, 1985)	Chondrodysplasia	Autosomal recessive	No	Type II collagen is abnormally soluble in neutral salt solutions as it has significantly more proteoglycans with longer proteoglycan monomers and longer chondroitin sulfate side chains with increased amounts of chondroitin-6-sulfate
Beagle (Rasmussen, 1971, 1972; Sande and Bingel, 1983; Campbell *et al.*, 1997, 2001)	Chondrodysplasia punctata	Unknown	No	
	Multiple epiphyseal dysplasia	Autosomal recessive	No	
	Osteogenesis imperfecta	**Unknown**	**No**	***COL1A2 mutation***
Bulldog (Louw, 1983)	Osteochondrodysplasia	Unknown	No	
Bull Terrier (Watson *et al.*, 1991)	Osteochondrodysplasia	Unknown	No	
Cocker Spaniel (Beachley and Graham, 1973)	Hypochondroplasia	Unknown	No	
Collie (Holmes and Price, 1957)	*Osteogenesis imperfecta*		No	
Dachshund (Drögemüller *et al.*, 2009)	***Osteogenesis Imperfecta***	**Unknown**	**No**	***SERPINH1 mutation***
Dunker (Rørvik *et al.*, 2008)	Multiple epiphyseal dysplasia	Unknown	No	
English Pointer (Whitbread *et al.*, 1983)	Enchondrodystrophy	Homozygous recessive	No	
Golden Retriever (Campbell *et al.*, 2000)	***Osteogenesis imperfecta***	**Unknown**	**No**	***COL1A1 mutation***
Great Pyrenees (Bingel and Sande, 1994)	Chondrodysplasia	Autosomal recessive	No	
Hygenhund (Rørvik *et al.*, 2008)	Multiple epiphyseal dysplasia	Unknown	No	
Irish Setter (Hanssen, 1992; Hanssen *et al.*, 1998)	Hypochondroplasia	Autosomal recessive	No	

Labrador Retriever (Carrig *et al.*, 1977, 1988; Goldstein *et al.*, 2010)	***Oculoskeletal dysplasia***	**Autosomal recessive**	**No**	***COL9A2 and COL9A3 mutations***
Miniature Poodle (Cotchin and Dyce, 1956; Gardner, 1959; Amloff, 1961; Lodge, 1966; Bingel *et al.*, 1986)	Achondroplasia	Autosomal recessive	No	Primary defect in sulfation pathway or increased activity of sulfatase enzymes
	Multiple epiphyseal dysplasia	Unknown	No	
	Pseudoachondroplasia	Unknown	No	
Mixed breed dog (Haskins *et al.*, 1984)	**Mucopolysaccharidosis VII**	**Autosomal recessive**	**Yes**	**β-D-glucuronidase deficiency**
Norwegian Elkhound (Bingel and Sande, 1982)	Chondrodysplasia	Autosomal recessive	No	
Plott Hound (Shull *et al.*, 1982; Spellacy *et al.*, 1983)	**Mucopolysaccharidosis I**	**Autosomal recessive**	**Yes**	**α-L-glucuronidase deficiency**
Samoyed (Meyers *et al.*, 1983; Aroch *et al.*, 1996)	Oculoskeletal dysplasia without hematological abnormalities	Autosomal recessive	No	
	Oculoskeletal dysplasia with haematological abnormalities	Unknown	No	
Scottish Terrier (Mather, 1956; Hay *et al.*, 1999)	Achondroplasia	Unknown	No	
	Idiopathic multifocal osteopathy	Unknown	No	
Scottish Deerhound (Breur *et al.*, 1989, 1992)	Pseudoachondroplasia	Autosomal recessive	No	
Shiba Inu (Suu, 1956, 1957, 1958; Ueshima, 1961; Hansen, 1968)	Short-spine syndrome	Unknown	No	

Table 7.3. Paediatric bone and joint conditions with a proven or presumed genetic aetiology. The conditions in bold are further discussed in the section on 'Molecular Genetics of Canine Disorders with Orthopaedic Manifestations' (after LaFond *et al.*, 2002; and Breur *et al.*, 2010).

Trait	Incidence	Breeds at risk	h^2	Sex	Comments
Paediatric bone conditions					
Craniomandibular osteopathy	1.4 per 100,000	Cairn Terrier, Scottish Terrier, West Highland White Terrier	–	M = F	Autosomal recessive in West Highland White Terriers C, D
Hypertrophic osteodystrophy	2.8 per 100,000	Boxer, Chesapeake Bay Retriever, German Shepherd Dog, Golden Retriever, Great Dane, Irish Setter, Labrador Retriever, Weimaraner	0.68	M > F	B, C, D, E
Panosteitis	2.6 per 1000	Afghan Hound, Akita, American Cocker Spaniel, American Staffordshire Terrier, Basset Hound, Bearded Collie, Bernese Mountain Dog, Boxer, Bull Terrier, Bulldog, Chesapeake Bay Retriever, Chow Chow, Dalmatian, Doberman Pinscher, English Setter, English Springer Spaniel, Giant Schnauzer, German Shepherd Dog, German Shorthaired Pointer, Golden Retriever, Great Dane, Great Pyrenees, Irish Wolfhound, Labrador Retriever, Mastiff, Neapolitan Mastiff, Newfoundland, Rhodesian Ridgeback, Rottweiler, Saint Bernard, Shar-Pei, Shih Tzu, Weimaraner, West Highland White Terrier	0.13	M > F	A, B, C, D, E, F
Paediatric joint conditions					
Osteochondritis dissecans (OCD) Shoulder	–	Bernese Mountain Dog, Border Collie, Bouvier, Boxer, Bullmastiff, Chesapeake Bay Retriever, Dalmatian, English Setter, German Short-haired Pointer, German Shepherd Dog, German Wire-haired Pointer, Golden Retriever, Great Dane, Great Pyrenees, Irish Wolfhound, Kuvasz, Labrador Retriever, Mastiff, Munsterland, Newfoundland, Old English Sheepdog, Rottweiler, Saint Bernard, Standard Poodle	0.10–0.70	M > F	Polygenic mode of inheritance B, C, D, E, F
OCD Elbow	**–**	**Chow Chow, German Shepherd Dog, Golden Retriever, Great Dane, Labrador Retriever, Newfoundland, Rottweiler**	**–**	**M > F**	**Polygenic mode of inheritance A, B, C, D, E, F**
Fragmented medial coronoid process	**–**	**Basset Hound, Bernese Mountain Dog, Bouvier, Bullmastiff, Chow Chow, German Shepherd Dog, Golden Retriever, Gordon Setter, Irish Wolfhound, Labrador Retriever, Mastiff, Newfoundland, Rottweiler, Saint Bernard**	**0.18–0.31**	**M > F**	**Polygenic mode of inheritance A, C, D, E, F**

Ununited anconeal process	**–**	**Basset Hound, Bernese Mountain Dog, Chow Chow, English Setter, German Shepherd Dog, Golden Retriever, Labrador Retriever, Mastiff, Newfoundland, Pomeranian, Rottweiler, Saint Bernard, Shar-Pei**	**–**	**M > F**	**Polygenic mode of inheritance A, C, D, E, F**
Hip dysplasia	**21.1–28.1 per 1000 (Morgan and Stavenborn, 1991)**	**Airedale, Alaskan Malamute, Bearded Collie, Bernese Mountain Dog, Bloodhound, Border Collie, Bouvier, Briard, Brittany Spaniel, Bulldog, Bullmastiff, Chesapeake Bay Retriever, Chow Chow, English Springer Spaniel, German Shepherd Dog, German Wire-haired Pointer, Giant Schnauzer, Golden Retriever, Gordon Setter, Great Dane, Great Pyrenees, Keeshond, Kuvasz, Labrador Retriever, Mastiff, Neapolitan Mastiff, Newfoundland, Norwegian Elkhound, Old English Sheepdog, Pointer, Portuguese Water Dog, Rottweiler, Saint Bernard, Samoyed, Tree Walking Coonhound**	**0.25–0.60**	**–**	**A, B, C, D, E, F**
Legg–Calvé–Perthes disease	–	Australian Shepherd, Cairn Terrier, Chihuahua, Dachshund, Lhasa Apso, Manchester Terrier, Miniature Pinscher, Pug, Toy Poodle, West Highland White Terrier, Yorkshire Terrier	–	M = F	Autosomal recessive or multifactorial with a high heritability A, B, D
OCD Stifle joint	–	Boxer, Bulldog, German Shepherd Dog, Golden Retriever, Great Dane, Irish Wolfhound, Labrador Retriever, Mastiff, Rottweiler	–	M > F	B, C, D, E, F
Patella luxation (medial and lateral)	–	Akita, American Cocker Spaniel, Australian Terrier, Basset Hound, Bichon Frise, Boston Terrier, Bulldog, Cairn Terrier, Cavalier King Charles Spaniel, Chihuahua, Chow Chow, Flat-coated Retriever, Great Pyrenees, Japanese Chin, Keeshond, Lhasa Apso, Maltese, Miniature Pinscher, Miniature Poodle, Papillon, Pekingese, Pomeranian, Pug, Shar-Pei, Shih Tzu, Silky Terrier, Standard Poodle, Toy Fox Terrier, Toy Poodle, West Highland, White Terrier, Wire-haired Fox Terrier, Yorkshire Terrier	–	M > F	A, B, C, D, F
OCD Talocrural joint	–	Labrador Retriever, Rottweiler, Bullmastiff	–	M<F	C, F
OCD Sacrum (Alexander and Pettit, 1967; Lang *et al.*, 1992; Hanna, 2001)	–	German Shepherd Dog	–	M > F	E

Heritability is denoted by h^2. Capital letters in 'Comments' column indicate the breed clusters to which the breeds affected with the condition have been assigned. A, Ancient Asian group; B, Herding–Sighthound group; C, Mastiff–Terrier group; D, Hunting group; E, Mountain group; and F, Miscellaneous (Parker *et al.*, 2005, 2007)

Table 7.4. Adult bone and joint conditions with a proven or presumed genetic aetiology. The conditions in bold are further discussed under 'Molecular Genetics of Canine Disorders With Orthopaedic Manifestations'.

Condition	Incidence	Breeds	Comments
Cranial cruciate ligament disease (CCLD) (Duval *et al.* 1999; Wilke *et al.*, 2006, 2009; Griffon, 2010)	**17.4 per 1000**	**Akita, American Staffordshire Terrier, Chesapeake Bay Retriever, Labrador Retriever, Mastiff, Neapolitan Mastiff, Newfoundland, Rottweiler, Saint Bernard**	**Newfoundland: heritability 0.15–0.27, recessive mode of inheritance with 51% penetration**
Fracture	–	Greyhound, Miniature Pinscher, Italian Greyhound, Papillon, Poodle, Shetland Sheepdog, Whippet (Ljunggren, 1971)	These breeds, except for the Shetland Sheepdog and Greyhound, are also at increased risk of fractures of the radius/ulna but not of the tibia/fibula
	–	Miniature breeds (Waters *et al.,* 1993; Welch *et al.*, 1997; Larsen *et al.*, 1999)	Fractures of the radius/ulna
	–	Brittany Spaniel, Cocker Spaniel, Rottweiler (Rørvik, 1993; Marcellin-Little *et al.*, 1994)	Humeral condyle fractures; incomplete ossification of the humeral condyle was suggested as a predisposing factor; may have a recessive mode of inheritance
Neoplasia	–	Doberman Pinscher, Great Dane, Greyhound, Irish Setter, Irish Wolfhound, Rottweiler, Saint Bernard, Scottish Deerhound (Bech-Nielsen *et al.,* 1978; Ru *et al.*, 1998; Phillips *et al.*, 2007; Rosenberger *et al.*, 2007)	Patterns of familial aggregation of osteosarcoma for the Saint Bernard have been identified
Intervertebral disc disease (IVDD, disc degeneration and calcification) (Ghosh *et al.*, 1975; Ball *et al.,* 1982; Verheijen and Bouw, 1982; Jensen and Christensen, 2000; Jensen *et al.*, 2008; Young and Bannasch, 2008)	–	Dachshund	Lifetime risk is estimated at 18–24%; heritability of disc chondroid degeneration with calcification is 0.47–0.87 An autosomal polygenic pattern of inheritance has been proposed; no dominance or sex linkage has been found
Spondylosis deformans (Langeland and Lingaas, 1995; Carnier *et al.*, 2004)	–	Boxer, Italian Boxer	Prevalence may be as high as 91% and 84%, respectively, for the two breeds
Diffuse idiopathic skeletal hyperostosis (DISH) (Woodard *et al.*, 1985; Morgan and Stavenborn, 1991; Kranenburg *et al.*, 2010)	–	Boxer	Prevalence 41%; may represent a severe manifestation of spondylosis deformans

Molecular Genetics of Canine Disorders with Orthopaedic Manifestations

Skeletal development and congenital bone and joint diseases

Several mutations that define the shape and size of dogs and may be associated with orthopaedic traits and diseases have been described in recent years and are documented in the OMIA (Online Mendelian Inheritance in Animals) database (OMIA, 2011). A single *IGF1* allele on chromosome 15 is a major determinant of small body size (Sutter *et al.*, 2007). All the small breed dogs studied had a single *IGF1* single nucleotide polymorphism (SNP) haplotype, which was nearly absent from giant breed dogs. Common orthopaedic traits that this group appears to be predisposed to include atlanto-axial luxation, patella luxation and Legg–Calvé–Perthes disease. A retrogene in the *FGF4* receptor contributes to the short-legged, chondrodysplastic phenotype in breeds like Dachshunds, Corgis, Pomeranians, Cocker Spaniels and Basset Hounds (Parker *et al.*, 2009). Dogs from this group are at risk of IVDD. In many breeds, dogs with the short-tailed phenotype (bobtail) are heterozygous for an ancestral *T*-box mutation (Haworth *et al.*, 2001; Hytönen *et al.*, 2009).

The molecular basis of two dysostoses has been reported. Brachyury (short tail) in Pembroke Welsh Corgis is caused by a *T*-box mutation (Haworth *et al.*, 2001). Homozygosity for the *T*-box mutation is lethal and is associated with vertebral defects and anorectal atresia (Haworth *et al.*, 2001; Indrebø *et al.*, 2008). It has been suggested that this mutation is also lethal in Swedish Vallhunds. The *T*-box mutation is present in dogs of many breeds with a short-tailed phenotype, but not all dogs with a short tail have this mutation (Hytönen *et al.*, 2009). The molecular basis of pre-axial polydactyly in Sapsaree dogs is caused by a mutation in the conserved intronic sequence of the *LMBR1* gene (Park *et al.*, 2004, 2008). Two mutations were identified: one for Korean breeds and one for Western breeds.

The molecular basis of several skeletal dysplasias has been reported. *Osteogenesis imperfecta* in dogs is caused by mutations in *COL1A1* (Campbell *et al.*, 2000), *COL1A2* (Campbell *et al.*, 2001), and *SERPINH1* (Drögemüller *et al.*, 2009). *COL9A2* and *COL9A3* mutations cause canine autosomal recessive oculoskeletal dysplasia (Goldstein *et al.*, 2010). Hereditary 1,20-dihydroxyvitamin D-resistant rickets in a Pomeranian dog was caused by a mutation in the vitamin D3 receptor gene (LeVine *et al.*, 2009). Mucopolysaccharidosis I and VII (MPS I and VII) are due to deficient activity of the glycosaminoglycan-degrading enzymes alpha-L-iduronidase and beta-glucuronidase, which cause abnormal bones and joints such as short stature, articular erosions and joint subluxations (Herati *et al.*, 2008). Gene therapy for MPS I and VII has been attempted experimentally (Herati *et al.*, 2008).

Paediatric bone and joint diseases

Hip dysplasia

HERITABILITY. The heritability of a complex or polygenic trait is estimated as the additive genetic variance divided by the total phenotypic variance (Falconer and Mackay, 1996) and is a feature of the particular pedigree (pure breed) in which it is estimated. For the hip score based on the ventrodorsal, extended hip joint radiograph (EHR) of the pelvis described by the OFA, heritability ranges between 0.2 and 0.3 (Breur *et al.*, 2001; Hou *et al.*, 2010); it reaches as high as 0.5–0.6 for the distraction index (DI) and dorsolateral subluxation (DLS) scores (Leighton, 1997; Todhunter *et al.*, 2003) in closed populations.

ESTIMATED BREEDING VALUES. in the 1960s, the OFA established a registry of inherited orthopaedic traits in dogs. Its initial mission was to provide radiographic evaluation, data management and genetic counselling to reduce the incidence of canine hip dysplasia (CHD). Since then, improvement in hip joint phenotypes in North American pure breed dogs has been modest (Kaneene *et al.*, 1997, 2009; Zhang *et al.*, 2009).

The estimated breeding value (EBV) is based on integration of genetic and phenotypic

information from each animal and its relatives, and yields better results for the selection of desirable traits than phenotypic selection alone. Selection of dogs for hip joint quality based on the EHR has resulted in predominant genetic improvement in the last 10 to 15 years, during which strong selection pressure has been applied to dogs at the Guiding Eyes for the Blind on the basis of phenotypes selected in the mid-1990s. The OFA implemented the best linear unbiased prediction (BLUP) method for the estimation of breeding values to apply additional pressure on hip joint conformation in 2004 (Zhang *et al.*, 2009).

We retrieved the pedigrees of 258,851 Labrador Retrievers, the major breed scored by the OFA (25% of total records) and available in their semi-open database. Of these, 154,352 dogs had an OFA score reported between 1970 and 2007. The remainder of the dogs (104,499) were the ancestors of those with OFA scores, and were used to build genetic relationships. The OFA hip score is based on a 7-point scale with the best ranked as 1 (excellent) and the worst CHD as 7. A mixed linear model was used to estimate the effects of age, sex and test year period and to predict the EBVs for each dog. The hip scores averaged 1.93 (± SD = 0.59) and the heritability was 0.21. A steady linear genetic improvement has accrued over the four decades. By the end of 2005, the total genetic improvement was 0.1 units, which is equivalent to 17% of the total phenotypic standard deviation (Hou *et al.*, 2010). The EBVs and inbreeding coefficients for these Labrador Retrievers were uploaded to a searchable web-based database Cornell University College of Veterinary at the Medicine (at http://www.vet.cornell.edu/research/bvhip/). Even though the OFA score data in this website are biased towards better hip scores, estimated breeding values that were derived integrate genetic relationships between the dogs with both good and poor hip quality. Breeders and prospective buyers of Labrador Retrievers can now select dogs from among a group chosen for qualities they prefer which also have the best genetic potential to produce offspring with good hip conformation. For a newly acquired pup, the breeding pair with the highest chance of producing offspring with good hip conformation can be selected. Estimated breeding values should be available for dogs of all breeds, with over 1000 individuals in the semi-open OFA database.

Multiple-trait modelling suggested that a single hip radiograph does not provide as much information about a dog's genetic potential as a combination of measurements of hip joint conformation (Todhunter *et al.*, 2003; Zhang *et al.*, 2009). Thus, a single hip measurement is insufficient to provide the optimal basis for breeding decisions. A combination of the Norberg angle (NA) and DLS score or DI provided a more accurate prediction of secondary hip osteoarthritis (OA) than a single hip radiographic trait predictor in young adult Labrador Retrievers, Greyhounds and their crossbreed offspring (Todhunter *et al.*, 2003). Nevertheless, the only currently available semi-open database for hip scores in dogs is based on the OFA score.

LOCI FOR CANINE HIP DYSPLASIA. Four radiographic measurements define what it is to be dysplastic in the dog: the DI, the DLS score, the NA and the OFA (EHR) score. Based on principal component analysis, the DI and DLS score reflect hip laxity, and the NA and EHR score reflect the chondro-osseous conformation of the hip joint (Ogden *et al.*, 2011). The two members of each pair are highly genetically correlated (~–0.9) but only moderately so between each pair (Zhang *et al.*, 2009).

Quantitative trait loci (QTLs) influencing CHD and hip OA have been identified independently on several canine chromosomes: CFA03 (Chase *et al.*, 2005), CFA04, 09, 10, 11, 16, 20, 22, 25, 29, 30, 35 and 37 (Todhunter *et al.*, 2005), and CFA01, 03, 04, 08, 09, 16, 19, 26 and 33 (Marschall and Distl, 2007). Chase *et al.* (2004) identified 2 QTLs on CFA01 associated with CHD measured by the NA in Portuguese Water Dogs. In Labrador Retrievers, this chromosome also harboured a QTL for CHD measured as the NA as well as a QTL on CFA02, 10, 20, 22 and 32 (Phavaphutanon *et al.*, 2009). Marschall and Distl (2007) identified a QTL for CHD on CFA01 in German Shepherd Dogs. Further, both Todhunter *et al.* (2005) and Marschall and Distl (2007) identified QTLs for CHD on CFA01, 04, 09, 10, 16 and 22 in Labrador Retrievers and German Shepherd

Dogs. Identification of QTLs for CHD on the same chromosomes and in a similar physical location in independent studies across breeds supports their existence and 'identical-by-descent' inheritance (for a summary of reported and unreported studies, see Table 7.5).

We genotyped 369 dogs at 23,500 SNP loci across the genome (using the Illumina CanineSNP20 BeadChip). The majority (301) of these dogs were from two breeds (the Labrador Retriever and Greyhound) and their crosses (F_1, F_2 and two backcross groups). The remaining 68 dogs were from five other breeds. Fine SNP mapping was also undertaken on DNA from 585 dogs genotyped at ~3300 loci spanning QTLs on four chromosomes (CFA03, 11, 29 and 30) for CHD and four chromosomes (CFA05, 18, 19 and 30) for hip OA (Mateescu *et al.*, 2008). We genotyped dogs carefully selected from our archive for both genetic and phenotypic diversity (Zhu *et al.*, 2009). Among all the genotyped dogs, 100 were genotyped on both platforms, which enabled us to integrate the two sets of genotypes. The total of ~800 dogs covered eight pure breeds (Labrador Retriever, Greyhound, German Shepherd, Newfoundland, Golden Retriever, Rottweiler, Border Collie and Great Dane) and the crosses between dysplastic Labrador Retrievers and non-dysplastic Greyhounds (Zhou *et al.*, 2010). A genome-wide association study accounting for the genetic relatedness between dogs revealed four quantitative trait nucleotides (QTNs) for the NA and two for secondary hip OA (Table 7.5). Some of these SNPs were located near genes already shown to be associated with human forms of OA.

Labrador Retrievers and German Shepherd Dogs with CHD exhibit delayed onset of ossification in the secondary centres of their femoral heads (Todhunter *et al.*, 1997). Chromosomes 1, 8 and 28 harbour putative imprinted QTLs for the age at onset of femoral head ossification in Labrador Retriever/Greyhound crossbreeds (Liu *et al.*, 2007). The role of imprinting in the expression of complex canine traits has yet to be explored.

Recently, Feldman *et al.* (2010) linked a locus on human chromosome 17q21 to developmental dysplasia of the human hip (DDH). A separate analysis of 101 Chinese families showed that the DDH locus was associated with the microsatellite marker D17S1820 (Jiang *et al.*, 2003); this is within the 4 Mb candidate region described by Feldman *et al.* (2010). This locus is syntenic to canine chromosome CFA09 in the 28–30 Mb interval where we localized a putative locus discovered through linkage analysis of SNP genotypes in 100 Labrador Retriever/Greyhound crossbreeds (Table 7.5). Marschall and Distl (2007) also located a locus for CHD on CFA09 at 52 Mb (Table 7.5).

MUTATION FOR CANINE HIP DYSPLASIA. Following fine SNP-based mapping (Zhu *et al.*, 2008), in 100 Labrador Retrievers, a 10 bp deletion haplotype in intron 30 of *FBN2* at 20.3–20.5 Mb on CFA11 was significantly (P = 0.007) associated with a worsening of CHD. In 143 dogs of five other breeds, this *FBN2* haplotype was also associated with a significant (P = 0.01) worsening of CHD and contributed 11–18% of trait variance (Friedenberg *et al.*, 2011). Mutations in the gene encoding *FBN2* have been associated with congenital contractural arachnodactyly in humans (Putnam *et al.*, 1995).

HIP OSTEOARTHRITIS (HIP OA). Hip dysplasia in humans and dogs leads inexorably to hip OA characterized by hip pain, lameness or limping, gait dysfunction and disability. Hip subluxation and laxity results in secondary hip OA. QTLs for hip OA secondary to CHD were mapped in Labrador Retrievers on CFA05, 18, 23 and 31 (Mateescu *et al.*, 2008) (Table 7.5). Chase *et al.* (2005) mapped a locus for hip OA in Portuguese Water Dogs on CFA03 (Table 7.5). Interestingly, no genetic variants associated with human hip OA over the last decade have been replicated in other studies (Limer *et al.*, 2009). Sixty-eight polymorphisms in *IL1A, IL1B, IL1RN, IL4R, IL6, COL2A1, ADAM12, ASPN, IGF1, TGFB1, ESR1* and *VDR* that had been associated with human knee and hip OA in previous studies were genotyped in over 1000 individuals with no strong association. Heterogeneity in human populations and the likely interaction between the inheritance of a primary complex trait like hip dysplasia, and its secondary effects on hip OA incidence and

Table 7.5. Summary table of loci putatively linked or associated with canine hip dysplasia. Canine chromosome number (CFA no.) is shown in column 1. The LOD score (logarithm of the odds, an estimate of how closely two loci are linked) is followed by map location in Mb in parentheses for linkage studies on 159 Labrador Retriever/Greyhound crossbreeds (Todhunter *et al.*, 2005) using MSS1 (Minimal Screening Set 1) and MSS2. The same statistics for linkage analysis on Labrador Retrievers are summarized for MSS1 and MSS2 (Phavaphutanon *et al.*, 2009). Statistics are from linkage analysis on 100 Labrador Retriever/Greyhound crossbreeds genotyped at 21,455 single nucleotide polymorphisms (SNPs) (Zhou *et al.*, 2010) and measured for four hip dysplasia phenotypes (the distraction index, DI; the dorsolateral subluxation score, DLS; the Norberg angle, NA; and the Orthopedic Foundation for Animals (OFA) hip score – or EHR, extended hip joint radiograph) and their principal components (PCs) for the left (L) and right (R) hips of these measurements. The results of the complete genome-wide association study (GWAS) reported in Zhou *et al.* (2010) are tabulated using *P* values instead of LOD scores. Statistics for multi-point linkage mapping for the principal components of the DI, DLS and NA on CFA11 and 29 used the same SNP genotypes and dogs reported in Zhu *et al.* (2008). The two right-hand columns summarize linkage analysis statistics reported by Chase *et al.* (2004, 2005) for the NA and acetabular osteophytes (OA) in Portuguese Water Dogs, and by Marschall and Distl (2007) in German Shepherd Dogs for a hip score.

CFA no.	MSS1 (crossbreed) (Todhunter *et al.*, 2005)	MSS1 and 2 cross	MSS1 and 2 LR (Phavaphutanon *et al.*, 2009)	Linkage analysis on 100 crosses	Fine mapping with ABI SNPLex (Zhu *et al.*, 2008)	GWAS (Zhou *et al.*, 2010)	Microsatellites (linkage) (Chase *et al.*, 2004, 2005)	Microsatellites (linkage) (Marschall and Distl, 2007)
1			3.1 (55)PC	3-5(49)NA/OFA			P < 0.08 (27)	2.1(83)modified EHR score
			2.3(70) PC				*P* < 0.08(111)	
2			2.0(70) PC	3.2-3.8(6)DLS				
			2.4(65)	3.0 (8)PC3				
3		2.4(61)PC		8(32)NA/OFA		7×10^{8} (75)NA	*P* < 0.002(45)	2(90)
4	2.2 (0) NA/ OFA	2.3-2.5(35 36 44)	2.2(0)	4.0(39)NAL				3.3(5)
				2.6(19)NAR				
				2.3(19)OFAR				
5		2.6(15) OA (Mateescu)		3.5(80)				
8				3.3(39)DLSL				2.3(29)
9	2.1(1)PC	2.3(1)	2.3(1)	3.6(51)DILR				2.2(14)
				2.4(31)NAL				
				3.4(31OAL				
				3(17)OFAL				
				2.5(17)OAR				
				3.3(35)PC7				

10	2.1(27)	2.3(53)NA/OFA	2.9(55)	3.7(19) PC3;2.8(35) DLS NAL PC1 2.4(19)PC4, OAR2.3(25) PC7			1(69)
11	2.6(63)	2.6(2)DLS/DI 2.5(32)PC	2.6(63)	2.3(60)DLS/DI, 2.8(60)OFA 3.5(60)PCI	8(12)PCI 10(51)PC3	3×10^6 (33)NA 9×10^7 (58)	
16	1.4(10)NA/ OFA 1.6(0)PC 2(0)NA/OFA		2(0)	2.9(19)multiple traits 3.5(57)PC6			2(35)
17				3.4(33)NA		5×10^8 (48)OA	
18			2.7(54) OA(Mateescu)	2.9(37,38)NA OFA			
19		1.5(3)OA		2.9(30)NA			4.3(46)
20	2.2(0)DLS/OC		3.3(30)PC 2.4(60)DLS/DI 2.2(8)PC	3.3(5)DI 3.1(56)NAR			
21		2.4(44)PC		3.2(35)PC6 3.2(35)PC6 2.4(39)PC1			
22	2(6)DLS/DI	2.2(43)DLS/DI	2.1(0) 2(6)	3.2(54)DI; 3.6(44)DLS 2.3(PC6)			1.2(4)
23		2.7(51)OA (Mateescu)					
25	1.6(12)NA/ OFA 1.6(18)PC		1.6(18)	3.6(50)NA 2.1(54)PCI OFAL			
26				2.9(5) 2.3(20)DIR			2(31)
29	2.8(9)DLS/DI	2.0(19)DLS/DI PC	2.8(9)	2.4(3.2)DIL	9(23)PCI		1(28)

Continued

Table 7.5. Continued.

CFA no.	MSS1 (crossbreed) (Todhunter *et al.*, 2005)	MSS1 and 2 cross	MSS1 and 2 LR (Phavaphutanon *et al.*, 2009)	Linkage analysis on 100 crosses	Fine mapping with ABI SNPLex (Zhu *et al.*, 2008)	GWAS (Zhou *et al.*, 2010)	Microsatellites (linkage) (Chase *et al.*, 2004, 2005)	Microsatellites (linkage) (Marschall and Distl, 2007)
	1.6(9)OCA/ DLS	2.7(10)DLS/DI		3.4(3.4)PC3 2.3(29.4)NAL	10(20)PC2			
30	2.0(18)DLS/DI	2.0(19)NA/OFA	2.0(18)	2.6(8)PC6 DIL		1×10^6 (14)NA		
	1.7(18)PC	2.3(20)NA/OFA 2.0(29)DLS/DI		3(30)PC7 3.3(23)PC7				
31		2.2(0) OA(Mateescu)		3.1(15)PC6				
32			2.4(5)PC	3(28)DI				
33				3.1(23)DI OAL 2(24)NAR 2(5)PC2.4				2.4(6)
35	1.6(0)	2(2)		2(18) worst OFA				
37	2(42)PC	2(6)PC	2(42)	2(9)PC5;2.4(4) PC2 best NA		4×10^5 (17)OA		

progression, will make the genetic dissection of human hip OA difficult. Dog populations may be more tractable for the localization of genes that affect the expression of OA, but the genetic architecture of the primary inherited trait should be addressed simultaneously.

A complementary approach to QTL linkage and association mapping for finding genes that contribute to the pathogenesis of CHD and hip OA is to use genome-wide expression arrays. Such studies can lead to the identification of genomic mutations or be used to identify genes within QTLs. Using the first-generation Affymetrix Canine GeneChip, we identified 32 genes as significantly differentially expressed in response to impact damage to articular cartilage when the false discovery rate was held to 10% (Burton-Wurster *et al.*, 2005; Mateescu *et al.*, 2005). Upregulated genes confirmed by quantitative PCR included *COX-2, MIG-6/Gene 33, DNCL1, LAM5* and *ATF3*. With the exception of *COX-2*, these genes were not previously considered to have a role in OA pathogenesis. Importantly, *DNCL1* and *MIG-6* mRNA levels were 3–4-fold higher in naturally degenerated articular cartilage in dysplastic dog hips compared with the site-matched, disease-free area from dogs at low risk for hip OA. For *DNCL1*, the increase in mRNA level was confirmed *in vivo*, where increased gene expression in articular cartilage lesions was found.

GENOMIC PREDICTION OF COMPLEX TRAITS. Because haplotype blocks in the canine genome are extensive, a genetic marker in linkage disequilibrium (LD) with causal genes for a complex trait can be used in marker-assisted selection. Until the genes underlying complex diseases such as CHD and hip OA are discovered, the genetic merit of animals can be estimated by genomic selection, which uses genome-wide SNP panels as markers and statistical methods that capture the simultaneous effects of large numbers of SNPs and relates them to their EBV. This SNP based genome-wide panel can be substituted for an individual EBV, and selection pressure based on genomic breeding value may exert further genetic improvement than the use of EBVs alone. Simulations by Stock and Distl (2010) for CHD in German Shepherd Dogs support this finding.

Two sets of dogs (six breeds) were genotyped with SNPs covering the entire canine genome (Zhou *et al.*, 2010). The first set contained 359 dogs upon which a predictive formula for genomic breeding value (GBV) was derived from their EBV of the NA and their genotypes at 22,000 SNPs (using the Illumina CanineSNP20 BeadChip) covering the entire genome. To investigate how well the formula would work for an individual dog with genotype only (without using EBV or phenotype), a cross validation was performed by masking the EBV of one dog at a time. The genomic data, when reduced to the best set of 100 SNPs, and the EBVs of the remaining dogs were used to predict the GBV for the single dog that was left out. The cross validation showed a strong correlation ($r > 0.7$) between the EBV and the GBV. A second set of dogs contained 38 new Labrador Retrievers and 15 dogs that were in the first set for the purpose of data quality verification (e.g. genotyping error) and imputation of missing SNPs. These 53 Labrador Retrievers were genotyped with 50,000 genome-wide SNPs from the Affymetrix Canine Array (13,000 overlapped with the Illumina array). The 38 new dogs had no pedigree relationship to the dogs in the first set, and thus were used to investigate how well the predictive formula would work for a general Labrador Retriever outside the dogs used to derive the predictive formula. The strength of the prediction dropped when the most influential SNPs in the Bayesian analysis were reduced below the optimum 100. This independent validation showed a strong correlation ($r = 0.5$) between the GBV for the Norberg angle (NA) and the true NA (no EBV was available for the new 38 dogs). Sensitivity, specificity, and positive and negative predictive value of the genomic data were all above 70%. Our analysis demonstrated that prediction of CHD from genomic data is feasible and can be applied for risk management of CHD, and for early selection for genetic improvement to reduce the prevalence of CHD in breeding programmes. The prediction can be implemented before maturity, at which age current radiographic screening programmes are traditionally applied, and as soon as DNA is available (Guo *et al.*, 2011).

Elbow dysplasia

Temwichitr *et al.* (2010) recently reviewed current knowledge concerning the incidence, biomechanics and genetics of elbow dysplasia, especially fragmented medial coronoid process (FMCP) in dogs. The mode of inheritance of the various forms of elbow dysplasia, including osteochondrosis and FMCP, remains undetermined, suggesting that it is a complex trait. In Labrador Retrievers, the heritability of elbow OA secondary to OCD or FMCP was estimated at 0.27 (Studdert *et al.*, 1991). When both osteochondrosis and FMCP were considered together, ranges were as high as 0.77 and 0.45 for male and female Labrador Retrievers and Golden Retrievers, respectively (Guthrie and Pidduck, 1990). Bernese Mountain Dogs are at 12-fold increased risk for elbow dysplasia. A heritable basis for elbow osteochondrosis in Rottweilers and Golden Retrievers has been reported. In Labrador Retrievers, the genetic risk for FMCP ranges from 18% to as high as 50% in some families (Ubbink *et al.*, 2000). Other breeds that are affected are the German Shepherd Dog, Saint Bernard, Great Dane and Newfoundland. Within-breed heritabilities for fragmented medial coronoid process were estimated to be 0.31 for Rottweilers (Maki *et al.*, 2004) and 0.18 in German Shepherd Dogs (Janutta *et al.*, 2006). Hip and elbow dysplasia have some genetic correlation in certain breeds (Maki *et al.*, 2000; Malm *et al.*, 2008) so that selection pressure exerted for one trait may influence the prevalence of the other trait. Selection based on EBVs for elbow dysplasia and its components would be likely to lead to faster reduction in prevalence and severity of the trait. Strong association of collagen genes with elbow dysplasia has been ruled out (Salg *et al.*, 2006).

Adult bone and joint diseases

Cranial cruciate ligament disease

Degeneration and/or rupture of the cranial cruciate ligament (CCLD) is the most common cause of acute and chronic clinical hind limb lameness in dogs. The majority of dogs with CCLD have a chronic disease course without a history of distinct trauma, similar to the non-contact type of ligament rupture in humans. Current data suggest that CCL rupture in dogs is a complex disease to which genetic predisposition and environmental factors contribute (Comerford *et al.*, 2004). Stifle instability following CCLD is associated with a consistent cascade of events, which include capsulitis, synovitis, articular cartilage degeneration, osteophytosis, bone sclerosis and meniscal injuries – all part of the osteoarthritic cascade. Bilateral disease may be as high as 50% in dogs.

HERITABILITY. Canine breeds predisposed to CCLD include the Newfoundland, Labrador Retriever, Rottweiler, Chow Chow and Mastiff. A heritability of 0.27 for rupture of the CCL has been reported in the Newfoundland breed (Wilke *et al.*, 2006). Nielsen *et al.* (2001) estimated heritability for stifle disease at 0.15 (palpation alone) to 0.27 (radiography). No EBVs for CCL rupture are available for dogs.

LOCI FOR CCLD. Wilke *et al.* (2009) discovered four putative QTLs that underlie CCLD in a pedigree of Newfoundland dogs at 68.3 Mb on CFA03, 32.7–33.2 Mb on CFA05, 32.6–33.1 Mb on CFA13 and 3.5–3.8 Mb on CFA24. Interestingly, we discovered a putative QTL for CHD at 61 and 75 Mb on CFA03, which spans the CCLD linkage interval (Table 7.5). Whether this locus is pleiotropic for both CHD and CCLD in large-breed dogs is as yet unexplored. In clinical practice, many large-breed dogs with CCL rupture have concomitant CHD. Although Maccoux *et al.* (2007) identified several upregulated interleukins in osteoarthritic tissue secondary to CCLD, the same group (Clements *et al.*, 2010) failed to find association between SNPs in 121 candidate genes and genomic DNA from dogs with OA secondary to hip and elbow dysplasia or CCLD.

Osteosarcoma

Recently, a major locus for osteosarcoma in the Greyhound and several other large-breed dogs was reported. Dogs carrying the susceptibility allele had an odds ratio of three compared with controls (Sigurdsson *et al.*, 2010). Osteosarcoma is discussed elsewhere in this book.

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8 Genetics of Cancer in Dogs

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Introduction

Scope of the chapter: cancer biology and genetics

Cancer is, in an old cliché, a multiplicity of different diseases. These diseases have a common theme in that they involve an imbalance between cell proliferation and cell death. Unfortunately for our understanding of the diseases, the triggers for this imbalance and the routes that lead from these initial triggers to the manifestation and survival of a tumour are highly varied and usually complex. They include environmental effects on the genome, such as those of chemical mutagens, UVB (medium-wave UV) and other genotoxins in damaging DNA; the effects of some viruses in disrupting the host genome or mimicking host genes involved with cell proliferation and its control; and problems triggered by host intrinsic factors such as repeated growth or inflammatory stimuli, chromosome instability or errors in DNA replication. Whatever the cause, changes in the genetic material and its expression are an essential part of the disease process. These include gain of function mutations, primarily in oncogenes, as well as losses of function – primarily of tumour suppressor genes. There is insufficient space here to give a thorough account of these processes and the reader is referred to a number of excellent textbooks, such as that of Weinberg (2006), and

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excellent web resources. A large number of genes are referred to in this chapter. For the ease of the reader, these are tabulated, with some of their properties, in Table 8.1.

Any consideration of cancer genetics has two parts: the presence or absence of predisposing factors in the germ line (an inherited component to the trigger or to vulnerability to an external trigger), and the genetic changes in somatic tissues that lead to tumour formation. There is a developing science describing the genetics of cancer predispositions in the dog, but this has yet to come to maturity. It will be tackled briefly below as it applies to each disease, as well as in the concluding paragraphs of the chapter. I shall look in more detail at what is known about somatic genomic changes and changes in gene expression in a variety of well-studied tumour types.

Canine cancer: comparison with humans

In the developed world, for dogs, as for humans, cancers are one of the most important causes of both morbidity and mortality. The classification of types of cancer in dogs is very similar to that in humans and follows closely the WHO/IARC (World Health Organization// International Agency for Research in Cancer) guidelines for human tumour classification (WHO/IARC, 2000–2005). The dog also has a relatively large body size, often displays responses to cytotoxic or other therapeutic agents comparable to those of humans, and has a relatively high natural incidence of several cancers with similar biology to that of human tumours. Dogs are long lived and are amenable to a variety of treatment options similar to those available to humans. They share human environments and are exposed to the same group of environmental carcinogens as their owners. As shown elsewhere in this book, they are very amenable to genetic analysis, displaying much less within-breed genetic heterogeneity than humans, so that single genes of major effect are often relatively easy to tease out, and have very long regions of linkage disequilibrium. The genes implicated in cancer (as other canine genes) are closer in sequence to their human orthologues than the equivalent murine genes. Medical surveillance of the canine population of developed countries is maintained at a high level so that even relatively rare diseases are likely to be observed and documented. Canine cancers are naturally occurring, and analyses can, in the first instance, be conducted on clinical populations rather than requiring experimental modelling or breeding colonies. Many molecular mechanisms of tumorigenesis are likely to be similar in human and dogs: for example, chromosomal and genomic instability have already been recognized as important phenomena in canine carcinogenesis. Epigenetic changes have received little formal investigation as yet, but are indicated by the growth inhibitory effect of histone deacetylase inhibitors on a number of tumour cell types. (See the sections on mammary carcinoma and mastocytoma.) Thus, dogs represent an important and extremely useful model to explore cancer biology and therapy.

Differences in tumour frequency by type

Despite similarities in tumour classification, the incidence of different types of tumour differs significantly between dogs and humans. For example, dogs in general are comparatively more prone to sarcomas of many types than humans. In man, soft tissue sarcomas account for about 1% of all adult malignant neoplasms, whereas, in dogs, soft tissue sarcomas account for more than 15% of all malignancies (Dobson *et al.*, 2002). In man, prostate cancer is the most common male tumour with a lifetime risk of diagnosis of about one in nine in the UK, and around half of all 50-year-olds brought to post-mortem having histological evidence of prostate cancer; in dogs, this is not a commonly diagnosed cancer – it is well below the top ten in occurrence – with about 0.6% of male post-mortems (all ages) reported as showing evidence of prostate cancer (Bryan *et al.*, 2007). There are also differences between humans and dogs in the detailed pathology associated with some tumours. For instance, renal cystadenocarcinoma and nodular dermatofibrosis (RCND) is a familial syndrome caused by loss of function of the Folliculin gene (*FLCN*) in dogs (Lingaas *et al.*, 2003). In this species, both skin and kidney involvement is seen in all affected animals. Kidneys are the primary site of cyst formation, but uterine leiomyomas are also

Table 8.1. Genes referred to in the text. Note that many of the acronyms used are explained in other entries in the table.

Gene symbol	Gene name	Gene class	Gene effect	Comments
ABL1	Abelson Leukaemia Virus Oncogene Homologue 1	Tyrosine kinase	Oncogene	Activated by fusion with *BCR1* (see *BCR*)
AKT1	Akt Murine Thymoma Viral Oncogene Homologue 1	Serine/threonine kinase	Oncogene	Also known as protein kinase B
ANGPT2	Angiopoetin 2	Diffusable ligand for TEK protein kinase	Endothelial growth suppressor	Antagonist of angiopoietin 1 (ANGPT1)
ASIP	Agouti Signalling Protein	Paracrine ligand for *MCR1*	Triggers production of pheomelanin instead of eumelanin	Reduces UVB shielding of melanocytes and other skin cells
BAD	BCL2-Associated Agonist of Cell Death – aka BCL-X	BCL2 (B cell lymphoma 2) family. Forms heterodimers with BCL2 and BCL-XL	Tumour suppressor gene	Promotes cell apoptosis by binding BCL-XL and BCL2, reversing their death repressor activity
BAK	BCL2-Antagonist/Killer 1	BCL2 family	Tumour suppressor gene	Localizes to mitochondria, and functions to induce apoptosis
BAX	BCL2-Associated X Protein	BCL2 family	Tumour suppressor gene	Localizes to mitochondria, and functions to induce apoptosis, expression regulated by TP53
BBC3	BCL2 Binding Component 3 – aka P53 Upregulated Modulator of Apoptosis (PUMA)	BH3 – only BCL2 family member	Tumour suppressor gene	Pro-apoptotic transcriptional target of p53 (a tumour suppressor protein encoded by *TP53*)
BCL2L1	BCL2-like 1 – aka BCLX	BCL2 family	Oncogene or tumour suppressor	Two alternative spliced forms; longer isoform acts as an apoptotic inhibitor and the shorter as an apoptotic activator
BCL2L11	BCL2-like 11 (apoptosis facilitator) – aka BIM	BCL2 family	Tumour suppressor gene	Inducible by Neuronal Growth Factor and Forkhead transcription factors
BCR	Breakpoint Cluster Region	Serine/threonine kinase and GTPase-activating protein for p21rac	Oncogene	Activates ABL1 fusion product in lymphomas
BID	BCL2 Homology Domain 3 – Containing Interacting Domain Death Agonist	Death domain binding agonist of BAX or antagonist of BCL2	Tumour suppressor gene	Cleavage by caspase 8 activates

Continued

Table 8.1. Continued.

Gene symbol	Gene name	Gene class	Gene effect	Comments
BIRC5	Baculoviral IAP Repeat Containing 5 – aka Survivin	Inhibitor of apoptosis (IAP) gene family	Oncogene	Gene normally expressed in fetal development
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homologue B1	raf/mil family of serine/threonine protein kinases	Oncogene	Mutations associated with many human and murine cancers
BRCA1	Breast Cancer 1	Nuclear phosphoprotein involved in maintenance of genome stability	Tumour suppressor gene	Mutation responsible for approximately 40% of human inherited breast cancers
BRCA2	Breast Cancer 2	DNA binding and histone acetylase	Tumour suppressor gene	Mutation responsible for many human inherited breast cancers
CADM1	Cell Adhesion Molecule 1 – aka ISGF4, TSLC1	Immunoglobulin superfamily	Pro-apoptotic/tumour suppressor	Nectin-like molecule localized at cell–cell contacts; associates with an actin binding protein
CCND1	Cyclin D1	Cyclin family	Oncogene	Regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition; interacts with tumour suppressor protein Rb
CDK4	Cyclin Dependent Kinase 4	Serine/threonine protein kinase	Oncogene	Phosphorylates RB1, activity required for cell cycle G1/S transition
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A – aka Wild-Type P53-Activated Fragment 1, WAF1	Inhibitor of CDK2 and 4	Tumour suppressor gene	Functions as regulator of cell cycle progression at G1
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A	Inhibitor of CD4 kinase	Tumour suppressor gene	Encodes p16(Ink4a) and p19(ARF) which stabilize p53 and inactivate cell cycle
CDKN2B	Cyclin Dependent Kinase Inhibitor 2B	Inhibitor of CD4 and CD6 kinases	Tumour suppressor gene	Encodes p15(Ink4b) which inactivates cell cycle
COX2	Cyclooxygenase 2 – aka PTGS2	Cyclooxygenase	Oncogene	Prostaglandin synthase involved in inflammation and mitogenesis
CTNNB1	Beta-Catenin	Cadherin-associated protein	Tumour suppressor gene	Anchors actin cytoskeleton; may be responsible for transmitting the contact inhibition signal
CXCR4	Chemokine (C-X-C Motif) Receptor 4	Seven transmembrane region receptor	Oncogene	Alpha-chemokine receptor specific for stromal-derived-factor-1 (SDF1)

DERL1	Derlin, Degradation in Endoplasmic Reticulum-Like 1	DER domain family	Cell survival factor/oncogene	Recognizes and promotes degradation of non-ubiquitylated misfolded proteins; over-expressed in many neoplasms
DLA DRB1/ DQA1/DQB1	Dog Leucocyte Antigens	Major histocompatibility complex type II (MHCII)	Allow recognition of tumour-associated antigens by immune system	Antigen presentation at cell surface
DLC1	Deleted In Liver Cancer Type 1	rho family of GTPase activating proteins (rhoGAP)	Tumour suppressor gene	Regulates small protein phosphorylation in a number of signal transduction pathways
EPHB2	Ephrin Receptor B2 – aka Elk Receptor Kinase (ERK)	Tyrosine kinase, ephrin receptor family	Oncogene/angiogenesis	Normal functions in cell–cell contact and cell morphology; ligand ephrin B is a transmembrane protein
ERBB2	Erythroblastic Leukaemia Viral Oncogene Homologue 2 – aka HER2/Neu	Tyrosine kinase of the epidermal growth factor receptor (EFGR) family	Oncogene	Has no ligand binding domain but activates other EGFs through heterodimer formation
ERFFI1	ERBB Receptor Feedback Inhibitor 1 – aka MIG6	Regulator of receptor trafficking	Tumour suppressor	Protein reduces EGFR signalling by driving EGFR into late endosomes and lysosome-mediated degradation after ligand stimulation
FGF2	Basic Fibroblast Growth Factor	FGF family of heparin-binding proteins	Oncogene and angiogenic factor	Possesses broad mitogenic and angiogenic activities; implicated in limb and nervous system development, wound healing and tumour growth
FHIT	Fragile Histidine Triad Gene	Bis-adenosine triphosphate hydrolase	Tumour suppressor gene	Involved in purine metabolism; human gene encompasses the fragile site FRA3B on HSA3 (human chromosome 3) (orthologue on CFA20 – canine chromosome 3)
FLCN1	Folliculin –aka Birt–Hogg–Dubé Gene BHD	Function unknown	Tumour suppressor gene	Loss of function in dogs causes RCND (renal cystadenocarcinoma and nodular dermatofibrosis)
FLT1	Fms-Related Tyrosine Kinase 1 – aka Vascular Endothelial Growth Factor Receptor (VEGFR) 1	Receptor tyrosine kinase of VEGFR family	Angiogenesis	Binds VEGFA, VEGFB and placental growth factor
FLT3	Fms-Related Tyrosine Kinase 3	Class III receptor tyrosine kinase	Oncogene	Regulates haematopoiesis

Continued

Table 8.1. Continued.

Gene symbol	Gene name	Gene class	Gene effect	Comments
FLT3LG	Flt3-Ligand – aka FLT3L	Helical and cystine knot protein ligand, macrophage colony stimulating factor (MCSF) family	Oncogene	Ligand to FLT3 receptor; controls development of dendritic cells
HGF	Hepatocyte Growth Factor	Plasminogen family of peptidases	Oncogene	Multifunctional cytokine acting on cells mainly of epithelial origin; stimulates mitogenesis, cell motility, and matrix invasion; central role in angiogenesis and tissue regeneration
HMGA1	High Mobility Group (HMG) Protein AT-Hook 1	Non-histone HMG protein	Upregulated in activation	Binds AT-rich DNA; role in opening chromosome structure, regulating transcription
HRAS	Harvey Rat Sarcoma (Ras) Viral Oncogene Homologue	Ras protein family of small GTPases	Oncogene	Functions in signal transduction; undergoes continuous cycle of de- and re-palmitoylation, regulating exchange between plasma membrane and Golgi apparatus
ITGAD	Integrin Alpha-D	Alpha integrin family	Cell surface marker, cell adhesion molecule; encodes cluster determinant CD11D	Myeloid-specific leukocyte integrin; associates with CD18 on macrophages
ITGAX	Integrin Alpha-X	Alpha integrin family	Cell surface marker, cell adhesion molecule; ; encodes cluster determinant CD11C	Myeloid-specific leukocyte integrin; associates with CD18 mainly on dendritic cells
ITGB2	Integrin Beta 2	Integrin beta chain family	Cell surface marker, cell adhesion molecule; encodes cluster determinant CD18	Leukocyte integrin beta chain; combines with a variety of alpha chains
JUN	Jun Activation Domain Binding Protein c-Jun	Transcription factor	Oncogene	Combines with c-Fos to make activating protein AP-1
KIT	Hardy–Zuckerman Feline Sarcoma Viral Oncogene Homologue, cKIT – aka cluster determinant CD117	Tyrosine kinase	Oncogene	Mast/stem cell growth factor receptor

KITLG	Kit Ligand – aka Stem Cell Factor (SCF) – aka Steel Factor	PDGF (platelet-derived growth factor) cytokine family	Oncogene	Activities may reflect a role in cell migration; transmembrane protein can be proteolytically cleaved to produce soluble form or function as cell-associated molecule
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homologue	Ras protein family of small GTPases	Oncogene	Early member of a number of signal transduction pathways; usually tethered to cell membrane
LTBP4	Latent Transforming Growth Factor Beta Binding Protein 4	Extracellular matrix protein	Regulates tissue organization	Binds transforming growth factor beta (TGFB) as it is secreted and targets the extracellular matrix
MAP2K1	Mitogen-Activated Protein Kinase Kinase 1 – aka MEK	Dual-specificity protein kinase family	Oncogene	Kinase that lies upstream of MAPK1
MAPK1	Mitogen-Activated Protein Kinase 1 – aka ERK2	MAP kinase family	Oncogene	Activated by upstream kinases including MAP2K1; upon activation, translocates to the nucleus and phosphorylates nuclear targets
MC1R	Melanocortin 1 Receptor	Seven-pass transmembrane G protein-coupled receptor	Oncogene	Receptor protein for melanocyte-stimulating hormone (MSH)
MDM2	Mdm2 P53 Binding Protein Homologue	Nuclear phosphoprotein	Oncogene	Binds and inhibits transactivation by tumour protein p53
MET	MET proto-oncogene	Tyrosine kinase	Oncogene	Hepatocyte growth factor receptor; also activated in papillary renal carcinoma
MMP9	Matrix Metallopeptidase 9	Matrix metalloproteinase	Role in tumour-associated tissue remodelling	Degrades type IV and type V collagens
MTOR	Mechanistic Target of Rapamycin	Serine/threonine kinase	Oncogene	Phosphatidylinositol kinase-related kinase, target for PI3K (phosphoinositide 3-kinase)
MUM1	Melanoma Associated Antigen (Mutated) 1 – aka Melan-A, MAA	Chromatin component	Oncogene	After DNA damage, MUM1 rapidly concentrates in vicinity of DNA damage sites and promotes cell survival
MYC	Myelocytomatosis Viral Oncogene Homologue	Transcription factor	Oncogene	Roles in cell cycle progression, apoptosis and cellular transformation
NRAS	Neuroblastoma RAS Viral Oncogene Homologue	Ras protein family of small GTPases	Oncogene	Functions in signal transduction; undergoes continuous cycle of de- and re-palmitoylation, regulating exchange between plasma membrane and Golgi apparatus

Continued

Table 8.1. Continued.

Gene symbol	Gene name	Gene class	Gene effect	Comments
NTRK1	Neurotrophic Tyrosine Kinase Receptor, Type 1 – aka TRKA	Tyrosine kinase receptor	Oncogene	On neurotrophin binding, phosphorylates itself and members of the MAPK pathway
PI3K	Phosphoinositide-3-Kinase	Pi3k protein is heterodimer of catalytic and adaptor proteins	Oncogene	Catalytic proteins may be one of four: PI3KCA, CB, CG or CD; PI3KCA is most strongly associated with cancer
PMAIP1	Phorbol-12-Myristate-13-Acetate-Induced Protein 1 – aka NOXA	BH3-only BCL-2 family member	Tumour suppressor gene	Pro-apoptotic transcriptional target of p53
PTEN	Phosphatase and Tensin Homologue	Protein tyrosine phosphatase	Tumour suppressor gene	Preferentially dephosphorylates phospho-inositide substrates
PTHLH	Parathyroid Hormone-Like Hormone – aka PTHrP	Parathyroid hormone family	Causative of humoral hypercalcaemia of malignancy	Regulates endochondral bone development and epithelial–mesenchymal interactions during the formation of mammary glands and teeth
RAD51	RAD51 Homologue (*S. cerevisiae*)	RAD51 protein family	Loss causes genomic instability	Highly similar to *Escherichia coli* DNA recombinase A; interacts with Brca1 and Brca2 proteins
RAF1	V-Raf-1 Murine Leukaemia Viral Oncogene Homologue 1	Serine/threonine protein kinase	Oncogene	A MAP kinase kinase kinase (MAP3K) that functions downstream of ras proteins
RB1	Retinoblastoma 1	Chromatin component	Tumour suppressor gene	Negative regulator of the cell cycle; active form is hypophosphorylated
SATB1	Special AT-Rich Sequence-Binding Protein 1	Homeobox gene	Oncogene – promotes metastasis	Protein binds nuclear matrix and scaffold as well as DNA–chromatin remodelling for transcription
SHC1	Src Homology 2 Domain Containing Transforming Protein 1	Redox protein	Longest isoform is tumour suppressor	Three isoforms, adapter proteins in signal transduction pathways
SLC2A1	Solute Carrier Family 2 Member 1 – aka GLUT1	Solute carrier 2 family member	Facilitates cell nutrition	Major glucose transporter in blood–brain barrier
SLIT2	Slit Homologue 2 (*Drosophila*)	Chemorepulsive factor	Tumour suppressor gene	Inhibits chemotaxic migration of various types of cells

STAT3	Signal Transducer and Activator of Transcription 3	STAT protein family	Oncogene	Mediates signal transduction in response to cytokines and growth factors including IFNs, EGF, IL5, IL6, HGF, LIF and BMP2
STAT5B	Signal Transducer and Activator of Transcription 5B (previously STAT5)	STAT protein family	Oncogene	Mediates signal transduction triggered by IL2, IL4, CSF1 and different growth hormones
STC1	Stanniocalcin 1	Homodimeric glycoprotein	Cell homeostasis	Role in the regulation of renal and intestinal calcium and phosphate transport and cell metabolism
TERT	Telomerase Reverse Transcriptase	Ribonucleoprotein polymerase	Oncogene-cell immortalization	Maintains telomere ends by addition of the telomere repeat TTAGGG
TP53	Tumour Protein P53	TP53/73 family	Tumour suppressor gene	Multifunctional transcription factor controlling cell cycle arrest, apoptosis, senescence, DNA repair
TYR	Tyrosinase (Oculocutaneous Albinism IA)	Tyrosinase family enzyme	Biomarker of melanocytes	Enzyme has tyrosine hydroxylase and dopa oxidase catalytic activities
TYRP1	Tyrosinase-Related Protein 1	Tyrosinase family enzyme	Biomarker of melanocytes	Plays important role in the melanin biosynthetic pathway
VEGFA	Vascular Endothelial Growth Factor	PDGF/VEGF growth factor family	Oncogene and angiogenic factor	Increases vascular permeability, promotes angiogenesis, cell growth and migration, and inhibits apoptosis of endothelial cells
WT1	Wilms Tumor 1	Zinc finger transcription factor	Oncogene	Role in normal development of the urogenital system; mutated in patients with Wilm's tumours
YES1	Yamaguchi Sarcoma Viral Oncogene Homologue 1	Scr family tyrosine kinase	Oncogene	Roles in signal transduction

seen in female RCND dogs. Pulmonary cysts occur but are relatively rare (Moe and Lium, 1997). In Birt–Hogg–Dubé syndrome (BHD), a genetically orthologous disease in humans, all affected individuals have skin nodules but only about 20–30% have kidney cysts, while a greater proportion have lung cysts (Gunji *et al.*, 2007). Leiomyomas are not reported in human BHD patients.

The Morbidity and Mortality Burden of Cancer in Dogs

The lack of general veterinary surveillance of causes of death in dogs anywhere in the world makes it difficult to obtain hard numbers addressing the burden of canine cancer. In the USA, a necropsy survey by Dorn (1976) showed that 23% of all canine non-trauma deaths were from cancer. There have been only a few more recent surveys of cancer incidence. An owner survey of 15,881 deaths of pedigree dogs in the UK showed 27% of deaths from cancer (Adams *et al.*, 2010); similar figures were recorded in retrospective necropsy studies in Germany (Eichelberg and Seine, 1996), although a smaller Danish survey gave only 14.5% as an equivalent figure (Proschowsky *et al.*, 2003); insurance data for 220,000 dogs up to 10 years of age from 1992 and 1993, and for 350,000 dogs up to 10 years of age from 1995 to 2000 in Sweden gave intermediate figures, with tumours accounting for 17.5% and 18% of deaths (Bonnett *et al.*, 1997, 2005). In several studies, mortality from cancer goes up markedly with age as a proportion of all deaths (Dorn, 1976; Bonnett *et al.*, 2005; Egenvall *et al.*, 2005a), although, in one UK study of claims for veterinary treatment for cancer, these peak by the age of 9–11 years and thereafter fall off somewhat, even after allowing for the age structure of the insured versus the total population (Dobson *et al.*, 2002).

Published surveys suffer from a number of intrinsic biases in their reference populations, as well as from disparities and inaccuracies in recording of cause of death. Thus, insurance databases usually exclude older animals and may show a bias towards pure breds; retrospective analyses of veterinary referral hospital cases are biased towards the animals of richer owners (causing both environmental/dietary biases and biases to pure-bred animals) as well as suffering other local biases; owner surveys are by their nature biased towards owners who are on registries or refer to particular websites – nearly always again showing biases towards pure-bred and pedigree animals. Nevertheless, a consensus exists that 20–30% of all canine deaths are connected with cancer. In addition, cancers self-evidently cause considerable morbidity to those that suffer them, and may require severe (sometimes mutilating) surgical, radiation and chemotherapeutic treatments. In the case of dogs, there is a particular challenge in deciding when aggressive treatment options are appropriate, as minimal palliative options may be preferable on welfare grounds. Thus cancers represent one of the largest veterinary challenges as well as being of great comparative interest.

Breed-specific Predispositions to Cancer

It has been noted many times that particular breeds of pedigree dog show excess incidence of particular types of tumours (Priester and Mantel, 1971). Boxers and some lines of Rottweiler have been reported to be particularly susceptible to many types of cancers (Priester, 1967; Peters, 1969; Richards *et al.*, 2001; Rivera *et al.*, 2009). In Rottweilers, a polymorphism in the *MET* oncogene has been suggested to be associated with this trait (Liao *et al.*, 2006). Recognized predispositions to particular tumours are compiled in Table 8.2. However, much of the information on some of these associations has remained anecdotal (or unsupported by the peer-reviewed literature), and only a few relative risk figures are available. Relative risks that have been calculated for particular cancers within breeds (or groups of breeds) are sometimes much higher than for human cancers in particular groups. For instance, osteosarcoma in the most at-risk giant breeds (Scottish Deerhound; Irish Wolfhound; Great Dane) is about 200 times more common than in smaller pedigree breeds such as the Cocker Spaniel or

Table 8.2. Breed-associated or familial tumours in dogs.

Tumour type	Breed(s)
Epithelial	
Gastric	Chow Chow (McNiel *et al.*, 2004a), Tervuren (Belgian Shepherd) (Lubbes *et al.*, 2009)
Mammary	Beagle (Schafer *et al.*, 1998), Cocker Spaniel, Dachshund (Frye *et al.*, 1967; McVean *et al.*, 1978), German Shepherd, Pointer (Frye *et al.*, 1967), Springer Spaniel (Egenvall *et al.*, 2005b; Rivera *et al.*, 2009)
Nasal	Airedale, Basset Hound, Collie, German Short-haired Pointer (Wilson and Dungworth, 2002), Scottish Terrier, Shetland Sheepdog
Oral (squamous cell carcinoma)	Labrador Retriever, Poodle, Samoyed (Dennis *et al.*, 2006)
Renal (cystadenocarcinoma)	German Shepherd (Lium and Moe, 1985; Moe and Lium, 1997; Lingaas *et al.*, 2003)
Urinary bladder (transitional cell carcinoma)	Beagle, Scottish Terrier (Raghavan *et al.*, 2004), Shetland Sheepdog, West Highland White Terrier, Wire-haired Fox Terrier (Knapp *et al.*, 2000)
Skin	
Anal sac adenocarcinoma	Alaskan Malamute, Dachshund, English Cocker Spaniel (Polton *et al.*, 2006), English Springer Spaniel, German Shepherd (Goldschmidt and Shofer, 2002)
Basal cell tumours	Bichon Frise, Cockapoo, Cocker Spaniel, English Springer Spaniel, Kerry Blue Terrier, Miniature Poodle, Shetland Sheepdog, Siberian Husky, West Highland White Terrier (Goldschmidt and Shofer, 2002)
Ceruminous gland carcinoma	Cocker Spaniel, German Shepherd (Goldschmidt and Shofer, 2002)
Intracutaneous cornifying epethilioma	German Shepherd, Lhasa Apso, Norwegian Elkhound, Standard Poodle, Yorkshire Terrier (Goldschmidt and Shofer, 2002)
Perianal (hepatoid) gland tumours	Beagle, Brittany Spaniel, Cockapoo, Cocker Spaniel, Lhasa Apso, Pekinese, Samoyed, Shih Tzu, Siberian Husky, Vizsla (Goldschmidt and Shofer, 2002)
Squamous cell carcinoma	Basset Hound, Keeshond, Standard Poodle (Goldschmidt and Hendrick, 2002; Goldschmidt and Shofer, 2002)
Squamous cell carcinoma (subungual)	Dachshund, Gordon Setter, Kerry Blue Terrier, Labrador Retriever, Rottweiler, Scottish Terrier, Standard and Giant Schnauzer, Standard Poodle (Goldschmidt and Hendrick, 2002; Goldschmidt and Shofer, 2002)
Trichoepithelioma	Airedale Terrier, Basset Hound, Bullmastiff, English Setter, English Springer Spaniel, Golden Retriever, Irish Setter, Standard Poodle, Miniature Schnauzer (Goldschmidt and Shofer, 2002)
Endocrine	
Pancreatic islet cells	Airedale Terrier, German Shepherd Dog, Irish Setter, Standard Poodle (Priester, 1974; Kruth *et al.*, 1982)
Pheochromocytoma (adrenal chromaffin cells)	Airedale Terrier, Wire-haired Fox Terrier (McNiel and Husbands, 2005)
Thyroid	Alaskan Malamute, Beagle, Boxer, Golden Retriever, Siberian Husky (Hayes and Fraumeni, 1975; Wucherer and Wilke, 2010)
Mesenchymal	
Brain tumours	Boston Terrier (glioblastoma), Boxer (Hayes *et al.*, 1975)

Continued

Table 8.2. Continued.

Tumour type	Breed(s)
Histiocytoma (cutaneous, benign)	American Pit Bull Terrier, Boxer, Boston Terrier, Bulldog, Bull Terrier, Cocker Spaniel, Dalmatian, Doberman Pinscher, English Cocker Spaniel, English Springer Spaniel, Jack Russell Terrier, Labrador Retriever, Miniature Schnauzer, Pug, Rottweiler, Scottish Terrier, Shar-pei, West Highland White Terrier (Goldschmidt and Shofer, 2002)
Histiocytic sarcoma	Bernese Mountain Dog, Flat-coated Retriever, Golden Retriever, Rottweiler (Moore, 1984; Padgett *et al.*, 1995; Affolter and Moore, 2000, 2002; Morris *et al.*, 2000; Moore *et al.*, 2006; Abadie *et al.*, 2009)
Haemangiosarcoma	German Shepherd (Appleby *et al.*, 1978; Prymak *et al.*, 1988; Srebernik and Appleby, 1991), Golden Retriever (Goldschmidt and Hendrick, 2002)
Melanoma (oral)	Chow Chow, Cocker Spaniel, Golden Retriever, Miniature Poodle, Pekinese/Poodle cross (Ramos-Vara *et al.*, 2000; Dennis *et al.*, 2006)
Melanoma (subungual)	Giant Schnauzer, Rottweiler, Scottish Terrier (Schultheiss, 2006)
Melanoma (eye/limbal)	Golden Retriever (Donaldson *et al.*, 2006)
Osteosarcoma	Large-breed dogs: Bullmastiff, English Mastiff, Great Dane, Great Pyrenees, Irish Wolfhound, Newfoundland, Rottweiler, Saint Bernard. Also Borzoi, Greyhound, Irish Setter, Golden Retriever, Labrador Retriever (Misdorp and Hart, 1979; Ru *et al.*, 1998; Egenvall *et al.*, 2007; Rosenberger *et al.*, 2007)
Haematopoietic	
Lymphoma/leukaemias	Boxer, Bulldog, Bullmastiff, Cocker Spaniel, Setter, many breeds (Priester, 1967; Edwards *et al.*, 2003; Lurie *et al.*, 2004)
Mast cell tumours	Breeds with Bulldog ancestry (Boston Terrier, Boxer, Bull Terrier) (Peters, 1969), Labrador and Golden Retrievers, Pug (McNiel *et al.*, 2004b), Weimaraner (Murphy *et al.*, 2004)
Plasmacytoma (cutaneous)	Airedale Terrier, Cocker Spaniel (Dennis *et al.*, 2006), Scottish Terrier, Standard Poodle (Goldschmidt and Shofer, 2002)

Dachshund or in small mixed-breed dogs (Ru *et al.*, 1998). In retrospective studies, Bernese Mountain Dogs experience types of histiocytic sarcoma at some 50 to 100 times the rate expected from the proportion of these breeds in the general population (Affolter and Moore, 2002), with a prevalence of up to 25% (Abadie *et al.*, 2009), while up to 50% of all tumours in the Flat-coated Retriever are also histiocytic (Morris *et al.*, 2000). The English Cocker Spaniel has been shown to be predisposed to anal sac gland carcinoma (ASGC), both in the USA and in three data sets in the UK, with mean odds ratios centring on 7.3 (95% confidence interval (CI_{95}) = 3.4–14.6) compared with the general dog population (Polton *et al.*, 2006). Many of the breed relative risks given in Table 8.2 are likely to be lower – although probably only twofold to threefold increases (based on web registries or insurance data).

Heritability estimates have been made for some cancers and vary widely for different tumours. Narrow sense heritability for osteosarcoma in the Scottish Deerhound has been calculated as 0.69 (Phillips *et al.*, 2007), for malignant histiocytic sarcoma in the Bernese Mountain Dog as 0.30 (Padgett *et al.*, 1995), and for gastric carcinoma in the Tervuren (Belgian) Shepherd as 0.09 (±0.02) (Lubbes *et al.*, 2009). On the basis of the likely identity by descent of the predisposing mutations within individual breeds, causative genes have been searched for in a number of cancers. Initially these searches concentrated on candidate

genes, but more recently whole-genome or transcriptome approaches have been used.

Genetics of Well-characterized Cancers

Renal cystadenocarcinoma and nodular dermatofibrosis (RCND) – a monogenic inherited cancer syndrome

RCND in certain Scandinavian, American and German lines of the German Shepherd Dog is characterized by the formation of numerous nodules of dense collagen fibres in the skin and subcutis, and multifocal renal cysts consisting of epithelial proliferations (Lium and Moe, 1985; Moe and Lium, 1997). The latter are malignant. The syndrome shows autosomal dominant inheritance. Candidate genes including *TSC1, TSC2, TP53, PDK1, KRT9, WT1, FH and NF1* were eliminated and the RCND locus mapped to chromosome 5 using linkage analysis in an extended pedigree with 67 F_2 individuals (Jónasdóttir *et al.*, 2000). Following refinement of the interval, the disease was shown to segregate with the mutation H255R in the canine folliculin/BHD gene *FLCN* (Lingaas *et al.*, 2003). Although this mutation is conservative in terms of amino acid charge, it is at an evolutionarily conserved histidine found in this position across all eukaryotes from yeast upwards. Furthermore, an examination of renal tumours in RCND-affected dogs showed second hits on the *FLCN* gene with predicted functional implications in many tumours (Bønsdorff *et al.*, 2009). Targeted mutation of the *FLCN* gene in mice gives rise to renal tubule hyperproliferation and polycystic kidney disease in heterozygotes. Tumours can be shown to lack folliculin protein (Hudon *et al.*, 2010). This then represents a paradigm case for the Knudson model of carcinogenesis through tumour suppressor gene loss.

Osteosarcoma

Canine osteosarcoma is similar to human osteosarcoma in its sites of occurrence (>75% in the appendicular skeleton, often in a metaphyseal location), and in the histology and metastatic potential of the tumour (Withrow *et al.*, 1991). Over the whole dog population, annual incidence is about eight times that in humans (at 7.9/100,000). But in those breeds listed as predisposed in the previous section, and some others listed in Table 8.2, more than 10% of all breed mortality may occur from this tumour. In humans, the tumour shows the highest incidence during the adolescent growth spurt. In dogs, the tumour is most common in giant breeds, and, in at least one study, prevalence is associated with height and, to a lesser extent, weight, although the highest incidence occurs later in life than is the case for the human cancer (Ru *et al.*, 1998).

Transcriptome analysis shows similarities in clustering between osteosarcomas in humans and dogs (Paoloni *et al.*, 2009). Characteristic changes in gene expression within tumours and tumour cell lines include over-expression of the *MET, HER2/ERBB2* and *TRKA* oncogenes and of *MDM2*, expression of a number of biomarkers such as the IL-11R alpha subunit, the hepatocyte growth factor cDNA *HGF*, the chemokine receptor CXCR4, and the glucose transporter SLC2A1 (GLUT1), with elevated levels of the phosphorylated form of the Akt1 serine/threonine protein kinase, which is a substrate for the phosphatase and tensin homologue PTEN (Ferracini *et al.*, 2000; Flint *et al.*, 2004; Fan *et al.*, 2008; Petty *et al.*, 2008; Sottnik and Thamm, 2010). PTEN is downregulated in many osteosarcoma cell lines, often accompanied by large deletions in (or containing) the *PTEN* gene (Levine *et al.*, 2002). *RB1* mutation and *MDM2* amplification have been recorded in a few osteosarcoma cell lines (Mendoza *et al.*, 1998). The tumour suppressor protein p53 also appears to be over-expressed in a proportion of tumours, but in different studies 30–84% of canine osteosarcoma cell lines and tumours have mutations in the *TP53* gene, usually in the DNA-binding domain, and in the form of single nucleotide polymorphisms (SNPs) rather than larger rearrangements (van Leeuwen *et al.*, 1997; Johnson *et al.*, 1998; Mendoza *et al.*, 1998; Setoguchi *et al.*, 2001). There is no evidence that these mutations originate in the germ line.

The karyotype of canine osteosarcomas has been studied by Thomas *et al.* (2009).

Individual tumours show complex rearrangements, in which recurrent amplifications and deletions occur, the former associated with the *MYC, KIT* and *HRAS* genes, the latter with *WT1, RB1* and *PTEN*. Comparisons between Golden Retriever and Rottweiler breeds showed eleven regions of the genome where copy number changes were significantly associated with disease in the breed, including regions spanning the *YES1* and *WT1* loci.

To look at genetic predispositions to osteosarcoma, genome-wide association studies (GWAS) are now being performed by several laboratories in breeds including the Greyhound, Rottweiler, Irish Wolfhound and some others, although these have not yet led to published associations.

Mammary carcinoma

Canine mammary carcinoma has an uneven geographic distribution in part because of varying practices around the world regarding neutering (spaying) of canine bitches. The lifetime risk of malignant mammary tumours increases rapidly with number of oestrus cycles over the lifetime of the dog. An early estimate is that in bitches spayed before their first oestrus cycles the risk is 0.05%. In those undergoing one oestrus cycle it is 8%, and in unspayed 2-year-old dogs (undergoing two to three oestrus cycles) it is 26% (Schneider *et al.*, 1996). So this type of tumour is much more common in countries that do not spay their dogs, or do so late, than in those where the spaying of non-breeding dogs occurs early. Exposure to combinations of exogenous synthetic oestrogens and high doses of progestins increases the incidence of mammary cancer, although either hormone alone does not (see review, Misdorp, 2002). Oestrogen and progestin receptor expression is variable, with tumours positive for both receptors considered luminal type, while those negative for both are considered basal type. Mixed-type tumours also have high levels of oestrogen receptors, especially of the beta receptor. Higher receptor levels are seen in benign than in malignant tumours (Donnay *et al.*, 1996; de Las Mulas *et al.*, 2005). Like human mammary carcinoma, the canine disease occurs in different locations, with ductal carcinoma the most common, followed by lobular carcinoma. About 30% of excised tumours are malignant (Misdorp, 2002). Tumours are diverse in histological appearance, with simple carcinomas the most common, but tubulopapillary adenocarcinoma and mixed-cell tumours, including stellate, spindle or large pleiomorphic cells, are also important. It is likely that some tumours are of myoepithelial origin. A range of sarcomatous lesions is also seen. It is therefore unlikely that mammary tumours have either a single common genetic origin or a common pathogenesis history. However, breed predispositions as well as susceptible lines within breeds have been noted (see Table 8.2).

A large number of studies have classified canine mammary tumours by the expression of specific genes or whole transcriptomes. Association of gene expression with classification by tumour subtype has been performed (Rao *et al.*, 2008; Pawlowski *et al.*, 2009; Wensman *et al.*, 2009). It is clear that the tumours behave in many respects like those of humans. Elevated expression of cyclinD1, loss of cyclin-dependent kinase inhibitors, loss or aberrant expression of *TP53, PTEN* and *STC1*, and elevated *COX2* expression are all often associated with malignancy (Inoue and Shiramizu, 1999; Doré *et al.*, 2003; Sfacteria *et al.*, 2003; Heller *et al.*, 2005; Klopfleisch and Gruber, 2009a; Lavalle *et al.*, 2009; Ressel *et al.*, 2009). Benign and metastatic mammary tumours have been classified according to the aberrant expression of oncogenes, with genes including *ERBB2, MYC, APC, SLIT2, MIG6, SATB1, SMAD6, MMP9, LTBP4, DERL1* and others associated with malignancy (Rutteman *et al.*, 1994; Yokota *et al.*, 2001; Restucci *et al.*, 2007; Nowak *et al.*, 2008; Wensman *et al.*, 2009; Amorim *et al.*, 2010a; Klopfleisch *et al.*, 2010 (review, and references therein); Król *et al.*, 2010). *RAD51, BRCA1* and *BRCA2* also have altered expression in many mammary adenomas and adenocarcinomas and their metastases, but the direction of the change (elevated or reduced expression) varies in different tumours (Nieto *et al.*, 2003; Sugiura *et al.*, 2007; Klopfleisch and Gruber, 2009b). Separate studies have shown splice variation or mutations in *BRCA1* and *BRCA2* genes in tumours (Sugiura *et al.*, 2007; Hsu *et al.*, 2010).

A proportion of mammary tumours also demonstrate a mutator phenotype: high-level microsatellite instability (>20% of loci examined showing instability) has been shown at a high proportion of examined loci in just over 10% of a group of mammary tumours from a mixture of breeds (McNiel *et al.*, 2007).

Recently, a larger association study looking at potential predisposing genes for mammary carcinoma in the English Springer Spaniel in Sweden has shown that there is an association with the tumour of germ-line SNP variants around both *BRCA1* and *BRCA2*, with risk genotypes at each locus carrying a relative risk of about four (Rivera *et al.*, 2009). For several of the other genes mentioned in the previous paragraph germ-line SNPs were present in the study population, but no such predisposing association with the tumour could be demonstrated. In the same group of dogs, a protective *MHC* Class II haplotype has been identified in the at-risk population (*DRB1*00101/DQA1*00201/DQB1*01303*), suggesting a role for the adaptive immune system in surveillance for this tumour (Rivera, 2010). GWAS are continuing in this breed.

Anal sac gland carcinoma (ASGC) in English Cocker Spaniels

ASGCs are adenocarcinomas arising from the apocrine secretory epithelium of the anal sac. They may be seen as models for human glandular carcinomas of the anogenital glands, of sweat glands, of Moll's gland and of some forms of mammary gland cancer. The level of differentiation of ASGC tumours is very variable. They have a high metastatic potential, initially to adjacent lymph nodes, but subsequently become systemic. E-cadherin expression is associated with longer survival times, while tumours producing parathyroid hormone-related protein (PTHrP) (leading to hypercalcaemia, polyuria and polydypsia) have poor survival.

ASGC may occur in any dog breed, but it is a significant problem in English Cocker Spaniels and, to a lesser degree, in other Spaniel types (English Springer and Cavalier King Charles) and in some other breeds (Goldschmidt and Shofer, 2002; Polton *et al.*, 2006). The tumours occur in older animals, with typical onset at 8 to 12 years of age. Historically, this has been described either as a condition of the older bitch or of the intact male dog. Recent work does not indicate a gender predisposition, but in the English Cocker Spaniel breed it has indicated a higher prevalence in neutered animals that is more marked in males (Williams *et al.*, 2003; Polton *et al.*, 2006). It has been noted that many (possibly all) ASGC-affected English Cocker Spaniels are related through a single individual at four to eleven generations from the investigated cases (Aguirre-Hernández *et al.*, 2010).

English Cocker Spaniels with the *DLA* type *DLA-DQB1*00701* are at higher risk of ASGC (odds ratio, OR = 5.4 CI_{95} 2.51–11.61), whereas *DLA-DQB1*02001* is protective (OR = 0.25, CI_{95} 0.10–0.63) (Aguirre-Hernández *et al.*, 2010; see also Chapter 6). However the predisposing DLA type is very common in Cocker Spaniels, and other factors must also determine tumour onset. Whole genome mapping of predisposing genes is now underway in this breed.

Histiocytic sarcoma

Histiocytic sarcomas in dogs include both CD11c- and CD11d-positive tumours: tumours of dendritic cells and those of macrophages (Affolter and Moore, 2000, 2002). The tumours are CD18 and MHC Class II positive and CD4 negative; cells are typically histiocytic in form, with simple nuclei and substantial cytoplasm, although they may also be pleomorphic or dedifferentiated. Both dendritic cell tumours and those of macrophages are rare in humans, with only a few hundred cases reported in the medical literature (see review, Kairouz *et al.*, 2007). CD11c-positive dendritic cell tumours are considerably less rare in dogs, but only reach high prevalence in a few breeds, including the Bernese Mountain Dog, the Flat-coated Retriever, the Rottweiler and Golden Retriever (see Table 8.2 and associated references). Interestingly, CD11d-positive histiocytic sarcomas – containing macrophages – have been found most commonly in the same group of breeds (Moore *et al.*, 2006), suggesting some similarities in genetic mechanism. Localized histiocytic sarcomas, sometimes

including spindle-like cells, are found either in the subcutis or embedded in deep muscle sites in the limbs, and are the most frequent type found in Flat-coated Retrievers. Metastasis is often to local lymph nodes; these cases were previously referred to as suffering from Malignant Fibrous Histiocytosis. In the Bernese Mountain Dog, a disseminated form of the tumour affects the viscera, and particularly the spleen, lungs and liver, as well as causing nodular or ulcerated cutaneous lesions (Morris *et al.*, 2000, 2002; Affolter and Moore, 2002; Abadie *et al.*, 2009). However, Flat-coated Retrievers do also develop the disseminated form of tumour, with most of these cases expressing CD11d (Constantino-Casas *et al.*, 2010). Median survival times for localized histiocytic tumours are typically less than a year post diagnosis, while for the disseminated tumour survival may be only 60–100 days.

Such clear breed predispositions to this tumour have sparked a hunt for the genes responsible. A very large pedigree analysis study has shown that segregation of the tumour is not compatible with recessive inheritance, with a better fit to autosomal oligogenic models (Abadie *et al.*, 2009). In a general survey of variations in the *CDKN2B* gene, there was no association between numbers of repeats in the gene and cases of histiocytic sarcoma in Flat-coated Retrievers (Aguirre-Hernández *et al.*, 2009). Dendritic cell differentiation involves signalling through tyrosine kinases cKIT, FLT3 and MET, and their partner ligands SCF, FLT3L and hepatocyte growth factor (HGF). Within tumours, *KIT, FLT3* and *MET* do not show mutations and there are no systematic changes in RNA amounts for either receptors or ligands (Zavodovskaya *et al.*, 2006). GWAS are in progress looking for predisposing genes using large cohorts, but results have not yet been published. Some cytogenetic analysis has been conducted both on arrays and on metaphases. In a single tumour there were whole chromosome gains for CFA3, CFA13 and CFA37, and a high-level amplification on CFA20. Part or whole chromosome losses were detected on CFA5, CFA12, CFA14, CFA16, CFA19, CFA21, CFA23, CFA26 and CFA32. CFA9 showed both gains and losses (Thomas *et al.*, 2008). The large sizes of the deleted regions prevented identification of individual genes.

Mastocytoma

Mast cells are a population of bone marrow-derived cells that have a role in surveillance and defence of tissues against pathogens. When mature (in the tissues), they are distinguished by the presence of large basophilic granules rich in histamine, proteases, inflammatory cytokines and heparin, which are released on activation of the cell by IgE binding or in tissue damage.

Tumours of mast cells (mastocytomas) are very common in dogs, and represent up to 20% of all tumours in the skin (O'Keefe, 1990). In general, these tumours originate in the dermis or subcutis. They are also sometimes found at extra-cutaneous sites, including the conjunctiva, salivary gland, nasopharynx, oral cavity, gastrointestinal tract and urethra. In most dogs, tumours are solitary, but in 5–25% of cases they are multiple. Disseminated, visceral or systemic mastocytosis almost always occurs only as a sequel to an undifferentiated primary cutaneous mast cell tumour. The risks associated with the tumour arise not only from systemic spread, but also from the activation and degranulation of mast cells that occurs in up to 50% of all mast cell tumours (O'Keefe, 1990). This can lead to ulceration and bleeding around the tumour site (or bleeding on surgical excision), to gastrointestinal ulceration and (unusually) to anaphylactic shock. Several clinical staging systems for mastocytoma have been used for prognostic purposes, and particularly to gauge the risk of metastatic spread, but these will not be described here.

The *KIT* proto-oncogene encodes a receptor tyrosine kinase, c-KIT or CD117, that is activated by the ligand stem cell factor (SCF). Through interactions with the NRAS, PI3K and MEK/ERK (MAPK/EPHB2) pathways, this kinase upregulates intracellular processes such as cell growth, division and migration (Fig. 8.1). SCF binding causes c-KIT dimerization, autophosphorylation and activation. The involvement of c-KIT mutations in the transformation of rodent mast cell lines, as well as in human mastocytoma, has been known for many years. In canine mastocytoma, CD117 expression is often elevated. Activating mutations have included duplications within the juxtamembrane segment that cause constitutive autophosphorylation and

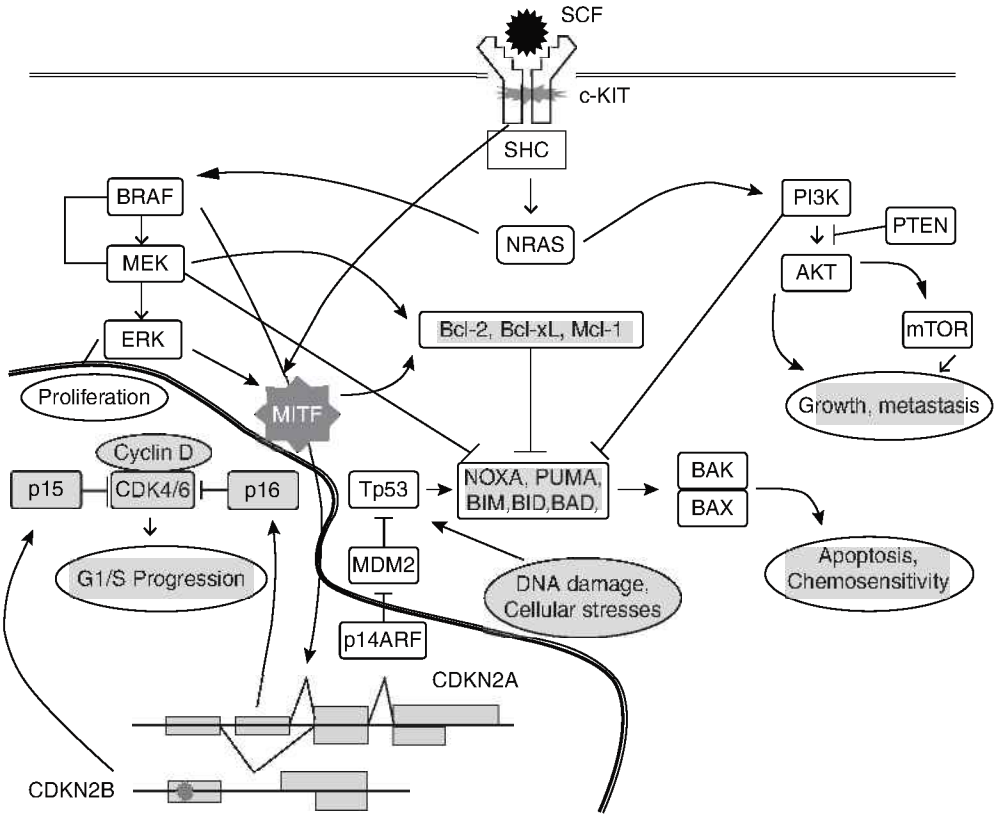

Fig. 8.1. Signal transduction and c-Kit, showing regions of duplications in mastocytoma. The *KIT* proto-oncogene encodes a receptor tyrosine kinase, c-KIT or CD117, that is activated by the ligand stem cell factor (SCF). The binding of SCF causes c-KIT dimerization, autophosphorylation and activation. The activated kinase, through interactions with the NRAS, PI3K and MEK/ERK (MAPK/EPHB2) pathways, upregulates intracellular processes such as cell growth, division and migration.

are associated with higher grade (more aggressive) tumours, as well as both deletions and substitutions in this region of the gene, some of which have also been shown to auto-activate c-KIT. Mutations in the *KIT* gene are not always present, however (London *et al.*, 1999; Ma *et al.*, 1999; Zemke *et al.*, 2002; Riva *et al.*, 2005; Ohmori *et al.*, 2008).

Gene expression and immunochemical studies both in tumours and cell lines have shown that tumours often have upregulated *MDM2*, dysregulated *P53*, and upregulated prostaglandin E2 (causing a further activation of the AKT pathway), while higher grade tumours express survival factors such as vascular endothelial growth factor (VEGFA), and have reduced cellular adhesion molecule CADM1 (TSLC1) expression (Wu *et al.*, 2006; Amorim *et al.*, 2010b; Taylor *et al.*, 2010). GWAS are now in progress in several breeds.

Specific inhibitors of c-KIT tyrosine kinase function, including imatinib mesylate, masitinib and bafetinib, have been used to treat canine mast cell tumours, and are effective against a proportion of them (Isotani *et al.*, 2008; London, 2009). These are competitive inhibitors of the ATP binding site of tyrosine kinases in the *KIT/ABL/PDGF* family, and are effective in the treatment of a range of tumours in which these molecules are active. As might be expected, the mutation spectrum of the *KIT* gene correlates with efficacy in the one available study.

(Peter *et al.*, 2010). AR-42 and vorinostat, two inhibitors of histone deacetylation, both restrict the growth of canine mastocytoma cell lines *in vitro*. *KIT* gene expression is downregulated by AR-42, suggesting an epigenetic basis for this growth inhibition. Downstream mediators of signal transduction, including Akt and STAT3/5, are also inhibited (Lin *et al.*, 2010).

Haemangiosarcoma

Haemangiosarcomas are tumours of the vascular endothelium in which neoplastic growth forms tumours intimately associated with the blood vessels and containing lacunae of blood. In humans, these tumours are rare, but occur most frequently in the liver and occasionally in atrial and other sites; in adults, they are associated with exposure to particular environmental toxins, including vinyl chlorides, arsenic and thorium dioxide. In dogs, the tumours are much more common. Visceral tumours are often located in the spleen or the right atrium of the heart, although other sites are not uncommon. Cutaneous haemangiosarcomas are also common. Post-mortem surveys suggest that up to 2% of all elderly dogs may have preclinical splenic haemangiosarcoma at the time of death, and in retrospective reviews of splenic abnormalities in UK, US and Australian referral practice 10–20% of all cases are haemangiosarcoma (Spangler and Cuthbertson, 1992). German Shepherd Dogs and, in the USA, Golden Retrievers show predisposition to visceral forms of the tumour, whereas cutaneous forms are associated with dogs with light-coloured short hair, such as Greyhounds, Whippets, Italian Greyhounds and Weimaraner dogs. In a recent health survey among Golden Retrievers in the USA, the lifetime risk of haemangiosarcoma was one in five (Tamburini *et al.*, 2009).

Haemangiosarcomas are extremely dangerous tumours. They show widespread metastasis through their intimacy with blood vessels; they are associated with disseminated intravascular coagulopathy and, paradoxically, because of excessive consumption of platelets and clotting factors, with bleeding. When the tumour ruptures, internal bleeding is often fatal. Somatic mutations have been found in *TP53* and *PTEN*, but not in any of the *RAS* genes or the von Hippel Lindau gene, *VHL* (Mayr *et al.*, 2002; Dickerson *et al.*, 2005; Tamburini *et al.*, 2010).

A number of genes have been shown to be over-expressed in haemangiosarcoma tumours, including *VEGFA*, basic fibroblast growth factor *FGF2* and their receptor genes, Angiopoetin2 (*ANGPT2*), *STAT3*, *RB1*, Cyclin D1 (*CCND1*) and Survivin (*BIRC5*). Whole transcriptome expression profiling of haemangiosarcoma tumour cells shows that they cluster separately from non-malignant endothelial cells, with a signature that includes genes such as those listed, and those involved in inflammation, angiogenesis, adhesion, invasion, metabolism, the cell cycle and signalling (Tamburini *et al.*, 2010). The Golden Retriever haemangiosarcoma expression profile signature is consistent between animals and has been distinguished from haemangiosarcoma tumours from other breeds (Tamburini *et al.*, 2009), although the other breeds in this case consisted of three dogs, each differing from both of the others. A set of GWAS has been performed in the Golden Retriever, and results from this are now being refined (Lindbladt-Toh and others, personal communication).

Melanoma

Melanoma is a tumour in which there is an uncontrolled proliferation of melanocytes. These cells produce either eumelanin (black or dark brown) or pheomelanin (yellow/red) pigments. In early development, they migrate from the neural crest, and take on important roles in the sensory organs (especially the eyes and ears), as well as in the pigmentation of skin and hair (Chapter 4). Once *in situ* in hair follicles, melanocyte proliferation and differentiation is coupled to the hair growth cycle, with activation in anagen (the main growth phase of the hair) (Botchkareva *et al.*, 2003). There are a number of developmental disorders of these cells that do not cause malignancies; in most cases, they cause defects in melanocyte distribution or survival, or melanocyte maturation and melanin production.

Melanoma is an important tumour of dog oral mucosa, skin, nail beds/digital (subungual)

and eye locations (both in the corneal limbus and iris, and in other uveal sites). At most of these locations, melanomas are typically highly aggressive and metastatic, although cutaneous melanoma in dogs may be less aggressive. Unlike the major human tumour that occurs after UVB damage of lightly pigmented skin, most canine melanomas occur spontaneously in darkly pigmented areas where melanocytes are very abundant. Melanomas of the limbus and cutaneous tumours that occur in light-coloured dogs are comparatively less common than in humans. Associations of melanomas with particular breeds, as listed in Table 8.2, are often associations with black coat colour so that, for example, at least half of all digital melanomas occur in black coat colour dogs (Henry *et al.*, 2005). Patterned breeds do not suffer melanoma at white skin locations (or in blue irises or other unpigmented tissues) as they do not have melanocytes there, but albino animals or those with colour dilution mutations can suffer amelanotic melanomas, as in these cases immature or non-melanin producing/transporting melanocytes may be present.

Around 20–40% of canine oral tumours are malignant melanoma (Brønden *et al.*, 2009). Estimates for melanoma abundance in skin locations range from just 4% of all skin malignancies in an Italian study, to 22% of malignancies at the same location in older work from the USA. Several intermediate figures have been derived from studies of other canine populations. It is likely that both breed and management of the animals contribute to these population differences.

Expression analysis of melanoma tumours and derived cell lines in the dog has shown that Melan-A (*MUM1* or *MAA*) and Tyrosinase (*TYR*) are reliable markers for these tumours, while the S-100 protein is also expressed in many tumours, although the male antigen MAGE, which is a typical antigen displayed by human tumours, is not expressed in canine melanoma (Stell *et al.*, 2009). Because melanomas have distinctive antigens they have been targets for vaccination using both dendritic cells activated *in vitro* against cancer antigens and a DNA vaccine encoding canine tyrosinase (Tamura *et al.*, 2008).

There is over-expression of *CDK4*, depletion or loss of *CDKN2A* and *PTEN* transcripts, and loss of the corresponding P16INK4A and PTEN proteins, as well as extra-nuclear accumulation of p53 protein in many tumours and cell lines. Less frequently, loss of transcripts and protein product of the *WAF1* gene, elevation of beta catenin expression or elevation of COX2 expression has been shown (Koenig *et al.*, 2002; Paglia *et al.*, 2009; Han *et al.*, 2010). An activating mutation in codon 599 of *BRAF* has been identified in the majority of cutaneous melanomas, but not in melanomas of mucosal origin in humans. No mutation in *BRAF* has been identified in dog oral melanomas though high levels of ERK phosphorylation suggest that dysregulation of the RAF/MEK/ERK pathway is present.

Familial predisposition has been suggested in cases of canine limbal melanoma (Donaldson *et al.*, 2006). In humans and mice, there are also inherited melanoma syndromes associated with at least six loci. High penetrance of familial melanoma is associated with mutations in *CDKN2A* and *CDK4*. Lower penetrance mutations occur in *MC1R, ASIP, TYR* and *TYRP1* (see review, Meyle and Guldberg, 2009).

Genetic predispositions to melanoma are being mapped in the Schnauzer and Poodle.

Lymphoma

Lymphomas or lymphosarcomas, cancers that arise from lymphocytes, account for one-quarter to one-fifth of canine malignant tumours. Tumours may carry markers showing their origin in T (and natural killer, NK) or B cell lineages, and may originate at different stages in lymphopoiesis. In the early stages, diagnosis of lymphoma may be difficult. In general, tumours can be recognized from reactive lymphocytes in an antigen response through their clonality, identified by DNA sequencing of the antigen-binding portions of immunoglobulin molecules or T cell receptor genes. Most lymphomas are monoclonal, but reactive proliferation of lymphocytes is polyclonal (Vernau and Moore, 1999). The classification of lymphomas in humans has traditionally been through cytology and cell-surface markers and, more recently, through chromosome rearrangements and transcriptome analysis. The current

WHO classification for human lymphosarcoma includes more than 30 tumour types, but only around five of these are common in adults. In dogs, lymphoma classification in most studies relies on B or T cell origin (see Chapter 6) and tumour location. Canine lymphomas largely reflect human non-Hodgkins lymphoma. Approximately 67% of canine lymphomas are B cell, 3% B + T cell and the rest T cell, but this varies by breed. Extranodal lymphomas and most leukaemias (including most chronic lymphocytic leukaemias) are of T cell origin, while plasma cell tumours are the most common tumours of B cell origin.

Breeds showing predispositions to tumours are given in Table 8.2. For a review, see Modiano *et al.* (2005a). The Boxer, Golden Retriever, Mastiff, Siberian Husky and Shih Tzu show a predisposition to T cell lymphoma (Lurie *et al.*, 2004), whereas the Basset Hound, Cocker Spaniel, German Shepherd and Rottweiler show a relative predisposition to B cell lymphoma (Modiano *et al.*, 2005b), suggesting that there is an inherited component to the tumour subtype.

Whole chromosome abnormalities that have been found in canine lymphoma include, most commonly, aneuploidy in chromosomes 11, 13, 14 and 31 (Hahn *et al.*, 1994; Thomas *et al.*, 2003a,b) as well as the sporadic involvement of other chromosomes (Winkler *et al.*, 2005). Human lymphoma contains cytogenetic abnormalities, including characteristic translocation chromosomes that vary with disease type. Chronic myelogenous leukaemia (CML) is characterized by the presence of the Philadelphia chromosome in which there is a reciprocal translocation t(9;22)(q34;q11) between the cellular copy of the Abelson leukaemia virus oncogene *ABL1* (often termed c-Abl) and the breakpoint cluster region gene (*BCR*). This gives rise to a highly expressed fusion protein with constitutive tyrosine kinase activity that activates multiple targets, including the RAS-RAF, MAPK, PI-3-kinase, STAT-5, c-Jun and c-Myc pathways. In chronic lymphocytic leukaemia (CLL), the *RB1* gene on HSA 13q14 is deleted hemizygously. In Burkitt's lymphoma, a form of acute lymphoblastic leukaemia (ALL), a t(8;14)(q24;q32) translocation places the MYC oncogene under control of the IGH enhancer. In canine lymphoma, Breen and Modiano (2008) have used fluorescence *in situ* hybridization of selected BACs, together with Western blotting techniques, to show the presence of each of these rearrangements in multicentric lymphomas of the appropriate cellular type, suggesting that the molecular basis and classification of these tumours are very similar in dogs to those in humans.

Mutations in the specific candidate genes *KIT*, fms-like tyrosine kinase (*FLT3*) and *N*- and *K-RAS* have also been found in a proportion of lymphomas (Sokołowska *et al.*, 2005; Usher *et al.*, 2009). *RAS* mutations occur in both acute myeloid leukaemia and ALL. *FLT3* mutations are characteristic of ALL, and like the mutations of mast cell tumours, are typically juxta-membrane domain duplications causing autophosphorylation and activation. TP53 protein over-expression is only rarely seen in canine non-Hodgkins lymphoma (or in lymphomas in other species) (Veldhoen *et al.*, 1998; Sokołowska *et al.*, 2005). The fragile histidine triad gene *FHIT* (at the fragile site *FRA3A*, and associated with human mammary, lung, oesophageal and gastric tumours) is deleted or shows reduced expression in canine lymphoma cell lines (Hiraoka *et al.*, 2009), while the P16INK4A protein is absent as the *CDKN2A* gene is deleted (commonly) or present but not expressed (rarely) in high grade lymphoblastic T-cell lymphomas (Fosmire *et al.*, 2007). Where these last two genes are present, reduced expression is associated with hypermethylation of the promoter. Hypermethylation of the Deleted in Liver Cancer 1 (*DLC1*) tumour suppressor gene has also been demonstrated in non-Hodgkins lymphoma of both B and T cell origin, although it was not associated with loss of expression in this study (Bryan *et al.*, 2009).

Several expression studies have been performed on canine lymphoma samples or lymphoma cell lines, and have shown numerous markers of cell activation or tumorigenesis that are elevated in lymphoma (or some classes of lymphoma) relative to reference samples, including those for *PTHLH* (the parathyroid hormone-like hormone or PTHrP), *MYC* (as expected from cytogenetic studies – this is associated with RB protein phosphorylation and inactivation), *BCL2L1 (BCL-XL), VEGFA* and *VEGFR1, TERT* (telomerase reverse transcriptase) and *HMGA1* (high mobility group protein)

(Yazawa *et al.*, 2003; Joetzke *et al.*, 2010; Wolfesberger *et al.*, 2007; Nadella *et al.*, 2008).

Genetic predispositions to lymphoma are being mapped in Mastiffs and Bullmastiffs, Cocker Spaniels and other breeds.

Conclusion

From this brief survey, it is clear that examples of most of the genetic phenomena that have previously been described in human cancer or its rodent models have emerged in canine cancer. There are examples of oncogenesis through: gain of function at several different levels in signal transduction cascades (see for example the section on mast cell tumours); loss of tumour suppressor genes, and the association of germ-line loss with inherited forms of cancer (for example in the renal cancer RCND); tumorigenesis associated with characteristic translocation chromosomes (see the section on lymphoma); genomic instability at either the chromosomal or the microsatellite level (see the sections on histiocytic sarcoma and mammary carcinoma); and there is limited but growing evidence for epigenetic phenomena.

The opportunities presented by the revolution in genetic technologies that is now taking place are only beginning to be exploited in our understanding of canine cancer. Large cohorts have already been collected to allow us to unravel the genes that predispose to many tumours: many of the early genome-wide associations have been performed. In some cases, refinement of loci is now taking place. In others, these preliminary mapping studies have shown that the number of cases required to obtain statistically significant associations is larger than anticipated. It seems likely that some of the diseases are more complex than was anticipated in the experimental design. The importance of the correct identification of tumour type, and of eliminating the effects of stratification, has also been a lesson from this work. None the less, we are now entering an exciting period in which the canine population structure will allow the dog to show its real strength as a cancer model. A number of novel cancer-predisposing genes seem to be about to emerge.

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9 Genetics of Neurological Disease in the Dog

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Introduction

Neurological disorders affect the body's nervous system and can be categorized according to the type of cause, the affected location or the type of dysfunction involved. The principal division is between central nervous system (CNS) disorders and peripheral nervous system (PNS) disorders. Genetic defects or other acquired abnormalities, including injuries and infections may affect the structural, biochemical or electrical properties of the CNS and PNS, resulting in a variety of symptoms, such as muscle weakness, paralysis, poor coordination, loss of sensation, seizures, confusion, pain and altered consciousness. The origin of the problem may also be in another body system that interacts with the nervous system, such as the cardiovascular system in the brain injury resulting from cerebrovascular disorders. The nature of neurological conditions varies from those that can be treated by medication, such as epilepsy, to others that are degenerative and eventually result in death.

Neurological disorders are common in both humans and dogs. According to an estimation by the World Health Organization (WHO) in 2006, neurological disorders and their sequelae affected one billion people worldwide. Although comprehensive epidemiological studies are still rare in canine disorders, a recent literature-based study of inherited defects in pedigree dogs identified the nervous system as one of the most common primarily affected systems (Summers *et al.*, 2010). A large number of different canine neurological conditions have been listed in online databases (List of Inherited Disorders in Animals,

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LIDA, http://www.vetsci.usyd.edu.au/lida; Canine Inherited Disorders Database, CIDD, http://www.upei.ca/~cidd/intro.htm; and Inherited Diseases in Dogs, IDID, http://www.vet.cam.ac.uk/idid). Many neurological conditions present in dogs also occur in humans and other species, and thus provide valuable access to research and treatment information. Canine medication, for example, is often referred to by human studies.

While most canine neurological conditions still remain uncharacterized on a molecular level, the annotation of the canine genome, followed by the advent of powerful genomic tools, are expediting new gene discoveries. The breed specificity of many neurological conditions suggests a strong genetic background, which facilitates gene mapping. Indeed, several new mutations have been discovered recently in both CNS and PNS disorders, and the goal of this chapter is to summarize some of these successes in genetic breakthroughs.

Epilepsy

Epilepsy is a group of chronic neurological disorders characterized by recurrent unprovoked seizures. Seizures are signs of abnormal, excessive or synchronous neuronal activity in the brain. There are over 40 distinct forms of epilepsy in humans, defined by such phenotypic criteria as age of onset, type of electroencephalographic (EEG) abnormalities, seizure characteristics and the type of stimulus that induces seizures (Engel, 2006; Engel *et al.*, 2006). The large number of underlying causes of seizures is reflected in the evolving epilepsy nomenclature (Berg *et al.*, 2010). The latest recommendation on epilepsy terminology – by the International League Against Epilepsy (ILAE) – classifies three major groups of epilepsy: 'genetic' (previously 'idiopathic'), 'structural/metabolic' (previously 'symptomatic') and 'unknown cause' (previously 'cryptogenic') (Berg *et al.*, 2010). In genetic epilepsy, the core symptom is seizure, which results from a known or presumed genetic defect. Structural/metabolic epilepsies are caused by another distinct structural or metabolic condition, including acquired disorders such as stroke, trauma or infection. In unknown epilepsy, the nature of the underlying cause is unknown and could be due to either a genetic defect or a separate as yet unrecognized disorder (Berg *et al.*, 2010).

Epilepsy includes two major types of seizures: generalized and focal. In generalized seizures, the thalamocortical circuitry is involved early in the attack and results in brain-wide synchronized firing of neurons, unconsciousness and often the violent shaking of body parts. In focal seizures, the synchronized activity is restricted to a single part of the cortex, and may or may not subsequently spread to recruit the thalamocortical pathways and result in secondary generalization. Focal motor seizures may be characterized by elementary motor events, which consist of a single type of stereotyped contraction of a muscle or group of muscles, or automatisms, such as chewing movements or running movements of the legs (Engel and Starkman, 1994; Engel, 2004).

Epilepsy is the most common chronic neurological disorder in dogs, with prevalence estimates typically varying from 0.5% to 5.7% and, in some breeds, such as Belgian Shepherds, reaching as high as 20% (Bielfelt *et al.*, 1971; Raw and Gaskell, 1985; Schwartz-Porsche, 1994; Knowles, 1998; Berendt *et al.*, 2002, 2008; Patterson *et al.*, 2003, 2005). There are different types and causes of epilepsy in dogs, but it is not usually differentiated into as many distinct syndromes as in humans. The lack of classification is partly due to the difficulty of seizure description and classification, and partly to the lack of routine use of the EEG in veterinary neurology clinics. With the exception of some symptomatic epilepsies, such as progressive myoclonus epilepsies (PMEs) (Lohi *et al.*, 2005b; Farias *et al.*, 2011) and benign familial juvenile epilepsy (BFJE) (Jokinen *et al.*, 2007a), most dogs with recurrent seizures are classified as having idiopathic epilepsy with focal or generalized seizures (Loscher *et al.*, 1985; Schwartz-Porsche, 1994; Berendt and Gram, 1999). Advances in brain imaging, molecular genetics and epidemiological studies are likely to improve understanding of the aetiology and the classification of canine epilepsy in the future.

Currently, all canine epilepsy genes for which information has been published are

related to symptomatic epilepsies. The first canine epilepsy gene, *NHLRC1*, was found in the Miniature Wire-haired Dachshund with canine Lafora disease (Lohi *et al.*, 2005b). This was followed by a number of other specific PME genes related to particular forms of neuronal ceroid lipofuscinoses (NCLs). Unlike symptomatic epilepsies, the clinical phenotype in idiopathic epilepsies (IEs) is often more variable and heterogeneous, even within a breed, complicating the establishment of well-phenotyped cohorts for genetic studies. However, ongoing clinical and genetic efforts are beginning to bring successes, and the first canine IE genes are being discovered in some breeds. To date, most of the known canine PME genes have been orthologues of the corresponding human epilepsy syndromes. The identification of canine epilepsy genes will provide novel candidates for common human epilepsies and establish physiologically relevant animal models so that the molecular mechanisms involved may be better understood. The following sections will summarize genetic studies in both symptomatic and idiopathic canine epilepsy.

Symptomatic epilepsy

All of the known canine epilepsy genes belong to a neurodegenerative group of autosomal recessively inherited diseases called progressive myoclonus epilepsies (the PMEs). In humans, PMEs afflict normal children, with progressively worsening and intractable myoclonus and epilepsy associated with dementia and premature death. From the pathogenic viewpoint, PMEs can be divided into non-lysosome and lysosome-related categories. The former category includes Lafora disease and the latter Unverricht–Lundborg disease, the action myoclonus-renal failure syndrome, and forms of NCLs, sialidosis and Gaucher disease (Shahwan *et al.*, 2005; Lohi *et al.*, 2006). Canine mutations have been found from the orthologues of the human Lafora disease and six different forms of NCLs.

The first canine epilepsy gene, *NHLRC1*, was found in Lafora disease (LD, OMIM No. 254780 in the Online Mendelian Inheritance in Man (OMIM) database at http://omim.org). This is an autosomal recessive PME and the severest form of teenage (adolescence)-onset epilepsy in humans. Patients suffer from an increasingly intractable seizure disorder paralleled by decreasing mental function, dementia and death within 10 years of the first symptoms (Minassian, 2002). LD is characterized by the accumulation of insoluble starch-like Lafora inclusion bodies (with less unbranched glycogen) that is found in various tissues, especially those with the highest glucose metabolism: the brain, heart, liver and skeletal muscle (Van Heycop Ten Ham, 1974). Human LD is caused by mutations in the *EPM2A* and *NHLRC1* genes, with evidence of a third locus (Minassian *et al.*, 1998; Chan *et al.*, 2003, 2004). A linkage analysis in pure-bred Miniature Wire-haired Dachshunds (MWHDs) from the UK mapped canine Lafora disease on chromosome 35 to a syntenic region with the human *NHLRC1* locus (Lohi *et al.*, 2005b). A sequence analysis of the canine single-exon *NHLRC1* gene revealed a tandem dodecamer repeat expansion mutation in affected dogs (Fig. 9.1). The GC-rich dodecamer repeat expansion prevents normal transcription of *NHLRC1*. The coding dodecamer repeat mutation represents the first repeat mutation outside the human genome. This particular dodecamer sequence was polymorphic and present across canine breeds, and its origin was predated beyond the Canidae to the time of the canid–arctoid split about 50 million years ago. Its recurrence and instability were indicated by the presence of the expansion mutation that was also found in an affected Basset hound with myoclonus epilepsy (Lohi *et al.*, 2005b). The affected dogs closely mimicked the clinical features and the histopathology of human Lafora disease, including the starch-like Lafora bodies in different tissues. The major clinical difference between canine and human LD patients was observed in the age of onset: while human LD sets in during the teenage years, all canine patients were clearly adults. Canine patients have provided access to several tissues and served as an important model for understanding the molecular pathogenesis of the disease in both species (Lohi *et al.*, 2005a,b).

Besides LD, several canine mutations have been found in other heritable PMEs called neuronal ceroid lipofuscinoses (NCLs). NCLs form

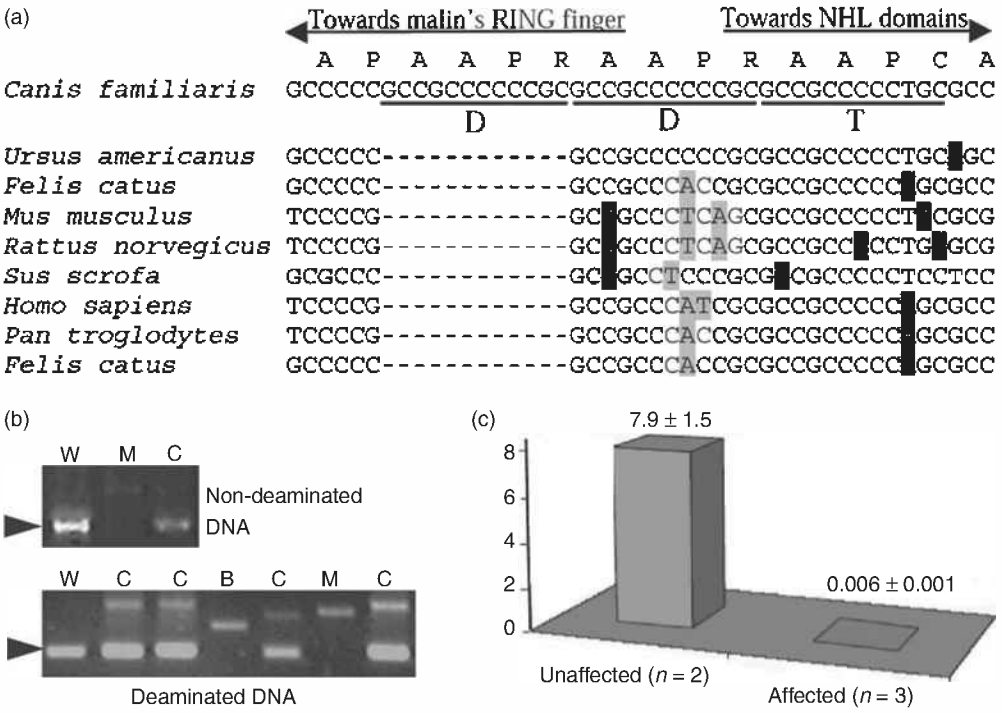

Fig. 9.1. (a) The unique canine *NHLRC1* dodecamer sequence and orthologues found on chromosome 35 in canine Lafora disease. The two identical dodecamer sequences are marked by D, and the third one, which differed only by a single base pair, by T. The dodecamer repeat is polymorphic across breeds and dogs usually have two to three copies of the D sequence in each chromosome. None of the other species tested had the same particular dodecamer repeat sequence in their genomes. (b) PCR amplification across the GC-rich dodecamer repeat sequence reveals the expansion mutation in the affected dogs. The affected Miniature Wire-haired Dachshunds (MWHD) had 19–26 copies of the D sequence. A Basset Hound with a milder phenotype had 15 copies of D. Key: W, wild-type; M, affected MWHD; C, carrier; B, affected Basset Hound; arrowheads, normal alleles; other bands, mutant alleles. (c) Total RNA was isolated from the skeletal muscles of the unaffected and affected dogs to determine the effect of the repeat mutation on the expression of the *NHLRC1* transcript. The expression study indicates that the dodecamer expansion mutation prevents the expression of the *NHLRC1* transcript in the affected dogs, resulting in the absence of the malin protein encoded by *NHLRC1*. The figure is reproduced from the original (Lohi *et al.*, 2005b) in *Science* by permission.

a group of progressive neurodegenerative lysosomal storage disorders characterized by the accumulation of autofluorescent lysosomal storage granules in the CNS and other tissues (Haltia, 2006; Kyttala *et al.*, 2006; Siintola *et al.*, 2006a; Jalanko and Braulke, 2009; Kohlschutter and Schulz, 2009). There is no effective treatment for NCLs, and patients suffer from seizures, loss of vision and progressive motor and cognitive decline, usually culminating in a persistent vegetative state and premature death (Wong *et al.*, 2010). The detailed causative mechanisms of the neurodegeneration in NCLs remain unclear, although a combination of oxidative stress and autophagy, leading to lysosome dysfunction-induced neuronal death has been proposed (Luiro *et al.*, 2006; von Schantz *et al.*, 2008; Bellettato and Scarpa, 2010; Saja *et al.*, 2010). The NCLs are classified clinically by age of onset into infantile (INCL), late-infantile, juvenile and adult-onset forms. They are additionally categorized according to the defective genes associated with the disease. There are also examples

of how different types of mutations in the same NCL-causing gene, such as in the case of the *CTSD*-mediated NCL (see below), produce partially defective proteins with residual biological activities, which can delay the onset of clinical signs until adulthood (van Diggelen *et al.*, 2001a,b). The genetic aetiologies of adult-onset NCLs – collectively referred to as Kufs disease – are as yet largely unknown (Kohlschutter and Schulz, 2009).

To date ten different forms of NCL have been described and eight genes, *CLN1* (alias *PPT1), CLN2* (alias *TPP1), CLN3, CLN5, CLN6, CLN7* (alias *MFSD8), CLN8* and *CLN10* (alias *CTSD)* have been definitively associated with NCL in human patients (Jalanko and Braulke, 2009). Canine NCLs have been reported in at least 18 different breeds (for information see the Canine Genetic Diseases Network website: http://www.caninegeneticdiseases.net/CL_site/basicCL.htm), and altogether eight NCL genes have been implicated in seven different breeds, including the Border Collie, English Setter, American Bulldog, Dachshund, American Staffordshire Terrier, Australian Shepherd and Tibetan Terrier. Six of the genes are orthologues of the corresponding human NCL genes. The first canine NCL mutation was found in *CLN5*, and this was followed by the discovery of mutations in *CLN8, CLN10* (alias *CSTD*), *CLN2*, CLN1 (alias PPT1), *CLN6, ARSG* and *ATP13A2*, as described in the following paragraphs. The last two recently identified canine NCL genes, *ARSG* and *ATP13A2*, have not yet been associated with human NCLs and provide novel candidates for mutation screenings.

The first canine NCL mutation was found in Border Collies by a linkage analysis and comparative genomics. A microsatellite-based analysis localized the gene to a region in CFA22 (canine chromosome 22) which was syntenic to that in human chromosome 13. This region contained a candidate gene, *CLN5*, a gene responsible for the Finnish variant of human late infantile NCL. Sequencing of the Cln5 revealed a recessive nonsense mutation (Q206X) within exon 4 in the affected dogs, resulting in a truncated protein product. The carrier frequency in a general Border collie population was estimated to be ~3.5% (Melville *et al.*, 2005). The onset and severity of the symptoms in affected Border Collies were variable but became observable after the first year of life. The clinical course included psychological abnormalities, ataxia and seizures, with increasing levels of agitation and hyperactivity, hallucinations and aggression. Some cases presented with complete blindness. Most of the affected dogs had failure to thrive and they rarely survived beyond 3 years of age (Studdert and Mitten, 1991). Although mutations in the *CLN5* gene in both humans and Border Collies result in highly similar clinical and biochemical features of the disease, the major symptomatic difference is that only a minority of human patients exhibit behavioural problems, whereas these symptoms are quite common in affected Border Collies (Jolly *et al.*, 1992; Jolly and Walkley, 1997; Santavuori *et al.*, 2001).

The second canine NCL mutation was found in a two-generation English Setter family with an autosomal recessive NCL that had already been described in the 1950s by the Norwegian veterinarian Nils Koppang (Koppang, 1973). English Setter NCL was mapped to CFA37 (Lingaas *et al.*, 1998) and to a syntenic region of the human *CLN8* gene. A mutation screening in the affected dog revealed a homozygous missense mutation (L164P) in the canine orthologue (Katz *et al.*, 2005a). The clinical symptoms in the English Setters closely mimic the clinical phenotype reported for Turkish patients with *CLN8* mutations (Ranta *et al.*, 2004). Affected dogs appear normal at birth, before beginning to exhibit NCL-like symptoms at 1–2 years of age. Symptoms include seizures, progressive motor and cognitive decline, and visual impairment (Koppang, 1973, 1988), and affected dogs typically die from intractable seizures in 2 years. The Turkish patients also showed a rapid progression with seizures, motor impairment, myoclonus, mental regression and loss of vision (Ranta *et al.*, 2004). The canine and Turkish patient phenotype is different from the Finnish NCL phenotype, also known as Northern epilepsy, which is associated with the R24G missense mutation in the *CLN8* gene (Ranta *et al.*, 1999). In Northern epilepsy, the onset and the progression are slower, with the first symptoms appearing between 5 and 10 years of age as frequent tonic–clonic seizures

followed by progressive mental retardation without significant impairment of vision. A spontaneous NCL mouse model, the *mnd* mouse, which has a frameshift mutation in the murine orthologue of *Cln8*, exhibits impairments in vision and in motor functions, without spontaneous seizures or cognitive impairment (Messer *et al.*, 1987; Ranta *et al.*, 1999). Comparison of the clinical features across species indicates that the English Setter NCL is more similar to human *CLN8*-associated NCL than to the Cln8-deficient mouse. However, all three species accumulate the lysosomal storage bodies containing large amounts of mitochondrial ATP synthase subunit c, suggesting a disruption of shared biochemical pathways (Katz *et al.*, 1994; Ranta *et al.*, 2001).

The third canine NCL mutation was found in American Bulldogs with a mutation in the Cathepsin D (*CSTD*) gene (Awano *et al.*, 2006a). The onset of this late-onset NCL is usually before 2 years of age and the affected dogs exhibit hypermetria and ataxia followed by progressive psychomotor deterioration and death before 7 years of age. Cytoplasmic autofluorescent storage material is present within neurons in the brains and retinal ganglion cells of affected dogs (Evans *et al.*, 2005). A genetic study revealed a homozygous missense mutation (M199I) in the *CSTD* gene in the affected dogs, resulting in a significant loss (>60%) of the *CSTD*-specific enzymatic activity in the brains of the affected dogs compared with control dogs. The frequency of the breed-specific M199I mutation was high (28%) in the breed. Mutations in the *CSTD* gene have been previously found in sheep and mice, with complete lack of enzymatic activity and more severe course of the disease (Koike *et al.*, 2000; Tyynela *et al.*, 2000). The milder clinical course in American Bulldogs could be due to the residual activity of the encoded enzyme.

The association of the *CTSD* gene in the human NCL was established after the canine discovery (Siintola *et al.*, 2006b). A complete loss of CTSD enzymatic activity in association with a protein truncation mutation was reported in four patients representing two different families with a severe congenital NCL. These patients exhibited perinatal seizure-like activity and died by 2 weeks of age (Siintola *et al.*, 2006b). In contrast, another study discovered that a compound heterozygosity of two missense mutations (F229I and W383C) in the *CTSD* gene resulted in a partial loss of enzymatic function in an adolescent patient who presented with later-onset NCL-like symptoms at an early school age (Steinfeld *et al.*, 2006). Together, these studies across species suggest that the degree of enzymatic deficiency dictates the age of onset and the progression of the NCL phenotype.

The fourth canine NCL gene, the orthologue of the human *CLN2*, was identified in an isolated Dachshund family (Awano *et al.*, 2006b). A candidate gene approach was utilized followed by a comparison of the clinical features and biochemical characteristics of the storage material between human and a 9-month-old male Dachshund. The dog exhibited a rapidly progressive course of disease including disorientation, ataxia, visual deficits, generalized myoclonic seizures and death at 12 months of age. An electron microscopical analysis of the brain sample revealed storage granules characteristic of the human late-infantile NCL caused by *CLN2* mutations. Additionally, the affected dog lacked detectable activity of the tripeptidyl-peptidase enzyme encoded by *CLN2*. These characteristics supported *CLN2* as a primary candidate for the disease, and a subsequent nucleotide sequence analysis revealed a single base pair deletion in exon 4, leading to a frameshift and premature stop codon. This truncation mutation explains the lack of enzyme activity in the affected dog. The affected Dachshund was homozygous for the mutant c.325delC allele; his sire and dam were heterozygotes, while 181 unrelated dogs, including 77 Dachshunds, were all homozygous for the wild-type allele. Collectively, these results propose the affected Dachshund as a model for *CLN2*-mediated NCL.

The most common form of infantile neuronal ceroid lipofuscinosis (INCL) is caused by mutations in the *CLN1* gene, which encodes the enzyme palmitoyl protein thioesterase 1 (PPT1) (Vesa *et al.*, 1995). *CLN1* participates in the intracellular palmitoylation processes, which are crucial for many cellular functions, such as membrane anchorage, vesicular transport, signal transduction and the maintenance of cellular architecture (Huang *et al.*, 2005; Resh, 2006). Numerous *CLN1*

mutations have been reported in patients with NCL (see the NCL Resource of University College London at http://www.ucl.ac.uk/ncl/cln1.shtml). Like the *CLN2* mutation discussed above, a *CLN1* mutation (the fifth canine NCL gene) was also found by a candidate gene approach in a 9-month-old Miniature Dachshund presenting with NCL-like signs, such as disorientation, ataxia, weakness, visual impairment and behavioural changes. Neurons of the affected dog contained autofluorescent lysosomal inclusions with granular osmiophilic deposit (GROD) ultrastructure characteristic of classical INCL. Resequencing of the canine orthologue of human *CLN1* revealed that the dog was homozygous for a frameshift mutation, a single nucleotide insertion in exon 8 (*CLN1* c.736_737insC), upstream from the catalytic site of the *CLN1* enzyme. Accordingly, brain tissue from the dog lacked *CLN1* activity. The dog was euthanized owing to worsening neurological signs at 14 months of age. This mutation was heterozygous in the parents, but was not found in any other screened Dachshunds, suggesting that it was a relatively recent sporadic event in the family (Sanders *et al.*, 2010).

Similar to previous *CLN1* and *CLN2* discoveries in Dachshunds, as suggested by the human NCL pathologies, Katz *et al.* (2011) identified a potential *CLN6* model (the sixth canine NCL gene) in an Australian Shepherd. A pet female Australian Shepherd was diagnosed with blindness, progressive ataxia and behavioural changes at 1.5 years and was euthanized at 2 years of age. Pathological analysis revealed enlarged lateral ventricles and apparent hypoplasia of the cerebellum. The cerebral cortex, cerebellum and retina contained massive accumulations of autofluorescent inclusions characteristic of the NCLs. The canine *CLN6* gene was screened for a mutation because of the clinical and pathological similarities to human *CLN6* deficiency causing a late-infantile form of NCL. Nucleotide sequence analysis of DNA from the affected dog identified a missense mutation (c.829T>C) in exon 7 of *CLN6*, resulting in a tryptophan to arginine amino acid change. Given that the mutation was not found in over 600 other normal Australian Shepherds, and the fact that the tryptophan is fully conserved across the 13 other vertebrates evaluated, the c.829T>C transition was suggested to be a strong candidate for the causative mutation. This was further supported later by the discovery of another affected dog from the same breed with the same homozygous mutation (Katz *et al.*, 2011). As in the Dachshunds discussed above, the same study also indicated that there is another form of NCL in the breed not associated with the current mutation.

A novel late-onset form of NCL sharing features with human Kufs disease was recently described in US and French American Staffordshire Terrier (AST) dogs previously diagnosed as having an inherited form of locomotor ataxia (Abitbol *et al.*, 2010). The affected AST dogs showed varying expressivity of the disease and presented late-onset and slow progress, absence of visual impairment, marked cerebellar atrophy, and accumulation of PAS (periodic acid-Schiff stain)-positive lipopigment in Purkinje cells and thalamic neurons. The disease in ASTs differs from most other NCLs in that the autofluorescent inclusions are primarily found in the thalamus and cerebellum (Narfstrom *et al.*, 2007), whereas autofluorescent inclusions are typically distributed throughout the brain in Kufs disease and other types of NCL (Jalanko and Braulke, 2009). The disease locus in ASTs was mapped to CFA9 (the seventh canine NCL gene) through combined association, linkage and haplotype analyses. A homozygous mutation was found in exon 2 of the arylsulfatase G (*ARSG*) gene, which causes an R99H substitution in the vicinity of the catalytic domain of the enzyme that decreases its sulfatase activity. A few healthy dogs (~2%) were also homozygous for the affected haplotype, suggesting a variable penetrance of the *ARSG* mutation. This study uncovered a novel sulfatase protein involved in neuronal homeostasis, and offers a unique opportunity to establish a functional link between lysosomal sulfatase activity and other CLN proteins. *ARSG* also represents a new candidate gene for human late-onset NCLs (Abitbol *et al.*, 2010).

The eighth, and the latest canine NCL mutation, was found in a recessive, adult-onset NCL present in Tibetan Terriers. The affected dogs have widespread distribution of autofluorescent inclusions in the brains (Alroy *et al.*,

1992; Katz *et al.*, 2005b, 2007) with specific accumulation of glial fibrillary acidic protein (GFAP, isoform 2) and histone H4 in storage bodies not previously reported for any of the NCLs (Katz *et al.*, 2007). A genome-wide association study identified this NCL locus to a 1.3 Mb region of canine chromosome 2, which contains canine *ATP13A2*. Sequence analysis indicated that NCL-affected dogs were homozygous for a single-base deletion in *ATP13A2*, producing a frameshift and premature termination codon (Farias *et al.*, 2011). Previously homozygous truncating mutations in human *ATP13A2* have been shown to cause Kufor–Rakeb syndrome (KRS), a rare neurodegenerative disease. The canine discovery suggests that KRS is also an NCL. Dogs and humans with *ATP13A2* mutations share many clinical features, such as generalized brain atrophy, behavioural changes and cognitive decline. However, other clinical features differ between the species. For example, affected Tibetan Terriers develop cerebellar ataxia, which is not reported in KRS patients, and KRS patients exhibit Parkinsonism and pyramidal dysfunction not observed in affected Tibetan Terriers. Further molecular investigations into the underlying causes of the phenotypic differences between species could shed light on the selective vulnerability of cell types, such as that of the dopamine cells to lysosomal dysfunction, and provide insights into the pathogenesis of Parkinson's disease and related disorders. *ATP13A2* also presents a novel candidate gene for human adult-onset NCLs, though the first screening of some Kufs patients suggests that it may not be a common cause (Farias *et al.*, 2011).

Idiopathic epilepsy

Although several mutations have been found in symptomatic epilepsies associated with specific neuropathologies, most dogs with spontaneous recurrent seizures are thought to have idiopathic (genetic) epilepsy without any identifiable underlying cause of seizures. In these dogs, neurological examination, haematology and biochemistry, dynamic bile-acid testing, serum ammonia, magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis are all normal or within normal range, though transient neurological deficits and changes on MRI may be observable as a result of prolonged seizures or status epilepticus (Mellema *et al.*, 1999). The typical age of onset of seizures in many breeds is between 2 and 4 years of age, but there is much variation from a few weeks or months to as late as 10 years of age (Heynold *et al.*, 1997; Jaggy and Bernardini, 1998; Berendt *et al.*, 2002, 2004; Jeserevics *et al.*, 2007).

The diagnosis of epilepsy and seizure type requires standardized study methods. The diagnosis of canine epilepsy is essentially clinical, based upon medical history, neurological examination and history of epileptic seizures. In humans, classification of epileptic seizures is standardized according to the guidelines of the ILAE Commission on Classification and Terminology. A similar concept in dogs, based upon the ILAE system, has been suggested (Berendt and Gram, 1999; Berendt *et al.*, 2004). Historically, focal seizures have been considered rare in dogs (Schwartz-Porsche, 1994), but this has recently been questioned, and it is being increasingly recognized that focal seizures are more common in dogs than was previously thought (Jaggy and Bernardini, 1998; Berendt and Gram, 1999; Kathmann *et al.*, 1999; Berendt *et al.*, 2008). Careful observations of seizures by both owners and veterinarians have revealed the focal onset, which may include an initial aura-like event (Berendt and Gram 1999; Berendt *et al.*, 2004, 2008; Jeserevics *et al.*, 2007). A focal seizure may or may not progress to a secondary generalized seizure. Many human patients with focal seizures have been shown to have focal intracranial pathology (Pitkanen and Sutula, 2002), though there is increasing evidence that focal seizures can also be idiopathic and that some are associated with specific genetic mutations (Gourfinkel-An *et al.*, 2004; Michelucci *et al.*, 2009; Striano *et al.*, 2011). In dogs, focal seizures are now frequently associated with idiopathic epilepsy with possible genetic causes, but further work on the electroencephalographic characteristics of canine seizures needs to be done to better define the seizure types (Jeserevics *et al.*, 2007).

The limited data available indicate interbreed variability in the severity of the epilepsy as defined by the occurrence of clustered seizures (CSs) and status epilepticus (SE). A study in 45 English Springer Spaniels found 38% of the tested dogs with CSs (Patterson *et al.*, 2005), while the prevalence of CSs in Border Collies was much higher (94%) (Hulsmeyer *et al.*, 2010). The overall proportion of SE in Border Collies (53%) correlated with the results of a study of 32 epileptic dogs in several breeds, which showed that 59% of dogs had at least one episode of SE (Saito *et al.*, 2001; Hulsmeyer *et al.*, 2010).

Approximately 20–30% of treated dogs have been said to respond poorly to treatment with phenobarbital and potassium bromide (Volk *et al.*, 2008). However, in a recent study in Border Collies treated with at least two antiepileptic drugs (AEDs), a much higher drug-resistant fraction (71%) was found (Hulsmeyer *et al.*, 2010). Though there are likely to be genetic reasons for drug resistance, the variable results may also be partly explained by the lack of a consistent definition of drug resistance and the different criteria used to assess the medical response (Dewey *et al.*, 2004, 2009; Govendir *et al.*, 2005; Kluger *et al.*, 2009).

A few studies on the epidemiology of canine epilepsy have been published, mainly based on data from retrospective hospital-based referral practices (Podell *et al.*, 1995; Berendt *et al.*, 2008). The high prevalence of epilepsy in certain canine families suggests a strong genetic component. Studies in Belgian Shepherds (Famula *et al.*, 1997; Oberbauer *et al.*, 2003; Berendt *et al.*, 2008), Keeshonds (Hall and Wallace, 1996; Goldstein *et al.*, 2007), Dachshunds (Holliday *et al.*, 1970), and British Alsatians (Falco *et al.*, 1974) all suggest a hereditary basis for seizures. In a study of 997 Belgian Shepherds, Famula *et al.* (1997) found that 17% had experienced at least one seizure, with an estimated high heritability of 0.77. A study of Irish Wolfhounds revealed a similar incidence of seizures (18.3%) with a heritability value of 0.87 (Casal *et al.*, 2006). High prevalences have also been estimated to exist in other breeds, including Labradors (Jaggy *et al.*, 1998; Berendt *et al.*, 2002), Bernese Mountain Dogs (Kathmann *et al.*, 1999), English Springer Spaniels (Patterson *et al.*, 2005), Viszlas (Patterson *et al.*, 2003) and Golden Retrievers (Srenk and Jaggy, 1996). Although the postulated modes of inheritance include autosomal recessive in some breeds (Keeshonds, Viszla, Beagles, Belgian Shepherds and Lagotto Romagnolos), most studies suggest a polygenic nature. In addition, a gender bias with an excess of males has been indicated in several breeds (Falco *et al.*, 1974; Srenk and Jaggy, 1996; Jaggy *et al.*, 1998; Kathmann *et al.*, 1999; Casal *et al.*, 2006). Besides genetic components, other factors, such as stress and sex steroids, are believed to contribute to the expression of epilepsy (Heynold *et al.*, 1997; Berendt *et al.*, 2008).

A large number of different types of epilepsies have been described in humans, and ongoing clinical and genetic studies are uncovering novel forms in dogs too. An example was described in the Lagotto Romagnolo breed in which a focal juvenile epilepsy with spontaneous remission resembled common benign human childhood epilepsies (Jokinen *et al.*, 2007a). A clinical evaluation of 25 Lagotto Romagnolo puppies from nine different litters identified simple or complex focal seizures at 5–9 weeks of age, followed by complete resolution by 4 months of age. The seizures consisted of whole-body tremor, sometimes with alteration of consciousness. The EEG revealed unilateral epileptic discharges in the central-parietal and occipital lobes, and the MRI was normal. During the months with epilepsy, the animals were often ataxic, but this also resolved completely as the seizures disappeared. There were no abnormalities in routine laboratory screenings of blood, urine and cerebrospinal fluid. The mode of inheritance was suggested to be autosomal recessive (Jokinen *et al.*, 2007a). We have recently confirmed this by identifying the recessive causative mutation and its related affected neuronal pathway (Seppälä *et al.*, unpublished). Remitting epilepsies are the most common forms of epilepsies with unknown molecular mechanisms. The discovery in this breed of dog provides now a unique model to study molecular mechanisms of spontaneous epilepsy remission in the developing brain.

Despite ongoing efforts, such as those of the LUPA project (a European research project for the study for animal models – specifically

dog models – of human diseases, see www.eurolupa.org), supported by new genomic tools to identify new genes in canine idiopathic epilepsies, there are currently only very few published studies available. Oberbauer *et al.* (2010) used a microsatellite-based approach with 410 markers in a population of 366 Belgian Shepherds, including 74 epilepsy cases, to identify six tentative quantitative trait loci (QTLs) in four chromosomes. However, none of those regions reached genome-wide significance, suggesting a polygenic nature of inheritance, inaccurate phenotypic classification of the study cohort or insufficient resolution of the markers.

Our preliminary unpublished data using the canine high-density single nucleotide polymorphism (SNP) chip arrays in several epileptic breeds, including Belgian Shepherds, are promising and indicate several new loci in the most common forms of canine epilepsy. It is likely that several new mutations will be revealed in the near future, which will provide new candidates for human studies, gene tests for breeders and the possibility of establishing important models for therapeutic trials and understanding the molecular mechanism of common epilepsies in both dogs and humans.

Cerebellar Abiotrophy

Cerebellar abiotrophy refers to a disease group known as cerebellar cortical abiotrophies (CCAs) (de Lahunta, 1990). Abiotrophy describes the idiopathic premature neuronal degeneration of the cerebellum. CCAs have been described in several dog breeds (de Lahunta, 1990), and typical clinical signs include ataxia, dysmetria, tremors, broad-based stance and loss of balance. The disease progresses often rapidly and causes difficulties for the affected dog in walking (Siso *et al.*, 2006).

Age of onset of clinical signs is variable across breeds and can be grouped into three general categories. Some breeds, like the Beagle, Miniature Poodle and Rough-coated Collie, have early onset at birth or as early as 3 to 4 weeks of age. Most breeds, including the Kerry Blue Terrier, Border Collie, Australian Kelpie and Labrador Retriever, show clinical signs from 6 to 16 weeks of age, while Gordon Setters and Brittany Spaniels represent later onset, which occurs from 6 to 30 months and 7 to 13 years of age, respectively (de Lahunta *et al.*, 1980; Tatalick *et al.*, 1993; Steinberg *et al.*, 2000). Pathological findings in CCAs are focused on the cerebellar cortex, with the loss of cortical Purkinje cells (PCs), followed by secondary changes in granular and molecular layers (Siso *et al.*, 2006). Primary degeneration rarely affects the cortical granule cells, although this has been described in some breeds (Sandy *et al.*, 2002; Jokinen *et al.*, 2007b). The involvement of other CNS structures has also been reported in some breeds, such as a cerebellar and extrapyramidal nuclear degeneration in Kerry Blue Terriers (de Lahunta and Averill, 1976) and a spinocerebellar syndrome in Brittany Spaniels (Tatalick *et al.*, 1993). A more systemic phenotype has been identified in Rhodesian Ridgebacks and Bernese Mountain Dogs, in which cerebellar degeneration is accompanied by a diluted coat colour and hepatic degeneration, respectively (Chieffo *et al.*, 1994; Carmichael *et al.*, 1996). The observed inter-breed variability of CCAs in onset, severity and histopathology suggests genetic heterogeneity (Manto and Marmolino, 2009a).

Human hereditary ataxias are also heterogeneous and characterized by progressive degeneration of the cerebellum and cerebellar connections, with a variable degree of involvement from extra-cerebellar structures (Manto and Marmolino, 2009b). In humans, the predominant inheritance patterns are autosomal dominant or recessive (Taroni and DiDonato, 2004; Anheim, 2011). Dominant ataxias have late onset at 30 to 50 years of age, whereas recessive ataxias tend to manifest earlier, at 20 years of age (Manto and Marmolino, 2009b). Over 25 causative mutations have been identified for at least 16 out of 28 known dominant spinocerebellar ataxias (SCAs), most of which are caused by expanded polyglutamine-coding CAG repeats in several different types of genes (Duenas *et al.*, 2006; Matilla-Duenas *et al.*, 2010). The number of the known recessive genes varies between ten and 20 depending on the classification criteria (Taroni and DiDonato, 2004; Manto and Marmolino, 2009b; Anheim, 2011). The suggested pathological mechanisms

in human ataxias are diverse, including the accumulation of protein aggregates, defects in the DNA-repair system, mitochondrial dysfunction and oxidative stress (De Michele *et al.*, 2004; Taroni and DiDonato, 2004; Manto and Marmolino, 2009b).

Most of the canine CCAs have been suggested to be autosomal recessive conditions (Deforest *et al.*, 1978; de Lahunta *et al.*, 1980; Thomas and Robertson, 1989; Steinberg *et al.*, 2000; Urkasemsin *et al.*, 2010), but the underlying genetic causes are not yet known. The first molecular characterization was reported in Coton De Tulear dogs with a neonatal ataxia, also called Bandera's neonatal ataxia (BNAt) (Coates *et al.*, 2002; Zeng *et al.*, 2011). In BNAt, the affected puppies are unable to stand or walk, with signs of intention tremor and head bobbing, but their development is otherwise normal. The BNAt locus has been mapped to CFA1, and a sequence analysis of a positional candidate gene revealed that the disease is caused by a homozygous retrotransposon insertion into exon 8 of *GRM1*, which encodes metabotropic glutamate receptor 1 (Coates *et al.*, 2002). Although both CCAs and BNAt cause ataxia, there is an important clinico-pathological difference: the Cotons are affected from birth with a more severe non-progressive ataxia without any CCA-like cerebellar pathology (Coates *et al.*, 2002).

We have recently successfully expanded the clinical and genetic study of a previous case report of early-onset progressive CCA in Finnish Hounds (FHs) (Tonttila and Lindberg, 1971). The first ataxia symptoms in the affected FH puppies occur at the age of 3 months, followed by a rapid progression and euthanization in a few weeks. The cerebellum shows massive neurodegeneration and primary loss of PCs, with a visible shrinkage in MRI. The disease was suspected to be autosomal recessive, and we have recently confirmed it by identifying the causative mutation in a genome-wide association study (K. Kyöstilä and H. Lohi, unpublished). The identified gene reveals a novel ataxia-related pathway and provides a candidate for mutation screenings in human and other species. Similar breakthroughs are likely in other breeds, and could uncover a series of novel canine ataxia genes that will reveal the true clinical and genetic overlap between human and canine diseases.

Besides CCAs, dogs have two other neurodegenerative diseases that share common clinical features, neuroaxonal dystrophy (NAD) and neuronal ceroid-lipofuscinosis (NCL). NCL is relatively more common than NAD and CCA in dogs (Jolly and Walkley, 1997). In all three disorders, most affected dogs exhibit progressive neurological signs with a lethal course. Histopathological lesions of the cerebellum are characterized by moderate-to-severe neuronal loss with disease-specific patterns. Canine NAD is characterized by severe axonal degeneration with numerous formation of spheroids throughout the CNS, whereas there is no such feature in CCA (Settembre *et al.*, 2008). NCLs are characterized by severe lysosomal storage of autofluorescent lipopigments in neurons and macrophages, various degrees of neuronal loss and astrocytosis. PC loss is most evident with CCA, while granule cell loss occurs with NCL. The neuronal loss with NAD is intermediate between that in CCA and NCL (Nibe *et al.*, 2010). There is also some evidence for different cellular pathways of neuronal loss in each disease (Nibe *et al.*, 2010). Cerebellar atrophy observed by MRI or CT (computerized tomography) has been a significant finding to suggest the diagnoses of these neurodegenerative disorders, but it is evident that the recent and the future genetic discoveries will aid in differential diagnostics.

Neonatal Encephalopathy

Encephalopathy is a term for any diffuse disease of the brain that alters brain function or structure. The myriad causes include metabolic or mitochondrial dysfunction, infectious or toxic agents, brain tumours or increased intracranial pressure, trauma, malnutrition, or lack of oxygen or blood flow to the brain. One of the severest forms of the disease involves neonatal encephalopathy in full-term or near-term infants. It is a significant contributor to mortality and morbidity in full-term infants, with a prevalence of 3.2–4.4 per 1000 live term births (Badawi *et al.*, 1998). Infants with neonatal encephalopathy suffer from a variety of

abnormalities in consciousness, tone and reflexes, feeding and respiration as well as intractable seizures with a poor prognosis (Aicardi, 1992; Guerrini and Aicardi, 2003; Wirrell *et al.*, 2005; Wachtel and Hendricks-Munoz, 2011).

Similarly, canine congenital encephalopathies comprise a broad range of developmental disorders, with clinical signs dependent upon the affected area of the brain (Battersby *et al.*, 2005; Caudell *et al.*, 2005; Aleman *et al.*, 2006; Chen *et al.*, 2008; Baiker *et al.*, 2009; Duque *et al.*, 2011). The first canine neonatal encephalopathy was described in the Standard Poodle breed (Chen *et al.*, 2008), which had shown a high incidence of fading puppies (Moon *et al.*, 2001). The affected puppies presented a characteristic progressive encephalopathy and seizures, referred to as a neonatal encephalopathy with seizures (NEWS). Specific neurological signs included a whole-body tremor, weakness and ataxia, which are characteristic of cerebellar dysfunction. Severe generalized clonic–tonic seizures developed between 4 and 6 weeks of age. The NEWS puppies were born smaller than their litter mates, and failed to develop normally even with the supplemental nutrition. Intractable seizures or declining neurological status compromised the life of the affected dogs, resulting in death before 7 weeks of age. Pathological analyses revealed reduced size of the cerebella, with dysplastic foci consisting of clusters of intermixed granule and Purkinje neurons (Chen *et al.*, 2008).

The NEWS gene was mapped to a 2.87 Mb segment on CFA36 using microsatellite markers in a 78-member Poodle family that included 20 affected dogs. Sequencing of the *ATF2* (activating transcript factor 2) gene revealed a homozygous c.152T>G transversion in exon 3, resulting in a methionine to arginine missense mutation at the conserved N-terminal domain of *ATF2* (Chen *et al.*, 2008). *ATF2* is a member of the basic region leucine zipper (bZIP) family of transcription factors (Maekawa *et al.*, 1989) and is involved in the regulation of a variety of vital cellular processes (van Dam and Castellazzi, 2001; Luvalle *et al.*, 2003; Bhoumik *et al.*, 2007; Bhoumik and Ronai, 2008; Vlahopoulos *et al.*, 2008). The segregation analysis of the *ATF2* mutation in a larger Poodle sample indicated that it is an autosomal recessive disease (Chen *et al.*, 2008).

Despite the experimental murine models that closely recapitulate the symptoms of canine NEWS (Reimold *et al.*, 1996; Maekawa *et al.*, 1999), *ATF2* mutations have not been associated with diseases of humans or domestic animals. Neonatal encephalopathy is often associated with intrapartum hypoxia, but new studies suggest that the majority of cases could be attributed to a variety of antepartum factors, including genetic risk (Badawi *et al.*, 1998; Scher, 2006). *ATF2* thus serves as a novel candidate gene for the study of such diseases.

Inflammatory Central Nervous System Diseases

Canine inflammatory CNS disorders can be divided into several categories, including necrotizing encephalitis, necrotizing vasculitis (steroid-responsive meningitis-arteritis, SRMA), eosinophilic meningoencephalitis and granulomatous meningoencephalitis. All of these disorders show aberrant immune responses against the CNS, while having unique histopathological features. Arriving at a differential diagnosis may be challenging, and requires a combination of findings on medical history, signalment and clinical and CSF analysis, possibly combined with advanced imaging and histopathology (Schatzberg, 2010; Talarico and Schatzberg, 2010). Common clinical features include dullness or lethargy, altered behaviour, proprioceptive and postural reaction deficits, circling, ataxia, decreased appetite and weight loss (Granger *et al.*, 2010; Schatzberg, 2010).

Strong breed predispositions exist for many of the inflammatory CNS disorders, suggesting a genetic susceptibility. Necrotizing encephalitis has been described in the Pug, Chihuahua, Maltese Terrier and Yorkshire Terrier breeds (Cordy and Holliday, 1989; Stalis *et al.*, 1995; von Praun *et al.*, 2006; Higgins *et al.*, 2008; Baiker *et al.*, 2009). The Beagle, Boxer, Nova Scotia Duck Tolling Retriever and Bernese Mountain Dog are predisposed to necrotizing vasculitis (Scott-Moncrieff *et al.*, 1992; Cizinauskas *et al.*, 2001; Redman, 2002; Behr

and Cauzinille, 2006) and the Rottweiler and Golden Retriever breeds have an increased risk of eosinophilic meningoencephalitis (Smith-Maxie *et al.*, 1989; Olivier *et al.*, 2010). Granulomatous meningoencephalitis has also been documented in a wide range of breeds (Bailey and Higgins, 1986; Munana and Luttgen, 1998). A novel form of meningoencephalitis with distinctive histopathological findings has recently been described in Greyhounds (Callanan *et al.*, 2002; Shiel and Callanan, 2008; Shiel *et al.*, 2010).

Despite a thorough history, examination and diagnostic tests of inflammatory CNS diseases, the aetiological agent remains unidentified in more than a third of dogs (Tipold, 1995). This is likely to reflect the limited understanding of some of the causes of these disorders and the lack of specific diagnostic tools. However, recent studies have begun to uncover the genetic causes, as evidenced by the identification of several new loci in necrotizing encephalitis in Pugs (Greer *et al.*, 2010b) and in necrotizing vasculitis in Nova Scotia Duck Tolling Retrievers (NSDTRs; Wilbe *et al.*, 2010). Additionally, a microarray-based analysis of the brains of the affected Greyhound puppies suggested a unique expression profile of more than 20 upregulated genes compared with control Greyhounds. Most of the upregulated genes were related to immune function, including pathways of viral infections and autoimmunity (Greer *et al.*, 2010a). These findings may highlight a common aetiology and pathogenesis for the breed-associated meningoencephalitis in Greyhounds, and further genetic studies with high-density SNP arrays are likely to reveal the associated susceptibility genes.

A necrotizing form of encephalitis (NME) was first recognized in Pug Dogs in the 1960s (Cordy and Holliday, 1989). Pathological features consist of a non-suppurative, necrotizing meningoencephalitis with a striking predilection for the cerebrum. Affected dogs are usually 6 months to 7 years of age, with a higher risk in young dogs. The disease may present an acute or chronic course. The acute form includes seizures, followed by abnormal behaviour, ataxia, blindness or neck pain, and its rapid progression may culminate in status epilepticus or coma within weeks. The chronic form is usually manifested as recurrent generalized or focal seizures with interictal neurological deficits, such as lethargy, ataxia, circling and blindness, which develop over a few months (Cordy and Holliday, 1989; Kobayashi *et al.*, 1994).

Population studies have suggested a high heritability with a possible immune aetiology in the NME of Pug Dogs (Levine *et al.*, 2008; Greer *et al.*, 2009). A recent genome-wide association scan with microsatellite markers mapped the NME locus in the dog leucocyte antigen (DLA) complex on CFA12 containing DLA-*DRB1*, DLA-*DQA1* and DLA-*DQB1* genes. A single homozygous high risk haplotype, DLA-*DRB1*010011/DQA1* 00201/DQB1*01501*, was revealed in the affected dogs with an odds ratio of ~12 (Greer *et al.*, 2010b). Besides the risk haplotype, at least three protective haplotypes were found, supporting the presumed immunological basis of the NME in the breed (Greer *et al.*, 2010b). The strong DLA class II association of NME in Pug Dogs resembles that of human multiple sclerosis (MS). Like MS, NME is more frequent in females than males, and appears to be a complex, low-incidence and inflammatory disorder with genetic and non-genetic factors. However, NME involves necrosis rather than demyelination as the prominent pathological feature, and has an earlier onset and faster disease course, thus resembling more an acute than a classical form of MS (Greer *et al.*, 2010b).

Other genetic associations in inflammatory CNS disorders were found in NSDTRs with SRMA (Wilbe *et al.*, 2010). The onset of SRMA is usually between 4 and 19 month of age, with neck pain, stiffness, depression, anorexia, fever and response to corticosteroid treatment (Anfinsen *et al.*, 2008). SRMA has been suggested to have an autoimmune origin, with two existing forms (acute and chronic) (Redman, 2002; Anfinsen *et al.*, 2008). In acute SRMA, dogs suffer from hyperaesthesia along the vertebral column, cervical rigidity, stiff gait and fever (Tipold and Schatzberg, 2010). In the chronic form, inflammation-induced meningeal fibrosis may obstruct CSF flow or occlude the vasculature (Tipold and Schatzberg, 2010). The characteristic lesion of SRMA is fibrinoid arteritis and leptomeningeal inflammation, consisting predominantly of neutrophils and scattered

lymphocytes, plasma cells and macrophages, and associated necrotizing fibrinoid arteritis (Tipold *et al.*, 1995). SRMA may occur concurrently with immune-mediated polyarthritis (IMRD), but it is unclear whether these two conditions are part of the same disease complex (Webb *et al.*, 2002; Hamlin and Lilliehöök, 2009).

Unlike in NME in Pug Dogs, a recent genetic study indicates that SRMA in NSDTRs is not associated with the MHC II locus, despite the suspected autoimmune origin (Wilbe *et al.*, 2009). However, a genome-wide association study revealed at least three loci in CFA8 and CFA32 (Wilbe *et al.*, 2010). All three loci include several immunologically relevant candidate genes. Interestingly, two of the SRMA loci overlap the immune-mediated rheumatic disease (IMRD) loci, suggesting that the two disorders may have common genetic factors (Wilbe *et al.*, 2010). Our ongoing resequencing efforts in the identified loci are likely to reveal new causative mutations that will aid in the understanding and diagnosis of SRMA and its related conditions.

Peripheral Nervous System Diseases

All the nervous tissue apart from the brain and the spinal cord is called the peripheral nervous system (PNS). The PNS partly consists of sensory fibres and motor neurons, and carries information from the rest of the body to and from the spinal cord and brain. Individual peripheral nerves control each muscle, and functional impairments of these nerves are often manifested as neuromuscular disorders. These disorders occur when a disease or infection damages the signal pathway from brain to nerve, causing a disease in the nerve (neuropathy or polyneuropathy), or in the neuromuscular junction (junctionopathy) or in the muscle (myopathy). Although the most common cause of PNS problems is injury, there are also several inherited forms associated with specific mutations. Some of the canine inherited PNS disorders with identified mutations and resemblances to corresponding human conditions are exampled in the following paragraphs.

Centronuclear myopathy

Centronuclear myopathies (CNMs) form a group of congenital myopathies (OMIM 160150) in which cell nuclei are abnormally located at a position in the centre of the cell, instead of in their normal location at the periphery, in skeletal muscle cells. Typical clinicopathological features include generalized muscle weakness, ptosis, ophthalmoplegia externa, areflexia and muscular atrophy affecting predominantly type 1 myofibres, with centralization of nuclei and pale central zones with variably staining granules. There are several types of CNMs, with varying age of onset and severity, including autosomal recessive, dominant and X-linked forms (Wallgren-Pettersson *et al.*, 1995). To date, three genes are known to be associated with a classical CNM phenotype. The X-linked neonatal form (XLCNM) is due to mutations in the myotubularin gene (*MTM1*), and involves a severe and generalized muscle weakness at birth (Laporte *et al.*, 1996). *MTM1* belongs to a large family of ubiquitously expressed phosphoinositide phosphatases that are implicated in intracellular vesicle trafficking (Laporte *et al.*, 2002, 2003). The autosomal dominant form results from mutations in GTPase dynamin 2 (*DNM2*) and has been described with early-childhood onset and adult onset (ADCNM) (Bitoun *et al.*, 2005). *DNM2* is a mechanochemical enzyme and a key factor in membrane trafficking and endocytosis (Praefcke and McMahon, 2004). Autosomal recessive centronuclear myopathy (ARCNM) has recently been associated with mutations in *BIN1*, encoding amphiphysin 2, which possesses an N-terminal BAR domain able to sense and bend membranes and an SH3 domain mediating protein–protein interactions (Itoh and De Camilli, 2006; Nicot *et al.*, 2007).

At least two naturally occurring canine CNMs have been characterized at clinical and molecular levels, both in Labrador Retrievers (Kramer *et al.*, 1976; Cosford *et al.*, 2008). The first recessive CNM mutation was found in a population of Labrador Retrievers with hypotonia, generalized muscle weakness, abnormal postures, stiff hopping gait and exercise intolerance, with signs of skeletal muscle atrophy and centralization of myonuclei (Kramer *et al.*,

1976; McKerrell and Braund, 1986; Gortel *et al.*, 1996; Bley *et al.*, 2002). This myopathy was transmitted as an autosomal recessive disease and was mapped to a 18.1 cM segment in CFA2 (Tiret *et al.*, 2003), followed by the identification of a tRNA-like short interspersed repeat element (SINE) insertion mutation in exon 2 of the *PTPLA* gene, which encodes a protein tyrosine phosphatase-like member A. The inserted SINE showed complex effects on the maturation of the *PTPLA* mRNA involving splicing out, partial exonization or multiple exon skipping (Pele *et al.*, 2005). *PTPLA* has not yet been associated with human CNMs.

The second CNM mutation was found in an isolated population of Labradors representing a genetic homologue of human XLMTM (X-linked myotubular myopathy, a well-defined subtype of CNM) with important clinical and pathological characteristics (Cosford *et al.*, 2008). The affected dogs were clinically normal at birth, but began to exhibit progressive generalized muscle weakness and atrophy beginning at about 2 months of age, and often required euthanasia between 3 and 6 months of age. A candidate gene sequencing study in the affected dogs revealed a homozygous missense variant in the *MTM1* gene, the human orthologue of XLMTM (Beggs *et al.*, 2010). The major difference between human and canine XLMTM concerns the progressivity of the disease, the human form being relatively non-progressive (Pierson *et al.*, 2005).

There is also an example of a canine mutation outside CNMs. One of the first canine neuromuscular mutations was found more than 20 years ago in association with Duchenne muscular dystrophy (DMD), the most common and the most severe form of the human muscular dystrophies (Cooper *et al.*, 1988). DMD is a recessive X-linked disease characterized by ongoing necrosis of skeletal muscle fibres with regeneration and eventually fibrosis and fatty infiltration. An X-linked myopathy with characteristics close to those of human DMD was identified with the lack of the *Duchenne* gene transcript and its protein product, dystrophin (Cooper *et al.*, 1988).

The identification of the mutations in the inherited neuromuscular disorders described above has established important canine models not only for the study of the pathogeneses and mechanisms of the diseases, but also for the use of these models in preclinical trials (Paoloni and Khanna, 2008). Results from rodent models cannot always be directly extrapolated to the human condition, and the identification and use of larger animal homologues will be of increasing importance. Established models of X-linked dystrophin-deficient muscular dystrophy in Golden Retrievers (Cooper *et al.*, 1988; Sharp *et al.*, 1992) and *PTPLA*-deficient Labrador Retrievers with CNM are being used in various preclinical and therapeutic trials (Sampaolesi *et al.*, 2006; Yokota *et al.*, 2009; Beggs *et al.*, 2010; Saito *et al.*, 2010).

Degenerative myelopathy

Canine degenerative myelopathy (DM) has been recognized as a spontaneously occurring, adult-onset spinal cord disorder of dogs (Averill, 1973). The disease has been diagnosed in several breeds without sex predilection (Averill, 1973; Braund and Vandevelde, 1978; March *et al.*, 2009; Miller *et al.*, 2009; Coates and Wininger, 2010). Symptoms usually begin at the age of 8 years with signs of spastic and general proprioceptive ataxia in the pelvic limbs, hyporeflexia and paraplegia (Coates and Wininger, 2010). A definitive diagnosis of DM is accomplished by the post-mortem histopathological observation of axonal and myelin degeneration (Matthews and de Lahunta, 1985; March *et al.*, 2009). Based on the histopathological findings which show nerve fibre loss in the thoracolumbar spinal cord, DM has been commonly referred to as a disease of the upper motor neuron system (Coates and Wininger, 2010). However, a recent study indicated that dogs with advanced DM have both upper and lower motor neuron disease (Awano *et al.*, 2009).

The progression of the canine DM disease is similar to that reported for the upper motor neuron dominant adult onset form of human amyotrophic lateral sclerosis (ALS) (Engel *et al.*, 1959; Hirano *et al.*, 1967). ALS refers to a group of adult-onset human diseases, in which progressive neurodegeneration affecting both the upper and lower motor neuron systems

causes advancing weakness and muscle atrophy, culminating in paralysis and death. Mutations in the superoxide dismutase 1 gene, *SOD1*, account for 20% of the familial ALS cases and 1–5% of the cases of sporadic ALS (Rosen *et al.*, 1993; Schymick *et al.*, 2007). *SOD1* functions as a homodimer, which converts superoxide radicals to hydrogen peroxide and molecular oxygen (Rosen *et al.*, 1993).

A genome-wide association study in Pembroke Welsh Corgis was performed to map the DM locus to a syntenic region of CFA31 that contains the canine orthologue of the human *SOD1* gene (Awano *et al.*, 2009). A homozygous E49K missense mutation was found in *SOD1*, with an incomplete penetrance, possibly due to modifier loci, environmental factors and/or inaccurate phenotyping because of the late onset and slow progression. The same mutation was also found in other breeds with DM (Awano *et al.*, 2009). DM-affected dogs mimic most of the features of the human *SOD1*-deficiency and could serve as valuable models for ALS in the evaluation of therapeutic interventions and investigation of the processes underlying the motor neuron degeneration.

Sensory ataxic neuropathy

Sensory ataxic neuropathy (SAN) is a recently identified neurological disorder in Golden Retrievers (Jaderlund *et al.*, 2007). It occurs during puppyhood, with signs of ataxia, postural reaction deficits and reduced or absent spinal reflexes. About half of the affected dogs are euthanized by 3 years of age. SAN is associated with reduced conduction velocities of nerve impulses in sensory nerves without muscle atrophy. Degenerative findings exist in both the CNS and PNS. Pedigree data enabled the tracing back to a founder female on the maternal side, suggesting a mitochondrial origin of the SAN (Jaderlund *et al.*, 2007).

To identify the mutation, the entire mitochondrial genome was sequenced, revealing a 1 bp deletion in the mitochondrial *tRNATyr* gene at position 5304 in the affected dogs (Baranowska *et al.*, 2009). The mutation was absent in other breeds and in wolves, and it was traced back to a common ancestor. A heteroplasmy analysis in blood and tissue samples demonstrated a reduced number of the non-mutated sequence in the affected dogs and in their cousins compared with distant relatives and other unrelated Golden Retrievers. The affected dogs also showed a compromised mitochondrial function – a common feature of mitochondrial pathology. These findings indicate a neurological disease caused by a mitochondrial gene in Golden Retrievers and provide the first example of mitochondrial mutation in dogs (Baranowska *et al.*, 2009).

Heterogeneous mitochondrial disorders are among the most common metabolic diseases, with over 250 known pathogenic mutations in humans, mostly in the tRNA genes (Chinnery *et al.*, 2000; Schaefer *et al.*, 2008). In mitochondrial diseases and in ageing, the uniformity of the mtDNA (homoplasmy) breaks and cells may simultaneously contain a mixture of wild-type and mutated mtDNA (heteroplasmy), which affects the manifestation of the disease in individuals (DiMauro and Hirano, 1993, 2009; Schon and DiMauro, 2007; DiMauro, 2010, 2011). The canine model with the affected *tRNATyr* gene provides a unique model to study the role of heteroplasmy and the complex interplay between the mitochondrial and nuclear genes in this devastating neurological disease of Golden Retrievers.

Polyneuropathy

Peripheral neuropathy is the term used for nerve damage of the peripheral nervous system, which may be caused either by diseases of by trauma to the nerves or the side effects of systemic illness. The four cardinal patterns of peripheral neuropathy are polyneuropathy, mononeuropathy, mononeuritis multiplex and autonomic neuropathy (Bertorini *et al.*, 2004; Auer-Grumbach *et al.*, 2006). Subtypes of inherited polyneuropathies can be classified as hereditary motor and sensory neuropathies (HMSN), hereditary motor neuropathies (HMN) and hereditary sensory (and autonomic) neuropathies (HSAN). These clinically heterogeneous phenotypes affecting the peripheral nerves

are grouped together as Charcot–Marie–Tooth (CMT) disease. More than 40 genes with distinct mutations have been described, mostly in autosomal dominant forms of CMT (Reilly and Shy, 2009). Currently, there is no drug therapy available for the human CMT disease (Reilly and Shy, 2009).

CMT diseases also occur in dogs and have been described in several canine breeds, including Great Danes, Rottweilers, Dalmatians, Alaskan Malamutes, Leonbergers, German Shepherds, Italian Spinonis, Bouvier des Flandres, Border Collies, Pyrenean Mountain Dogs, Miniature Schnauzers and Greyhounds (Drögemüller *et al.*, 2010; Granger, 2011). The first genetic defect has been recently elucidated in the juvenile form of CMT in Greyhounds (Drögemüller *et al.*, 2010). These dogs suffer from an early-onset severe chronic progressive mixed polyneuropathy with onset between 3 and 9 months of age. The initial symptoms in the affected dogs include exercise intolerance and walking difficulties, followed by a progressive severe muscle atrophy, ataxia and dysphonia. Neurological signs in affected dogs include progressive ataxia and tetraparesis, delayed proprioceptive placing reactions, hyporeflexia, distal limb muscle atrophy and inspiratory stridor. With disease progression, proprioceptive deficits and laryngeal involvement appear (Drögemüller *et al.*, 2010).

Pedigree analysis of the affected dogs indicated a monogenic disease, and the gene was mapped to CFA13 in a genome-wide association study. The associated region uncovered a positional candidate gene, *NDRG1*, which causes hereditary motor and sensory neuropathy-Lom (Lom after the town in Bulgaria where the initial cases were found) in humans (CMT4D). A mutation analysis revealed a 10 bp deletion in canine *NDRG1* exon 15, causing a frameshift (Arg361SerfsX60) that results in a truncated protein. The *NDRG1* transcript and protein were reduced or absent in a peripheral nerve biopsy of an affected Greyhound. These findings identified a causative mutation for a juvenile polyneuropathy – and the first genetically characterized canine CMT model, which provides a resource for therapeutic trials for human *NDRG1*-associated CMT disease and the possibility of a better understanding of the molecular mechanisms and pathobiology of the disease in general (Drögemüller *et al.*, 2010).

Conclusion

The number of successful genetic studies in canine neurological diseases has increased rapidly over the past few years, and there is reason to believe that there will continue to be new breakthroughs. Clearly, these studies have been informative not only for dogs but also in the understanding of our own diseases. The general overview of studies in the field that has been presented in this chapter warrants several conclusions.

First, the identification of a large number of genes in almost as many distinct neurological diseases demonstrates that the extensive variety of aetiologically unique disorders in humans exist also in dogs. For example, nine different PME genes have been discovered in nine distinct PMEs, including both early- and late-onset disorders.

Secondly, the clinical phenotypes of dogs and humans resemble each other closely, and the fact that the majority of the affected genes have been orthologues indicates shared aetiologies. However, there are also some important clinical differences commonly seen in relation to the onset or the development of a particular pathology that may reflect species-specific biological differences in the timing of development, function and sensitivity of the neuronal systems. Some of the clinical differences could also be caused by different types of mutation found across species. The understanding of molecular backgrounds of the observed differences should be highly informative for the overall explanation of disease aetiologies.

Thirdly, the entire spectrum of inheritance patterns, including autosomal dominant and recessive, X-linked, mitochondrial and polygenic forms, is present in canine neurological disorders, and they mostly follow the patterns observed in the corresponding human conditions. For example, all of the canine PMEs characterized so far have been autosomal recessive, as are human PMEs. In some cases, the penetrance of the mutation has been incomplete, suggesting additional genetic or

environmental factors behind the disease. Additionally, a particular type of common repeat mutations unravelled in certain human CNS disorders, such as the polyglutamine repeats in spinocerebellar ataxia, have not yet been found in dogs. Future studies will inform whether this is a reflection of a unique species-specific difference or a structural polymorphism yet to be found in dogs, as was the case with the dodecamer repeat mutation in the Dachshund's Lafora disease.

Fourthly, the prevalence of neurological diseases varies significantly between breeds – from isolated cases up to two-digit carrier frequencies. This suggests either the recent and local appearance of some of the mutations in some of the breeds, or that they are the result of specific breeding practices. Notably, most CNS and PNS disorders and their related mutations are breed specific, though there are a few mutations, such as that for degenerative myopathy, that are found in several breeds. Furthermore, the same breed may have several forms of the same disease. For example, at least two genetically different forms of the NCL disease exist in Dachshunds and Australian Shepherds. Thus, new PME genes are yet to be discovered and these studies remain important future research tasks.

Fifthly, although the breakthroughs in canine neurological disorders owe much to the annotation of the canine genome and the availability of genomic tools, it is clear that prior genetic or clinico-pathological knowledge of human CNS and PNS conditions has informed several successful candidate gene screens in small sample sets. Comparative clinical and genetic studies are clearly warranted in future studies as well.

Sixthly, canine studies have revealed completely new loci, genes and molecular pathways and, in at least one case (*ATP13A2*-deficient NCL dogs), even a significant overlap between different disease groups, thereby establishing important large animal models for the better understanding of the disease mechanisms in neurological diseases. The novel genes have also provided candidates for mutation screenings in human conditions. For example, the two new canine NCL genes, *ATP13A2* and *ARSG*, represent candidates for human late-onset forms of NCLs that have remained poorly characterized to date. Several examples demonstrate that the canine models recapitulate the human syndromes better than experimental rodent models. The identified canine models therefore also serve as clinically and physiologically relevant models for novel therapeutic trials, such as in the case of centronuclear myopathies in Labrador Retrievers.

Finally, the identification of new genes has enabled the development of genetic tests for dog breeding purposes. These tests will help to diagnose and monitor the carrier frequencies of the mutations and, if systematically used in breeding programmes, to eradicate at least some of the devastating neurological diseases. It is also worth stressing here the value and importance of careful clinical studies and detailed phenotyping to improve the accuracy of genetic testing. The availability of an ever-growing number of specific DNA tests will also help in veterinary diagnostics – differential diagnostics is often a challenge due to clinical similarities in related conditions and so the genetic dissection of syndromes will provide new tools for improved diagnostic practices.

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10 Genetics of Eye Disorders in the Dog

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Introduction

Inherited forms of eye disease are arguably the best described and best characterized of all inherited diseases in the dog, at both the clinical and molecular level. A large number of excellent texts have been compiled that describe the clinical characteristics of the enormous number of canine eye disorders, many of which are inherited and associated with particular breeds. However, the genetic basis of many of these disorders has yet to be dissected to any significant depth. This chapter focuses on those conditions that have something to teach the interested geneticist in terms of the mode of inheritance and, in most cases, the causal mutation(s). At the time of writing, close to 25 different mutations have been documented in the scientific literature that are associated with an inherited ocular disorder in the dog (Table 10.1), and several more conditions have been described very well at the genetic and clinical levels although their causal mutations remain, for now, elusive. Conditions for which an association with particular breeds is largely anecdotal have been omitted, although the genetic basis of many of these will undoubtedly be unravelled over the coming years, thanks to the increasingly sophisticated genetic resources that are now available for the dog.

The canine eye diseases that have been characterized at the molecular level and that are the focus of this chapter are all the result of spontaneous mutations that occurred historically in founding animals, but in many cases have become relatively frequent within certain breeds owing to the well-documented practices, such as high levels of inbreeding and popular sire effects, that are commonplace in the world of domestic dogs. These conditions are distinct from those that are secondary to specific, often extreme, physical conformations that have been actively selected for by dog breeders. Examples of these conditions include eyelid disorders such as entropion, which is an inversion of the lid margin towards

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Table 10.1. Genes associated with inherited eye disorders in the domestic dog.

Disease	Locus or abbreviation	Gene	Breed	Reference
Cone–rod dystrophy	CRD3	*ADAM9*	Glen of Imaal Terrier	Goldstein *et al.* (2010c); Kropatsch *et al.* (2010)
Primary lens luxation	PLL	*ADAMTS17*	Multiple, mainly terrier breeds	Farias *et al.* (2010); Gould *et al.* (2011)
Cone degeneration	CD	*CNGB3*	Alaskan Malamute	Seddon *et al.* (2006)
Cone degeneration	CD	*CNGB3*	German Shorthaired Pointer	Sidjanin *et al.* (2002)
Dwarfism with retinal dysplasia (oculoskeletal dysplasia)	DRD2 (OSD2)	*COL9A2*	Samoyed	Goldstein *et al.* (2010a)
Dwarfism with retinal dysplasia (oculoskeletal dysplasia)	DRD1 (OSD1)	*COL9A3*	Labrador Retriever	Goldstein *et al.* (2010a)
Hereditary cataract	HC, EHC	*HSF4*	Staffordshire Bull Terrier, Boston Terrier, French Bulldog	Mellersh *et al.* (2006b)
Hereditary cataract	HC	*HSF4*	Australian Shepherd	Mellersh *et al.* (2009)
Collie eye anomaly	CEA	*NHEJ1*	Collies	Parker *et al.* (2007)
Cone–rod dystrophy		*NPHP4*	Standard Wire-haired Dachshund	Wiik *et al.* (2008b)
Photoreceptor dysplasia	PD	*PDC*	Miniature Schnauzer	Zhang *et al.* (1998)
Rod–cone dysplasia	RCD3	*PDE6A*	Cardigan Welsh Corgi	Petersen-Jones *et al.* (1999)
Rod–cone dysplasia	RCD1	*PDE6B*	Irish Setter	Suber *et al.* (1993)
Rod–cone dysplasia	RCD1	*PDE6B*	Sloughi	Dekomien *et al.* (2000)
Progressive rod–cone degeneration	PRCD	*PRCD*	Multiple breeds	Zangerl *et al.* (2006)
Rod–cone dysplasia	RCD2	*RD3*	Collie	Kukekova *et al.* (2009)
Autosomal dominant progressive retinal atrophy	ADPRA	*RHO*	English Mastiff	Kijas *et al.* (2002)
Congenital stationary night blindness	CSNB	*RPE65*	Briard	Aguirre *et al.* (1998); Veske *et al.* (1999)
X-linked progressive retinal atrophy	XLPRA2	*RPGR*	Mixed-breed dogs	Zhang *et al.* (2002)
X-linked progressive retinal atrophy	XLPRA1	*RPGR*	Siberian Husky, Samoyed	Zhang *et al.* (2002)
Cone–rod dystrophy	CORD1 (CRD4)	*RPGRIP*	Dachshund	Mellersh *et al.* (2006a)
Early retinal degeneration	ERD	*STK38L*	Norwegian Elkhound	Goldstein *et al.* (2010b)
Canine multifocal retinopathy	CMR	*VMD2/ BEST1*	Great Pyrenees, English Mastiff, and Bullmastiff	Guziewicz *et al.* (2007)
Canine multifocal retinopathy	CMR	*VMD2/ BEST1*	Coton de Tulears	Guziewicz *et al.* (2007)
X-linked progressive retinal atrophy	XLPRA3	unknown	Border Collie	Vilboux *et al.* (2008)
Cone–rod dystrophy	CRD1	Unknown	Pit Bull Terrier	Kijas *et al.* (2004)
Cone–rod dystrophy	CRD2	Unknown	Pit Bull Terrier	Kijas *et al.* (2004)

the globe that results in the hairs at the lid margin coming into contact with the cornea, and ectropion, the converse of entropion, where the lower lids become everted exposing conjunctival tissue. Another eye disorder that is very common in breeds that have been selectively bred to have excessive facial folds is trichiasis, where normal facial hair comes into contact with the cornea and may cause conjunctivitis, keratitis and corneal ulceration. These conditions, and many others, are likely to be genetically complex, and although well described at the clinical level have not been subject to rigorous genetic investigation; they will not, therefore, be discussed further in this chapter.

Why have so many inherited eye disorders been described in the dog? A principal reason is that the eye is very accessible, and much of it can be examined in detail using non-invasive techniques, making it relatively easy to detect abnormalities, even if they do not impair vision significantly. A schematic diagram illustrating the basic anatomy of the normal eye is shown in Fig. 10.1. There are clinical screening schemes in place in many countries that offer breeders the opportunity to screen their dogs, usually before they are bred from, for disorders known to be inherited in their breed. One such scheme is the British Veterinary Association (BVA)/Kennel Club/International Sheep Dog Society Eye Scheme that operates in the UK (http://www.bva.co.uk/canine_health_schemes/Eye_Scheme.aspx). This scheme covers 11 inherited eye disorders in over 50 breeds of dog. The disorders and breeds covered by the scheme are listed on two different schedules: those on Schedule A are diseases and breeds where the condition is known to be inherited, and there is robust evidence to support its inheritance, whereas those on Schedule B are conditions and breeds that are currently under investigation. The European College of Veterinary Ophthalmologists (ECVO) Scheme (http://www.ecvo.org/) is in use in seven European countries, and individual ECVO diplomates work in accordance with the scheme in other countries to control presumed inherited diseases of the eye and its adnexa. In the USA, the Canine Eye Registration Foundation (CERF) (http://www.vmdb.org/cerf.html) is a national registry of dogs certified free of heritable eye disease by members of the American College

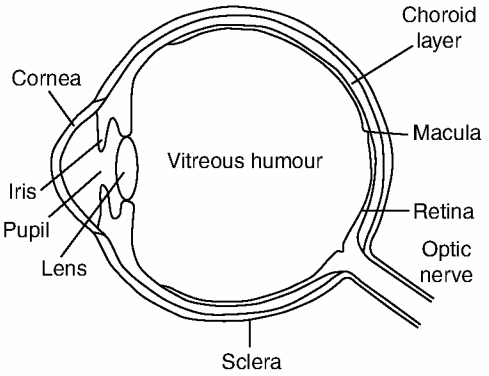

Fig. 10.1. Schematic diagram illustrating the basic anatomy of a normal eye.

of Veterinary Ophthalmologists. The three schemes listed above, and many other comparable schemes in place around the world, differ incrementally from one another in the precise ways in which they are operated, but they all serve to document and register dogs affected with, and free from, inherited eye diseases. Each dog that is clinically examined under any of these schemes receives a certificate on which the results of the examination are recorded; the findings are also recorded in the relevant registry/database, thus providing a wealth of data regarding the clinical characteristics and incidence of inherited eye disorders in different breeds of domestic dog.

The ease with which the eye can be examined and the widespread use of international clinical screening schemes help to explain why so many inherited disorders have been identified and described in the dog. Many of these disorders have also been characterized at the molecular genetic level, so it is also interesting to consider why it has been possible to identify the causal mutations of so many canine eye diseases compared with other categories of canine disease. Now that a high-quality genome sequence and high-density single nucleotide polymorphism (SNP) arrays are available for the dog, it is very likely that many genes with novel ocular function will be identified in the coming years. However, at the time of writing, and with a small number of notable exceptions, most of the mutations associated with inherited eye disorders in the dog reside in genes previously implicated in similar conditions in

other species. The canine mutations were identified either because the gene concerned was a good functional candidate for the disease under investigation, or because they were identified as good positional candidates based on the results of a genome-wide linkage or association study.

It seems, therefore, that the dog has benefited from the advanced understanding that many genes that have been identified are associated with inherited ocular diseases in mice and humans. Humans rely to an enormous extent on vision, using our eyes as our main means of sensory input. Any disease or condition that impairs our ability to see is, therefore, hugely debilitating. Consequently, enormous amounts of research funding and effort have been spent on the study of inherited forms of blindness in humans and in common model organisms such as the mouse. Since the first human eye disease-associated mutation was reported in 1990 (Dryja *et al.*, 1990) close to 170 different genes have been associated with inherited retinal diseases alone, according to the RetNet Retinal Information Network website (http://www.sph.uth.tmc.edu/retnet/home.htm), and for many genes multiple different mutations have been identified. Among the 50,000 or so coding single nucleotide variants that were rediscovered during the 1000 Genomes project (Durbin *et al.*, 2010) a disproportionately large fraction were associated with eye diseases, which was thought to be due to the extent to which medical genetics research has focused on this field and, also, to the fact that, although debilitating, eye disorders probably do not affect the biological fitness of individuals to the same extent as do other categories of disease. All this means that there are many more candidate genes for canine inherited eye diseases than for most other categories of disease. As a result, the dog has already provided several models of human ocular disease based on spontaneously occurring mutations, and promises to provide many more.

Hereditary Cataract

The lens is the transparent, biconvex, avascular structure in the anterior segment of the eye that is responsible for the refraction of light to be focused on the retina. The lens consists of a nucleus, cortex and capsule and is suspended by many dense zonular ligaments which are attached to the capsule and connect between the ciliary body and the lens equator. Transparency is a crucial property of the lens, and this is achieved, in part, by the absence of light-scattering organelles within the lens fibres. New lens fibres are generated from the equatorial cells of the lens epithelium, which elongate, synthesize crystallin and finally lose their nuclei as they become mature lens fibres. The crystallins, which make up over 90% of the proteins in the lens, are specially adapted to contribute to the maintenance of transparency by forming soluble, high-molecular weight aggregates that need to stay in solution for the duration of an individual's life.

Cataracts are simply defined as opacities of the lens and can develop for a variety of reasons, including advanced age, the secondary effects of other diseases such as diabetes or progressive retinal atrophy, and trauma. Primary or hereditary cataracts (HC) are common among dogs and are a leading cause of blindness. HC has been reported in as many as 97 different breeds (Rubin, 1989; Davidson and Nelms, 2007), with around 60 breeds being reported as at increased risk compared with mixed-breed dogs (Gelatt and Mackay, 2005). Hereditary cataracts reported in different breeds vary with respect to their anatomical position within the lens, their age of onset and their progressive or stationary nature, although within a breed cataracts usually display marked breed specificity in type.

Despite the large number of breeds affected by HC, only a single gene, the transcription factor gene *HSF4*, has been implicated in the development of cataracts in dogs to date. *HSF4* belongs to a family of heat shock transcription factors that regulate the expression of heat shock proteins in response to different stresses, such as oxidants, heavy metals, elevated temperatures and bacterial and viral infections (Nakai *et al.*, 1997). Different mutations in *HSF4* have been reported to cause both human autosomal dominant and recessive cataracts (Bu *et al.*, 2002; Smaoui *et al.*, 2004; Forshew *et al.*, 2005), and studies in mice have shown that *HSF4* is

required for normal fibre cell differentiation during lens development (Fujimoto *et al.*, 2004; Min *et al.*, 2004). The position of mutations within the *HSF4* gene and the corresponding protein that have been reported to cause autosomal and recessive forms of cataracts in different species is shown in Fig. 10.2. Disruption of the gene leads to the development of cataracts via multiple pathways, including the downregulation or loss of post-translational modification of different crystallin proteins (Shi *et al.*, 2009). A single recessive nucleotide insertion in exon 10 of the gene (CFA5 g.85286582_85286583insC), which causes a frameshift and introduces a premature stop codon, is responsible for an early onset, bilaterally symmetrical and progressive form of HC in the Staffordshire Bull Terrier (Fig. 10.3) (Mellersh *et al.*, 2006b). This cataract starts to develop from a few months of age and invariably progresses to total cataract within 2–3 years if left untreated (Fig. 10.4) (Barnett, 1978). The identical mutation is also shared by the Boston Terrier, in which it causes the clinically identical early-onset hereditary cataract (EHC), one of two genetically distinct forms of cataract known to affect this breed (Barnett, 1978; Curtis, 1984); the mutation associated with the clinically more variable, late-onset hereditary cataract (LHC) in this breed has yet to be identified (Mellersh *et al.*, 2007). The same mutation has also been identified in a small number of French Bulldogs with a clinically identical cataract (Mellersh, unpublished).

A single nucleotide deletion at the same position in *HSF4* (CFA5 g.85286582delC) has also been associated with HC in the Australian Shepherd. The form of cataract caused by the insertion identified in the Staffordshire Bull Terrier and related breeds is recessive, highly penetrant, early onset, highly progressive and uniform. In contrast, the form of cataract observed in the Australian Shepherd that is caused by the deletion just described has a dominant or codominant mode of inheritance, is not completely penetrant and is typically associated with a posterior polar subcapsular cataract that also has a variable age of onset. It is highly likely that other mutations associated with the development of cataracts are co-segregating in the

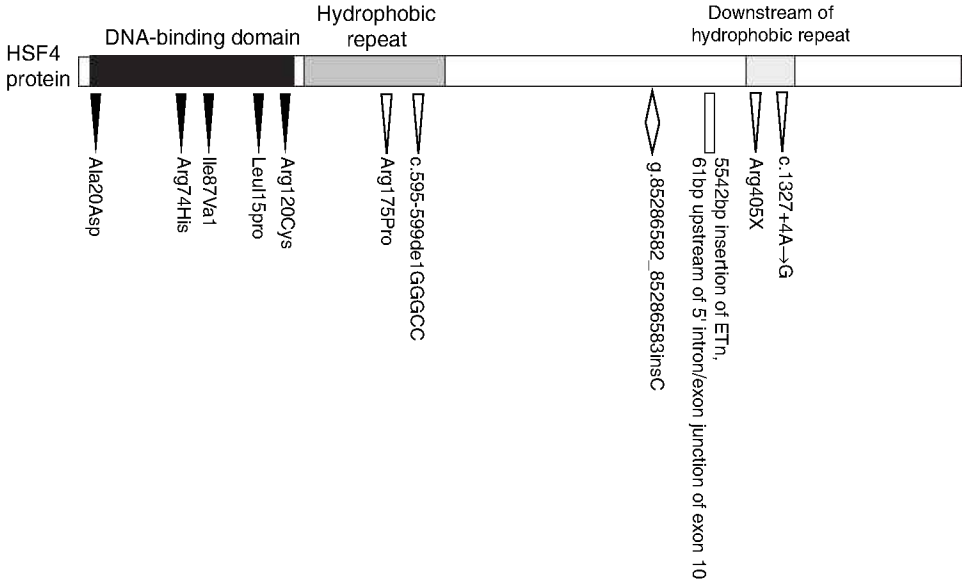

Fig. 10.2. Schematic diagram indicating domains of the HSF4 (heat shock factor 4) protein and the position of mutations reported to cause autosomal and recessive forms of cataracts. Mutations indicated by solid black symbols are dominant and those indicated by grey symbols are recessive. Mutations reported in different species are indicated by differently shaped symbols: triangles (human), rectangle (mouse), and diamond (dog). ETn, early transposable element (Source: Mellersh *et al.*, 2009).

```
Protein (clear)    A  P  P  P  L  S  V  A  V  V  Q  A  I  L  E  G  K  G  N  F  S  P  E  G  P  R  N  A  Q  Q  P  E  P
DNA (clear)        GCC CCC CCC CCA CTG TCC GTG GCT GTG GTG CAG GCC ATC CTG GAA GGG AAG GGG AAC TTC AGC CCC GAG GGG CCC AGG AAT GCC CAA CAG CCT GAA CCA
                                   ↓
DNA (affected)     GCC CCC CCC CCC ACT GTC CGT GGC TGT GGT GCA GGC CAT CCT GGA AGG GAA GGG GAA CTT CAG CCC CGA GGG GCC CAG GAA TGC CCA ACA GCC
Protein (affected) A  P  P  P  T  V  R  G  C  G  A  G  H  P  G  R  E  G  E  L  Q  P  R  G  A  Q  E  C  P  T  A
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Fig. 10.3. The *HSF4* gene exon 9 insertion (CFA5 g.85286582_85286583insC) associated with hereditary cataract in the Staffordshire Bull Terrier and Boston Terrier. The DNA sequence and corresponding amino acids are indicated for clear and affected dogs. The inserted C nucleotide is in bold text and is indicated with a black arrow. The insertion generates a frameshift which introduces a premature stop codon, indicated by the grey shading. The 27 incorrect amino acids that are coded for as a result of the frameshift are underlined.

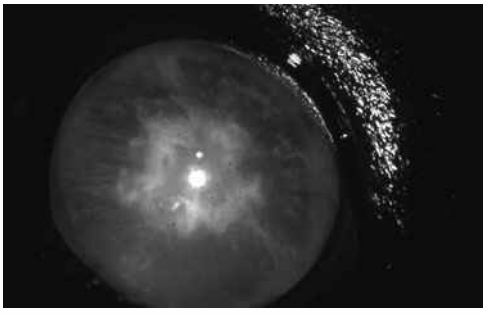

Fig. 10.4. Developing cataract in a 2-year-old Staffordshire Bull Terrier. Photo courtesy of the Animal Health Trust.

Australian Shepherd population because not all the dogs with bilateral posterior polar subcapsular cataract carry a copy of the *HSF4* deletion (Mellersh *et al.*, 2009).

HSF4 has been excluded from involvement in the development of HC in a long list of breeds, including the Alaskan Malamute, American Cocker Spaniel, Havanese, Belgian Shepherd Tervuren and Groenendael, Dachshunds, English Cocker Spaniels, English Toy Terrier, Finnish Lapphund, Golden Retriever, Griffon Bruxellois, Kromfohrlander, Jack Russell Terrier, Lapponian Herder, Miniature Schnauzer, Miniature Pinscher, Nova Scotia Duck Tolling Retriever, Rottweiler, Samoyed, Schnauzer and Tibetan Mastiff (Mellersh *et al.*, 2006b, 2009; Engelhardt *et al.*, 2007; Müller *et al.*, 2008; Oberbauer *et al.*, 2008; Müller and Distl, 2009). The paucity of canine cataract mutations that have been reported in the literature, compared with those associated with, for example, inherited retinal degenerations in the dog, is testament to the fact that HC is probably a genetically complex disorder in most breeds of dog and that studies to date have not included the analysis of sufficient numbers of cases and controls to identify DNA variants associated with the disease. A recessive mode of inheritance has been suggested for congenital cataracts and microphthalmos in the Miniature Schnauzer (Gelatt *et al.*, 1983) as well as cataracts in the Entlebucher Mountain Dog (Spiess, 1994), the Bichon Frise (Wallace *et al.*, 2005) and the American Cocker Spaniel (Yakely, 1978). In contrast, an autosomal dominant mode of inheritance with a high degree of penetrance has been suggested for the pulverulent (dust-like) form of cataract observed in the Norwegian Buhund (Bjerkås and Haaland, 1995); and autosomal dominant with variable penetrance has been suggested for inherited posterior polar subcapsular cataracts in Labrador and Golden Retrievers (Curtis and Barnett, 1989), although current anecdotal evidence indicates that in the Labrador cataracts could also be inherited as an autosomal recessive trait. Evidence of inheritance has been reported for a handful of other breeds, including the Leonberger, Jack Russell Terrier and Chow Chow, although the precise mode of inheritance has rarely been identified (Collins *et al.*, 1992; Heinrich *et al.*, 2006; Oberbauer *et al.*, 2008).

Primary Lens Luxation

Primary lens luxation (PLL) is not a disease of the lens itself, but rather an inherited deficiency of the lens suspensory apparatus, the zonule, which is a system of fibres that suspend the lens from the ciliary body, maintaining it on the visual axis and in contact with the anterior surface of the vitreous body. In dogs affected with PLL, ultrastructural abnormalities of the zonular fibres are already evident at 20 months of age (Curtis, 1983), long before the lens luxation that typically

occurs when the dogs are 3–8 years old as a result of degeneration and breakdown of the zonules, which cause the lens to be displaced from its normal position within the eye (Curtis and Barnett, 1980; Curtis *et al.*, 1983; Curtis, 1990; Morris and Dubielzig, 2005). In the majority of cases, the dislocated lens will pass into the anterior chamber (Fig. 10.5) where its presence is likely to cause acute glaucoma.

The condition has been recognized as a canine familial disorder for more than 100 years (Gray, 1909, 1932), and is encountered at high frequency in several terrier breeds and in some other breeds with probable terrier co-ancestry (Willis *et al.*, 1979; Curtis and Barnett, 1980; Curtis *et al.*, 1983; Curtis, 1990; Morris and Dubielzig, 2005). PLL is recessively inherited in the Tibetan Terrier (Willis *et al.*, 1979) and inheritance has been suggested to be recessive in the Shar-Pei and other western terrier breeds in which it has been studied (Sargan *et al.*, 2007). A mutation in *ADAMTS17* has been described as the cause of PLL in three breeds: the Miniature Bull Terrier, the Lancashire Heeler and the Jack Russell Terrier. The mutation is a G → A substitution at c.1473+1, which destroys a splice donor recognition site in intron 10 and causes exon skipping that results in a frameshift and the introduction of a premature termination codon (Fig. 10.6) (Farias *et al.*, 2010). The great majority of PLL-affected dogs are homozygous for the mutation, but a small minority are heterozygous, leading to speculation that carriers, of some breeds at least, might be at increased risk of developing the condition compared with dogs that are homozygous for the wild-type allele (Farias *et al.*, 2010). *ADAMTS17* is one of 29 known mammalian members of the ADAMTS family of genes which encode secreted metalloproteases that proteolytically modify extracellular structural proteins. Mutations in a variety of *ADAMTS* genes have been associated with a diverse set of human diseases, including Ehlers–Danlos syndrome (Colige *et al.*, 1999) and Weill–Marchesani syndrome (Dagoneau *et al.*, 2004). The canine *ADAMTS17* splice site mutation is shared by at least 17 different breeds, many of which are terriers or terrier-type breeds, but some of which have more diverse origins (Gould *et al.*, 2011). Some breeds that are known to be at increased risk of PLL, such as the Shar-Pei, do not carry the same *ADAMTS17* mutation as the terrier breeds, indicating that their form of the disease must be genetically distinct, although it is clinically similar (Gould *et al.*, 2011).

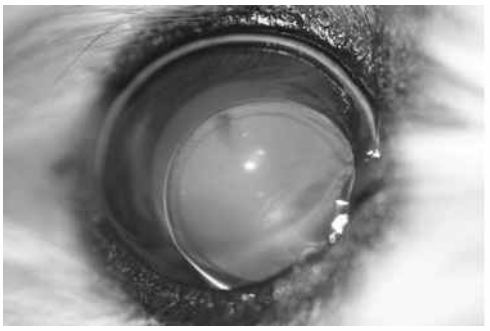

Fig. 10.5. Anterior lens luxation in a dog. Photo courtesy of the Animal Health Trust.

Diseases of the Retina

Inherited forms of retinal disease are among the best clinically and genetically characterized genetic conditions in the dog. Retinal disorders can be categorized in various ways, and the way in which they have been described in this chapter, which is summarized in Fig. 10.7, is certainly not the only way to partition them. Most methods of classification will, however, broadly take into account the typical stage of development or age of onset of the disease, the cells that are typically affected and whether the disease becomes progressively more severe during the dog's lifetime or whether it is more or less stationary. Here, the retinal disorders have been broadly divided into two main categories; the *degenerative* conditions where the retina develops normally and then degenerates during the dog's lifetime, and the *developmental* or *dysplastic* diseases in which the retina develops abnormally.

Degenerative retinal disorders

The majority of retinal diseases that have been described in the dog are degenerative

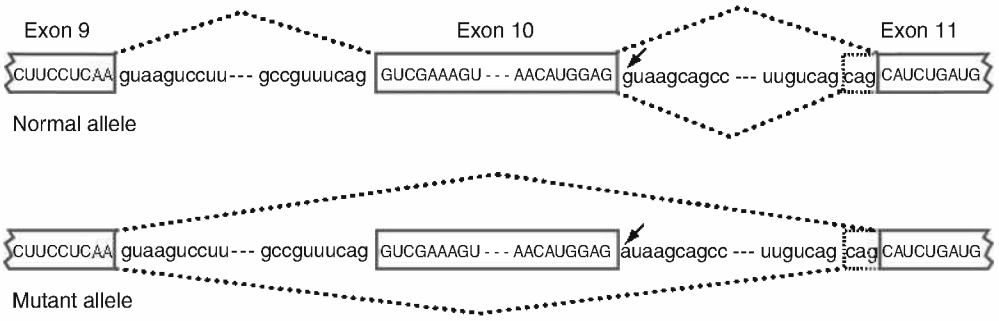

Fig. 10.6. Exon splicing patterns of transcripts from normal and mutant *ADAMTS17* alleles. In mRNA from the normal allele, exon 9 is spliced to exon 10 which is spliced to each of the two alternative splice acceptor sites at the 5´ end of exon 11. In RNA from the mutant allele, exon 10 is skipped and exon 9 is spliced to the alternative exon 11 splice acceptor sites. Arrows indicate the position of the transition at *ADAMTS17:c.1143+1*.

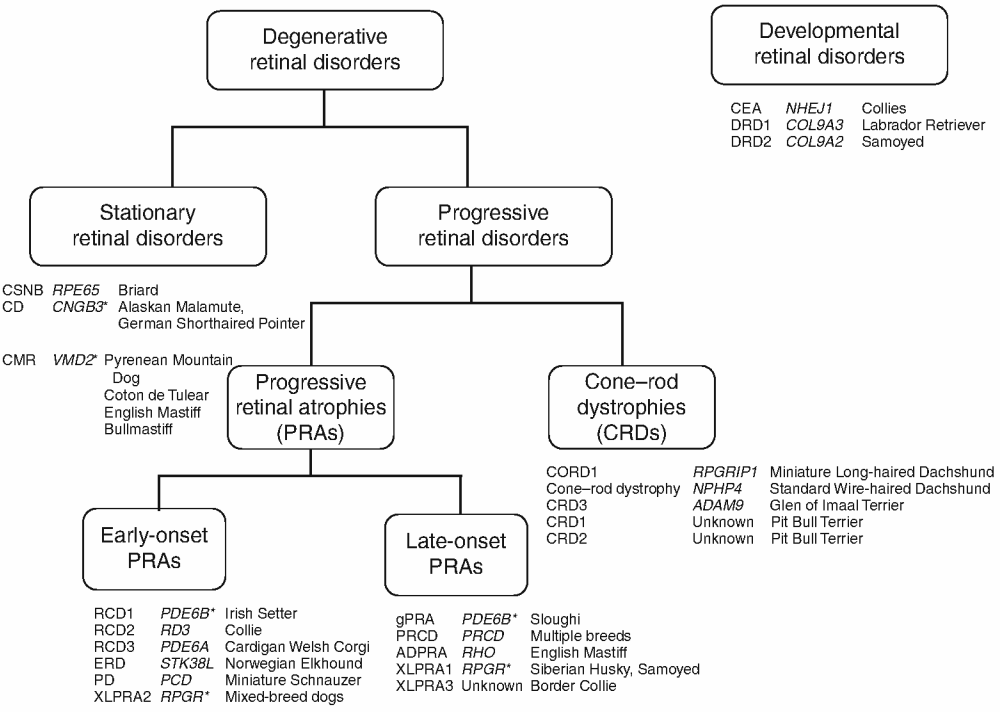

Fig. 10.7. Categorization of canine retinal disorders, showing the locus or abbreviation, gene and breed/s affected for each disorder. Different mutations in the genes marked with an asterisk account for genetically distinct conditions.

conditions and these are described first. Some degenerative conditions are characterized by an inevitable increase in severity over time, invariably culminating in complete loss of vision, whereas other conditions are characterized by a pathology that does not deteriorate throughout life. These two broad clinical categories of disease are described below under the headings *progressive* and *stationary*, respectively.

Progressive retinal disorders

Progressive retinal atrophy (PRA) and cone–rod dystrophy (CRD) are collective terms for two broad forms of progressive, bilateral degenerative diseases that affect the retinal photoreceptor cells.

Progressive retinal atrophy

In general, PRAs are characterized by initial loss of rod photoreceptor function, followed by that of the cones, and for this reason night blindness is the first significant clinical sign for most dogs affected with PRA. Visual impairment in bright light invariably follows, accompanied by characteristic changes to the fundus that are visible upon ophthalmoscopic investigation. Typical changes include attenuation of the blood vessels of the retina, increased reflectivity of the tapetal layer as a result of retinal thinning and atrophy of the optic disc. In many dogs, secondary cataracts develop, which might become extensive enough to obscure the retina and require the use of electroretinography (ERG) for diagnosis. Whereas most dogs show the same ophthalmoscopic abnormalities, the age at which these abnormalities develop varies considerably between breeds and genetically different forms of PRA can be broadly divided into early- and late-onset forms.

EARLY-ONSET FORMS OF PRA. Early-onset forms of the disease are typically expressed between 2 and 6 weeks of age, the period of postnatal retinal differentiation in dogs, and are characterized by the abnormal development of the rod and cone photoreceptors. Three well-characterized, genetically distinct forms of autosomal recessive, early-onset retinal degeneration are rod–cone dysplasia type 1 (RCD1), rod–cone dysplasia type 2 (RCD2) and early retinal degeneration (ERD) (Acland *et al.*, 1989). RCD1, which affects Irish Setters from approximately 25 days after birth and culminates at about 1 year when the population of rods and cones is depleted, is caused by a nonsense mutation at codon 807 of the gene encoding the beta subunit of cGMP phosphodiesterase (*PDE6B*), an essential member of the phototransduction pathway (Suber *et al.*, 1993). This mutation was the first mutation responsible for any form of PRA to be identified in the dog. An 8 bp insertion after codon 816 in the same gene causes a genetically distinct form of PRA in the Sloughi which has a later age of onset than the Irish Setter form, with the first signs of visual impairment not being noticed until dogs are between 2 and 3 years of age (Dekomien *et al.*, 2000). PRA in the Cardigan Welsh Corgi, termed rod–cone dysplasia 3 (RCD3), is also caused by a mutation in a subunit of cGMP phosphodiesterase, this time the alpha subunit, which results in a disease with a comparable age of onset to that of RCD1 (Petersen-Jones *et al.*, 1999). The genetically distinct RCD2 segregates in Rough and Smooth Collies (Wolf *et al.*, 1978) and is caused by an insertion in *RD3* that results in a stretch of altered amino acids and an extended reading frame (Kukekova *et al.*, 2009). Mutations in *RD3* have been associated with retinal degeneration in both humans and mice (Friedman *et al.*, 2006).

Whereas the early-onset forms of PRA, RCD1 and RCD3 described above, were among the first canine inherited diseases to be characterized at the molecular level, the mutation responsible for the similarly early-onset condition of ERD (early-onset degeneration) has only very recently been identified. This condition, which was originally described in Norwegian Elkhounds (Acland and Aguirre, 1987), and was first mapped more than 10 years ago (Acland *et al.*, 1999), is caused by an exonic SINE (short interspersed element) insertion in the gene *STK38L* (Goldstein *et al.*, 2010b). Although known to have neuronal cell functions, *STK38L* has not previously been associated with abnormal photoreceptor function, and being associated with such a disease in dogs establishes this gene as a potential candidate for similar diseases in other species, including man.

A different form of early-onset PRA affects Miniature Schnauzers. Histologically this disease is evident from a very early age, when the normal retina is nearing the end of postnatal differentiation, and as it affects both rods and cones it is termed photoreceptor dysplasia (PD) (Parshall *et al.*, 1991). This disease has been associated with a missense mutation in codon 82 of the phosducin gene (*PDC*) that causes a non-conservative substitution of Arg to Gly in close vicinity to the residue (Glu 85) that directly

interacts with the βγ-subunits of transducin (Zhang *et al.*, 1998). However, not all PRA-affected Miniature Schnauzers are homozygous for this mutation, indicating that at least two non-allelic forms of PRA segregate in this breed; the form associated with the PDC mutation has hence been termed Type A PRA (Aguirre and Acland, 2006).

The early-onset forms of PRA described above are all caused by mutations in autosomal genes. In contrast, a mutation in the X-linked retinitis pigmentosa GTPase regulator gene (*RPGR*) causes a very severe form of PRA, known as XLPRA2, that has been described in mixed-breed dogs (Zhang *et al.*, 2002). The XLPRA2 mutation is a two-nucleotide deletion that results in a frameshift that significantly changes the predicted peptide sequence by leading to the replacement of many acidic glutamic acid residues with basic arginine residues and results in the premature termination of the protein 71 amino acids downstream. Unlike the genetically distinct relatively late-onset XLPRA1 that is described below, the phenotype associated with the frameshift mutation in XLPRA2 is very severe and is manifested during retinal development. ERG abnormalities are evident by 5–6 weeks of age and cell degeneration is present by 4 months, suggesting that the mutant protein has a toxic gain of function that severely compromises the early stage of development of the photoreceptors.

LATE-ONSET FORMS OF PRA. The late-onset forms of PRA are degenerations of photoreceptors that have completed normal development. Whereas the genes implicated in early-onset diseases are those necessary for the correct development of photoreceptors, those associated with later-onset forms of disease are those that are necessary for the long-term maintenance and function of photoreceptors.

Progressive rod–cone degeneration (PRCD) is a late-onset form of PRA that affects multiple breeds. Before characterization of this disease at the molecular level, elegant inter-breed crosses were undertaken to determine that the phenotypically similar diseases that were segregating in multiple breeds, including the Miniature Poodle, the English and American Cocker Spaniels, the Labrador Retriever, the Australian Cattle Dog, the Nova Scotia Duck Tolling Retriever and the Portuguese Water Dog, were in fact allelic (Aguirre and Acland, 1988, 2006). However, when PRCD-affected dogs were mated to PRA-affected dogs of the Border Collie, Basenji and Italian Greyhound breeds the progeny were normal, indicating that these breeds are affected by genetically distinct forms of disease. The PRCD locus was mapped to a large region on CFA9 (canine chromosome 9) in 1998 (Acland *et al.*, 1998) before the canine genome sequence was available and while the tools available to investigate the canine genome were relatively unsophisticated. The whole-genome radiation panels that were available at the time, and that would have been useful to investigate any other region of the genome, did not significantly help to locate the mutation because they were both TK1 (thymidine kinase 1) selected (Priat *et al.*, 1998), and as TK1 was tightly linked to the PRCD locus it was difficult to order positional candidate genes within the PRCD-critical region. However, the fact that a genetically identical disease segregated in so many breeds proved to be invaluable as it allowed the use of linkage equilibrium mapping across affected breeds to considerably narrow the PRCD-associated region (Goldstein *et al.*, 2006); it led to the eventual identification of a single nucleotide substitution in the second codon of a previously unknown gene that is now known to be the cause of PRCD in at least 18 different breeds (Zangerl *et al.*, 2006). Intriguingly, an identical homozygous mutation was identified in a human patient with recessive retinitis pigmentosa, the human equivalent of PRA, and established the novel retinal gene, *PRCD*, as an important gene for the maintenance of rod photoreceptor structure and function across species.

A genetically distinct, late-onset PRA has been described in the English Mastiff. This disease is unique, to date, among canine inherited retinopathies in that it is inherited as an autosomal dominant disease, and is caused by a single non-synonymous C → G transversion at nucleotide 11 of the rhodopsin gene (*RHO*) that changes Thr-4 to Arg (T4R). Dogs carrying the *RHO* mutation have normal photoreceptor-specific ERG function at 3 to 6 months of age, but by 13 months these responses are abnormal.

In young affected dogs, retinal structure, rhodopsin expression and photoreceptor activation are normal; disease progression is characterized by regions of initial focal photoreceptor degeneration surrounded by areas of structurally normal retina which, interestingly, is very similar to the phenotypes of humans with *RHO* mutations (Kijas *et al.*, 2002). This mutation, originally identified in the English Mastiff, has also been identified in PRA-affected Bullmastiffs, but it has not been identified in any other breeds to date (Kijas *et al.*, 2003).

A different mutation in *RPGR* from that associated with XLPRA2 (described above) is responsible for a sex-linked form of late-onset form PRA that was originally described in the Siberian Husky (Acland *et al.*, 1994) and is known as XLPRA1. The mutation, which has also been identified in the Samoyed, is a five-nucleotide deletion that causes a frameshift and an immediate premature stop; the truncated protein lacks 230 C-terminal amino acids, which causes a slight decrease in the isoelectric point (Zhang *et al.*, 2002). The photoreceptors of dogs that carry this mutation develop normally (in contrast to those of dogs with XLPRA2) and remain morphologically and functionally normal until young adulthood, indicating that the C-terminal of the protein from the *RPGR* gene is not essential for the functional and structural differentiation of rods and cones.

All of the progressive, late-onset retinal disorders described behave, more or less, as single-gene conditions, caused by highly penetrant mutations. There is, however, some evidence that environmental modifiers may play a role in some of these diseases, causing phenotypic variation between and within breeds (Aguirre and Acland, 2006).

A different form of late-onset, X-linked PRA has been described in the Border Collie (Vilboux *et al.*, 2008). This condition is genetically distinct from both XLPRA1 and XLPRA2, although as yet the causal mutation has not been identified. The disease has an age of onset of around 3 years and is characterized by ophthalmoscopic and ERG abnormalities that strongly suggest this is a rod-led retinal degeneration.

Late-onset forms of PRA have been described in several other breeds for which the causal mutations are currently unknown. An autosomal recessive mode of inheritance has been proposed for a rapidly progressing retinal degeneration reported in the Tibetan Spaniel (Bjerkås and Narfström, 1994). This condition has a typical age of diagnosis of between 4 and 7 years, and affected dogs usually become blind within a year of initially presenting with early clinical signs of night blindness. PRA has also been described in the Tibetan Terrier, although the disease has an earlier onset in this breed compared with the Tibetan Spaniel. Night blindness and ophthalmoscopic signs of tapetal hyperreflectivity, as less light is absorbed by the atrophic retina, could be detected in dogs of around 1 year old, and ERGs recorded from affected dogs, compared with those of clinically normal dogs of the same age, did not reveal appreciable abnormalities until affected dogs were 10 months old. In contrast, histopathological findings included patchy disorientation and disorganization of the outer segments of rods and cones in affected dogs as young as 9 weeks (Millichamp *et al.*, 1988).

Cone–rod degenerations

Cone–rod dystrophies are disorders predominantly of cones, with rods becoming affected later. CRDs have ophthalmoscopic changes that are very similar to those of PRA, and detailed ERG studies that measure both cone- and rod-specific responses are required to distinguish between the two types of condition. For this reason, several disorders were initially described as PRAs – to be later reclassified when extensive ERG investigations were undertaken.

One such disorder is a form of retinal degeneration that has been described in the Miniature Long-haired Dachshund (MLHD). The disease was originally Described as an early-onset, autosomal recessive PRA, with all affected dogs within an inbred research colony displaying ophthalmological abnormalities that were detectable by ERG by 6 weeks of age and at 25 weeks by fundoscopy; the dogs became blind by the time they were 2 years of age (Curtis and Barnett, 1993). A subsequent electroretinography study identified an initial reduction of the cone photoreceptor function, which led to the condition being reclassified as a cone–rod dystrophy (CRD), rather than a rod-led PRA, and the disease was termed CORD1, for cone–rod degeneration 1 (Turney *et al.*, 2007).

The same condition has also been referred to as *CRD4* by others, for cone–rod degeneration 4 (Aguirre and Acland, 2006). Later findings by Lhériteau *et al.* (2009) were also consistent with the condition being a CRD.

Using the same colony of dogs, CORD1 was mapped to a large region on CFA15 and a mutation in *RPGRIP1* was identified that co-segregated completely with CORD1 in the research colony (Mellersh *et al.*, 2006a). The mutation is a 44 bp insertion of an A29 tract flanked by a 15 bp duplication in exon 2 of the gene; it creates a frameshift and introduces a premature stop codon early in exon 3. Mutations in *RPGRIP1* have been associated with Leber congenital amaurosis (LCA) (Dryja *et al.*, 2001), retinitis pigmentosa (RP) (Booij *et al.*, 2005) and CRD (Hameed *et al.*, 2003) in humans, as well as with inherited retinal abnormalities in mice (Zhao *et al.*, 2003); this suggests it plays an important role in visual function. The gene product's precise role is not currently understood, but it is thought to anchor regulatory complexes at the photoreceptor connecting cilium, which acts as a bridge between the inner and outer segments of photoreceptor cells (Roepman *et al.*, 2000) as well as having functions in disk morphogenesis (Zhao et al., 2003) and in the structure of the ciliary axoneme (Hong *et al.*, 2001). *RPGRIP1* also interacts with *NPHP4*, a gene that has been associated with a genetically distinct form of early-onset CRD segregating in the Standard Wire-haired Dachshund (Ropstad *et al.*, 2007a,b; Wiik *et al.*, 2008a,b, 2009). Within the research colony of MLHDs, there was complete correlation between the *RPGRIP1* genotype and phenotype of the dogs with respect to their CORD1 phenotype, whereas in the pet MLHD population this was not the case (Miyadera *et al.*, 2009). Outside the colony there was considerable variation in the age of onset of retinal degeneration in dogs that were homozygous for the *RPGRIP1* insertion (*RPGRIP1*$^{-/-}$), which has also been identified in other breeds, including the English Springer Spaniel and the Beagle. However all *RPGRIP1*$^{-/-}$ Beagles and MLHDs showed reduced or absent ERG cone responses, even in the absence of ophthalmoscopic abnormalities, a finding that has also been corroborated by Busse *et al.* (2011).

Together, these findings suggest that additional mutations are involved which modify the age of onset of ophthalmoscopic abnormalities associated with the *RPGRIP1* mutation. Because the original research colony used was developed from a very small number of dogs, it is a real possibility that the colony was fixed for these additional loci which, therefore, went undetected until the more outbred pet population was investigated. The mutation in *NPHP4* described above that causes an early-onset cone–rod dystrophy in Standard Wire-haired Dachshunds (Wiik *et al.*, 2008b) was not present in the Dachshund studies by Miyadera, enabling that mutation to be excluded. A recent association study using *RPGRIP1*$^{-/-}$ MLHDs that had either early- or late-onset CORD1 has indeed revealed a second locus that segregates with early-onset disease (K. Miyadera, Cambridge, 2010, personal communication), indicating that early-onset CRD in MLHDs is more likely to be a digenic condition, and that the *RPGRIP1* insertion alone causes a late-onset CRD, although ERG abnormalities may be detected early in life.

Another form of canine cone–rod dystrophy to be characterized at the molecular level is CRD3, for cone–rod dystrophy 3, that segregates in the Glen of Imaal Terrier. This disease becomes evident ophthalmoscopically in affected dogs as young as 3 years of age, and progresses to end-stage retinal degeneration over several years. Very recently, the causal mutation has been identified by two research groups almost simultaneously, as a large genomic deletion of *ADAM9* (a disintegrin and metalloprotease domain, family member 9) that removes exons 15 and 16 of the ADAM9 transcript (Goldstein *et al.*, 2010c; Kropatsch *et al.*, 2010) and generates a premature stop codon that is predicted to result in a truncated protein that lacks critical domains. This finding established CRD3 as a true orthologue, and a potentially useful model, of the similar human condition CORD9 in which four distinct *ADAM9* mutations have been found (Parry *et al.*, 2009).

Two additional, early-onset, cone–rod degenerations have been described, both of which were originally identified in separate dogs of Pit Bull Terrier ancestry (Kijas *et al.*, 2004). These conditions are known as CRD1

and CRD2, for cone–rod dystrophy type 1 and 2, respectively, and are known to be non-allelic diseases despite the fact they are both characterized by very early and severe dysfunction of both cones and rods, with cone function being consistently more severely impaired than that of rods (Kijas et al., 2004). The mutations responsible for these two conditions have not been identified.

Stationary retinal disorders

The forms of both PRA and CRD described above are all inherited retinopathies that are characterized by increasing severity and decreasing visual function over time. Progressive retinal changes during the dog's lifetime invariably lead to complete blindness.

The first non-progressive retinopathy to be well characterized was described in the Briard by Narfström *et al.* (1989) as stationary and congenital, resulting in it being termed congenital stationary night blindness (CSNB). Since the initial report, the disease was also described as having a progressive component (Wrigstad *et al.*, 1994), which led to it also being called a hereditary retinal dystrophy. However, CSNB and hereditary retinal dystrophy were later both shown to be caused by a four-nucleotide deletion in exon 5 of the *RPE65* gene, indicating that they are genetically identical conditions (Aguirre *et al.*, 1998; Veske *et al.*, 1999). *RPE65* is involved in the conversion of all-*trans*-retinoids to 11-*cis*-retinoids and, in its absence, the visual cycle is interrupted, resulting in a lack of visual pigment (Bok, 2005). This canine disease has a very characteristic clinical phenotype; affected dogs have profound visual impairment present from at least 5–6 weeks of age, but remain ophthalmoscopically normal, at least for the first 3–4 years of life. Older dogs may show subtle retinal abnormalities indicative of a slowly progressive retinal degenerative process. Both cone- and rod-mediated ERG responses are abnormal and rod photoreceptor function is virtually absent, even though the photoreceptor cells are initially healthy (Aguirre *et al.*, 1998). It was the unique absence of visual function in dogs with healthy rod photoreceptors that was observed in CSNB-affected dogs that led to landmark studies in the field of retinal gene therapy. Sub-retinal injections of adeno-associated virus vectors expressing *RPE65* resulted in the restoration of rod photoreceptor function and improved visual function, first in dogs (Acland *et al.*, 2001; Le Meur *et al.*, 2007) and subsequently in humans (Bainbridge *et al.*, 2008; Hauswirth *et al.*, 2008; Maguire *et al.*, 2008).

Cone degeneration (CD) is also different from other progressive disorders in that early-onset cone degeneration occurs in the absence of the subsequent rod degeneration that characterizes cone–rod dystrophies. In CD, which was originally described in Alaskan Malamutes (Rubin *et al.*, 1967), affected puppies develop day blindness and photophobia between 8 and 12 weeks of age, when retinal development is normally completed in dogs, although these clinical signs only occur in bright light and the dogs remain ophthalmoscopically normal throughout their entire lives. Cone function starts to deteriorate by the age of 6–12 weeks and is unrecordable in adult dogs (Aguirre and Rubin, 1975). Rod photoreceptors, however, remain functionally and structurally normal throughout the animal's life. A large genomic deletion that removes all exons of *CNGB3*, the gene that encodes the β subunit of the cone cyclic nucleotide-gated cation channel, has been identified in CD-affected Alaskan Malamute-derived dogs, although there is evidence that the condition might be genetically heterogeneous in this breed, as some dogs have been identified with clinical signs of day blindness that lack the *CNGB3* deletion (Seddon *et al.*, 2006). A missense mutation in the same gene has been detected in German Short-haired Pointers affected with a clinically identical allelic disorder (Sidjanin *et al.*, 2002). These findings have established CD as an orthologue of human achromatopsia, a condition also known as rod monochromacy or total congenital colour blindness that shares many of its clinical features with CD and has also been associated with mutations in *CNGB3* (Kohl *et al.*, 2000; Sundin *et al.*, 2000). The potential of these orthologues has recently been demonstrated by the successful restoration of cone function and

associated phototopic vision in both of the canine achromatopsia models by gene replacement therapy (Komaromy *et al.*, 2010).

Another inherited retinal disorder that is generally non-progressive is canine multifocal retinopathy (CMR), a disease that has been recognized in several breeds, particularly Pyrenean Mountain Dog, Coton de Tulear, English Mastiff and Bullmastiff (Grahn *et al.*, 1998; Guziewicz *et al.*, 2007). Ophthalmoscopic changes are usually evident in affected dogs before the age of around 4 months and are characterized by multifocal areas of retinal elevation that contain sub-retinal accumulation of serous fluid. Retinal elevations can remain static for several years, whereas multifocal outer retinal atrophy is often seen in older animals. Two different variants in the bestrophin gene (*BEST1*, alias *VMD2*) have been identified as likely causal mutations for CMR in the dog. In Pyrenean Mountain Dog, English Mastiff and Bullmastiff dogs, a C73T mutation in exon 2 causes a premature translation termination that limits the open reading frame to 25 codons, compared with 580 codons in the wild-type mRNA; in Coton de Tulears dogs, a G482A transition changes an evolutionarily conserved glycine residue to aspartic acid. These mutations establish CMR as a novel animal model for Best macular dystrophy (BMD) in humans – an autosomal dominant, childhood retinal disease also caused by mutations in the bestrophin gene (Lorenz and Preising, 2005; Xiao *et al.*, 2010).

Developmental Diseases

Retinal dysplasia is the term used to denote disorderly proliferation and imperfect differentiation of the developing retina; it can be subdivided into focal, multifocal, geographic and total types. Focal and multifocal types are manifested as linear folds and 'rosettes' of tissue in the inner (sensory) retinal layer, whereas in geographic forms there are larger areas of defective retinal development that appear as large irregular or horseshoe-shaped areas of mixed hyper- or hypo-reflectivity in the central retina. Total or generalized forms of retinal dysplasia have been described as an inherited trait in several breeds, including the Bedlington Terrier (Rubin, 1968), Sealyham Terrier (Ashton *et al.*, 1968), Labrador Retriever (Barnett *et al.*, 1970) and Yorkshire Terrier (Stades, 1978), and they are associated with complete detachment of the abnormal neuroretina from the retinal pigment epithelium, which results in blindness of affected eyes. All forms of retinal dysplasia are congenital and non-progressive. Retinal dysplasia appears to be inherited as an autosomal trait, at least in those breeds where sufficient numbers of individuals have been studied to reliably estimate the mode of inheritance (Macmillan and Lipton, 1978; Crispin *et al.*, 1999; Long and Crispin, 1999). To date, the genetics of retinal dysplasia have not been characterized at the molecular level in any breeds, and no mutations have been associated with this condition.

Forms of syndromic retinal dysplasia have been reported in the Labrador Retriever (Nelson and MacMillan, 1983; Carrig *et al.*, 1977, 1988) and the Samoyed (Meyers *et al.*, 1983). Homozygous affected dogs had short-limbed dwarfism and a range of ocular changes characterized by complete retinal detachment and cataract, whereas heterozygous dogs had only focal or multifocal retinal lesions (Carrig *et al.*, 1977, 1988). Breeding studies determined that these two disorders are non-allelic (Acland and Aguirre, 1995), and they were termed DRD1 (dwarfism with retinal dysplasia type 1, Labrador Retriever) and DRD2 (Samoyed), respectively; the conditions have also previously been referred to as OSD1 and OSD2 – for oculoskeletal dysplasia. Mutations have recently been associated with both disorders; a 1 bp insertional mutation in exon 1 of *COL9A3* is associated with DRD1, and a 1267 bp deletion in the 5´ end of *COL9A2* co-segregates with DRD2. Both mutations affect the COL3 domain of their respective genes, the expression of which is reduced in affected retinas (Goldstein *et al.*, 2010a).

Another complex congenital defect of the retina is collie eye anomaly (CEA), although retinal involvement is secondary to the primary ocular defects associated with this disorder. The primary phenotypic element of the disorder is regional hypoplasia of the choroid, the highly vascular layer underlying the retina. Associated retinal lesions,

known as colobomas, are often detectable ophthalmoscopically, as are tortuous retinal vessels and multiple retinal folds in a minority of cases (Parker *et al.*, 2007). CEA, which segregates in several herding breeds with Collie ancestry, was mapped to a large region of CFA37 that included over 40 genes (Lowe *et al.*, 2003); subsequently, the fact that the disorder segregates in multiple, closely related breeds was used to reduce the size of the critical disease-associated region and to pinpoint the causal mutation to a 7.8 kb intronic deletion in the *NHEJ1* gene, which spans a highly conserved binding domain to which several developmentally important genes bind (Parker *et al.*, 2007).

Other Conditions

The diseases of the lens and retina described above represent all the inherited eye conditions in the dog for which causal mutations have been identified. Many other ocular conditions have been reported to be more common in certain breeds than others, which is indicative that they have a genetic component. However, a rigorous estimate of the mode of inheritance has been undertaken for relatively few of these conditions. To list comprehensively all of the eye conditions that have been reported in dogs is outside the scope of this chapter, so the remainder of the conditions described is restricted to those conditions for which an estimate of the mode of inheritance or the heritability has been reported.

Persistent hyperplastic primary vitreous

Persistent hyperplastic primary vitreous (PHPV) is a congenital, non-progressive condition which results from the abnormal regression of the fetal hyaloid vasculature. The condition is rare, but is seen more commonly in Staffordshire Bull Terriers, in which it has a presumed autosomal recessive mode of inheritance (Curtis *et al.*, 1984; Leon *et al.*, 1986). PHPV and persistent hyperplastic tunica vasculosa lentis (PHTVL) have also been described in detail in the Doberman (Stades *et al.*, 1991).

Glaucoma

Glaucoma is the term used to describe a group of conditions that result in increased intraocular pressure, with damage to the retinal ganglion cells and their axons, leading to vision loss and blindness. Glaucoma is commonly divided into congenital, primary and secondary types, depending on the aetiology of the condition. Congenital glaucoma is rare in the dog (Barnett *et al.*, 2002), and secondary glaucoma, which is the most common form of the condition observed in the dog, arises as a result of antecedent or concurrent ocular disease, so is not itself inherited, although the primary, causal condition might be. Primary glaucoma occurs in the absence of any other ocular disease, and, therefore, usually has a genetic component. It can occur in the presence (closed-angle glaucoma) or absence (open-angle glaucoma) of an abnormal, narrowed or closed opening into the ciliary cleft, which prevents the efficient drainage of aqueous humour from the posterior chamber of the eye, through the pupil into the ciliary cleft via openings between the pectinate fibres. Goniodysgenesis is the most common cause of primary glaucoma in dogs, and refers to the presence of abnormal, irregularly shaped or imperforate sheets of pectinate fibres.

Glaucoma has been reported to be more prevalent than average in several breeds, including the American Cocker Spaniel, the Bassett Hound, the Shar-Pei, the Norwegian Elkhound and the Boston Terrier (Wyman and Ketring, 1976; Bjerkås *et al.*, 2002; Gelatt and Mackay, 2004; Oshima *et al.*, 2004). A strong and significant correlation between goniodysgenesis and glaucoma was reported in the Great Dane, and the same study reported a high heritability for goniodysgenesis, suggesting that glaucoma may be heritable in this breed (Wood *et al.*, 2001). A similarly significant association has been reported between pectinate ligament dysplasia and adult-onset primary glaucoma in the Flat-coated Retriever, for which the heritability was estimated to be approximately 0.7 (Read *et al.*, 1998; Wood *et al.*, 1998). Autosomal recessive, primary open-angle glaucoma (POAG) has been very well characterized in the Beagle (Gelatt *et al.*, 1977a,b, 1981; Gelatt and Gum, 1981;

Mackay *et al.*, 2008), and mutations in the myocilin (*MYOC*) gene have been excluded from association with the condition in this breed (Kato *et al.*, 2009) and also in the Shiba Inu dog (Kato *et al.*, 2007). To date, no mutations have been identified that are associated with inherited glaucoma in any breed of dog.

Summary

At the time of writing, 24 different mutations have been associated with inherited eye disease in the domestic dog, and more are likely to have been identified by the time this chapter goes to press. This number far exceeds those associated with any other category of disease, meaning that inherited eye diseases are arguably better understood, at both the clinical and genetic level, than any other type of canine disease. The dog has already played an important role in emerging therapies for inherited blindness in humans, and similarities in disease phenotype and eye structure and function between dog and man, together with the increasingly sophisticated genetic tools that are available for the dog, mean that the dog is likely to play an ever increasing role both in our understanding of the normal functioning of the eye and in our ability to treat inherited eye disorders.

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11 Canine Cytogenetics and Chromosome Maps

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Introduction

A fundamental prerequisite for the development of a comprehensive and effective genome map for an organism is the ability to demonstrate that all chromosomes are represented in such a map. This, in turn, requires that all chromosomes comprising the karyotype of the organism under investigation can be recognized as separate entities, and thus necessitates that the organism has been studied at the cytogenetic level. The term 'physical chromosome map' refers to the description of genomic markers whose corresponding chromosomal location has been defined using one of a variety of physical mapping techniques. Depending on the approach used, the location of these markers may be described either by their assignment to a whole chromosome or, more precisely, by regional assignment to a cytogenetically visible chromosome band. The emergence of robust genome sequence assemblies and whole-genome sequencing strategies for many species is adding a new level of complexity to chromosomal studies, with molecular cytogenetics now reporting data in the context of corresponding nucleotide coordinates rather than band location. This chapter will provide a historical summary of advances in conventional and molecular cytogenetics of the domestic dog (*Canis familiaris*, CFA), beginning with the development of standardized chromosome nomenclature. This will be followed by an overview of the resources currently available for molecular cytogenetics studies of both normal and abnormal canine karyotypes. We will summarize the application of molecular cytogenetic approaches to canine cancer and comparative genomics, and conclude with a brief historical overview of canine meiotic linkage mapping resources.

Dog Chromosome Nomenclature – A Historical Perspective

The chromosome number of the domestic dog was first determined in 1928 from studies of

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meiotic cells by Minouchi (1928), and was later confirmed by Gustavsson (1964) using cultured lymphocytes. The diploid karyotype comprises 38 pairs of acrocentric autosomes, a large submetacentric X chromosome and a small metacentric Y chromosome (Fig. 11.1). Conventional Giemsa staining allows precise identification of only the sex chromosomes, due to their size and morphology, and of chromosome 1 (CFA1) by virtue of its size. Since the remaining autosomes gradually decrease in size, reliable recognition of conventionally stained homologous pairs is an impossible task, and presented a significant hindrance to the early advancement of canine genome analysis.

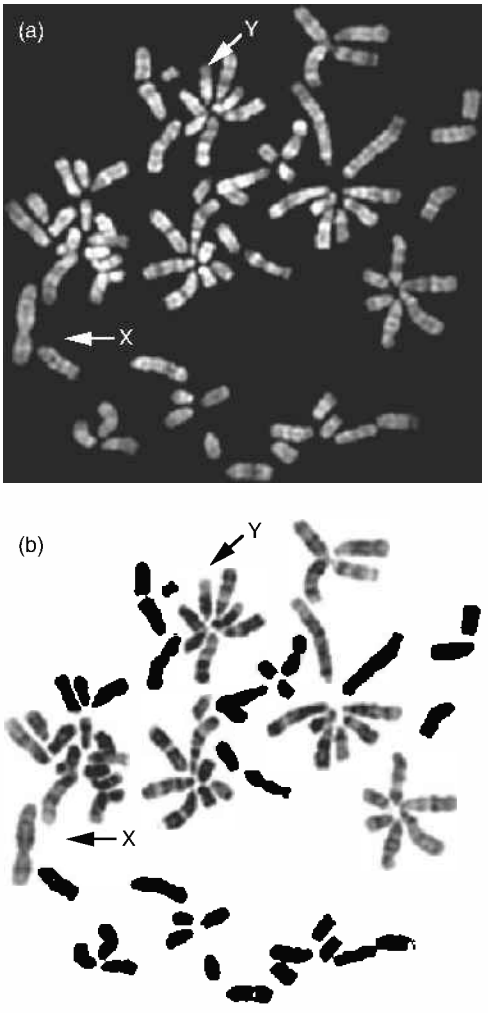

Fig. 11.1. Metaphase chromosomes of the dog: (a) metaphase preparation from a healthy male dog stained with the fluorescent stain DAPI; (b) inverted DAPI-stained metaphase preparation, revealing banding similar to that of conventional GTG banding (with Giemsa stain). The X and Y chromosomes are indicated.

In 1994, an international committee was established to promote the development of a standardized chromosome nomenclature for the domestic dog. Using conventional GTG (Giemsa stain) banding, the committee was able to establish a consensus partial karyotype that included the largest 21 autosomal chromosome pairs and the sex chromosomes (Świtoński *et al.*, 1996), numbered according to the system proposed two decades previously by Selden *et al.* (1975). The committee concluded that the development of a complete standard karyotype would require the use of molecular cytogenetic reagents, based upon the application of fluorescence *in situ* hybridization (FISH) techniques. A further advance towards a description of the entire G-banded dog karyotype was made by Reimann *et al.* (1996), who were able to orientate correctly all chromosomes with the aid of a centromeric repeat probe. Their findings were in agreement with the standardization committee for chromosomes 1–21 and also described an extended nomenclature of the canine karyotype for the remaining 17 chromosomes, proposing a revised GTG-banded ideogram at the 460-band level. A set of whole chromosome paint probes (WCPP) was generated for the dog using bivariate flow sorting (Langford *et al.*, 1996); this was used by the committee as a common resource and allowed unequivocal identification and standardized numbering of all 38 autosomes. These recommendations were later endorsed by the International Society of Animal Genetics (ISAG) DogMap workshop held in Minneapolis, Minnesota in 2000. The WCPP were also used in reciprocal chromosome painting analysis to identify evolutionarily conserved chromosome segments (ECCS) shared between the human and domestic dog genomes (Breen *et al.*, 1999a; Yang *et al.*, 1999), providing the first genome-wide assessment of the structural organization of the canine genome compared with that of the human genome.

The expanding utilization of FISH analysis in canine genome studies called for the use of

fluorochrome-based banding techniques (e.g. DAPI-banding) to facilitate concurrent chromosome identification. Using sequential application of the WCPP panel on individual dog metaphase spreads, a series of DAPI-banded karyotypes was generated in which the numbering of the chromosomes matched that endorsed by ISAG (Breen *et al.*, 1999b). A 460-band ideogram with five grey levels (Fig. 11.2) was proposed (Breen *et al.*, 1999b) to facilitate the accurate cytogenetic assignment of FISH-mapped loci, and has since become the accepted system for defining karyotype chromosome nomenclature in the domestic dog.

Molecular Markers and Chromosome Maps

Once dog karyotype nomenclature had been defined, the emphasis moved towards the generation and characterization of molecular markers to act as reference points for canine genome analyses. Following the development of several independent resources based on bacteriophage and plasmid cloning vectors, the first large-insert canine genome library (RPCI-81) was developed from a male Doberman Pinscher (Li *et al.*, 1999), representing ~157,000 recombinant bacterial artificial chromosome (BAC) clones with a mean insert size of 155 kb, providing 8.1-fold genome coverage. Unlike small insert clones, BAC clones constitute ideal single-locus probes for FISH analysis and provide excellent tools for robust and efficient chromosome identification. Thomas *et al.* (2003a) defined a panel of 41 BAC clones from the RPCI-81 library to act as markers for unequivocal identification of each dog chromosome. Subsequently, Courtay-Cahen *et al.* (2007) reported a panel of 80 RPCI-81 clones for simultaneous identification of the 38 dog autosomes within a single FISH assay, based on the profiles generated by hybridization of up to three independent BAC clones for each chromosome.

A second dog BAC library (CHORI-82, http://bacpac.chori.org/library.php?id=253) was constructed from a female Boxer, comprising 198,000 clones with a mean insert size of ~170 kb, and providing ~10× genome coverage. This library was utilized in the next-generation integrated cytogenetic/radiation-hybrid map for the dog (Breen *et al.*, 2004), which comprised 4249 markers, including 1760 BAC clones. Of these, 851 BAC clones were also chromosomally assigned using FISH analysis, resulting in robust anchor points between the radiation hybrid and cytogenetic maps at ~3.5 Mb intervals throughout the genome. BAC end-sequence data from CHORI-82 clones also provided the framework for the construction of a 7.5× coverage genome sequence assembly for the domestic dog (Lindblad-Toh *et al.*, 2005; Chapter 12). DNA sequence and positional data for these BAC clones are publicly available through several online resources (for example, the UCSC [University of California Santa Cruz] Dog (*Canis lupus familiaris*) Genome Browser Gateway at: http://genome.ucsc.edu/cgi-bin/hgGateway?org=Dog&db=canFam2), and allow direct selection of genomic markers representing chromosomal locations and/or gene sequences of specific interest for downstream studies.

The utilization of BAC clones in the development of the canine genome sequence assembly afforded extensive opportunities for the application of these markers as resources for evaluation of both normal and abnormal karyotypes, and for the interpretation of these observations directly in context with the corresponding DNA sequence. Thus, as genome-integrated molecular cytogenetic reagents have replaced conventional methods, bands have been superseded by base pairs. By reference to the canine genome sequence assembly, two panels of cytogenetically validated BAC clones were developed that tiled each dog autosome and the X chromosome. The first comprised 39 chromosome-specific clone panels distributed at uniform intervals of ~10 Mb (275 clones) (Thomas *et al.*, 2007); see Plate 11. This was followed by a second panel distributed at ~1 Mb intervals throughout the genome (2097 clones) (Thomas *et al.*, 2008). The chromosomal location and relative order of each clone were defined using multicolour FISH analysis, resulting in robust frameworks for anchoring the dog genome assembly and the cytogenetic map, with over 95% of the FISH-mapped clones showing unique hybridization at the chromosomal location predicted from the genome assembly. The remaining clones included probes with unique

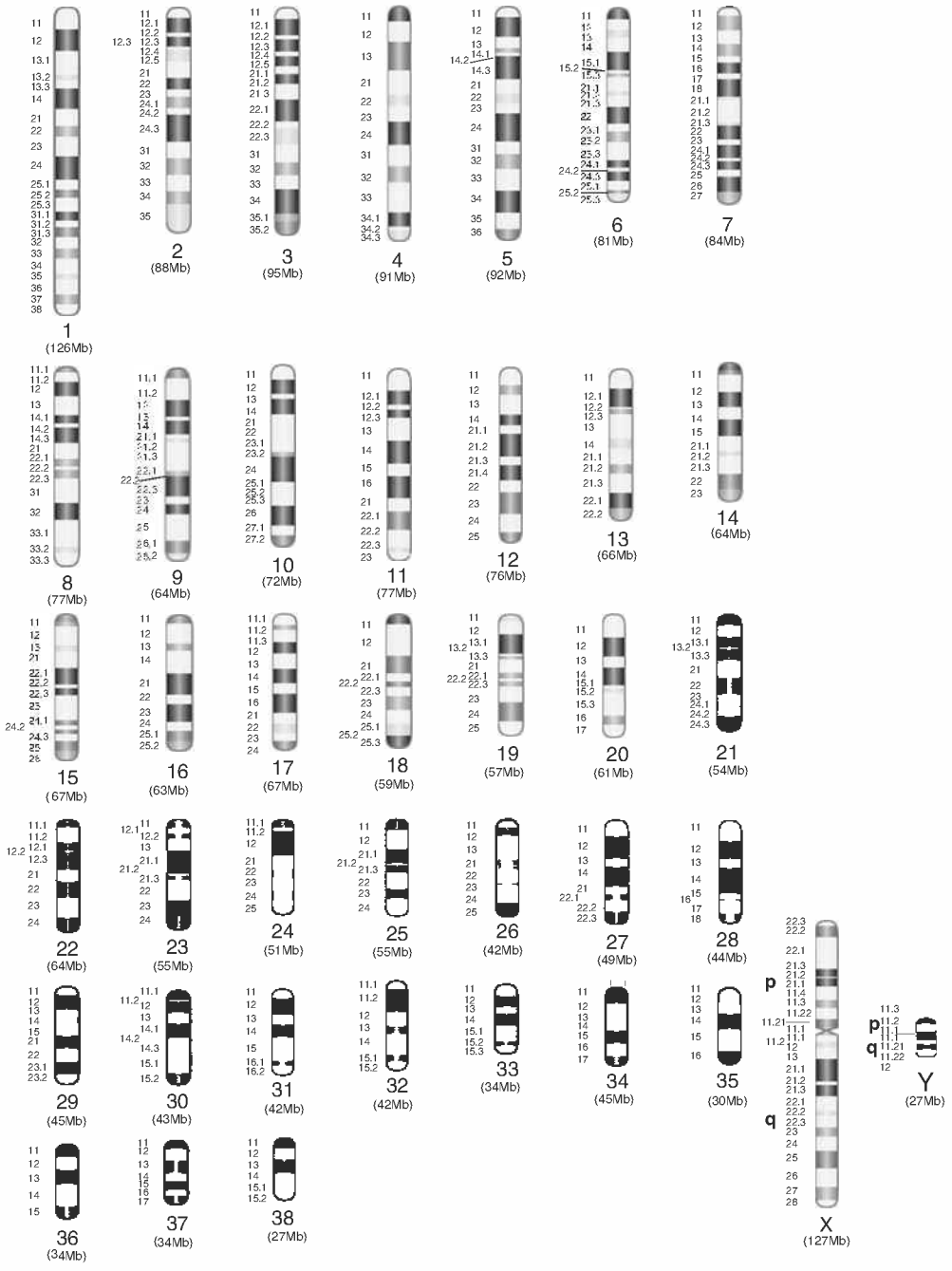

Fig. 11.2. DAPI-banded ideogram of the dog at the 460-band resolution, with chromosomes numbered according to the recommendations of the International Committee for the Standardization of the Karyotype of the Dog (Świtońksi *et al.*, 1996; Breen *et al.*, 1999b). The size of each chromosome in megabase pairs is given in parentheses and is based on the canine genome sequence assembly data (Lindblad-Toh *et al.*, 2005). Since the sequence assembly was generated from a female dog, the physical size of CFAY is based on an earlier study of flow-sorted chromosomes (Langford *et al.*, 1996).

1

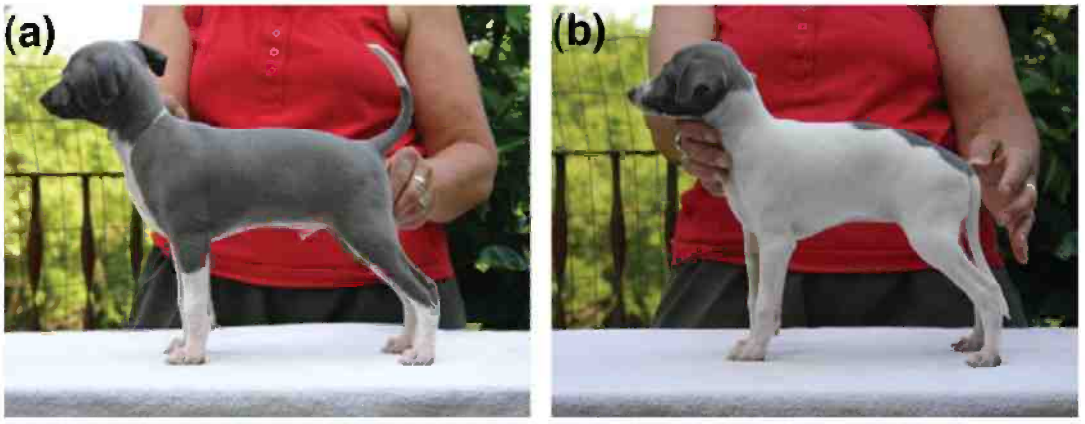

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Plate 1. (a) An Italian Greyhound – K^B/K^B (or K^B/k), *d/d*, s^i/s^i – puppy with a blue coat (due to the dilution of eumelanin) and the 'Irish' pattern of white-spotting. (b) Its sibling – K^B/K^B (or K^B/k), *d/d*, s^w/s^w – has a blue coat and extreme white spotting.

Plate 2. (a) A Dachshund – K^B/K^B (or K^B/k), a^t/a^t, *M/m* – with the merle pattern, referred to as dapple in this breed. It also carries the a^t allele of *ASIP*, resulting in 'tan points' above the eyes, on the sides of the muzzle, on the chest and on legs and feet. (b) This Dachshund – K^B/K^B (or K^B/k), *b/b*, a^t/a^t – is brown (a dilution of eumelanin) with 'tan points' (pheomelanin).

Plate 3. All three retrievers carry the dominant black allele of *CBD103* (K^B). The action of K^B can be modified in different ways. Recessive loss-of-function *TYRP1* alleles (*b*) cause the dilution of black to brown pigment in the Labrador retriever, and a recessive loss-of-function *MC1R* allele (*e*) causes uniformly yellow coat in the Golden retriever.

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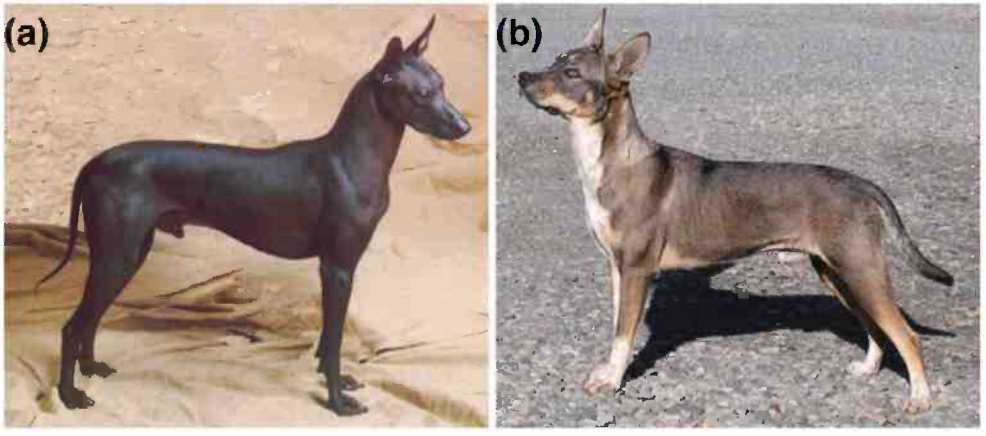

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Plate 4. In the Saint Bernard, long hair is recessive to short hair (Crawford and Loomis, 1978) and is caused by an *FGF5* mutation (Drogemuller *et al.*, 2007b).

Plate 5. Long hair in the Afghan Hound is not due to the $FGF5^{59F}$ mutation associated with long hair in other breeds (Cadieu *et al.*, 2009).

Plate 6. The Xoloitzcuintle, also known as the Mexican hairless dog, has both (a) hairless – Hr^{Hr}/hr^{+} – and (b) coated – hr^{+}/hr^{+} – varieties.

Plate 7. The interaction between coat length and wire hair in Dachshunds. (a) Shorthaired wires – *L/L* (or *L/l*), Wh^{Wh}/Wh^{Wh} – have coarse, bristly coats while (b) longhaired (soft) wires – *l/l*, Wh^{Wh}/Wh^{Wh} – have softer coats. Both shorthaired and longhaired wires have facial furnishings.

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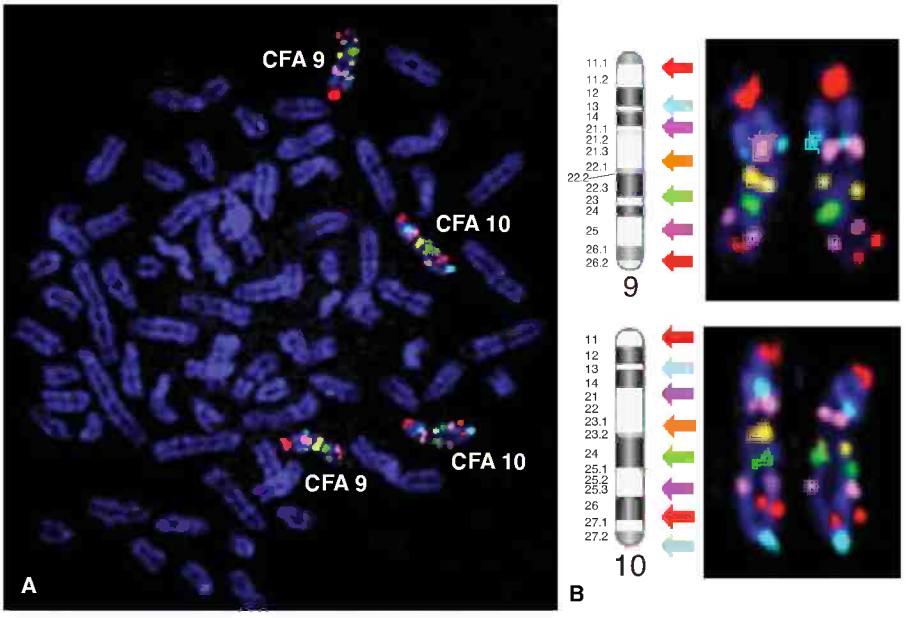
Plate 8. (a) Wavy-coated and (b) curly-coated varieties of the Portuguese Water Dog used in a genome-wide study to identify an association between *KRT71*^{151W} and curly hair.
Plate 9. A dorsal stripe resulting from the inverted orientation of hair follicles along the dorsal midline is a dominant trait characteristic of the Rhodesian Ridgeback.
Plate 10. The recessive ripple coat pattern in a litter of newborn Weimaraner puppies.

11



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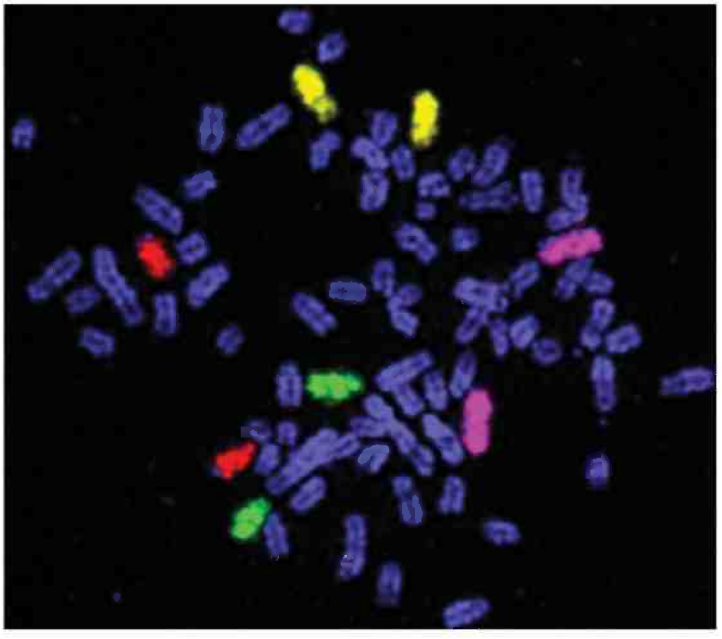

Plate 11. Chromosome-specific BAC 'tiling' to generate a coloured bar-code. BAC clones were selected at approximately 10Mb intervals along the length of CFA 9 (seven clones) and CFA 10 (eight clones). Each clone was labelled with one of five spectrally resolvable fluorochromes, and all clones for both CFA 9 and CFA 10 were hybridized simultaneously to a normal dog metaphase preparation. (a) BAC probe signal is visible only on the two CFA 9 and 10 homologues. (b) Enlarged and aligned homologues of CFA 9 and CFA 10 from panel (a) shown alongside ideograms of both chromosome.

Plate 12. Multicolour pseudo-painting using pools of BAC clones spaced at 1Mb intervals along four dog chromosomes and labelled with a common fluorochrome-conjugated nucleotide. In this example a metaphase spread from a healthy female dog has been hybridized with BAC pools representing CFA 4 (purple), 8 (gold), 13 (green) and 16 (red).

but contradictory mapping locations, and others exhibiting multiple hybridization profiles in FISH analysis. At least a proportion of these apparent anomalies appeared to reflect natural genomic polymorphisms among individual dogs/breeds and they represent interesting resources in their own right for the investigation of canine genome architecture.

With the resolution of metaphase-based FISH analysis being limited to ~4 Mb, physical ordering of BACs from the 1 Mb panel was largely reliant on the evaluation of mapping data from interphase nuclei. This limitation on resolution confers the advantage of allowing chromosome-specific pools of BAC clones to be used as an effective and efficient substitute for conventional WCPP. Historically, WCPP have been generated either by micro-dissection of single chromosomes, or from cultured cells of normal donors using bivariate flow-sorting technology to capture multiple copies of each chromosome into a separate collection tube, based on its unique sequence composition. This material is then amplified using degenerate PCR followed by incorporation of a fluorescent nucleotide. The resulting FISH probe may then be used to assess both the numerical and structural status of that chromosome in any individual for which metaphase spreads are available. Flow sorting and micro-dissection techniques present technical challenges, are time-consuming and generate relatively small quantities of probe. Although representing a marked reduction in actual chromosomal coverage compared with conventional WCPP, chromosome-specific BAC pools generate comparable image data to those of their traditional counterparts (Thomas *et al.*, 2005) and so represent an appropriate substitute for most applications (see Plate 12). Moreover, they are both simpler and less time-intensive to generate, and are more readily accessible to the typical molecular biology laboratory. Since they are derived from cloned DNA sequences rather than viable cells, they also represent an effectively unlimited resource.

The uniform distribution and genomic integration of the 1 Mb BAC clone panel provides a valuable tool for defining the DNA sequence composition of cytogenetic landmarks. Application of BACs from the CFAX panel to male dogs identified clones mapping within the canine pseudoautosomal region (PAR) shared by the X and Y chromosomes (Thomas *et al.*, 2008). Targeted selection and FISH mapping of additional clones selected from the genome sequence assembly subsequently narrowed the boundary of the canine PAR to a 32 kb region within a single BAC located ~6.6 Mb from CFA Xptel, which was refined further to a 2 kb region by computational analysis of DNA sequence data from CFAX (Lindblad-Toh *et al.*, 2005) and CFAY (Kirkness *et al.*, 2003). This combined *in situ/in silico* study (Young *et al.*, 2008) provides a worked example of the approach that may now be utilized for precise definition of both constitutional and tumour-associated chromosomal break points, as well as those occurring during speciation.

Detection of Constitutional Chromosome Aberrations

The identification of chromosome abnormalities in the dog using conventional cytogenetics is highly challenging, and thus historical studies are limited both in number and scope. Reports of constitutional numerical chromosome abnormalities are uncommon and restricted to sex chromosome aneuploidy, generally XO, XXX, XXY and XX/XXY in intersex dogs (Johnston *et al.*, 1985; Mellink *et al.*, 1989; Chaffaux and Cribiu, 1991; Mayenco-Aguirre *et al.*, 1999; Świtoński *et al.*, 2000). Published studies of constitutional structural chromosome abnormalities in the dog are also limited. While there are few reports describing Robertsonian translocations (centric fusions) in canine patients (reviewed by Larsen *et al.*, 1979), the chromosomal composition of such aberrations is not clear as these studies were reliant on conventional cytogenetics, and were confounded further by the use of different chromosome nomenclatures. In a study by Mayr *et al.* (1986), karyotype analysis of 112 randomly selected dogs representing 31 breeds revealed seven carriers of Robertsonian translocation between CFA1 and an undefined small autosome. With the advent of molecular cytogenetic markers for the dog, Schatzberg *et al.* (1999) described the application of FISH analysis using small-insert genomic clones from CFAX to identify

male dogs affected with Duchenne muscular dystrophy, as well as carrier females, according to whether their respective X chromosomes showed deletion of the dystrophin gene locus located at CFA Xp21. Beyond this, canine constitutional genomic aberrations have received little attention compared with those associated with tumour pathogenesis.

Canine Cancer Cytogenetics

Human cancers are frequently associated with the presence of non-random chromosome aberrations, which may be numerical and/or structural in nature, and include: numerical abnormalities of entire chromosomes (e.g. trisomies and monosomies); partial chromosome numerical abnormalities (e.g. duplications and deletions of sub-chromosomal regions); and structural abnormalities (e.g. translocations). Early conventional cytogenetic analyses indicated that aneuploidy and bi-armed derivative chromosomes, resulting from centric fusions, are also common features of canine cancer cells (Mellink *et al.*, 1989; Mayr *et al.*, 1990a,b; Nolte *et al.*, 1993; Tap *et al.*, 1998). While the acrocentric morphology of normal canine autosomes makes centric fusions easy to recognize, detailed characterization of these aberrant chromosome structures is largely intractable to conventional banding analysis. Reimann *et al.* (1994) advanced these findings by showing, using *in situ* hybridization of a telomere-specific probe, that bi-armed chromosomes in canine mammary tumours are dicentric, as a result of head-to-head telomeric fusions of acrocentrics. Through the application of dog chromosome-specific paint probes, Tap *et al.* (1998) revealed that in mammary carcinoma cell lines some bi-armed chromosomes are isochromosomes and some arose by centric fusions. Perhaps the largest conventional cytogenetic studies of canine cancers are those reported by Hahn *et al.* (1994) and Reimann *et al.* (1999). Hahn *et al.* (1994) presented cytogenetic findings from 61 cases of dog lymphoma and noted that 30% of their cases demonstrated chromosome translocations and 70% demonstrated aneuploidy. Reimann *et al.* (1999) reported the cytogenetic investigation of over 200 solid tumours of different histological types and noted a high incidence of aberrations involving CFA2 and CFAX, with over 75% of the chromosomes comprising the karyotype observed in aberrations. The reliance on conventional cytogenetics precluded the ability to characterize these aberrations in detail and to define their genomic content.

The advent of molecular cytogenetic technology has revolutionized human cancer studies, and the canine field has followed closely behind, utilizing a spectrum of approaches based on FISH analysis and comparative genomic hybridization (CGH). FISH analysis is a direct means to examine genomic aberrations in chromosomes prepared from a cancer specimen, and permits evaluation at the level of individual cells from within the entire tumour cell population. Using the appropriate reagents in FISH analysis, whether single-locus probes or WCPP, gross numerical and structural aberrations of the tumour genome may be identified. Comprehensive evaluation of chromosome architecture is, however, reliant on the availability of viable cells as a starting material for the generation of metaphase chromosome preparations in which structural defects may be investigated in detail. Furthermore, evaluation of a tumour presenting with complex aberrations may require the application of an extensive series of FISH probes. In contrast, within a single experiment, CGH analysis permits a detailed and accurate genome-wide analysis of DNA copy number status, and is based on competitive hybridization between differentially labelled tumour DNA versus normal reference DNA derived from a healthy donor. Deviations from the expected 1:1 fluorescence ratio of tumour and reference DNA indicate genomic imbalance at the corresponding locus in the tumour specimen. As CGH analysis is an indirect technique that utilizes DNA isolated from tumour cells, it is not reliant on the availability of viable cancer cells; hence it is applicable to archival case materials in the form of frozen or fixed tissue specimens. It is, however, restricted to the identification of abnormalities resulting in net gain or loss of genomic material, and is unable to detect balanced structural changes. The combination of both FISH and CGH analysis of the same tumour

case thus represents a strategy that maximizes the opportunities for comprehensive identification of chromosome aberrations.

As in the human field, canine CGH analysis was performed initially using metaphase chromosomes from clinically healthy donors as the immobilized DNA target for competitive hybridization between tumour and reference DNA. This method was used in the identification of tumour-associated DNA copy number aberrations in 25 canine multicentric lymphomas (Thomas *et al.*, 2003a), but, by its nature, was both time and labour intensive, and restricted to the definition of genomic imbalances at the level of cytogenetic bands. The development of cytogenetically-validated and genome-integrated single-locus probe sets for the dog enabled progression to microarray-based CGH analysis, in which the metaphase chromosome template is replaced by BAC clone DNA. Since the location of each BAC clone within the genome assembly is known, copy number imbalances in the tumour may now be interpreted directly in terms of their precise DNA sequence composition and gene content. A prototype platform for canine array-based CGH (aCGH) analysis was generated with just 87 unique cytogenetically assigned, genome-anchored BAC clones from the RPCI-81 library (Thomas *et al.,* 2003b). The genomic distribution of arrayed clones was targeted to four chromosomes (CFA11, 13, 14 and 31) that had been shown previously to exhibit recurrent DNA copy number imbalance in dog lymphoma (Thomas *et al.*, 2003a), and was supplemented with BACs containing the canine orthologue of 25 genes implicated in human cancers (Thomas *et al.*, 2003c). The first comprehensive genome-wide CGH microarray (Thomas *et al.*, 2005) comprised 1158 clones distributed at ~2 Mb intervals. of which 84.3% were cytogenetically assigned by FISH analysis to their unique chromosomal location. Overall 98.5% of arrayed clones were anchored within the recently generated 7.5× canine genome sequence assembly (Lindblad-Toh *et al.*, 2005), so reinforcing the robust relationship between the assembly and the cytogenetic map. As the dog genomics community transitioned to the use of the CHORI-82 library to maximize opportunities for integration of data from complementary studies, both the 10 Mb and, subsequently, the 1 Mb BAC probe sets from this library were developed into microarray platforms for aCGH analysis (Thomas *et al.*, 2007, 2008). Unlike previous canine aCGH platforms, the utilization of CHORI-82 BAC clones selected strategically from the genome sequence assembly permitted direct assessment of the gene content of regions of DNA copy number imbalance as well as extrapolation of these data to the genomes of other species. This opportunity was extended by the supplementation of both these aCGH platforms with additional BAC clones containing the canine orthologue of 53 genes that have been shown in earlier studies to be intimately associated with a wide range of human cancers, thereby enabling their copy number status to be evaluated in canine patients. Importantly, each arrayed clone was, owing to its nature, also available as a FISH probe for direct interrogation of the copy number and structural organization of the corresponding DNA sequence in individual cells derived from both fresh and archival specimens.

The application of aCGH analysis has enabled accurate and detailed characterization of recurrent DNA copy number aberrations in a wide variety of canine cancer types, including intracranial tumours (Thomas *et al.*, 2009a), osteosarcoma (Thomas *et al.*, 2005, 2009b; Angstadt *et al.*, 2011), lymphoma (Thomas *et al.*, 2001, 2003c, 2011), leukaemia (Culver *et al.*, personal communication) and malignant histiocytosis (Hedan *et al.*, 2011). From these studies, it has become increasingly evident that tumours of the same histological type in human and canine patients present with equivalent cytogenetic lesions (Breen and Modiano, 2008; Modiano and Breen, 2008; Thomas *et al.*, 2009a, 2011). Furthermore, FISH-based assays are now becoming available for detecting recurrent aberrations in canine tumours that are known to be diagnostic of that same form of cancer in human patients (Cruz *et al.*, 2011). The presence of these evolutionarily conserved cytogenetic changes suggests a conserved pathogenesis and reinforces the tremendous potential of a 'one medicine' approach to the definition of novel diagnostic, prognostic and therapeutic strategies in the expanding field of molecular oncology.

Next-generation Cytogenetic Resources

BAC-array platforms have largely been superseded by the development of microarrays comprising short synthetic oligonucleotide sequences that offer increased resolution and flexibility. Currently, there are two commercially available platforms for oligonucleotide aCGH of canine specimens: a 385,000-feature array of probes 50 to 75 nucleotides in length spaced evenly at mean intervals of 4.7 kb throughout the dog genome assembly (Nimblegen, Madison, Wisconsin); and a 180,000-feature array comprising repeat-masked probes of ~60 nucleotides distributed at ~13 kb intervals (Agilent Technologies, Santa Clara, California). Figure 11.3 shows the sequential application of the

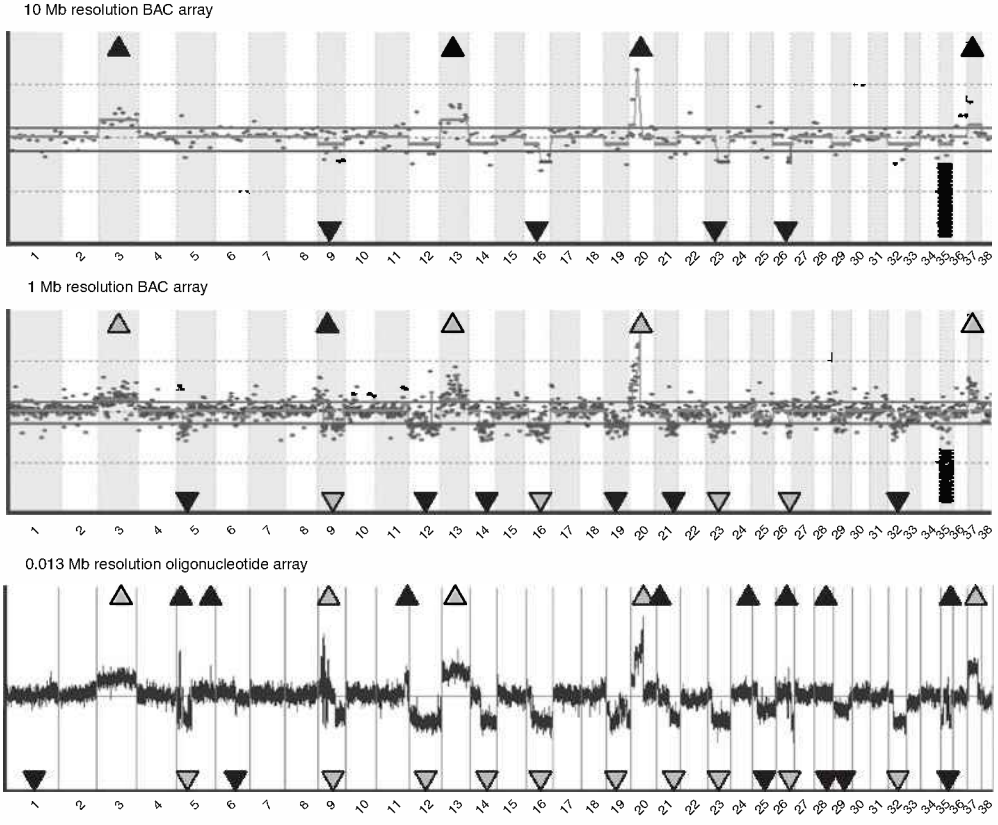

Fig. 11.3. Sequential application of canine CGH (comparative genomic hybridization) arrays of increasing resolution to the detection of tumour-associated DNA copy number aberrations. Top to bottom shows the same cancer specimen when profiled using a 10 Mb spacing (BAC – bacterial artificial chromosome), 1 Mb spacing (BAC) and 13 kb spacing (Agilent oligonucleotide) array platform. Arrowheads show the location of major genomic DNA copy number gains (arrowheads pointing up) and losses (arrowheads pointing down) in the tumour. For each of the three profiles, aberrations that were newly detected by that array platform are denoted by black arrowheads. Those that were evident from lower resolution platforms are shown in grey. When profiled at 10 Mb intervals, four major DNA copy number increases and four DNA copy number losses were detected. At 1 Mb resolution, one additional gain and six additional losses became evident, while, at 13 kb spacing, the number of major gains and losses detected increased to a total of 13 and 16, respectively. Numerous smaller aberrations also become evident with increasing array resolution.

10 Mb and 1 Mb resolution BAC array platforms and the Agilent oligonucleotide array to the analysis of the same canine tumour specimen. This demonstrates the relative increase in the density of arrayed sequences – corresponding to an elevated genomic resolution – with each successive platform revealing copy number aberrations that were intractable to the previous iteration. The challenge therefore now largely becomes one of manipulating, interpreting, distributing and archiving the extensive volume of data arising from studies such as these (for example Seiser *et al.*, 2011; Thomas *et al.*, 2011), particularly when large patient cohorts are involved.

Oligonucleotide array platforms have also been used for the investigation of natural genetic variation in domestic dog populations, in order to move towards a greater understanding of the mechanisms leading to the extensive morphological and behavioural variation exhibited by this species. Chen *et al.* (2009) investigated natural genomic variation in nine dogs of different breeds using the commercially available Nimblegen array, with a female Boxer as the common reference sample. This approach defined 155 variants within 60 genomic regions, consistent with data from earlier human and mouse studies, and showed how these findings can be used to investigate the evolutionary relationships between breeds. In contrast to Chen *et al.* (2009), Nicholas *et al.* (2009) developed a custom array that was targeted heavily to a defined subset of the genome. Using a series of computation methods, Nicholas *et al.* (2009) estimated that approximately 4.2% of the canine genome reference sequence (Lindblad-Toh *et al.*, 2005) comprises segmental duplications and associated copy number variants (CNVs). A small subset of these were investigated further using FISH analysis of BACs containing known duplications; these revealed distinct multiple hybridization sites when probed onto a canine fibroblast cell line. From these data, a high-density oligonucleotide microarray was designed (Nimblegen) representing all predicted canine segmental duplications and spanning a total of ~137 Mb of genomic sequence with an average probe spacing of 200 bp. Application of this array in aCGH analysis against a common female Boxer reference sample identified a total of 3583 CNVs among 17 morphologically diverse domestic dog breeds and a grey wolf. These two studies provide valuable data for the interpretation of genomic duplications and their gene content in the context of observed phenotypic variation within and between members of the Canidae.

Comparative Cytogenetics of the Canidae

The domestic dog belongs to the Canidae, a family believed to have diverged from other carnivore families approximately 55–60 million years ago (Graphodatsky *et al.*, 2008). Within the extant Canidae, divergence from a common ancestor is reported to have commenced approximately 10 million years ago (Wayne, 1993; Graphodatsky *et al.*, 2008). Previous studies have indicated that the family is divided into two major groupings, the 'dog-like' and 'fox-like' canids (Bininda-Emonds *et al.*, 1999; Graphodatsky *et al.*, 2001). More recent genetic data, generated as part of the dog genome sequence project (Lindblad-Toh *et al.*, 2005), have suggested that the family may be refined into four major phylogenetic groups represented by the fox-like canids (including the raccoon dog), the grey and island fox species, the South American canids and the wolf-like canids (including the domestic dog; Ostrander, 2007). Conventional cytogenetic studies of the 34 extant species comprising the Canidae revealed considerable variation in chromosome number and morphology, with their karyotype architecture ranging from $2n = 34$ (+ B chromosomes) in the red fox (*Vulpes vulpes*) to $2n = 78$ in the wolf-like canids, including the domestic dog (Wayne, 1993). The family has thus undergone a relatively high rate of karyotype evolution and so offers an exciting opportunity for the use of detailed molecular cytogenetic evaluation to assess chromosomal evolution that occurred during speciation.

Whole-chromosome reciprocal chromosome painting and comparative chromosome banding have been performed on numerous species within the Canidae. In addition to the domestic dog ($2n = 78$), these studies have primarily involved the fox-like canids such as the red fox ($2n = 34 + B$ (0–8)), Chinese raccoon dog (*Nyctereutes procyonoides procyonoides*,

$2n$ = 54 + B (0–4)), Japanese raccoon dog (*Nyctereutes procyonoides viverrinus*, $2n$ = 38 +B (0–8)) and the Arctic fox (*Alopex lagopus*, $2n$ = 48–50). These studies suggest that an apparent gross pattern of whole-arm (single segment) chromosome rearrangements have taken place during speciation within the Canidae (Yang *et al.*, 1999; Graphodatsky *et al.*, 2000, 2001; Nie *et al.*, 2003). Notable exceptions to the presence of single-segment shuffling was evident for CFA1, 13 and 19, each of which correspond to two chromosome segments of the red fox, raccoon dog and Arctic fox; and, while CFA18 is represented by a single conserved segment in the raccoon dog, this too is split across two segments in the red fox and Arctic fox (Graphodatsky *et al.*, 2000, 2001, 2008). In a recent study by Duke Becker *et al.* (2011), the 10Mb-resolution BAC map of the domestic dog (Thomas *et al.*, 2009) was overlaid onto the karyotypes of 11 wild canids. Conserved evolutionary breakpoint regions (EBRs) shared between their karyotypes were identified using targeted BAC panels spaced at ~1-Mb intervals. This study identified new EBRs and refined the boundaries of known EBRs. In addition, the locations of the EBRs were noted to be consistent with regions of the domestic dog genome that undergo breakage in a variety of cancers.

An interesting feature of the karyotypes of several species within the Canidae is the presence of B chromosomes, supernumerary chromosomes within a karyotype that may vary in their number, and which have been described in a wide range of species, primarily plants and insects (Jones, 1975; Jones and Díez, 2004). The role of B chromosomes and their variability in number is still unknown, though in some non-mammalian species it has been suggested that B chromosome numbers may contribute to the formation of aberrant meiotic products, potentially leading to reduced fertility (Camacho *et al.*, 2000, 2004). Early studies of B chromosomes in the raccoon dog indicated that they were composed of telomeric-like sequences (Wurster-Hill *et al.*, 1988), while more recent studies indicated the presence of nucleolar organizer region (NOR)-like sequences (Szczerbal and Świtoński, 2003). B chromosomes of the red fox, however, have been shown to be rich in centromeric-like sequences (Yang *et al.*, 1999). Preliminary FISH analysis, using chromosome paints and/or single-locus probes, has suggested that canid B chromosomes may share ancestry with small (<1 Mb) autosomal DNA segments and therefore contain active genes (Trifonov *et al.*, 2002; Graphodatsky *et al.*, 2005; Yudkin *et al.*, 2007). Duke Becker *et al.* (2011) noted that B chromosomes of wild canids share orthology with autosomes of the domestic dog, including several cancer-associated genes. These data suggest that these supernumerary elements may represent more than inert passengers within the cell. A more detailed assessment of the genome organization of these supernumerary chromosomes will be needed to evaluate their potential significance further. The dog genome sequence assembly will undoubtedly play a key role in advancing these studies by providing a common reference point for the other canids.

Canine Linkage Maps

Meiotic linkage maps have acted as key resources for understanding the genomic architecture of an organism, and development of a genomic chromosome map for the domestic dog was one of the initial goals of the canine genome mapping project (Ostrander *et al.*, 2001; Moran and James, 2005). Loci that exist in close proximity on the same chromosome do not assort independently at meiosis, because the frequency of genetic exchange by crossover between sequences located on homologous chromosomes is reduced as their physical separation decreases. The crossover rate between specific loci, based on the proportion of recombinant genotypes present in offspring, therefore reflects the distance between them. This forms the basis for the construction of linkage maps and the utilization of these maps for localization of the genes responsible for traits of specific interest.

The generation of a meiotic linkage map requires large multigeneration 'reference families' in which the relationships between individuals are known. Ideally, comprehensive three-generation pedigrees should be utilized so that it becomes possible to determine 'phase', i.e. whether a given set of alleles is

inherited from the maternal or paternal chromosomes, assuming a lack of recombination. Linkage mapping also requires an extensive panel of well-characterized genetic markers for genotyping analysis, which must be sufficiently polymorphic to enable alleles to be tracked accurately through the multiple generations of the pedigree.

Early canine linkage maps were constructed primarily using polymorphic microsatellite markers comprising short tandem dinucleotide repeat sequences (Mellersh *et al.*, 1997, 2000; Neff *et al.*, 1999; see also Ostrander *et al.*, 2001 for a detailed review). Subsequent efforts were directed towards the integration of meiotic linkage data with physical mapping data from genome-wide cytogenetic and radiation-hybrid analysis of the same markers (Breen *et al.*, 2004; Hitte *et al.*, 2005). The most comprehensive canine linkage map available to date is based on 3075 markers, in which the total length of sex-averaged linkage maps of all canine autosomes and CFAX is 2085 cM (Wong *et al.*, 2010; Canine Genetic Linkage Map from the University of California Davis Veterinary Genetics Laboratory at: http://www.vgl.ucdavis.edu/dogmap/). The availability of canine whole genome sequence assemblies (Kirkness *et al.*, 2003; Lindblad-Toh *et al.*, 2005; Chapter 12) now provides an extensive resource of polymorphic markers for the characterization of heritable traits using linkage analysis and for the integration of mapping data from multiple approaches.

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12 Canine Genomics

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Introduction

Dogs are attractive for genetic research for many reasons. They share many common genetic diseases with their human owners, and a clear breed predisposition is often seen, which suggests that genetic risk factors predispose certain breeds to certain diseases. Dogs also share a large fraction of their environment with their owners, and therefore are likely to be exposed to many of the same kind of environmental risk factors. In addition, the unique breeding history of the domestic dog (Chapter 3) makes this species ideally suited for genetic and genomic studies. Recent breed creation, with tight bottlenecks, has led to reduced heterogeneity within breeds and relatively little recombination since the last bottleneck. Because of these advantageous factors, an international consortium was able to successfully petition the National Human Genome Research Institute (NHGRI) for the creation of a high-quality canine genome sequence with the goal of enhancing the gene mapping of

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canine diseases and of undertaking comparative genomic studies of mammals (Fig. 12.1). In 2004, the dog became the fifth mammalian organism to have a high-quality draft sequence made publicly available. Both the genome and other resources generated by the canine research community have led to an avalanche of morphology and disease gene identification since the identification of the first canine disease gene of human relevance: the narcolepsy gene (Li *et al.*, 1999).

In this chapter, I summarize the existing genomic resources for the dog, the improved understanding of genome evolution that has been gleaned through comparative analysis of the dog with other mammals (Lander *et al.*, 2001; Waterston *et al.*, 2002; Gibbs *et al.*, 2004), as well as the current understanding of the haplotype structure in dogs, and the resources and strategies associated with trait and disease gene mapping. We expect that all of these resources and tools will yield many findings relevant to both human and companion animal health in the next few years and beyond.

Genomic Resources

Early resources

By the mid-1990s, scientists such as Don Patterson and others had already begun marketing the dog as a model for human disease. As they were contemplating how to create the

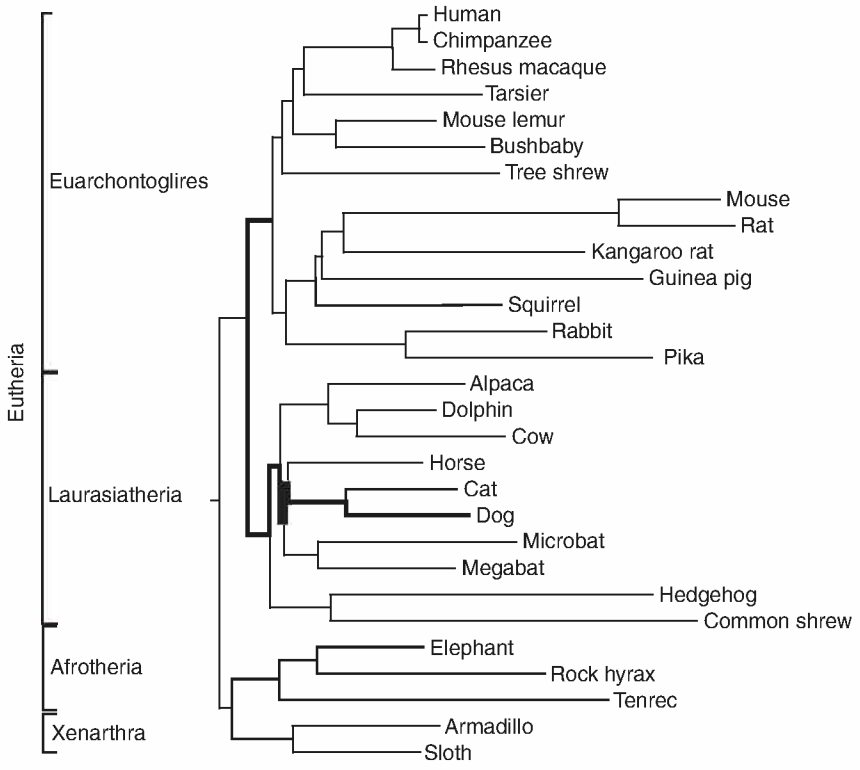

Fig. 12.1. Cladogram of the eutherian mammals. The dog is the first sequenced organism from the Laurasiatheria clade. The human, mouse, rat and chimp (Euarchontoglires clade) were sequenced before the dog. Note that dogs are evolving faster than humans but more slowly than rodents. Many additional mammals have been sequenced since the dog, with the purpose of further annotation of the human genome.

genomic resources necessary for gene mapping, much work went into making maps of general utility. An early achievement was the karyotypic delineation of the dog's 38 chromosomes, most of which are small and acrocentric and therefore challenging to tell apart (Breen *et al.*, 1999; Chapter 11). Several maps were also generated to start ordering the genome sequence in relationship to the canine chromosomes, and for comparing gene order with that in humans. A radiation hybrid (RH) map of the dog genome includes markers from over 10,000 genes, 3000 microsatellites and bacterial artificial chromosome (BAC) ends (Breen *et al.*, 2001, 2004; Hitte *et al.*, 2004). Two deep BAC libraries (Li *et al.*, 1999) and a multiplexed set of microsatellite markers spanning the genome at 8 cM density (Guyon *et al.*, 2003; Clark *et al.*, 2004) were available early on, while a more extensive linkage map, including 3000 microsatellites, has been developed recently (Wong *et al.*, 2010). In 2003, a low-coverage draft sequence (~1.5×) from a Standard Poodle was generated (Kirkness *et al.*, 2003). This resource was exceptionally useful for identifying short pieces of sequence from genes of interest. It also led to the discovery that the canine genome contains a large number of recently active mobile elements called SINEC-Cfs (a carnivore-specific SINE – short interspersed nuclear element – family), several of which are involved in the development of phenotypic traits (Clark *et al.*, 2006; Gray *et al.*, 2010). However, a 1.5× sequence can only be expected to cover ~78% of the genome, and most of the sequence is present in smaller pieces (a few thousand bases connected together).

A high-quality draft genome sequence

Soon, a high-quality draft consisting of 7.5× coverage was generated by the NHGRI-funded genome project. This assembly covered ~99% of the 2.4 Gb genome of a single female Boxer. Both the remarkably high quality of the genome sequence and the speed at which it was produced reflected continuing improvements in sequencing and assembly technology since the initiation of the human genome sequence project, which took approximately 10 years to complete. Whole genome shotgun (WGS) sequencing, the strategy employed for the canine genome, was perfected from that used for the private human genome project (Venter *et al.*, 2001). In WGS, the genome is randomly fragmented and reads are generated from each of the ends of the fragments. Using sequence overlaps, the reads are then rejoined into contiguous segments (contigs), much as one would assemble a jigsaw puzzle. Because the process of assembling the genome is more straightforward if the two copies of each chromosome are as alike as possible, a female Boxer was chosen based on the low levels of heterozygosity observed in an earlier study (Parker *et al.*, 2004). A female was chosen to ensure equal coverage of the X chromosomes and the autosomes. Half of the bases in the resulting assembly were found to reside in contigs larger than 180 kb, and, for the majority of genes, the full gene sequence was contained within a single contig. In contrast, the contigs in the mouse assembly were on average only 25 kb long. As part of the assembly process, groups of contigs were ordered, oriented and joined into supercontigs using the information from paired reads. Half the bases in the assembly were found to reside in supercontigs that were longer than 45 million bases (45 Mb) – and greater in length than the 17 Mb supercontigs seen in the mouse. Thus, just one or two supercontigs are needed to construct most canine chromosomes (Fig. 12.2). The high quality of the dog genome assembly can be attributed to higher quality data, better assembly algorithms and, critically, a 'cooperative genome' with lower levels of repetitive and duplicated sequence than are found in both the mouse and human genomes. To place the sequence on the canine chromosomes, a combination of RH mapping and fluorescence *in situ* hybridization (FISH) data was utilized (Breen *et al.*, 2004; Hitte *et al.*, 2004). There was an excellent agreement between the assembly and the RH and FISH data and, in total, 97% of the sequence could be placed on chromosomes in this way. The resulting assembly was called CanFam2.0.

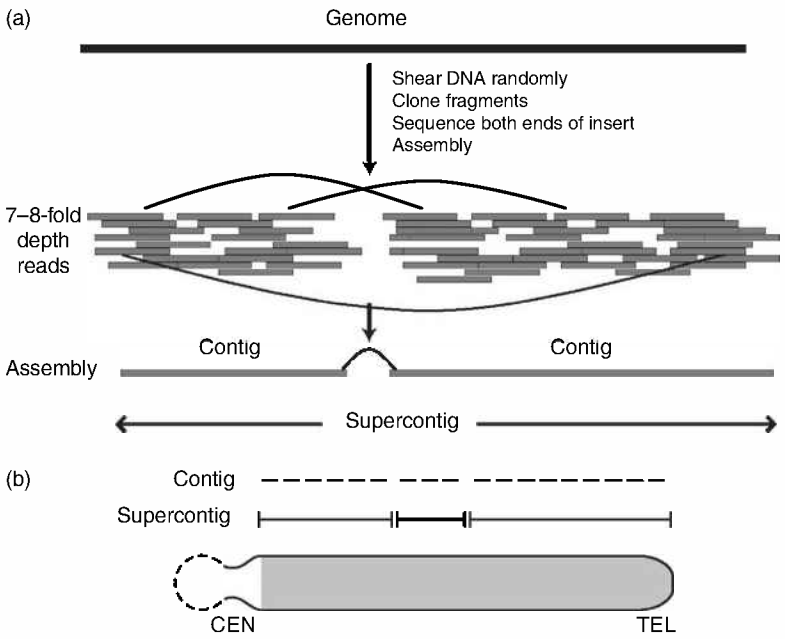

Fig. 12.2. The principle of whole genome shotgun (WGS) sequencing. (a) In WGS sequencing, the genome is randomly fragmented. Fragments of different sizes (typically 4, 10, 40 and 200 kb) are cloned into plasmids, Fosmids or BACs (bacterial artificial chromosomes), and the inserts end sequenced. The resulting reads are joined together by sequence overlap (contigs) and linked together into larger structures (supercontigs) using the pairing of the two end reads from single fragment. To achieve coverage of more than 95% of the genome sequence, every position in the genome was sequenced on average 6–8 times using the Sanger technology, in which a ~700 bp average read length was possible. Now, 80× Illumina sequencing is the standard for mammalian genome sequencing and is based on the shorter read lengths of ~100 bp used in this technology. (b) The canine genome is further mapped on to chromosomes using FISH (fluorescence *in situ* hybridization), resulting in only a few supercontigs per chromosome based on the high contiguity of this assembly. CEN, centromere; TEL, telomere.

An improved genome assembly

While the bulk of the CanFam2.0 assembly approaches finished quality, it does have a few lower quality features. Several megabase-sized regions (comprising less than 1% of the genome in total) are clearly unreliable, probably mostly due to over-collapse of segmental duplications or to haplotype differences between the maternal and paternal chromosomes of the sequenced Boxer. In addition, there are many small gaps in the genome caused by an extremely elevated GC content. Unfortunately, many of these gaps overlap promoters and first exons, making several thousands of gene annotations incomplete.

To enhance the genome sequence further, a special effort was made to improve poor-quality regions in a targeted fashion (Pirun *et al.*, unpublished). This effort resulted in a marked improvement: the contiguity increased by 50% (half of the gaps were filled), 60% of the problematic regions were fixed, and 98% of the ENCODE regions were brought to near-finished quality. While this effort improved the genome sequence considerably, the GC-rich regions overlapping first exons were not substantially improved. Instead, transcriptome sequencing (RNA-Seq) efforts are now being employed to improve gene annotation specifically (see next section). The resulting improved assembly is called CanFam3.0.

Genome and Gene Evolution

The genome landscape

For any comparative analysis across species, knowing what portion of an organism's genome corresponds to that in another organism is critical to laying the foundation for further analysis (Fig. 12.3). Regions of the dog and human genomes that share a common ancestry are called segments of *conserved synteny* and are identified by assessing sequence similarity. Their common ancestry reveals similarities and changes in each of their genomes since their evolutionary split. Large-scale conserved synteny analyses show general relationships across a large region, but also allow for smaller rearrangements, and are especially useful for pairing orthologous genes (derived from a common ancestral gene) and identifying gene family expansions. Finer-scale synteny maps examine small intra-chromosomal changes in detail, allowing examination of evolution at the base pair level. Comparison of the dog with other mammals usually shows a good correspondence for ~94% of the dog genome, whereas for the chicken, a non-mammalian species, only ~76% of the dog genome is easily matched, owing to the ~350 million years of evolution since their common ancestor. When examining the map of conserved synteny between dogs and other mammals, it is clear that the canine genome has undergone relatively little change. Specifically, dogs have fewer inter-chromosomal rearrangements than rodents, although they still have significantly more than humans (230 dog–human conserved segments at 500 kb resolution versus 310 dog–mouse segments). In contrast, the rate of intra-chromosomal reshuffling has been similar in the human and dog lineages. Given that dog is an outgroup to the primate and rodent lineages in the evolutionary tree (Fig. 12.1) (Kirkness *et al.*, 2003; Thomas *et al.*, 2003; Froenicke, 2005), we see clearly that rodents have undergone the highest number of genomic rearrangements relative to humans (with exceptions such as human chromosome 17 and dog chromosome 9).

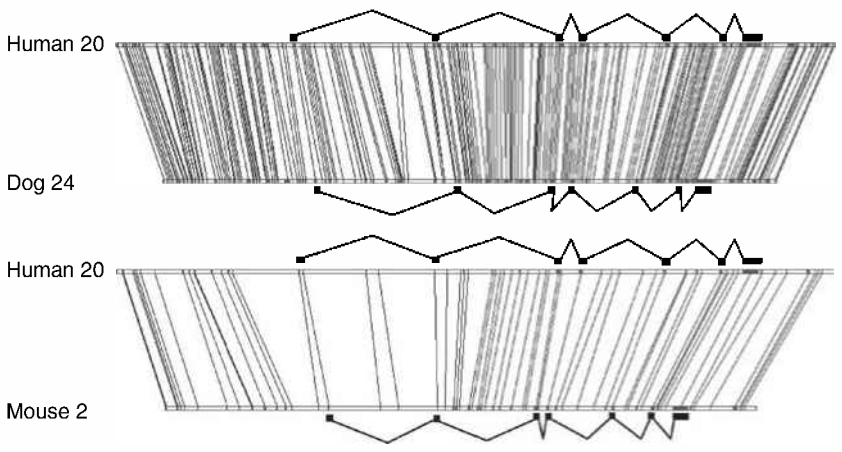

Fig. 12.3. The human and dog genomes are more similar to each other than either one is to the mouse genome. The segments of genomes of the dog, human and mouse that have evolved from the same segment in the common ancestor line up well, as can be seen from a 300 kb region on human chromosome 20, dog chromosome 24 and mouse chromosome 2. Note that more uniquely alignable sequences exist between the dog and human (more closely spaced anchors), and that both the dog and mouse genomes are smaller than the human genome. These types of alignments are used to generate maps of conserved synteny between species (covering ~94% of the dog genome) and are very useful for translating information about genes (black cartoon) between species (figure modified from Wade *et al.* (2006), by kind granting of permission by Cold Spring Harbor Press).

The euchromatic portion of the canine genome is ~2.4 Gb (1 Gb is one billion bases), which is approximately 450 Mb (19%) smaller than the human genome, and 150 Mb (6%) smaller than the mouse genome. This size difference is noticeable within the average segment of conserved synteny and can be attributed to two different factors (Fig. 12.3): a lower rate of repeat insertions in the dog genome relative to both human and mouse, and rates of ancestral base deletion that are approximately equal in the dog and human lineages, but higher in the mouse (Lindblad-Toh *et al.*, 2005). Consequently, and despite our more recent common ancestry with the mouse, the human genome shares approximately 650 Mb more ancestral sequence with the dog than with the mouse. Thus, the dog genome sequence is likely to be closer to the ancestral eutherian mammalian genome than is that of either the human or the mouse.

With an average sequence divergence of ~0.3 substitutions per site, the human and the dog are also more similar to each other on the sequence level than either one is to the mouse. So, in a typical region of the genome, the rate of divergence in the dog genome is 20% faster than in the human genome, but 50% slower than in the mouse genome. The reduced rate of divergence in the human lineage compared with the dog lineage, and in the dog lineage compared with the mouse lineage, is consistent with the previously described correlation between lower mutation rates, lower metabolic rates (Martin and Palumbi, 1993; Gillooly *et al.*, 2005) and longer generation times (Laird *et al.*, 1969; Li *et al.*, 1987).

As in all sequenced mammals, there is significant variation in the nucleotide divergence rate across the dog genome (coefficient of variation = 0.11 for 1 Mb windows, compared with the 0.024 expected under a Poisson distribution) (Waterston *et al.*, 2002; Kirkness *et al.*, 2003; Gibbs *et al.*, 2004). This regional variation is significantly correlated across regions of conserved synteny in the dog, human and mouse genomes, but the strength of the correlation appears to decrease with total branch length (therefore the correlation in the mouse is weaker than that in the human and dog), and some lineage-specific differences can be seen in certain regions of the genome (Lindblad-Toh *et al.*, 2005; Mikkelsen *et al.*, 2005). Similarly, in the dog and human genomes, GC content averages 41% but varies from ~25 to 60% (10 kb windows), whereas in the mouse a slightly higher GC content (42%) and less regional variation are observed. As in other mammals, GC content in the dog correlates with both chromosomal position and divergence rate (Hardison *et al.*, 2003; Rodin and Parkhomchuk, 2004; Yang *et al.*, 2004; Webber and Ponting, 2005).

In addition to changing the size of a genome, the insertion of repetitive sequence elements plays a role in evolution in changing genomic functions. In fact, up to ~50% of mammalian genomes consists of repeats of three main types: long interspersed nuclear elements (LINEs), SINEs and long terminal repeats (LTRs). The dog genome generally has fewer and older LINE and LTR repeats than the human and mouse genomes, but the carnivore-specific SINE family (defined as SINEC_Cf) appears active. Consequently, the presence or absence of a SINE at specific loci is a form of genomic variability occurring much more frequently in dogs than in humans (Kirkness *et al.*, 2003).

Gene annotation and evolution

A thorough understanding of protein-coding and non-coding transcripts is essential for understanding both the dog genome itself and its evolutionary relationship with those of other mammals, as well as for identifying disease mutations. The evolutionary changes in a species can depend on changes in protein sequence, which can be driven by species-specific positive selection, gene expansions through local genome duplications, as well as regulatory changes caused by changes in either non-coding RNAs or other regulatory elements.

The original gene annotation of the high-quality draft canine genome identified ~19,300 protein-coding genes, a considerably lower number than the ~22,000 human genes in the Ensembl (http://www.ensembl.org/) gene set (Ensembl build 26) available at the time. By using conserved synteny and the orthologous relationships between genes in the human, mouse, rat and dog species, several scientists have revised the canine gene count by a few hundred genes (Clamp *et al.*, 2007; Derrien

et al., 2007, 2009). The excess of human genes with no dog orthologue reflects the relative lack of gene expansions in the dog.

While, in general, dogs have few gene family expansions, some large gene families do exist. The largest number of dog-specific genes is seen in the histone H2Bs and the alpha-interferon families, which cluster in monophyletic clades when compared with their human homologues. The expansion is particularly striking for the interferons, and, while there is likely to be no direct correlation, this should be kept in mind when studying the many inflammatory diseases present in the dog. A third well-known case of dog gene expansion is the olfactory receptor genes described in Chapter 17 and by Robin *et al.*, 2009).

An alternative route to gene evolution is through positive selection of whole proteins or specifically important amino acids. Positive selection on genes is typically measured by Ka/Ks (variants that cause amino acid changes versus those that are silent). A comparison of the relative evolutionary constraints across ~14,000 orthologous genes common to the human, dog and mouse showed that the relative rate of evolution (strength of selection) between different groups of genes was highly correlated in the three species. In contrast, the absolute rate of evolution (total number of substitutions) was significantly higher in the dog lineage than in the human lineage but lower than in mouse lineage, reflecting the rates of neutral evolution discussed above (Lindblad-Toh *et al.*, 2005). In dogs, no single family of genes showed overwhelming accelerated evolution, but some positive selection was observed in genes related to metabolism and in some types of nervous system-related genes. The nervous system genes evolving quickly in the dog also appeared to evolve quickly in humans, suggesting similar selection pressures and possibly convergent evolution in dogs and humans.

However, as mentioned previously, one of the major challenges of the canine genome sequencing effort was the GC-rich regions surrounding first exons that lead to sequence gaps. Unfortunately, these gaps often overlap exons and lead to incomplete and inaccurate gene models. To try to circumvent this problem, RNA-Seq is currently being generated for ~20 tissues, with the goal of improving the genome annotation. An advantage of RNA-Seq data is that it not only identifies coding transcripts but also identifies the majority of long non-coding transcripts, such as lincRNAs (large intergenic non-coding RNAs) (Guttman *et al.*, 2010).

Short microRNAs (miRNAs) have been shown to play an important role in gene regulation in many mammalian species (Muljo *et al.*, 2010). Unfortunately, no extensive effort to identify canine miRNAs has been performed to date, but with high-throughput sequencing available these resources are likely to become accessible.

Functional conservation within mammalian genomes

In addition to the transcription units of a mammalian genome, a large number of additional signals is encoded in the genome. Many of these are likely to be regulatory in nature and are so far poorly characterized. Interspecies comparison is one of the most versatile and powerful methods for identifying and studying the evolution of such functional elements present in a genome (Kellis *et al.*, 2004; Kok *et al.*, 2005; Richards *et al.*, 2005). A comparison of related species (such as many diverse mammals) identifies genomic features that are functionally conserved between the species (Waterston *et al.*, 2002; Gibbs *et al.*, 2004).

For mammals, the human and mouse genome comparison set the stage for finding human conserved elements by estimating that a little over 5% of the human genome showed excess conservation (Waterston *et al.*, 2002). With protein-coding genes comprising just ~1.5% of the genome, this suggested an additional 3.5% contained in unknown functional elements. However, with just two mammalian species, it was impossible to precisely define the boundaries, extent or function of the vast majority of these elements (Miller *et al.*, 2004). When compared with the dog genome, it was clear that most conserved elements are common to the genomes of all placental mammals. The most highly conserved non-coding elements (constituting 0.2% of the genome) were enriched near developmental genes, suggesting that these genes are under complex regulation. This pattern is seen across all vertebrates surveyed to date (Dermitzakis *et al.*, 2004;

Lindblad-Toh *et al.*, 2005; Ovcharenko *et al.*, 2005). Later comparisons with the first marsupial genome (opossum) showed that, while 99% of the genes were shared, only ~80% of non-coding conserved elements were shared between marsupials and placental mammals, suggesting that regulatory elements may be driving evolution.

With the 29 eutherian genomes project, the ability to pinpoint conserved (or constraint) elements in the human genome was dramatically increased (Lindblad-Toh *et al.*, 2011). Altogether, 3.6 million elements, accounting for ~4.2% of the human genome, were detected at a 10% false-discovery rate (Fig. 12.4). By detection of evolutionary signa-

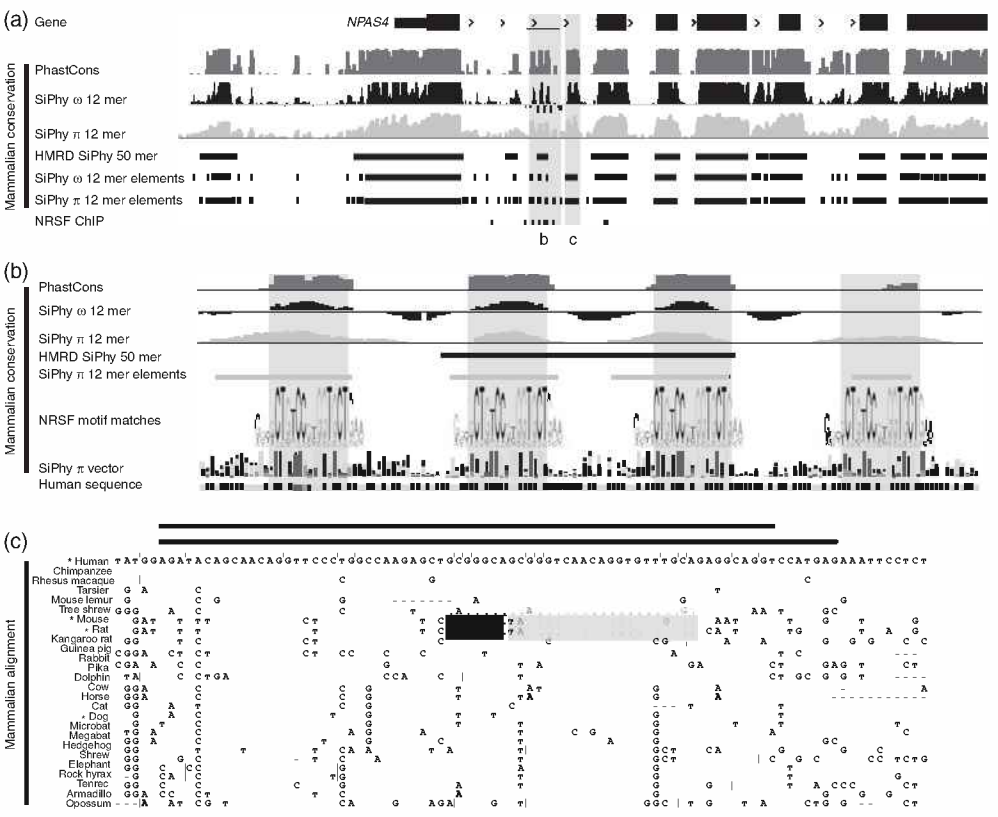

Fig. 12.4. An example of the improved annotation on non-coding conserved elements possible with comparative genomic analysis of 29 eutherian (placental) mammals. (a) The neurological gene *NPAS4* (for neuronal PAS domain protein 4) has many constrained elements overlapping introns and the upstream intergenic region. Note that the shaded box b contained only one constrained element using human/mouse/rat/dog genomes (HMRD), while the analysis of 29 mammalian sequences revealed four smaller elements. (b) These four constrained elements in the first intron correspond to binding sites for the NRSF (neuron-restrictive silencer factor) transcription factor, which is known to regulate neuronal lineages. (c) Another 70 bp constrained element in the first intron, marked as the shaded box c in the top panel (a), was not detected in the HMRD analysis owing to unusually high divergence in the mouse and rat, but is highly constrained in all other mammals and was therefore detected with sequences from 29 mammals. Due to the fact that the 2× coverage genome only covers ~85% of the sequence, four species of placental mammals are randomly lacking sequence coverage at this locus (note that the opossum also listed here is marsupial rather than placental). PhastCons and SiPhy, methodologies for detecting conserved elements in multiple alignments; ChIP (ChIP-Seq), chromatin immunoprecipitation with sequencing (figure modified from Lindblad-Toh *et al.*, 2011. The publisher kindly granted permission).

tures and through comparison with large-scale experimental data sets, candidate functions could be identified for up to 60% of constrained bases. The new elements reveal a small number of new coding exons, 10,000 regions of overlapping synonymous constraint within protein-coding exons, hundreds of candidate RNA structural families, and nearly a million elements overlapping conserved candidate promoter, enhancer and insulator regions. These data sets also revealed specific amino acid residues that have undergone positive selection, large numbers of non-coding elements that are novel to the mammalian lineage, and hundreds of primate- and human-accelerated regions that have changed based on positive selection in these lineages. Human polymorphic sites follow the evolutionary pressures observed in the mammalian clade, suggesting that the same will be true in dogs and that the genome annotation based on comparative genomics of many mammals will be relevant for studies aiming to reveal mutations affecting canine biology and disease. While this annotation is human centric, the features have been lifted on to CanFam2.0 and CanFam3.0, and are available from the Broad Institute at http://www.broad-institute.org/ftp/pub/assemblies/mammals/dog/conservedElements/. They should be a useful resource when evaluating variants within disease-associated regions for their potential to be the causative mutation.

Gene Mapping Facilitated by Breed Structure

The basics: a SNP map

To fully utilize the potential for trait mapping offered by the canine breed structure, researchers need a large, uniformly spaced marker set. The full genome sequence of the dog made it possible to develop an extensive single nucleotide polymorphism (SNP) map of ~2.5 million SNPs. To capture the diversity of the dog population, the Boxer sequence was compared with ~100,000 sequence reads from each of nine dog breeds, and ~22,000 sequence reads from each of four grey wolves and a single coyote. In addition, the Boxer sequence was compared with partial sequence of the 1.5× Poodle assembly (Kirkness *et al.*, 2003). Finally, the variation found in the Boxer genome (between the two copies of each chromosome pair) was catalogued. Taking a random set of SNPs from the 2.5 million SNP map, on average ~72% of SNPs are polymorphic in any specific breed. With a mean spacing of 1 SNP / 1 kb across the genome, several SNPs are typically found per gene. Overall these SNPs will be useful for all canine genetics, including genome-wide association mapping and more targeted fine mapping (Lindblad-Toh *et al.*, 2005).

Canine haplotype structure reflects breed history

The unique breed structure of the domestic dog is the result of two major population bottlenecks that have created a very distinctive haplotype pattern within dog breeds (Fig. 12.5). The first bottleneck, which occurred at the domestication of dogs from wolves (Vilà *et al.*, 1997; Wayne and Ostrander, 1999), echoes humanity's own bottleneck during migration out of Africa. Across all dogs, the genome fractures into short haplotype blocks (<100 kb), similar in size to those found in humans (e.g. Daly *et al.*, 2001; Gabriel *et al.*, 2002; Wall and Pritchard, 2003; Frazer *et al.*, 2007). The second bottleneck, the creation of breeds in the last few hundreds of years, have resulted in 'breed-derived' haplotypes that extend for megabases within dog breeds.

The domestication bottleneck, which probably occurred as multiple domestication events, is strongly supported by the SNP rates observed in dog breeds. For random breeds versus the Boxer assembly (dog versus dog) the rate of about one SNP per 900 bp (Fig. 12.6a) is considerably lower than the SNP rate between the dog and wolf (1/580 bp (Lindblad-Toh *et al.*, 2005)). The only outlier breed in this analysis, the Alaskan Malamute (1/790 bp), is known to be of Asian origin (Parker *et al.*, 2004), suggesting that it might have more wolf ancestry and therefore a higher divergence. The SNP rates for the dog compared with wolf (1/580 bp) and coyote (1/420 bp) are considerably smaller than

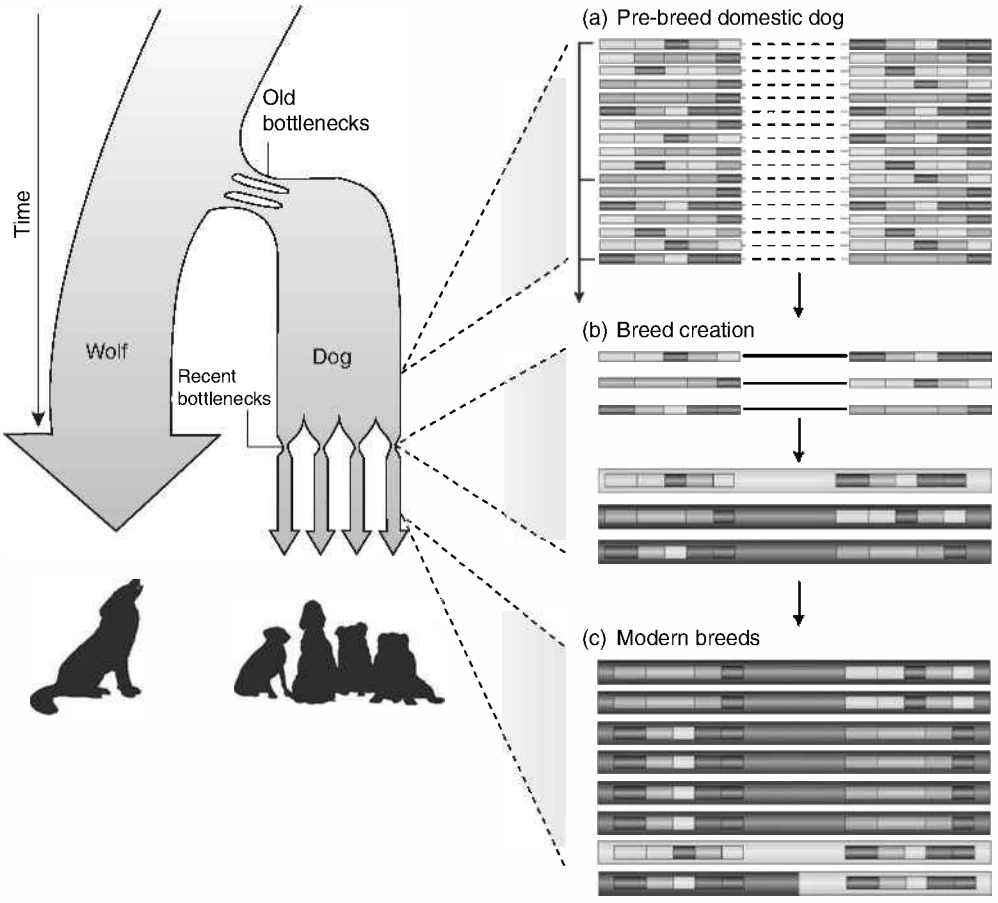

Fig. 12.5. Canine population history is reflected by the haplotype structure of the dog. Two population bottlenecks in dog population history, one old and one recent, shaped haplotype structure in modern dog breeds. First, the domestic dog diverged from wolves ~15,000 years ago, probably through multiple domestication events. Within the past few hundred years, modern dog breeds were created. Both bottlenecks influenced the haplotype pattern and linkage disequilibrium (LD) of current breeds. (a) Before the creation of modern breeds, the dog population had the short-range LD expected, given its large size and the long time since the domestication bottleneck. (b) In the creation of modern breeds, a small subset of chromosomes was selected from the pool of domestic dogs. The long-range patterns carried on these chromosomes became common within the breed, thereby creating long-range LD. (c) In the short time since breed creation, these long-range patterns have not yet been substantially broken down by recombination. Long breed haplotypes, however, still retain the underlying short ancestral haplotype blocks from the domestic dog population, and these are revealed when one examines chromosomes across many breeds (figure modified from Karlsson and Lindblad-Toh, 2008. The publisher kindly granted permission).

the ~1/70 bp calculated between the human and chimpanzee (Mikkelsen *et al.*, 2005), reflecting the recent common ancestry of these canid species.

Comparison of the two parental chromosomes in the sequenced Boxer revealed a distinctive pattern that alternated long regions of near total homozygosity with equally long regions of high heterozygosity (Fig. 12.6b), suggesting that the Boxer genome is a composite of haplotypes, which are either identical or very different. Many of the homozygous

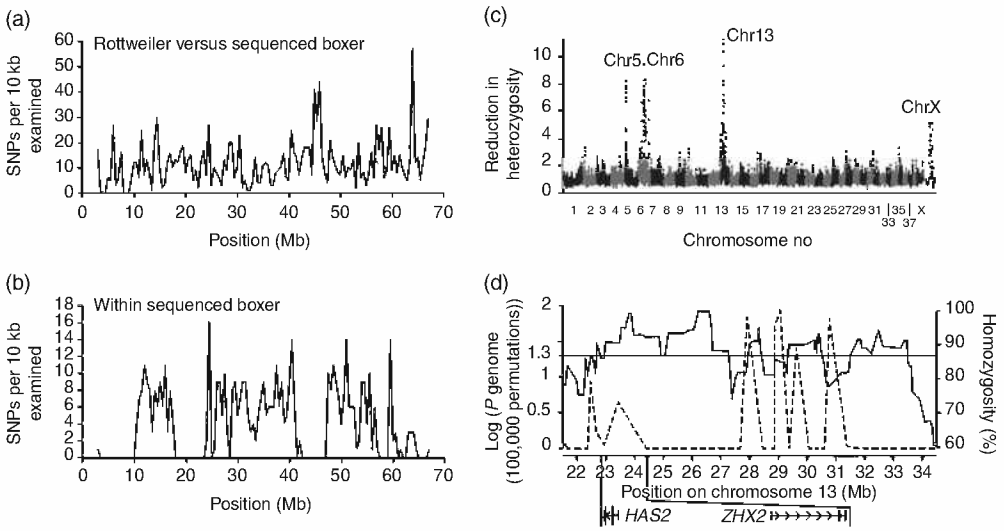

Fig. 12.6. SNP (single nucleotide polymorphism) distribution is strikingly different within and between breeds (a) The SNP distribution on chromosomes 17 based on comparison of random reads from a Rottweiler to that of the Boxer assembly, and (b) within the Boxer assembly, comparing the two alleles. Within individuals, roughly half of the genome is present in large homozygous regions. Within a breed, regions of homozygosity are also present, including smaller regions mostly caused by drift, and regions larger than 1 Mb primarily caused by selection. (c) An example of a sweep linked to the hyaluronan-filled thickened skin in Shar-Peis. This sweep is detected as a 10-fold reduction of heterozygosity on chromosome 13 when comparing Shar-Pei ($n = 50$) with 24 other canine breeds ($n = 230$). (d) The same mutation leading to increased hyaluronan production also predisposes to a periodic fever syndrome. In this region of chromosome 13 the fever association (solid line) is interspersed with the signals of selection (dotted line, % homozygosity). *HAS2, hyaluronic acid synthase 2* gene; *ZHX2*, zinc fingers and homeoboxes protein 2 gene (source: Olsson *et al.*, 2011).

haplotype regions are several megabases long and, in total, more than half of the genome lies in homozygous blocks. The SNP rate in the heterozygous regions matches that observed for other breeds (1/900 bp), suggesting that the variation in the sequenced Boxer is not unlike that of other dogs.

Furthermore, early studies of breed diversity and linkage disequilibrium (LD) suggested that all breeds had a similar haplotype structure. To expand on this, the genome sequence and SNP map were augmented with a carefully designed, large-scale experiment (6% of the genome divided among ten 15 Mb randomly chosen regions) to study the haplotype structure of dog breeds (Lindblad-Toh *et al.*, 2005). To assess the diversity of the dog population, ~1300 SNPs found in these regions were genotyped in 24 diverse breeds, with one dog from each breed. To examine within-breed variability, the same SNP set was genotyped in 20 dogs from each of ten breeds (200 dogs in total). Complete resequencing in the first ~10 kb of each region in the 24 diverse breeds, including 509 SNPs across a total of 79 kb, provided sufficient density to capture small haplotype blocks expected across the dog population. Ten breeds representing different breed groups (Parker *et al.*, 2004) with diverse population histories were selected. Within all ten breeds, homozygosity extended over long distances, in a manner similar to that already observed in the Boxer. The first 10 kb of a region was completely homozygous in 38% of cases ($n = 645$), and every dog examined was homozygous in at least one of the ten regions. From the total of 244 homozygous 10 kb regions, 46% maintained homozygosity out to 1 Mb and 17% out to 10 Mb. Sampling the Boxer genome at similar intervals showed an

almost identical pattern of homozygosity, suggesting that the long haplotypes in the Boxer genome are typical of almost all dog breeds (Lindblad-Toh *et al.*, 2005).

The long haplotypes found within breeds should also lead to extensive LD, making genome-wide association mapping within dog breeds more efficient than in human populations. In each dog breed, all ~1300 SNPs across the ten 15 Mb regions were genotyped to measure the change in r^2 (a bi-allelic measure of LD taking allele frequencies into account) with distance. Within each breed, LD initially declined sharply and then extended at an intermediate level for several megabases. This extent of LD was roughly 100× longer than seen in humans and slightly longer than that in inbred mice. The only tested breed with substantially shorter LD was the Labrador Retriever, and this is likely to be due to its large population size. Among the ten different regions tested, no major variation in the extent of LD was observed (Lindblad-Toh *et al.*, 2005). A recent study of LD using the 174,000 SNP panel described below shows a varying degree of LD across breeds and also some local variation in the recombination rate (Fig. 12.7; Axelsson *et al.*, 2012). However, most breeds fall within the realm of LD described originally.

In contrast, across the whole dog population, LD is roughly 100-fold shorter than when measured within breeds. In fact, the extent of LD is shorter than that observed in humans (Gabriel *et al.*, 2002). As with the within-breed analysis, little variation in the extent of LD was found among the ten randomly chosen loci. This short LD is expected, given that the dog population has a larger size than the human population, and that more generations have passed since dog domestication than since the human migration out of Africa. The fact that LD plateaus at an intermediate level within dog breeds suggests that the long, breed-derived haplotypes are a mosaic of shorter, ancestral haplotype blocks (Fig. 12.5c). Consequently, at any point, different breed-specific haplotypes may share the same ancestral allele, thereby lowering the measured LD. Using the ten very densely genotyped 10 kb regions, the haplotype pattern within dog breeds was compared with the pattern observed across the domestic dog population, as represented by a single dog from each of 24 breeds. The mosaic

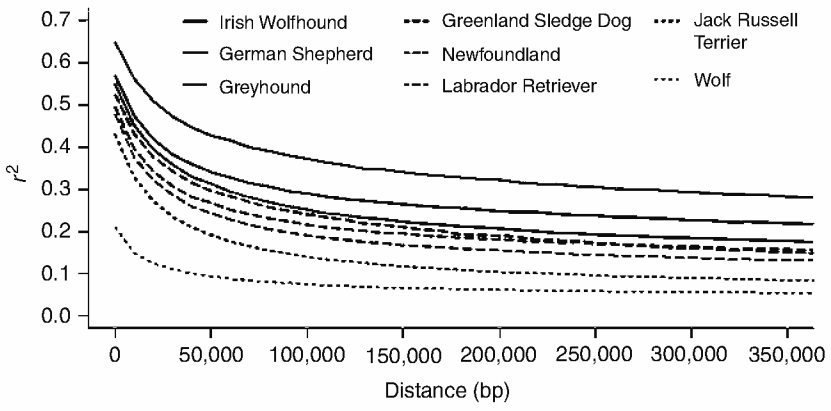

Fig. 12.7. Linkage disequilibrium (LD) is similar for most breeds of dog but also correlates with population history. In each dog breed tested for the genome project, all 1300 SNPs (single nucleotide polymorphisms) across ten 15 Mb regions were genotyped to measure the change in r^2 (a bi-allelic measure of LD taking allele frequencies into account) with distance. When looking at array data, as shown here, for most breeds LD is initially high, then quickly drops to an intermediate level that stays above unlinked as far as 5–15 Mb (Axelsson *et al.*, 2012). Trends in the strength of LD agree with the population history of the seven breeds tested here, with the most severe bottleneck reported in the Irish Wolfhound and less severe bottlenecks in Labrador Retrievers and Jack Russell Terriers.

pattern is clearly evident when analysing the across-dog population, with each 10 kb region containing a few short ancestral haplotype blocks, each with two to five haplotypes. In each ancestral block, the major haplotype dominates with an average frequency of 55%, suggesting that many breed-derived haplotypes will share this ancestral haplotype.

Selection and drift shape the genome

With strong, recent selection, large megabase-sized regions may be fixed or at very high frequency within particular populations (Sabeti *et al.*, 2002; Bersaglieri *et al.*, 2004; Grossman *et al.*, 2010). At the other extreme, complete homozygosity in certain regions of the genome (within a breed) may happen by random chance (drift) based on the tight population bottlenecks. In dogs, it is likely that both forces have been at work on the genome. In an analysis of homozygosity within the ten regions analysed as part of the genome project, the heterozygosity of 20 individuals of the same breed was measured for haplotypes of varying size, from the small ancestral haplotype blocks to the entire ten 15 Mb regions. In no case did the heterozygosity drop below 20% for any of the 100 kb, 1 Mb and 15 Mb regions (Lindblad-Toh *et al.*, 2005). So among the ten regions tested, which comprised 6% of the genome, there was no evidence for sufficiently strong selection to drive the breed-derived haplotype to fixation. Alternatively, an ancestral haplotype, although under selection, might occur on multiple breed-derived haplotypes, thereby masking the signature of selection. In contrast, when the ancestral haplotype blocks were examined, within-breed homozygosity was observed in 13% of blocks. This reflects the population history, as the fraction of completely homozygous loci is proportional to the inbreeding (F) coefficient of each breed: e.g. $F = 0.12$ should result in 12% of loci having coalesced. Thus, the mosaic pattern of the breed-derived haplotypes causes a fraction of the genome to be homozygous within a breed.

More recently, genome-wide analysis using 174,000 SNPs from many breeds found that as much as 1.2% of the canine genome resides in completely homozygous regions that are 1 Mb or larger (Vaysse *et al.*, 2011); this is similar to what has been reported with smaller, albeit genome-wide, SNP sets (Karlsson *et al.*, 2007; Akey *et al.*, 2010; Boyko *et al.*, 2010; vonHoldt *et al.*, 2010). To assess the influence of drift on random fixation of long haplotypes, this study used a coalescent model fitted to real data to estimate that, on average, ~25% of the genome lies within a homozygous block of >100 kb in an average breed. This suggests that it may be difficult to distinguish the signal of a selective sweep from background variation at this resolution. However, an important result of this analysis was that longer segments, particularly those over 1 Mb, are not expected to occur as a result of drift alone, and hence are more likely to reflect selection (Vaysse *et al.*, 2011). Our fundamental understanding of the canine genome was therefore already correct during the genome project, but more precise studies can be performed with genome-wide SNP arrays or whole genome resequencing. Naturally, functional genetic variation may reside both within selected regions and within regions arising from genetic drift, making all homozygous regions potential candidates for harbouring phenotypic or disease mutations.

Strategies and Tools for Trait Mapping

Strategies and power calculations

As the prevalence of different diseases is highly variable between dog breeds, one must assume that genetic risk factors have been enriched within breeds, and predispose them to specific diseases. To map traits in dogs one must determine the appropriate strategy based on the type of trait that one wants to map and what is known about its prevalence in different breeds. For example: traits may be monogenic and shared by many breeds, perhaps fixed in some breeds while inherited in other breeds; they may be monogenic and rare in general, perhaps selected specifically in a breed; or they may be just really complex traits of diseases. Depending on the scenario, the following options are possible:

1. *Mapping across breeds directly by GWAs or mapping within breeds followed by fine mapping across breeds* will be the optimal strategy for simple traits that are likely to be shared across breeds, such as coat colour traits or distinct morphologies, for instance brachycephaly.
2. *Selection mapping* will be useful for simple traits that have been under strong selection within one or a few breeds.
3. *GWAs within a breed followed by fine mapping, primarily within the same breed*, will need to be employed for many complex traits such as cancer. For the last scenario, fine mapping can be performed across breeds, but only a portion of loci are likely to be shared across breeds for the very complex diseases. It is also likely that regions homozygotized by drift, or selected, will play a role in complex traits, and that one of the challenges in canine disease mapping will be teasing apart the contribution of disease risk coming from segregating and fixed loci.

While most of these applications will benefit from increased marker numbers, the most straightforward scenario – mapping within one breed followed by fine mapping across breeds – does not require the same depth of markers and has been studied in more detail as part of the genome project. This strategy was supported by a pre-genome success story: in copper toxicosis (van de Sluis *et al.*, 2002), the derived haplotypes encompassing the disease allele were identified as shared segments of 50–150 kb in multiple breeds. In addition, shared ancestral haplotypes across nine breeds from different groups have been reported for the multi-drug resistance (MDR1) locus (Neff *et al.*, 1999).

The long LD observed within a breed immediately suggests that roughly 50- to 100-fold fewer markers might be needed for a GWA in dogs (5000–10,000 SNPs) than in humans (~500,000 SNPs) (Sutter *et al.*, 2004). However, the moderate level of homozygosity within a breed, as well as the lack of association for some markers based on the underlying shared ancestral haplotypes, might make association mapping within breeds slightly noisier than the long LD would suggest. Using real data from the ten random regions of the genome (Lindblad-Toh *et al.*, 2005), as well as data from coalescent simulations, we were able to show that the absolute majority of SNPs (in segregating disease mutations) can be captured by association using this marker density, but that association is detected much more cleanly if haplotypes, rather than single SNPs, are used as markers for the association. Thus, to be able to form at least two SNP haplotypes within 500 kb windows (where little recombination can be expected) a set of ~15,000 SNPs or more would be desirable. This marker density would work in the majority of breeds, except for Labrador Retrievers, where the LD is shorter and the haplotype diversity larger. It is, however, worth noting that this marker density was not expected to be sufficient for GWAs *across* breeds (Lindblad-Toh *et al.*, 2005).

Therefore, a breed with a high disease risk and sufficiently long LD should be identified first. A genome-wide scan using >15,000 SNPs could then be performed using 100–200 affected and 100–200 unaffected unrelated individuals for many traits conferring a >5-fold increased risk. Such a mapping effort would be expected to yield one or more associated regions of 0.5–1.0 Mb in size, where further fine mapping within the breed might be difficult without using large numbers of offspring. Secondly, because related breeds that share the same phenotype may share the same causative haplotype, using the shared ancestral haplotypes present on breed-derived haplotypes to further narrow the disease-associated region is the optimal approach. The fact that derived haplotypes of up to 100 kb are frequently shared between breeds suggests that, with a limited number of affected and unaffected individuals from two or three additional breeds, one could rapidly narrow the disease-associated region to contain only a few genes and thereby limit the amount of mutation detection needed. Three proof-of-principle studies using the first canine SNP array supported this model for association: the mapping of the white spotting locus present in many breeds (Karlsson *et al.*, 2007), the ridge in Rhodesian and Thai ridgebacks (Salmon Hillbertz *et al.*, 2007)

and the hairless phenotype present in Chinese Crested and Mexican hairless dogs (Drögemüller *et al.*, 2008).

SNP arrays for trait mapping

Several generations of canine SNP arrays have been generated based on available pricing and resources. The most recent SNP array is the canine Illumina HD array, which contains ~174,000 SNPs. This array was designed by the LUPA Consortium, a European collaboration of canine geneticists and veterinarians from 20 institutions in 12 countries, with the goal of mapping canine disease genes of human relevance. This novel array has higher density than previous arrays, and was designed only after holes in the old SNP map had been filled by targeted resequencing of four pools containing multiple samples of a single dog breed (Irish Wolfhound, West Highland White Terrier, Belgian Shepherd and Shar-Pei) and one pool of wolf samples. In total, we discovered 4353 additional high-quality SNPs using this method. The ~174,000 high-quality SNPs are distributed with a mean spacing of 13 kb, and only 21 gaps larger than 200 kb. Of these loci, 172,115 are validated for SNP genotyping and 1547 are used only for intensity analyses, which can detect the presence of copy-number variants. This is a significant improvement compared with the largest previously existing array, which had ~50,000 well-performing SNPs with a mean spacing of 47 kb and 1688 gaps larger than 200 kb. The improvement in coverage is particularly striking on the X chromosome, where >75% of 100 kb windows do not contain SNPs on the previous array, but <5% of windows do not contain SNPs on the Illumina CanineHD array.

Lessons learned about trait mapping

Using the early arrays with 20,000–50,000 SNPs, across-breed mapping has worked for a number of morphological traits shared by many breeds, and sweep mapping has identified more unique morphological traits. Within-breed mapping has found both monogenic trait and disease genes, as well as some polygenic traits. Many more exciting preliminary results are still to be published. For complex traits, a proof-of-principle paper mapped genes for a systemic lupus erythmatosus (SLE)-like disorder in the Nova Scotia Duck Tolling Retriever using the 22k SNP array (Wilbe *et al.*, 2010).

However, for several complex traits, the higher density of the novel 174,000 SNP array is proving advantageous, probably because the mosaic haplotype pattern does tend to make associations of uneven strength between SNPs within a region, and haplotype detection and association are easier with a denser SNP set.

Other findings, many unpublished at this time, point to the fact that the original power calculations were correct in terms of suggesting that only a few hundred samples can map real complex traits, including cancer and inflammatory diseases. However, for many of these traits, it is also clear that investigators should not stop at only a few top hits, but rather increase sample sizes to allow multiple loci to become significant findings, thus pointing to complex trait mechanisms and cooperative disease pathways as opposed to single gene mechanisms.

Finding Causative Mutations

Once associated loci have been identified the chase for the actual mutation is on. In some respects, finding the offending genes and pathways may be enough to suggest a biological function and treatment targets. Still, by identifying the actual mutation, its mechanistic effect can be studied in greater detail and utilized for more direct treatment targets. In canine genetics, as in human genetics, many association signals fall outside coding genes, making the identification of causative mutations more challenging. To overcome this, deep targeted sequencing of the associated regions will be important (Mosher *et al.*, 2007; Wade *et al.*, 2009; Olsson *et al.*, 2011), and needs to be followed by careful analysis of the massive amount of variants found utilizing the best possible annotation of

the canine genome. For example, a 100 kb associated region might contain 100 variants and, by selecting the ~ 5% of variants overlapping transcribed or conserved bases, one might be able to perform functional analysis primarily of these five variants. Further annotation of variants (i.e. by epigenomics) may suggest candidate functions.

Future Genomic Technologies and their Application to Canine Genomics

The world of genomics is changing fast, with massively parallel sequencing technologies becoming available to everyone at reasonable costs. These technologies will bring about a continuously increasing speed of discovery, but will also pose new challenges and requirements of expertise in computational biology and bioinformatics in canine genetics laboratories, owing to the gigantic amounts of data delivered. While genome-wide association mapping in hundreds of dogs per breed may be the method of choice for mapping segregating disease loci for some time, sequencing will be important when identifying the actual mutation, with study of the expression differences driving tumour initiation and progression. This will also allow the study of sweep signals or loci fixed by drift within breeds, as well as providing information about domestication and other evolutionary processes within the dog.

Conclusion

The canine genome is of very high quality, although 6 years after its initial release a perfect annotation is still missing. In the past 6 years, the world of dog genetics has been revolutionized by the genome and the tools built on it. Finally, anything that could previously only be done in humans or mice can now also be accomplished in dogs. With only a small number of traits mapped and published in 2005, the number has quadrupled in early 2011, and the chances are that it will further increase by one or two orders of magnitude in the next few years.

The findings resulting from canine disease mapping are likely to offer an increased understanding of biological processes and pathways, and the evolutionary process, including the effect of selection on health and disease and, perhaps of the highest societal value, a much better understanding of canine and human health, with the long term hope of better prevention and therapy strategies.

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13 Genetics of Canine Behavioural Disorders

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Introduction

The domestic dog has gained great momentum as a model organism for studying evolution (Boyko, 2011), development and behaviour (Neff and Rine, 2006). With its wealth of phenotypic diversity the dog is clearly a valuable model for studying both breed-specific behaviours and maladaptive behaviour within a simplified genetic structure. The persistence of breed-specific behaviours such as herding, pointing, tracking and hunting in the absence of training or motivation suggests that these behaviours are, at least in part, controlled at a genetic level (Spady and Ostrander, 2008). This chapter focuses on the growing relevance of the domestic dog as a model for investigating the genetic basis of behavioural disorders. We will briefly evaluate the dog's validity as a model for studying behaviour from a genetic standpoint and then summarize recent advances in canine genetics research involving behavioural pathology.

Since the domestication of the dog (*Canis familiaris*), it has been a constant friend, guardian and worker for humans as we have spread across the globe. As early as 1872, scientists such as Charles Darwin noted that canine behaviour embodied many of the same attributes as their human companions. In his work, *The Expression of the Emotions in Man and Animals*, Darwin (1872) drew parallels between humans and their animal companions across many different classes of emotional expression – observations that have lost nothing in their impact and utility after over nearly a century and a half of research. Notably, he remarked this about fear reactions in the dog:

> I have seen a dog much terrified at a band of musicians who were playing loudly outside the house, with every muscle of his body trembling, and panting for breath with widely open mouth, in the same manner as a terrified man does.

Some 80 years later, John L. Fuller of the Jackson Memorial Laboratory in Bar Harbor, Maine, began publishing articles on the field of 'behaviour genetics' in animals such as the dog and mouse (Scott and Fuller, 1965). The purpose of these studies, performed in the context

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of the long-range project later known as 'Genetics and the Social Behaviour of Mammals', was to assess the factor of heredity in behaviour. In brief, members from five dog breeds were intensely studied by the group: Basenjis, Beagles, American Cocker Spaniels, Shetland Sheepdogs, and Wire-haired Fox Terriers. Numerous forms of observational experiments were carried out, including: similarity/difference observations between breeds, and observations of behavioural development, cross-fostered puppies (between different breeds), and home versus kennel rearing. In addition, Fuller and colleagues carried out a cross between two very distinct breeds: Basenjis and Cocker Spaniels. They created a three-generation pedigree, including backcrosses, to compare inheritance patterns of different physical and behavioural traits, as well as assess variation within breeds, hybrids and litters. Among many other observations, the researchers concluded that heredity plays an important role in dog behaviour, and that 'genetic differences in behaviour can be as reliably measured and analysed as can hereditary differences in physical size' (Scott and Fuller, 1965). They further pointed out that it was unusual for any breed to be fixed for a behavioural trait.

We have chosen not to concentrate on the genetics of general behaviours (i.e. non-pathological behaviours), which were discussed in the previous edition of this book (Houpt and Willis, 2001). There remains great interest in research into the genetic basis of breed differences in behaviour (Spady and Ostrander, 2008), although there has not been extensive published work in this area over the past decade – although there have been recent exceptions.

For example, when attempts were made to identify quantitative trait loci (QTLs) for phenotypes stereotyped by breed, behaviours were included in the analysis (Jones *et al.*, 2008). In this work, 2801 dogs among 147 breeds were genotyped for 1536 single nucleotide polymorphisms (SNPs), and genotypes were regressed against sex-averaged scores for each phenotype (e.g. height, weight) based on breed standards and other information. Each breed was scored for behaviours (pointing, herding, boldness and trainability) by a dog trainer or from the literature (excitability), and several QTLs were significantly associated with behaviours. For instance, a SNP on chromosome 15 was associated with boldness with an adjusted genome-wide level of significance of $P = 0.001$ (Jones *et al.*, 2008). QTLs for excitability (adjusted $P = 0.01$) and herding (adjusted $P = 0.05$) mapped to the same region, which also was strongly correlated with height, weight and size. Excitability was correlated with body size ($r = -0.8$; $P < 10^{-12}$), while boldness, a phenotype that is not defined, was not. Figure 13.1 shows the frequency distribution of the associated boldness allele on chromosome 15 across the breeds. The gene in this region, that for insulin-like growth factor 1 (*IGF1*), had previously been associated with body size in small dogs (Sutter *et al.*, 2007). While these findings may suggest progress in the mapping of behaviour-related traits, they bring up issues related to definition of phenotype, especially given the reliance on expert opinion for scoring the traits, as opposed to objective and reproducible criteria. Once intermediate phenotypes – which it is hoped will include measurable biomarker correlates – are identified for these behavioural phenotypes, further progress in genetic analysis may ensue. Another area receiving research focus over the last 10 years involves the recognition that social cognition may be a trait correlated with domestication in dogs (Hare *et al.*, 2002) and in silver foxes (Hare *et al.*, 2005). For the latter, the development of genomic tools may facilitate genetic analysis of this complex trait (Kukekova *et al.*, 2007), and the next decade may see progress in understanding how behaviour can be shaped by focused genetic selection during domestication, as has long been recognized with physical characteristics (Belyaev *et al.*, 1981). Progress in this area has been nicely reviewed by Kukekova *et al.* (2006).

Behavioural disorders are an often under-recognized source of morbidity and mortality for dogs (Overall, 1997). Behavioural problems are the most common reason that dogs are relinquished to shelters (Patronek *et al.*, 1996; Salman *et al.*, 2000). Surveys of dog owners show that concerns about behaviour are extremely common, and that dogs presenting to behavioural clinics are seen primarily for disorders of aggression, followed by elimination (soiling) problems and fear-based disorders (Overall, 1997). Comprehensive population-based epidemiology is sorely missing for

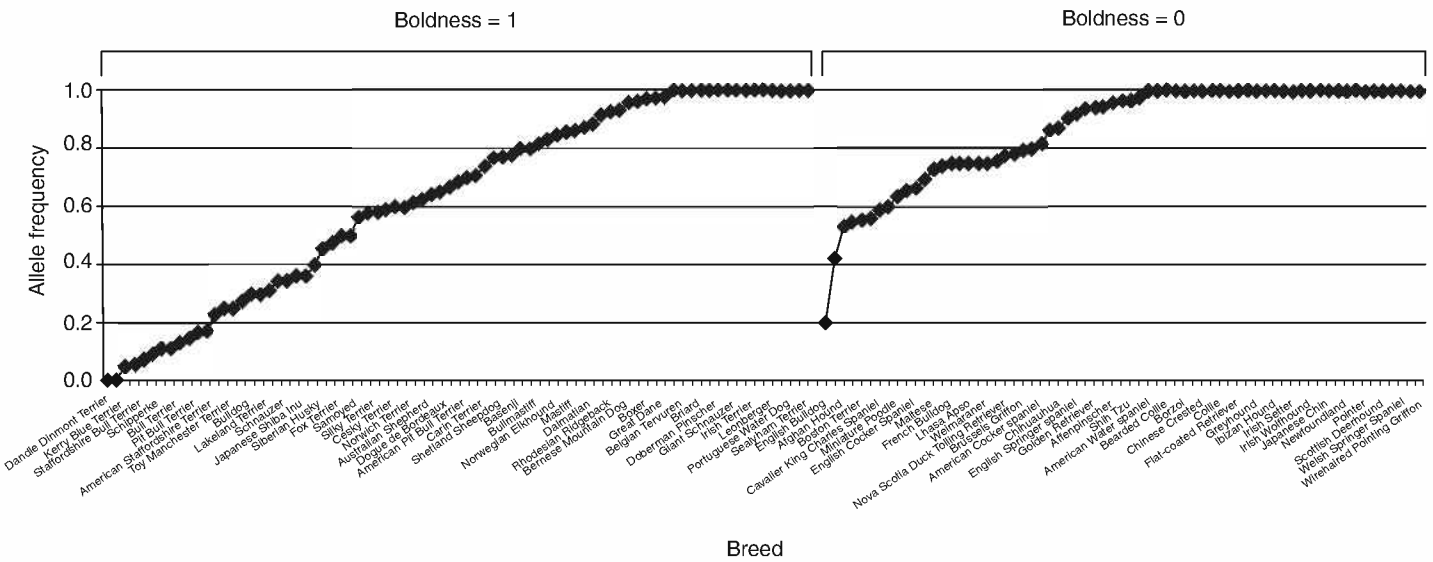

Fig. 13.1. QTL (quantitative trait locus) analysis of canine boldness. A total of 146 breeds were scored either 0 or 1 for boldness (one additional breed was not scored), and 2801 dogs were genotyped to identify loci for boldness. A SNP on chromosome 15, chr15_44134426, was associated with this phenotype with a corrected genome-wide *P* value of 0.001. The allele frequency for the G allele of this SNP is depicted for each breed and sorted by boldness status. The average allele frequency for the boldness = 1 group was 0.63, compared with 0.86 for the boldness = 0 breeds (data derived from Supplemental Tables 1 and 5 from Jones *et al.*, 2008).

estimates of the prevalence and incidence of all canine behaviour disorders, as are estimates of heritability. Most estimates of frequency come from surveys of clinical environments, which can often lead to overestimates in baseline rates, but also to underestimates for disorders in which dog owners may avoid presenting for evaluation and treatment.

Genetics research into behavioural problems in the dog during the 1960s and 1970s focused mainly on the thorough investigation of a line of 'nervous' pointers, starting in the Dykman laboratory at the University of Arkansas, with research carried out predominantly by Murphree (Murphree and Dykman, 1965; Dykman *et al.*, 1969; Murphree *et al.*, 1969, 1974, 1977; Murphree and Newton, 1971). In sum, a line of pathologically 'nervous' pointer dogs was developed through selective breeding, and compared with a control line of the same breed. Most notably, the nervous dogs demonstrated severe timidity and fearfulness (freezing/immobility bordering on catatonia) towards humans, but not towards other dogs. Because of this response, Dykman proposed that the nervous pointer line could be a model for anthropophobia (interpersonal relations phobia, or social phobia) (Dykman *et al.*, 1979), though whether or not this was relevant given the distinction between social relationships between dogs and dogs, when compared with relationships between dogs and people, remains in question.

Nevertheless, research on the nervous pointer lines continued over the next couple of decades, primarily by Uhde and colleagues at the Unit of Anxiety and Affective Disorders at the National Institute of Mental Health (NIMH) at Bethesda in Maryland, who characterized different biological attributes of these dogs (Klein *et al.*, 1988; George *et al.*, 1994). For example, Uhde *et al.* (1992) found that nervous dogs demonstrated lower body weights, lower weight/height body ratios, and lower insulin-like growth factor 1 (IGF1) serum levels compared with normal-behaving controls. Interestingly, it was also discovered that approximately 75% of the line of nervous pointers also suffered from bilateral deafness (demonstrated by complete absence of brain stem auditory evoked response), though it appeared that nervous dogs still responded the same to fear-invoking stimuli (i.e. human interaction), regardless of hearing status (Steinberg *et al.*, 1994). The most recent publication on the nervous pointer line is regarding its use as potential model for studying progressive juvenile hereditary deafness and neuronal retrograde degeneration (Coppens *et al.*, 2005).

In addition to the biological characterization of the nervous pointer lines, researchers also attempted pharmacological intervention (Tancer *et al.*, 1990; Uhde *et al.*, 1994). Importantly, Tancer and colleagues found that, although three nervous pointers showed marked improvement to short-term treatment with the anti-panic medication imipramine and not placebo, chronic administration did not modify any abnormal behaviour in the affected dogs (Tancer *et al.*, 1990). This emphasized the importance of the thorough evaluation of potential models of human anxiety disorders and how results from such studies need to be taken in the context of the model under study.

Another group of dogs provided a naturally occurring model of what was termed childhood hyperkinesis in humans, which is currently called Attention Deficit/Hyperactivity Disorder (ADHD). These dogs, characterized by resistance to inhibitory training, impulsiveness and low frustration tolerance, also show deficiencies in focusing on tasks, and came from several breeds (Corson *et al.*, 1980). Subsequent work focused on Beagle × Telomian crosses that were notably hyperactive, distractible and more genetically homogenous (Ginsburg *et al.*, 1984). These dogs were found to respond to amphetamine compounds in a manner similar to human children with ADHD, further lending credence to this model. Unfortunately, there has been little published work on these dogs in a quarter of a century, although there has fortunately been renewed interest in characterizing impulsive/hyperactive and inattentive behaviours in dogs (Vas *et al.*, 2007; Lit *et al.*, 2010).

In the 1980s, researchers also sought further information on the biological foundation of behaviour and its heredity. In their review of animal behaviour genetics, Wimer and Wimer (1985) assert a point as relevant now as it was then: 'Because of the complex nature of social systems, it has not been feasible to subject them to genetic analysis; instead, the approach

has been to manipulate genotype and study the effects on social systems', although 'sometimes major gene effects on behaviour are discovered by accident, then subsequently exploited in the search for mechanisms'. Regarding the nervous pointers, these authors observe that it was not clear what human behavioural disorder the animal phenotype parallels.

While studies before 1990 focused primarily on understanding behaviours intrinsic to specific lines or breeds of dogs, the past 20 years have seen more emphasis on behaviour in clinical settings with a concomitant exploration of treatment options. This was a logical next step in the field given the interest in pathological behaviour in the dog – particularly involving fears and phobias – as well as obsessive–compulsive disorder-like behaviours described in the nervous pointers and other dogs (Tuber *et al.*, 1982; Luescher *et al.*, 1991; Overall, 1998). For example, the study by Tancer *et al.* (1990) mentioned above sought to investigate the behavioural effects of chronic treatment of the tricyclic antidepressant (TCA) imipramine in the genetically nervous pointers. In addition, another TCA, clomipramine, was investigated by many groups for its effects on compulsivity and other anxiety behaviours. One group found that, within 1–12 weeks, 75% of dogs remaining in their study of compulsive tail chasing demonstrated a 75% or greater reduction in tail-chasing behaviour (Moon-Fanelli and Dodman, 1998). Similar clinical results were also seen by others in dogs with this disorder (Seksel and Lindeman, 2001; Overall and Dunham, 2002), as well as in those with other anxiety disorders (Crowell-Davis *et al.*, 2003; Gruen and Sherman, 2008). Interestingly, the use of clomipramine for canine anxiety disorders was found to be efficacious enough to allow its manufacturer, Novartis, to develop a canine-specific formulation, Clomicalm®, which was approved by the US Food and Drug Administration in 1998 (NADA #141-120). The interest in treatment prospects for canines highlights the desire of the veterinary field to develop better treatments for thoroughly characterized anxiety disorders commonly encountered in the clinic (Overall, 1997).

The ability to use antidepressant medications commonly used in humans for the treatment of similar anxiety disorders in dogs supports the fundamental hypothesis that there is at least some underlying neurobiology that is shared between the two species in the systems affected by these disorders. Here we thus see a confluence of scientific motivations towards common ends: by the veterinary community to identify effective pharmacological treatments for anxiety disorders commonly seen in the clinic, and for researchers investigating the neurobiological basis of psychiatric disease in humans. In light of the latter, the NIMH held a special workshop to discuss the development of animal models for anxiety disorders, which was reported by Shekhar *et al.* (2001). In addition to evaluating existing and new animal models and study approaches, the workshop also sought to examine how these models relate to clinical anxiety symptoms and syndromes and how they might have an impact on the research field. They concluded that it is unlikely that researchers will be able to develop a comprehensive animal model that accurately reflects the relative influences of factors contributing to human neuropsychiatric disease, but did point out that the dog may be an important naturalistic model for determining genetic susceptibility to certain discrete anxiety syndromes that demonstrate unique behavioural, epidemiological and treatment-response profiles, suggesting different underlying neurobiological aetiology.

Naturally Occurring Behaviour Disorders as Phenotypes for Genetic Studies

The study of laboratory rodents has provided a strong foundation for understanding the circuitry, physiology and neurochemistry of important aspects of behavioural repertoires such as feeding and fear. But these time-tested paradigms may not be useful for understanding pathological or maladaptive conditions, such as anxiety. For example, the vigilance and avoidance demonstrated by rodents that is an advantageous behavioural attribute in prey species would be seen as highly maladaptive in social species like humans or dogs (Overall, 2000). The rodent may thus provide a strong genetic model for studying gene function

(in the context of transgenic or knockout models) and pharmacological model for studying the effect of compounds on certain exploratory behaviours; however, it is probably not the most ethologically relevant organism for naturalistic modelling of the discrete neurobiological syndromes that comprise a specific suite of physiological behaviours (Shekhar *et al.*, 2001). It has been argued that the dog may be a more effective animal model for studying maladaptive behaviours given the spontaneous presentation of symptomatology without the need for genetic or neurochemical manipulation (Overall, 2000). Further, when comparing these behaviours with human psychiatric disorders, the face, predictive and construct validity of the canine disorders make them compelling models in their own right (Overall, 2000). Representative examples of canine behaviour disorders are shown in Table 13.1. Genetic studies have been carried out for selected phenotypes within each of the three categories represented in the table, and will be discussed in more detail in the next section. This representative list highlights the challenge of defining what is a behavioural disorder and what is part of a natural suite of behaviours. For example, aggression may be considered a normal part of a predatory repertoire, and certainly components of aggressive behaviours in the dog are construed as recapitulating truncated aspects of normal hunting behaviour (Overall, 1997). In contrast, a behaviour disorder such as dominance aggression may be understood as a problematic expression of impulsivity involving control in social situations with humans (Overall, 2000) that is maladaptive and out of context, and thus disordered, especially when it occurs in a breed where there is selection against such behaviours (van den Berg *et al.*, 2006). What is not currently clear is whether this type of aggression represents a symptom of a broader behavioural syndrome or a single phenomenological entity.

Table 13.1. Representative examples of canine behaviour disorders.

Behaviour disorder	Examples
Aggression	Dominance aggression, fear-based aggression, inter-dog aggression
Anxiety	Noise phobia, compulsive disorder, separation anxiety
Hyperactivity	Hyperkinesis

There are additional challenges to the study of canine behavioural disorders. First, phenotyping for behavioural traits or disorders often requires either: (i) direct laboratory observation; or (ii) the use of questionnaires filled out by the dog's owner. Secondly, locus-specific genetic manipulation (transgenic or knockout modelling, informative breeding, etc.) is socially unpalatable in community-based samples and is currently not feasible on a large scale in colony populations. Finally, a limited amount is known about canine psychobiology. However, a critical feature of the dog in terms of genetics is its foundation of pure breeds. This greatly facilitates gene-mapping efforts, as discussed in other chapters. The excitement about this last feature is itself dependent upon an assumption that behavioural disorders will be monogenic. Genetic research into human behavioural traits, such as psychiatric disorders, should temper this optimistic supposition. Studies in humans have shown that such traits are polygenic (International Schizophrenia Consortium, 2009), and that isolated human populations, somewhat analogous to homogenous dog breeds, do not offer great advantage over outbred populations. It is likely that canine behavioural disorders will also be polygenic, with susceptibility alleles that contribute modest effects and interact with each other and with environmental factors to influence the development of phenotypes.

A major challenge to understanding the genetics of canine behavioural disorders lies in the phenotypes themselves. Unlike cancers, autoimmune disorders and endocrinopathies, there are no physical findings, histological observations, or blood tests that can be used to diagnose these conditions. Direct observation by researchers using standardized diagnostic criteria is the most sensible approach, but is often hampered by the situational and context-specific nature of the most common canine behavioural disorders. Another approach involves the use of owner questionnaires for phenotyping. There are two main possibilities for questionnaire development.

The first method is to adapt human-based questionnaires for their utility in assessing

canine behavioural disorders, as has been attempted for inattention and hyperactivity. Using a questionnaire designed to survey human parents about their infants' attention deficits and hyperactivity, Vas *et al.* (2007) distributed their questionnaire to the owners of a total of 220 household pet dogs representing 69 different breeds. They concluded from their results that modified human questionnaires administered to dog owners were a valid means of measuring attention deficit and activity in dogs. The small number of dogs per breed (a maximum of ten) precluded any comment on breed-specific patterns of behaviour.

A second method for phenotyping dogs for behavioural traits is through the use of questionnaires developed specifically for surveying canine behaviour in general. One such questionnaire has been developed for evaluating clinical anxiety disorders in dogs (Overall *et al.*, 2006). The questionnaire specifically aims to objectively quantify observed responses by owners of their dogs' responses to specific, discrete situations or stimuli. The primary focus of this questionnaire is on anxiety disorders (separation anxiety and noise phobia), aggression and stereotyped/ritualistic behaviours, and it relies on standardized methodology and criteria (Overall, 1997). This approach substitutes personal interpretations of dog behaviour (e.g. 'the dog is nervous') with unambiguous measures of the frequency, intensity and severity of otherwise non-specific signs. For example, the respondent is asked whether the dog exhibits any of a number of behaviours during a thunderstorm. These include salivation, defecation, urination, destruction, escape, hiding, trembling, vocalization, pacing and freezing. The frequency of response is also recorded (e.g. 0–40% of the time, 40–60% of the time, etc.). A distribution of phobic responses to thunderstorms in a sample of Border Collies is shown in Fig. 13.2, from which it is clear that most subjects cluster at responses either occurring all of the time, or between 0% and 40% of the time. Respondents are questioned regarding exposure to the fearful stimulus, disallowing 'unaffected' status for noise phobia in dogs without requisite exposure. Similarly, for aggression, the nature of the questionnaire facilitates assessment of the intensity of response (i.e. how many of the following occur in specific situations: snarl, lift lip, bark, growl, snap, bite or withdrawal/avoidance) as well as severity of response (a measure of the occurrence of these behaviours across 52 categories, such as what happens when the dog is in a yard and an unknown dog passes). Given the rather concrete nature of the observations, this approach is amenable to repeated measurement in a dog, thus facilitating estimates of the stability of a behaviour. Comparisons across breeds, sex, or age groups are easily made, and treatment response can be monitored (Overall *et al.*, 2006).

Other groups have also developed questionnaires for assessing canine behaviour and

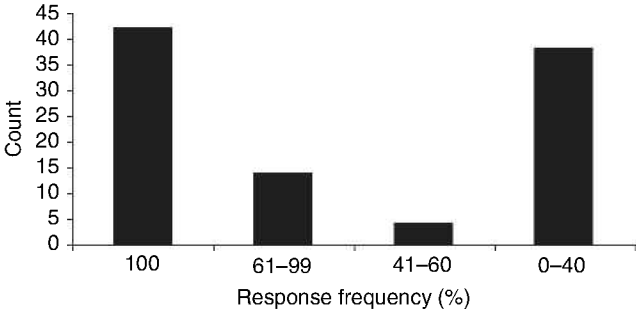

Fig. 13.2. Questionnaire-based assessment of noise phobia in dogs. A behaviour questionnaire (Overall *et al.*, 2006) was administered to 98 Border Collies and the scoring of the noise phobia question focusing on thunderstorms is shown. Owners were asked how often their dog responded with one or more of ten behaviours during a thunderstorm. Dogs were scored only if they were regularly exposed to storms. The observed frequencies are not estimates of prevalence, as the dogs were in part recruited for a genetic study of noise phobia (Yokoyama and Hamilton, unpublished data).

temperament both for use in research and for evaluating dogs trained for working duties; the most commonly used is the 'Canine Behavioural Assessment and Research Questionnaire' (CBARQ) developed by Hsu and Serpell (Serpell and Hsu, 2001; Hsu and Serpell, 2003). CBARQ incorporates measures of 'owner impression', in which owners are asked to give their opinion of their dog's 'aggressiveness', for example. An unanswered question for all of the phenotypic assessments mentioned above is how reliable the traits are for genetic studies.

Gene Mapping of Behavioural Disorders in the Dog

Genetics in the dog has been characterized by a long history of traditional mapping approaches, particularly in diseases demonstrating Mendelian inheritance, or in fixed traits. One notable example among gene-mapping projects involved the linkage analysis and comparative mapping of canine progressive rod–cone degeneration (PRCD), which not only located the disease-causing mutations in dogs but also identified the same mutation in a human patient with retinitis pigmentosa, thereby demonstrating the potential strength of the canine in mapping disease loci relevant to humans (Acland *et al.*, 1998; Zangerl *et al.*, 2006). In addition to the mapping of morphological and disease traits, there has also been interest in mapping the genes underlying behaviour. This interest was exemplified by the creation of a Newfoundland-Border Collie hybrid colony in the early 1990s as part of the 'Dog Genome Initiative' (Ostrander and Giniger, 1997).

Narcolepsy

Perhaps the strongest example to date of canine research in the behavioural disorder realm is that of canine narcolepsy. Described in the early 1970s (Knecht *et al.*, 1973; Mitler *et al.*, 1974), narcolepsy–cataplexy in dogs demonstrates pathology analogous to that of human narcolepsy. The phenomenon of cataplexy is primarily triggered by positive emotions; in dogs, excitation due to the presentation of a plaything, food or water most frequently elicits attacks, which result in atonia, or the loss of muscle tone. In addition to this face validity, canine cataplexy has also been shown to be responsive to imipramine, as seen in human cataplexy (Babcock *et al.*, 1976), thus demonstrating predictive validity in narcoleptic dogs.

In 1976, Stanford University scientists established a colony of narcoleptic dogs to evaluate the pathophysiology of the disorder (Riehl *et al.*, 1998). Doberman Pinschers and Labrador Retrievers were bred to create a colony of dogs that transmitted narcolepsy. The mode of transmission was described as autosomal recessive with one allele and full penetrance. Although narcolepsy observed in colony dogs had a very similar presentation to that in humans, early research established that it did not appear to be associated with the DLA (dog leucocyte antigen) region as had previously been demonstrated in humans, where HLA (human leucocyte antigen) association is strong (Mignot *et al.*, 1995). Over 20 years after the creation of its narcoleptic dog colony, the Stanford group published their discovery of the gene causing the disorder (Lin *et al.*, 1999). The researchers used linkage analysis to localize the causative allele on chromosome 12 – after including additional data from non-colony pedigrees of Dachshunds and Dobermans. They eventually localized the causative gene on chromosome 12 to be hypocretin (orexin) receptor 2 (*HCRTR2*).

The causative mutations in the *HCRTR2* gene that appear to be responsible for narcolepsy in these pedigrees were subsequently identified (Hungs *et al.*, 2001). The hypocretin (also called orexin) family of related proteins is an intriguing candidate for sleep disorders. The hypocretin excitatory neurotransmitters were only discovered a year before their linkage to narcolepsy in dogs, and were previously thought to be involved in appetite regulation. Neurons in the lateral posterior hypothalamus are responsible for the production of hypocretin-1 and hypocretin-2 (HCRT-1 and HCRT-2), which are derived proteolytically from a precursor protein. The HCRTR-2 receptor is located in the arcuate and paraventricular nuclei of the hypothalamus, as well as in the

nucleus accumbens, raphe nuclei, cerebral cortex and other brain structures (Cao and Guilleminault, 2011). Gene deletion studies in mice indicate that animals deficient for HCRTR-2 or HCRT experience non-rapid eye movement (non-REM) sleep 'attacks', accompanied by a disruption of wakefulness, with HCRT–/– mice showing more severe cataplexy-like REM sleep than HCRTR-2–/– mice (Willie *et al.*, 2003). In mice, animals with overexpression of hypocretin exhibit non-REM fragmentation of sleep episodes accompanied by REM sleep reduction (Willie *et al.*, 2011).

Studies of hypocretin pathway genes have not yet been found to be associated with narcolepsy in humans (Ólafsdóttir *et al.*, 2001). Though some might argue that lack of human association brings into question the construct validity of these canine colonies in the context of modelling human narcolepsy, the canine discovery has clearly opened up the field for research in pathways that may otherwise have never been investigated with regard to this disorder. For example, when the brains and cerebrospinal fluid (CSF) of dogs with familial narcolepsy and sporadic narcolepsy were analysed, it was found that hypocretins were undetectable in the latter, but unaltered in the former (Ripley *et al.*, 2001). This suggests heterogeneity in the pathophysiology of narcolepsy, and this is supported by the clinical differences noted between familial and sporadic narcolepsy (Ripley *et al.*, 2001).

The challenges of complex traits

A brief discussion of findings in several non-behavioural phenotypes may illuminate how the problem of heterogeneity will affect genetic studies of behavioural traits. For example, a study that describes a comparison of 25 Golden Retrievers with atopic dermatitis with 23 healthy matched controls showed a weaker association signal (Wood *et al.*, 2009) than seen in recent genome-wide association studies (GWAS) of canine morphological traits and monogenic disorders. Specifically, the study involved genotyping ~22,000 SNPs in the 48 samples, with atopic dermatitis diagnosis being derived from clinical signs, compatible history and the elimination of other aetiologies for pruritus (DeBoer and Hillier, 2001). The strongest SNP association finding was significant at the genome-wide level, occurring at a SNP marker named rs24872415 that lies within an intron of the predicted gene *SORCS2* on chromosome 3, with a P value of 1.27×10^{-6} and a corresponding odds ratio of 9.5. The researchers then sought to determine whether this SNP and the next most associated 39 SNPs would also be associated in additional samples. They genotyped 659 dogs from eight breeds (82.4 dogs per breed range 35–193; breeds were the Boxer, German Shepherd Dog, Labrador, Golden Retriever, Shiba Inu, Shih Tzu, Pit Bull and West Highland White Terrier). They found that the associated SNP rs24872415 was not now associated with atopic dermatitis, with a P value of 0.72 (Wood *et al.*, 2009). Interestingly, this SNP showed association only in the group of 57 German Shepherd Dogs (P value = 0.0002, with an odds ratio of 16.3), but in no other subgroup, such as 106 additional Golden Retrievers. While the initial finding may have represented a false positive finding, it could have also suggested prominent heterogeneity, with locus-specific associations between breeds. The lack of genome-wide significant findings at any SNP when all samples and breeds were used further supports this notion. An analysis of the same samples focusing on SNPs in 'known' genes for atopic dermatitis showed similarly heterogeneous results, with different variants showing associations to different breeds (Wood *et al.*, 2010).

Similarly, a GWAS for a canine systemic lupus erythematosus (SLE)-related disease complex in a primary sample of 81 affected and 57 control Nova Scotia Duck Tolling Retrievers reported a marginally significant top finding in the initial GWAS for all SLE-related disease (Wilbe *et al.*, 2010). In this study, dogs were deemed affected by immune-mediated rheumatic disease (IMRD) by suffering pain across several extremity joints, showing stiffness and exhibiting symptoms of symmetrical polyarthritis for at least 2 weeks. Dogs were regarded as having antinuclear antibody (ANA)-positive IMRD following a positive ANA titre. After genotyping ~22,000 SNPs and carrying out association analysis, an association P value of 1.5×10^{-6} was found, which was significant

at a genome-wide P value of 0.02 following permutation of the data, a statistical approach that allows empirical determination of significance in the setting of large-scale hypothesis testing. By increasing the final sample size to 324 dogs of the same breed, an association P value of 3.3×10^{-9} was observed, with an accompanying odds ratio of 3.8, suggesting a small genetic effect that required a larger sample for detection of the genetic signal.

These examples show the challenges facing the study of disorders with complex genetics, even when relatively straightforward phenotyping procedures are in place. Atopic dermatitis and SLE-related disease, like behavioural traits, show variable clinical signs without a single feature of history or physical examination that defines the presence of the disorder. The lessons from these disorders should be directly applicable to gene-mapping efforts with behavioural disorders, and initial indications with non-pathological behaviour suggest that this will be the case (Jones *et al.*, 2008).

Candidate gene association studies

Aggression

Before the widespread use of GWAS for gene mapping, researchers investigating behaviour disorders focused on the 'usual suspects' – i.e. genes related to neurotransmitters and the enzymes involved in their production and metabolism – that have been investigated in humans and rodents with regard to similar behavioural disorder phenotypes. In particular, many studies in the dog have focused on aggression. Aggression research in a number of animal models systems implicates the neurotransmitter serotonin (Francesco-Ferrari *et al.*, 2005; Frankle *et al.*, 2005). Attempts to extend this line of investigation into dogs suggest that serotonin also plays a role in the species. For instance, post-mortem brains for eight German Shepherd Dogs or Shepherd mixes that displayed numerous severe bites against their owners were compared with eight matched dogs that did not display similar behaviour (Badino *et al.*, 2004). The affected dogs were assessed via case histories and owner-completed questionnaires that assessed aggression in many contexts, and it was determined that predatory behaviour was not involved. Plasma membrane homogenates were obtained from hippocampus, thalamus, frontal cortex and hypothalamus, and serotonin binding was measured; aggressive dogs consistently showed higher serotonin binding activities when compared with control dogs. This observation suggests widespread deficits in synaptic serotonin, or increased serotonin turnover (Badino *et al.*, 2004).

Based on research similar to that described above, aggressive behaviour in dogs has been studied in a series of genetic investigations. One example of such work is shown by van den Berg *et al.* (2006), who have focused on an approach in which the measure of aggression was derived from impressions from owners regarding human-directed aggression collected during in-person interviews. Specifically, owners were questioned about whether their dogs exhibited aggressive behaviour towards people and whether they showed aggressive behaviour towards other dogs. This status resulted in classification of a dog's response to a human as non-aggressive, threatens or bites. When these researchers assessed this characteristic in 159 Golden Retrievers recruited because they had presented with aggressive behaviour, as well as in 166 of their first-degree relatives, a measure of heritability (h^2) of this score (non-aggressive = 1, threatens = 2, bites = 3) was found by variance component methodology to be 0.77 and 0.81 for human-directed and dog-directed aggression, respectively. This suggests a prominent genetic contribution to this trait, despite the rather imprecise nature of the phenotype (Liinamo *et al.*, 2007). As human-directed aggression was the presenting problem for the cases, the linkage and association study was focused on this phenotype. For the linkage component, they examined nine pedigrees from which they genotyped 31 affected and 65 unaffected Golden Retrievers for ten DNA variants in the serotonin receptor genes *HTR1A, HTR1B* and *HTR2A*, and the serotonin transporter gene *SLC6A4*. A parametric affecteds-only linkage analysis under autosomal dominant and autosomal recessive models was carried out, with assumptions of disease allele frequency and penetrance of 0.1

and 0.01, respectively. The pedigrees could theoretically generate maximum logarithm of odds (LOD) scores of 2.8 and 5.3 for dominant and recessive models, respectively. Linkage analyses demonstrated no significance linkage, with the maximum observed LOD score of 0.26 for *HTR1A* under the dominant model (van den Berg *et al.*, 2008).

Within the same study, van den Berg *et al.* (2008) carried out a case-control association study, selecting 42 dogs from their pedigrees and eight additional dogs as affected. For controls, 18 dogs with low aggression scores and 25 uncharacterized dogs were selected. Forty-one SNPs within one million base pairs (1 Mb) of *HTR1A, HTR1B, HTR2A* and *SLC6A4* derived from genome-wide SNP arrays were chosen for analysis. The strongest association was at a SNP (BICF2P855402) about 960,000 base pairs (960 kb) from *HTR1B*. The allele frequency for the minor allele in cases was 0.12, while it was 0.02 in controls, resulting in an association *P* value of 0.009. This was not considered significant, as the threshold for significance given the number of SNP analyses would be $P < 0.001$ in order to adjust for multiple comparisons (van den Berg *et al.*, 2008). While this work suggests that these particular variants/genes are unlikely to play a significant role in canine aggression phenotypes, the small sample size may have precluded detection of modest genetic effects. For example, if the sample had been twice as large, but the SNP allele frequencies had remained the same in cases and controls, the resulting *P* value would have been < 0.0001. Another issue may have involved the misclassification of controls. The inclusion of just a few true cases carrying the 'risk' allele among the unscreened controls would have been sufficient to reduce allele frequencies between cases and controls to non-significant levels. These two issues are likely to provide challenges to all studies of behavioural disorders in the dog.

Other research groups have examined aggressive phenotypes, without great success. A study of 62 SNPs within the gene boundaries or in proximity to 16 neurotransmitter genes, including serotonin and dopamine receptors, a glutamate transporter, and neurotransmitter synthetic/degradation enzymes, were investigated in 50 aggressive and 81 non-aggressive English Cocker Spaniels, with several SNPs reported to show association with the phenotype (Våge *et al.*, 2010). In this work, owners of dogs presenting for veterinary check-ups or for behavioural consultation or euthanasia secondary to behavioural problems completed questionnaires. The questionnaire consisted of 49 items grouped as: (i) unacceptable behaviour towards humans; (ii) fear; (iii) barking; and (iv) aggression towards other dogs. All items were scored on a 5-point scale. Combined with interview and veterinarian data, each dog was then scored on a separate 4-point scale ranging from 1 (non-aggressive) to 4 (very aggressive), with dogs scoring 3-4 being designated as aggressive for the analysis. It might be argued that responses to the questionnaire may be simply due to poor owner attention to the dogs, poor training, or the presence of provoking dogs, all of which would be unlikely as a result of genetic factors. While possible, the estimated heritability of some of these behaviours suggests that these phenocopies could reduce power, but that genetic effects may still be detectable. For dogs to be classified as aggressive towards humans, they had to score biting through the skin of humans and/or frequent growling (i.e. often to always) (Våge *et al.*, 2008). In fact, nearly half of the aggressive dogs were euthanized, highlighting the severity of the problems experienced by the owners of those dogs. While some would suggest that aggression may have been selected for and thus be a natural behaviour of some dogs, it is clearly problematic and considered disordered behaviour in some contexts. The allele frequencies between the cases and controls were compared, and then the results adjusted to take into account the number of tests that had been performed. Significant associations were found with four SNPs in regions of the dopamine D1 receptor (*DRD1*), two SNPs in the serotonin 1D receptor (*HTR1D*), one SNP in the serotonin 2C receptor (*HTR2C*) and five SNPs in a glutamate transporter (*SLC6A1*). The single best finding led to a *P* value of 0.02, and occurred in the predicted coding sequence of *DRD1*, although it is predicted not to change the primary amino acid sequence. Many of the findings with multiple SNPs within the same gene were ascribed to linkage disequilibrium, where alleles at SNPs

near to one another showed prominent correlation. These findings are consistent with the results of van den Berg *et al.* (2008) with regard to *HTR1A*, *HTR1B*, and *HTR2A*, although the differences in breed and phenotype between the two studies prevent precise comparisons between them.

Similar work focusing on multiple DNA variants in eight neurotransmitter-related genes was carried out in 77 Shiba Inus recruited from clinical sources and from a breed publication for a study of aggression. Owners completed a behavioural questionnaire with 26 items, including four aggression items (Takeuchi *et al.*, 2009). For example, with the question 'does the dog show aggression towards unfamiliar guests?', the respondent replies using a 1–5 scale of frequency. The researchers used factor analysis of 24 of the items, resulting in eight factors, of which two met their threshold for representing ≥10% of the variance. One of these factors was termed 'aggression to strangers', and loaded primarily with three aggression items (aggression to guests, children, other dogs), as well as with barking items. A second factor loaded on excitability and possessive aggression, among other items, and was designated as a 'reactivity' factor. Fifteen variants were genotyped and their association with the first two factors calculated. A SNP in *SLC1A2*, a glutamate transporter, showed association ($P = 0.0006$) with the 'aggression to strangers' factor when taking multiple comparisons into account (i.e. a 'Bonferroni correction' of $P = 0.05/(2 \text{ factor tests} \times 15 \text{ SNPs})$, or 0.0017, would correct for 30 tests).

A fundamental problem for complex phenotypes like aggression will be in defining the behaviour of interest. It is difficult to compare any of the studies of aggression described above, given the great differences in phenotypic assessment. While each study may report a specific genetic association, the great differences in defining the phenotype between them renders any reasonable conclusions about the genetics of aggression impossible to make.

Disorders of attention

A number of publications catalogue the detection of DNA variation in various dopamine-related genes and receptors in the dog genome (Hashizume *et al.*, 2003; 2005; Ito *et al.*, 2004; Masuda *et al.*, 2004; Takeuchi *et al.*, 2005; Våge and Lingaas, 2008). Studies have also been performed examining the role of such variation in behaviour disorders. For example, the variable number tandem repeat (VNTR) present in the dog dopamine D4 receptor gene (*DRD4*) has been associated with ADHD in humans, and was therefore investigated for a role in canine activity–impulsivity. As described above, Vas *et al.* (2007) modified a human ADHD questionnaire and distributed it to pet owners. Scores were calculated from six inattention items and seven activity–impulsivity items. An example of such an item is 'Its attention can be easily distracted', which is rated on a scale of 0–3, corresponding to never, sometimes, often and very often.

In one study of 189 German Shepherds (among which were 138 males and 51 females, 102 pets and 87 police dogs), Hejjas *et al.* (2007a) genotyped the *DRD4* VNTR, and observed two alleles of the exonic repeat (termed 2 and 3a), differing by 12 base pairs in length. They found that in police German Shepherds the 2/2 genotype was associated with lower scores on the activity–impulsivity subscale than were either the 2/3a or rare 3a/3a genotypes ($P = 0.018$). This lower score was not seen in the pet German Shepherds ($P = 0.90$). The allele frequency of the 2 allele was higher in the police dogs (0.71) when compared with the pet dogs (0.61), which was a significant difference ($P = 0.03$). These results highlight the potential effect that within-breed stratification may have on the results of association studies, a point that has been discussed in the literature (Quignon *et al.*, 2007; Chang *et al.*, 2009). For example, two subgroups within a breed, differing by geographic origin or functional selection (e.g. working dogs versus show dogs), may lead to detectable genetic differences. If the frequency of a trait differed between these groups, confounding by population stratification would occur.

Although the *DRD4* association with activity–impulsivity was not replicated in a group of 59 Belgian Tervurens, Hejjas *et al.* (2007b) did go on to find a nominal association between a different variant in *DRD4* and the attention-deficit subscale, as well as nominal associations

between variants in the dopamine transporter (*SLC6A3*) and dopamine β-hydroxylase (*DBH*) genes and attention deficit. None of these genes, as well as the gene for tyrosine hydroxylase (*TH*), were associated with the activity–impulsivity subscale. The inconsistency of findings between the two studies, which used identical phenotyping procedures, again highlights important pitfalls in complex trait genetic studies. The relatively small studies, as well as the low prior probability for the candidate gene, have an impact on the power to detect association. The analysis of multiple phenotypes raises the possibility of false positive findings. Finally, the study of different breeds can also be affected by locus and allelic heterogeneity.

Genome-wide association studies

The results from the hypothesis-driven candidate gene studies described in the previous subsection (Candidate gene association studies) highlight the genetic and phenotypic complexity of behavioural phenotypes such as aggression, and strongly suggest that methods beyond the assessment of hypothetical candidate genes are required when investigating the genetic basis of these phenotypes, primarily owing to the limiting requirement of pre-existing knowledge of the underlying neurobiology. The advent of technology that allows the genotyping of SNPs on a large scale provides such a method for making an unbiased assessment of association between a phenotype and DNA variation covering much of the genome. This subsection will describe how this method has just started to be applied to behavioural disorders.

Canine compulsive disorder

With regard to GWAS of behavioural disorder phenotypes, one publication reports the results of a study in Doberman Pinschers for canine compulsive disorder (CCD). Ninety-two cases and 68 controls were recruited from Doberman Pinscher clubs or in response to magazine articles (Dodman *et al.*, 2010). The dogs were evaluated using a survey focusing on symptoms of flank or blanket sucking. Data were collected with regard to age of onset, frequency and duration of sucking bouts, and level of impairment (Moon-Fanelli *et al.*, 2007). This phenotype is reported as being nearly exclusive to Doberman Pinschers. The blanket sucking behaviour involves the mouthing and sucking of fabric, while in flank sucking there is similar repetitive behaviour involving a flank region. Both behaviours tend not to have detrimental effects, although about 17% of affected dogs identified from a survey either required surgical procedures to remove obstructions or experienced dermatological lesions (Moon-Fanelli *et al.*, 2007); nearly three-quarters of the affected dogs had only blanket sucking, while 10% had both behaviours. According to owner survey, the primary trigger for the behaviour appears to be inactivity. An advantage of the behaviour (as far as the owner is concerned) is the ease with which it is recognized, although the question of whether it is a problematic or impairing behaviour is a matter of debate.

The dogs collected by Dodman *et al.* (2010) were analysed across 14,700 SNPs. Three SNPs on chromosome 7 withstood permutation for empirical significance, a procedure in which the data are permuted tens of thousands of times after randomly switching case and control designation. The most significant SNP had a raw P value of 7.6×10^{-7} and a permuted P value of 0.013, allowing the researchers to account for the large number of statistical tests performed. The most associated SNP after fine mapping with 84 SNPs in the 1.7 Mb region surrounding the associated SNP is located within the cadherin 2 gene (*CDH2*; Fig. 13.3). Interestingly, this locus has not appeared in previous linkage studies of human obsessive–compulsive disorder (OCD), although the relatively small numbers and statistical power of these studies are small. There are no current published GWAS of OCD, and no *CDH2* candidate gene studies of OCD have been published, so it may be premature to draw conclusions regarding the relevance of the *CDH2* gene association with CCD to analogous behaviour in humans. More importantly, the phenomenology of flank and blanket sucking as an analogue of OCD has limitations. OCD is characterized by obsessions and compulsions, and it is difficult to accurately assess obsessions in the dog. In humans, compulsive behaviours tend to be

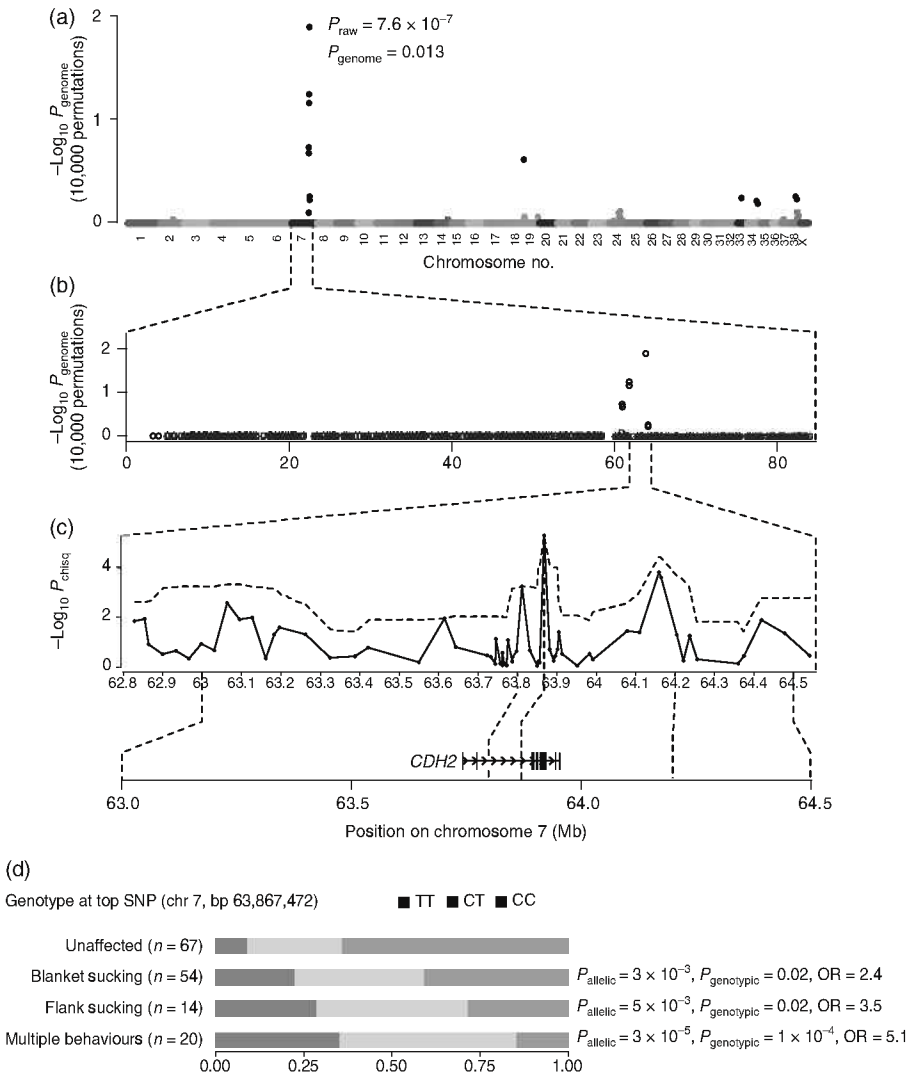

Fig. 13.3. Genome-wide association study (GWAS) of canine compulsive disorder. Doberman Pinschers with canine compulsive disorder (CCD, $n = 92$) were compared with 68 control dogs in a GWAS utilizing 14,700 single nucleotide polymorphisms (SNPs) (Dodman *et al.*, 2010). The Manhattan plot shown in (a) identifies a locus on chromosome 7 as reaching genome-wide significance after permutation testing, with taller peaks indicating stronger statistical significance. The raw *P* value from the association test ($P_{raw} = 7.6 \times 10^{-7}$), and after 10,000 permutations, the empirically derived *P* value (P_{genome}) was observed as 0.013, which is smaller than the threshold P_{genome} value of 0.05. Panel (b) focuses on chromosome 7, showing a relatively broad P_{genome} peak along the chromosome, which is depicted in megabase (Mb) units. Eighty-four SNPs were genotyped across 1.7 Mb in a total of 94 CCD dogs and 73 controls, and the results of fine mapping are shown in panel (c). This shows single SNP analyses with the solid line, with association *P* value haplotypes consisting of 2 to 6 SNPs shown with the dashed line. The overall strongest finding occurs within the predicted *CDH2* gene at base pair 63,867,472, with the strongest region of association in a 400 kb interval (vertical dashed line). Panel (d) shows that the SNP allele conferring risk for CCD is more frequent in the dogs with multiple symptoms, suggesting a more severe phenotype (reprinted by permission from Macmillan Publishers Ltd: *Molecular Psychiatry* (15:8–10), © 2010).

accompanied by significant anxiety, particularly if there are attempts to prevent the person from carrying them out. In the proposed canine model, it is reported that in 85–90% of dogs it is easy to interrupt the sucking activities, and that the behaviours did not interfere with normal quality of life for 77–84% of affected dogs (Moon-Fanelli *et al.*, 2007). However, these owners found that many of the dogs would immediately resume the behaviour, leading researchers to assume that there it has a compulsive aspect. The GWAS highlights the feasibility of identifying genetic risk loci for behavioural phenotypes, and paves the way forward for investigators focusing on these prevalent phenotypes in dogs.

Outlook

There are numerous lessons from studies of psychiatric disorders in humans that can inform investigations of behavioural disorders in the domestic dog. Despite relatively large sample sizes, genetic heterogeneity of human populations and inconsistency in disease phenotyping of psychiatric disorders have made the identification of genetic susceptibility loci for disorders such as depression, schizophrenia, panic disorder and autism a challenging project. Recent GWAS studies in humans have suggested that novel genes contribute risk towards these disorders (International Schizophrenia Consortium, 2009; Weiss *et al.*, 2009; Shyn *et al.*, 2011), but few of these candidates were previously thought to play a role based on known aetiology, and they would demonstrate novel roles in disease should they be replicated and shown to contribute risk to the disorder. The canine offers the possibility of overcoming some of the obstacles facing human genetic studies in psychiatric disorders by allowing the discovery of genetic variants that may influence predisposition towards behaviour disorders, thus contributing vital knowledge to an important field of research.

The two principal challenges for canine geneticists seeking to establish an understanding of behaviour disorders in the dog involve: (i) having the technical tools to assay the genome; and (ii) identifying reliable and valid phenotypes for genetic analysis. The first challenge has been largely overcome, as there are currently excellent array-based tools for fairly comprehensive analysis of DNA in the dog genome (Karlsson *et al.*, 2007; Ke *et al.*, 2011), which can be easily used with saliva-derived DNA (Yokoyama *et al.*, 2010), and allow large-scale and efficient sample collection. Additionally, the growing use of novel high-throughput sequencing technologies will soon make feasible whole-genome sequencing of cohorts of dogs for studies of traits, as well as transcriptomic analyses that will catalogue the complement of canine genes. Many of the post-GWAS tools used by human geneticists for exploring genetic data, including meta-analyses, pathway analyses and analyses of epistasis, are available to canine geneticists.

The second challenge is more daunting. Currently, there is little agreement in the field of veterinary behaviour regarding the diagnostic assessment and classification for behavioural disorders. For example, at least five different schemes exist for defining aggression in the dog, each with up to a dozen subcategories (Houpt and Willis, 2001), making it impossible to determine whether two different groups purporting to study aggression are accessing the same trait. As evidenced in the studies reviewed above, most investigators rely upon ratings scales, questionnaires or observational approaches that they have developed over long study of the disorders. While this reflects great expertise, it does not foster comparability between studies. The field would benefit from a systematic evaluation of the validity of the current phenomenological construction of behaviour disorders, with assessment of reliability and longitudinal coherence. While likely to require prolonged discussion and multiple field trials, such a process could result in a standardized criteria for each of the behavioural disorders faced by dogs and their owners and clinicians, allowing researchers to collaborate on reliably determined phenotypes. Without this, the field will not make any headway.

An important corollary to the complexity of the phenotype is the presumed complexity of the genetic architecture of behaviour, as various levels of heterogeneity will complicate genetic approaches. For example, given the

many factors that may influence behaviour, it is probable that non-genetic phenocopies may exist that would reduce the power to detect a true genetic signal. This would be the case if investigators jointly examined flank/blanket sucking (described above) and tail chasing as a single entity – canine compulsive disorder, as the latter behaviour is often found to occur in the context of serious neurological disorders such as epilepsy (Dodman *et al.*, 1996). Other sources of heterogeneity may also prove a challenge, including allelic heterogeneity, where different alleles at the same genetic locus confer risk to different individuals, pedigrees or breeds. Similarly, different genetic loci may confer risk to different individuals or breeds, a phenomenon termed locus heterogeneity. This has been observed in the non-behavioural complex trait of atopic dermatitis, where significant genetic associations to specific genes were restricted by breed (Wood *et al.*, 2010). Issues of penetrance and variable expressivity, which occur even in monogenic traits, are likely to complicate analysis of complex behavioural traits. Final challenges to the genetic study of behavioural disorders involve our insufficient understanding of the hundreds or thousands of genes influencing these disorders and the high likelihood of complex gene–gene interactions, as well as the pervasive effect of random genetic processes (Ruvinsky, 2010) and our lack of knowledge of how numerous environmental factors modulate the function of contributing genes.

Research into behaviour disorders of the dog has not attained great attention from public and private funding agencies, probably due in part to the complexity of the problem and stigma. With the countless dog owners who step forward with their suffering dogs to participate in genetics studies, it is hoped that we can continue to develop the discoveries that will benefit our canine patients, pets, and co-workers.

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14 Biology of Reproduction and Modern Reproductive Technology in the Dog

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Introduction

Wild canid species have only one oestrus cycle per year and are strictly seasonal, whereas in the domestic dog most bitches have two oestrus cycles per year, some even three. The domestic dog, consequently, has been considered to be non-seasonal. Some of the

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more recently domesticated breeds of dogs, notably the African breed the Basenji, have, however, retained much of the original canid reproductive pattern and have one cycle a year. Male Basenjis, like male wolves and foxes, reduce their sperm production and testicular size during the non-breeding seasons. Almost all Basenji pups in the northern hemisphere are born in the period November to January (Wikström and Linde Forsberg, 2006). If a Basenji is relocated to the opposite hemisphere, its breeding pattern will change to the autumn and winter seasons there. For the majority of other breeds of domestic dogs it has been shown that, although they usually have a cycle twice a year, significantly more matings occur during winter (December to February), with spring (March to May) in second place, and the fewest matings in summer. Consequently, most litters are born in the spring (Tedor and Reif, 1978; Wikström and Linde Forsberg, 2006; Gavrilovic *et al.*, 2008; Berglundh *et al.*, 2010; Lindfors, 2011). Autumn breeders, such as the Basenji, also include the Chow Chow and some of the other old, native Spitz breeds (Tedor and Reif, 1978). Chow Chow bitches have two cycles a year, but the autumn cycle is more functional, with easier and more fertile matings, and 60% of Chow Chow pups in Sweden are born in the period October to December (Wikström and Linde Forsberg, 2006). During the warm season, fertility in general is lower and litter size smaller (Gavrilovic *et al.*, 2008).

The differences in breeding patterns found in different breeds indicate a genetic basis for the persisting reproductive seasonality observed among domestic dogs, even though environmental factors and human preference also play a certain role. In addition to the characteristic monoestral cyclicity pattern of the canid species, with just one cycle and a long inter-oestrus interval, another peculiarity compared with most other mammalian species is the extended oestrous period, and the great variation in its length between individual bitches (7 to 27 days or more).

The dog is being used in medical research as a model for humans but the lack of understanding of the differences in the reproductive pattern, and hormonal effects between humans and dogs, led in the early days to some classic misconceptions, for instance regarding the tumorigenic effects of progestagens on mammary glands. More recently, however, the dog has proved to be a useful model in studies on human prostatic function and dysfunction. Canine genome sequences are now available (www.ncbi.nlm.nih.gov/genome/guide/dog), and this has made it possible to begin the identification also of the genes involved in sexual development (Meyers-Wallen, 2009). The domestic dog is also used as a model in research aiming to preserve the many species of wild canids that are threatened by extinction; these projects are popularly referred to as 'The Frozen Zoo'.

This chapter aims to summarize the basic reproductive physiology of the dog, including the latest discoveries within this field, and also to give an update on the applications of new reproductive technologies in this species.

Reproductive Endocrinology

The reproductive events, in both the male and the female dog, are orchestrated from the hypothalamus, which, in response to some as yet partly unknown stimuli, produces and releases gonadotrophin-releasing hormone (GnRH) which, in turn, influences the pituitary gland to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotrophic hormones induce ovarian follicular development and ovulation in the bitch, and testicular development, androgen production and spermatogenesis in the male. The hypothalamic–pituitary–gonadal axis is regulated via intricate feedback mechanisms whereby the gonadal hormones, having reached a certain concentration via negative feedback, downregulate further release of GnRH, and thus of FSH and LH.

Reproduction in the Male

The reproductive organs of the male dog consist of the testicles with the epididymides and the vas deferens, the prostate gland, the urethra and the penis. The testicle contains the seminiferous tubules, which produce

spermatozoa, and the interstitium with the Leydig cells, which produce steroids – particularly testosterone – in the sexually mature individual. The epididymis consists of a single long duct in which the spermatozoa, during their passage along it, undergo maturational changes and obtain the capacity for motility. The distal part of the epididymis, the cauda, is the storage site for the matured spermatozoa. Prostatic fluid constitutes the major portion of the ejaculate, and contains several enzymes, cholesterol and lactate. The penis consists of a pelvic part, and the glans penis, which is some 5–15 cm long, depending on the breed and size of the dog. The glans penis has two cavernous parts, the bulbus glandis and the pars longa glandis, which fill with blood during sexual arousal and create an erection. The size of the bulbus glandis prevents the intromission of the erect glans penis. The dog, therefore, has a penile bone, located dorsally of the urethra, which enables coital intromission of the non-erect penis. (Fig. 14.1).

Testicular descent

During fetal development, the bipotential primordial germ cells migrate to the gonadal ridge, located caudal to the kidneys, where they differentiate into ovaries or testes. In males, a gubernaculum testis, a mesenchymal structure, will develop at the caudal pole of the fetal testis and extend via the inguinal canal towards the scrotum. Structural changes in this gubernaculum are essential in the process of testicular descent, which takes place in two phases. During the first phase the extra-abdominal part of the gubernaculum increases in length and volume and extends past the inguinal canal, dilating this and creating the processus vaginalis, and incorporating the intra-abdominal part. During the second phase, the gubernaculum is transformed into a fibrous structure, thus enabling the testis to descend into the scrotum. At the time of birth, the testes are located halfway between the kidney and the deep inguinal ring and at day 3–4 after birth they pass through the inguinal canal; they reach their final position in the scrotum at day 35–50 after birth (Johnston and Archibald, 1974; Baumans, 1982; see also Chapter 15).

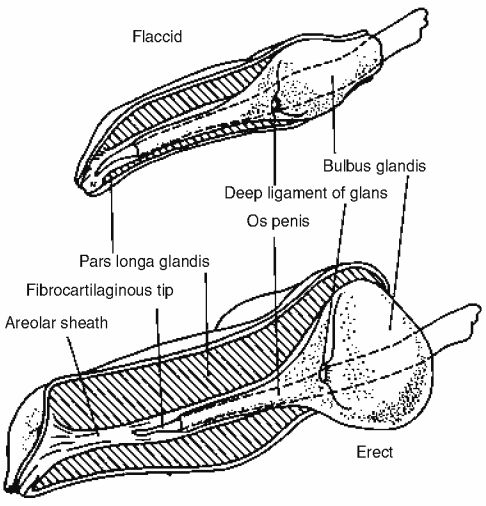

Fig. 14.1. Schematic representation of the changing relationship of the glans and os penis during erection in the dog (from Grandage, 1972).

Maldescent of one or both testicles into the scrotum is called cryptorchidism, and is a not uncommon condition in the dog. Dogs with bilateral cryptorchidism are infertile, while those with one descended and one cryptorchid testicle are fertile. Cryptorchid testicles suffer an increased risk of developing neoplasia. Testis descent is regulated by many different genes, including those directly controlling testosterone, i.e. *AR, INSL3, GREAT* and *CGRP* (Meyers-Wallen, 2009). It is considered likely that cryptorchidism in dogs is inherited as an autosomal recessive trait, which is influenced by modifying genes and also by environmental factors, although no such gene has so far been identified in the dog (Gubbels *et al.*, 2009; Chapter 15). Dolf *et al.* (2008) found a shift in sex ratio of 7.1% in favour of males in litters with one cryptorchid pup and of 10.9% in litters with two cryptorchid pups. In litters from carrier males mated to carrier bitches, Gubbels *et al.* (2009) likewise found an increased number of male pups per litter, a decreased number of female pups and, in addition, an increased litter size in comparison with litters from non-carrier parents. Other inherited disorders of sexual development described in the dog are XX sex reversal and Persistent Müllerian Duct Syndrome (Meyers-Wallen, 2009).

Puberty, sexual maturity and senescence

Puberty in the male dog usually occurs at between 6 and 12 months of age (Harrop, 1960). It is thought to depend mainly on size, with larger breeds developing more slowly than smaller breeds, and it is not unusual that males of the giant breeds are 18 months or more old before they can be used for breeding. Age at puberty is also likely to be influenced by both genetic and environmental factors, such as nutrition. Attainment of puberty in the male dog is not obvious – as in the bitch experiencing her first oestrus – but is a rather protracted process involving not only the display of sexual behaviour but also the beginning of sperm production, and maturation during epididymal transit, as well as the storage of mature spermatozoa in the tail of the epididymis. The ejaculates from young dogs contain high percentages of abnormal spermatozoa (Taha *et al.*, 1981). Andersen and Wooten (1959) found that male dogs usually become sexually mature 2 to 3 months after they have reached adult body weight. Takeishi *et al.* (1980) reported that Beagles reached puberty at 6 months, but optimal sperm production was first seen at 15–16 months of age. Few data are available on the effects of ageing on sexual activity in and fertility of the male dog. A physiological lowering of the level of testosterone, and the common occurrence of benign prostatic hyperplasia and prostatitis with age will reduce fertility. Although 14-year-old dogs have been known to sire litters, it is not unusual that male dogs over 10 years of age have a lowered fertility, and attempts to freeze semen from dogs over 9 years old may often yield disappointing results, even though the dogs may still be fertile if they are mating naturally.

Spermatogenesis/spermiogenesis

The production of spermatozoa is a continuously ongoing process throughout the fertile life of the male dog. The tubuli seminiferi in the testicles are lined by the spermatogonia, and the supporting and nurturing sertoli cells. In the sexually mature dog the spermatogonia undergo a series of mitoses resulting in primary spermatocytes which, in turn, divide by meiosis to become haploid round spermatids. The process of differentiation of the round spermatids into spermatozoa is called spermiogenesis. It includes the condensation of the DNA in the nucleus of the spermatid and the formation of the compact sperm head. Formation of the acrosome (a cap-like structure that develops over the anterior half of the sperm head) takes place and the mitochondria are arranged into the sperm midpiece, as well as the microfilaments growing into the sperm tail. The duration of the cycle of the seminiferous epithelium is 13.9 days in the dog. During epididymal transit, which takes around 15 days, the spermatozoa mature and a residual cytoplasmic droplet moves from a proximal to a distal position along the midpiece; the spermatozoa also acquire the capacity for motility. The entire process of spermatogenesis, from spermatogonium to mature spermatozoa, takes 62 days (Davies, 1982; Amann, 1986).

Mating

Surprisingly little research has been done on mating behaviour in the dog (e.g. Beach and LeBoeuf, 1967; Hart, 1967; LeBoeuf, 1967; Beach, 1968, 1969; Fuller and Fox, 1969; Beach and Merari, 1970; Daniels, 1983; Ghosh *et al.*, 1984). From these few studies, it is apparent that most female dogs in oestrus demonstrate clear-cut mating preferences, which tend to persist from one breeding season to the next. Bitches also differ in their degree of attractiveness to the males. Sexual selectivity is influenced by social experience and familiarity, and individuals that are accepted as playing partners are often not the same ones as those accepted for mating. Pups reared from weaning to sexual maturity in isolation show deficient copulatory behaviour. Most of the canid species are monogamous breeders. This can, for instance, be seen in wolf packs, in which only the alpha couple mate and live in a lifelong relationship. The monogamous breeding pattern is the reason for the comparatively small sperm production in the dog, as there has been no evolutionary advantage in sperm competition between males. The sexual promiscuity rather than pair-bond

mating usually seen among dogs is considered to be a domestication phenomenon (Kretchmer and Fox, 1975).

The mating procedure in canid species is different from that in all other species studied in that after mounting it includes a copulatory tie (Fig. 14.2) which usually lasts for from 5 to 20 min. Although it may seem irrational considering that a bitch usually allows mating and can conceive over a period of 7 days or more and be mated by several males, this tie presumably must serve a purpose as it has remained despite apparent drawbacks, such as vulnerability to attacks during the act.

The ejaculate

The dog ejaculates in three distinct fractions. The first fraction is emitted during courting and mounting of the female, and consists of from 0.5 to 7 ml of clear prostatic fluid. The second, sperm-rich, fraction is emitted after intromission; emission begins before accomplishment of the copulatory tie and continues for a couple of minutes. The volume is from 0.5 to 3 ml and it contains the major portion of the spermatozoa. Its colour is whitish with an intensity that varies depending on the sperm concentration. The third fraction, again, consists of prostatic fluid. It is emitted during the major part of the tie, and its volume can be up to 30 to 40 ml in the larger breeds. The accomplishment of the tie is not necessary for the attainment of pregnancy, but it increases the chances of conception.

The ejaculate contains between 100×10^6 and 5000×10^6 spermatozoa, depending on the size of the dog. The percentage of abnormal spermatozoa should not exceed 20–40% and motility should be at least 70% (Feldman and Nelson, 2004). The relative significance of different types of sperm defects in the dog has been little studied. It seems to be generally agreed, however, that dog spermatozoa with proximal droplets lack fertilizing capacity, while those with distal cytoplasmic droplets function normally. It has been suggested that a higher number of spermatozoa may to some extent compensate for a higher percentage of abnormal spermatozoa (Linde-Forsberg and Forsberg, 1989). Semen quality may vary between breeds and was found to be generally poor in Irish Wolfhounds (Dahlbom *et al.*, 1995, 1997).

Sperm capacitation and the acrosome reaction

Spermatozoa must go through a process of capacitation to be able to undergo the

Fig. 14.2. The copulatory tie.

acrosome reaction and thereby acquire the capacity to fuse with and fertilize an ovum. A crucial step in capacitation is the phosphorylation of membrane proteins. The process of capacitation is coordinated in the oviductal isthmus where the sperm cells attach to the tubal epithelium. Tyrosine phosphorylation of sperm head proteins and capacitation are delayed in spermatozoa in close contact with oviductal epithelium (Petrunkina *et al.*, 2004). The spermatozoa then detach from the epithelium while displaying a hyperactivated pattern of motility characteristic of the capacitated stage, with an accentuated curvilinear line velocity and lateral head displacement. The protein tyrosine phosphatase (PTP) PTPN11 and the dual-specificity phosphatases (DSPs) DUSP3 and DUSP4 are present in dog spermatozoa and have a positive role in the regulation of motility. PTPN11 is mainly found in the post-acrosomal region of the head, DUSP3 within the acrosome and DUSP4 mainly in the sperm tail. The subcellular distribution of these phosphatases suggests that they probably have their specific roles in sperm (González-Fernández *et al.*, 2009). Capacitation time varies between species, and has been found to be 3 to 7 hours for dog spermatozoa when studied in different culture media *in vitro* (Mahi and Yanagimachi, 1976, 1978; Tsutsui, 1989; Yamada *et al.*, 1992, 1993; Guérin *et al.*, 1999). Rota *et al.* (1999) found that the preservation of dog spermatozoa by chilling (using an extender) and then rewarming, or by freezing and thawing, significantly shortened the time for capacitation-like changes from 4 hours in fresh semen to 2 hours in the preserved samples.

The acrosome reaction is necessary for a spermatozoon to acquire its fertilizing capacity. It is triggered by an intracellular rise of Ca^{2+}. The Ca^{2+} channels open under the influence of progesterone. During the acrosome reaction, the apical and pre-equatorial domains of the sperm plasma membrane fuse with the outer acrosomal membrane, leading to a release of the acrosomal contents, including the hydrolytic enzymes that are necessary for the spermatozoon to be able to penetrate the zona pellucida of the oocyte and accomplish fertilization.

Daily sperm production

Daily sperm production in the dog has been found to be 12–17 × 10^6 spermatozoa per gram of testis parenchyma (Davies, 1982; Olar *et al.*, 1983). The volume of the testicular parenchyma, the total number of spermatozoa and the ejaculate volume show a distinct correlation with body weight (Günzel-Apel *et al.*, 1994). Daily sperm production, therefore, normally varies with the size of the dog. It is generally considered that mature, healthy dogs can accomplish a mating every second day without a decrease in ejaculate volume or number of spermatozoa (Boucher *et al.*, 1958).

Reproduction in the Female

The genital organs of the bitch consist of the two ovaries which contain the oocytes, and the tubular genital ducts, i.e. the oviducts, the bi-cornuate uterus with a short uterine body, the cervix, the vagina and the vestibulum (Fig. 14.3). The latter is quite large in this species, and is able to accommodate the bulbus glandis of the male during the copulatory tie. In the female, all of the oocytes are present already from birth, unlike in the male in which the spermatozoa are produced by the testicles throughout the dog's fertile life.

Puberty, sexual maturity and senescence

Puberty in the bitch appears, in most breeds, not to depend on day length, an exception being the Basenji which usually has its cycle only in the autumn. Puberty seems to be related to size and weight, in that it occurs when the bitch has reached around 85% of the adult weight, and, consequently, bitches of the smaller breeds in general have their first oestrus at an earlier age than those of the larger breeds. Under the influence of the sexual hormones, the growth plates of the long bones close, and little further growth will take place after this time. Most bitches reach puberty between 6 and 15 months of age, but

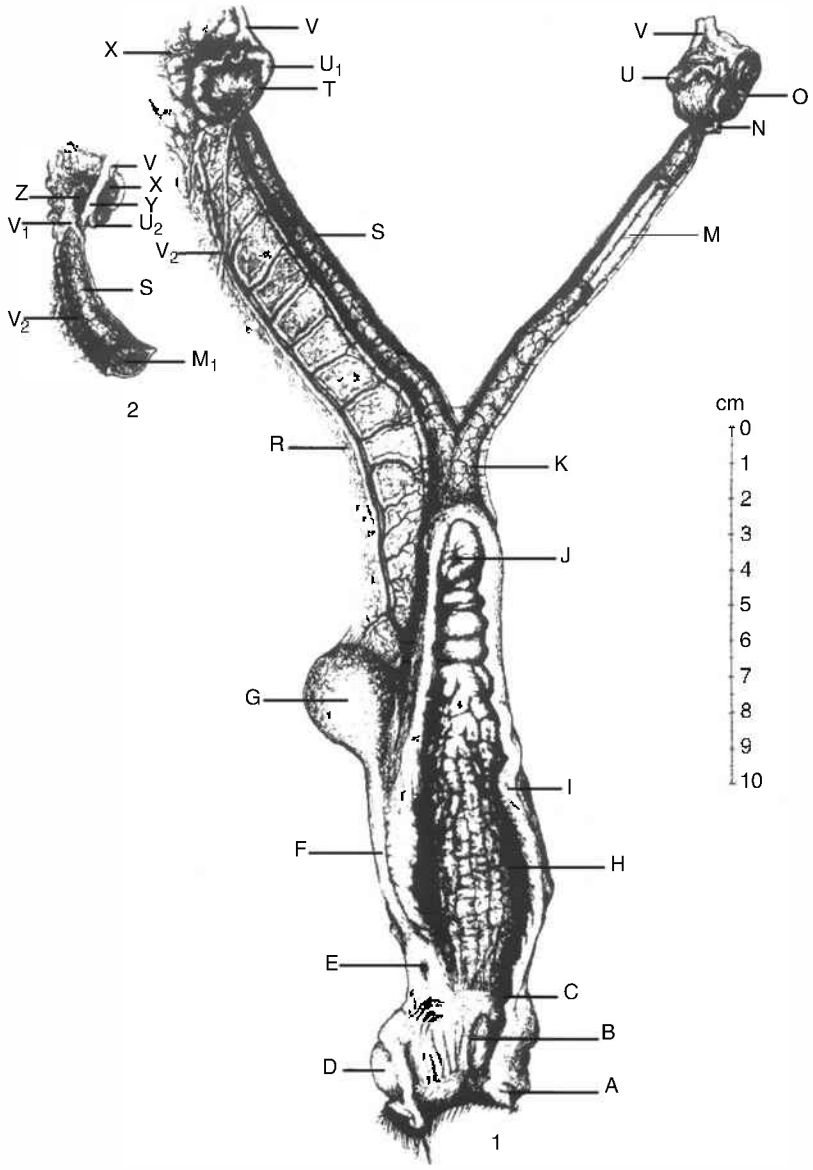

Fig. 14.3. The genital organs of the bitch (from Andersen and Simpson, 1973). A. vulva; B, vestibulum; C, cingulum; D,; E, urethral orifice; F, urethra; G, urinary bladder; J, external os of cervix; K, body of uterus; M and S, uterine horns; U, oviduct.

some, especially of the large breeds, not until 18–20 months of age. During the pubertal oestrus, circulating hormone levels are often low and fluctuating, causing absence of or incomplete ovulation, and the bitch may not show standing oestrus. Senescence is considered not to occur in the dog. Bitches continue to have oestrus cycles and, if mated, may become pregnant all their lives, even though their fertility decreases considerably with age and the inter-oestrus intervals may be prolonged in the older bitch.

The oestrus cycle

The oestrus cycle of the bitch is classically divided into four stages: prooesterus, oestrus, metoestrus and anoestrus. Some prefer the terminology dioestrus instead of metoestrus for the luteal period. Prooestrus is considered to begin on the day when a vaginal haemorrhage can first be seen from the turgid vulva. Prooestrus lasts on average for 9 days, but can be as short as 3 days or as long as 27 days. The beginning of prooestrus is gradual and a precise first day is often difficult to assess with certainty. The bitch is inviting the male, but is not ready to mate. The vulval turgidity and the haemorrhage subside towards the end of prooestrus. In oestrus, by definition, the bitch allows mating, usually for a period of 9 days, but some only for 2 or 3 days and some for as long as 21 days. In metoestrus the bitch rejects the male again. The progesterone-stimulated uterine epithelium desquamates as the progesterone concentration subsides over 2 to 3 months. The endometrial repair process is completed after 4.5 to 5 months (corresponding to the human menstruation cycle). Anoestrus lasts for 1 to 9 months, depending on whether the bitch has one, two or three cycles per year. The interval between two oestrus periods is usually prolonged by around 2 months after a pregnant cycle (Linde-Forsberg and Wallén, 1992). Breed differences in cycle length have been described, but are controversial and difficult to discriminate from familial and individual variations (see Willis, 1989).

The bitch is a spontaneous ovulator, i.e. mating is not necessary for release of LH and subsequent ovulation. With the great individual variation in the length of prooestrus, and the uncertainty about which exact day it starts, it is obvious that it is not possible to determine the fertile days of the bitch's cycle accurately if the timing is based on the days from onset of prooestrus. Some bitches may ovulate as early as day 3 to 4, and others as late as day 26 or 27 from the beginning of prooestrus. The only consistent relationship is the time from the LH peak until the onset of ovulation: ovulation in most bitches begins 24 to 72h after the LH peak (Concannon *et al.*, 1989; Lindsay and Jeffcoate, 1993). All ova are not released simultaneously, and ovulation may take from 24 to 96h. Unlike most other mammals, the dog ovulates primary oocytes that are at the germinal vesicle (GV) stage, and meiotic resumption occurs after about 48h spent in the oviduct (Reynaud *et al.*, 2005). *In vivo*, canine oocytes therefore mature in the oviducts and there is a multilayered and tight cumulus mass around the oocyte, which is seen to expand as the oocyte matures, a process that takes 2 to 5 days to complete (Holst and Phemister, 1971; Mahi and Yanagimachi, 1976; Tsutsui, 1989; Yamada *et al.*, 1992, 1993).Transit of the oviduct takes 5–10 days (Andersen and Simpson, 1973; Tsutsui, 1989). Fertilization occurs during this passage, in the distal part of the oviduct, when the oocytes reach the MII (metaphase II) stage, 56–60h post ovulation (Tsutsui 1989; Reynaud *et al.*, 2006). Mature canine ova may remain alive and fertilizable for 2 to 4.5 days (Concannon *et al.*, 1989; Tsutsui, 1989). Canine spermatozoa have been observed to fuse with immature oocytes but this is an exceptional occurrence (Reynaud *et al.*, 2005). Canine spermatozoa have been reported to survive in the uterus of the female for at least 4 to 6 days, and in one case 11 days, after a single mating (Doak *et al.*, 1967). Theoretically, thus, the bitch could conceive after one mating from about 1 or 2 days before until about 7 or 8 days after the LH peak, a period referred to as 'the fertile period' (Fig 14.4). Available data suggest that the most fertile days are from 2 to 5 days after ovulation, i.e. from 4 to 7 days after the LH peak when the oocytes have all been released and have matured and are ready to be fertilized, a period referred to as 'the fertilization period' (Fig. 14.4).

Vaginal exfoliative cytology is used to identify the stage of the oestrus cycle of the bitch. The thickness of the vaginal mucous membrane increases from 2–3 to 20–30 cell layers owing to the rising oestradiol levels during prooestrus, with a lag time of 3–6 days (Linde and Karlsson, 1984). The cells change during prooestrus from small parabasals, with a high nucleus to cytoplasm ratio to larger intermediary cells, which still have a large nucleus, and then to the fully cornified superficial cells, which usually are irregular in shape and sometimes have folded borders and either contain a small pycnotic

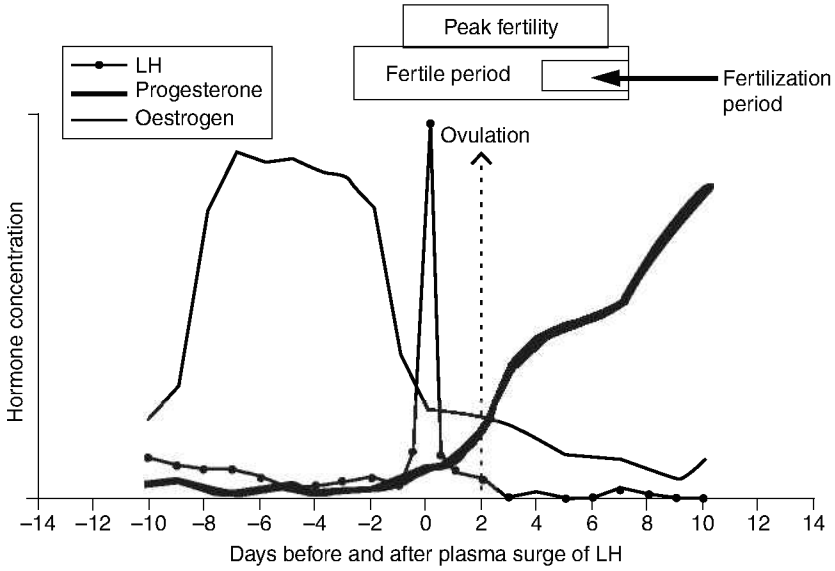

Fig. 14.4. Schematic representation of the changes in plasma progesterone, oestrogen [oestradiol] and luteinizing hormone (LH) in relation to ovulation, and the fertile and fertilization periods of the bitch (from England and Pacey, 1998).

nucleus or are anuclear. Maximal cornification can be seen from late prooestrus or early oestrus, and this remains during the period of the abrupt fall in oestradiol and rise in progesterone preceding ovulation, and throughout oestrus. In metoestrus, there is a quick shift from merely superficial cells to intermediary cells and parabasals. Characteristic of metoestrus is the appearance of a large number of polymorphonuclear leucocytes. The changes of the vaginal cells are caused by oestradiol and not by progesterone. Vaginal cytology can, therefore, not be used to determine whether, or when, the bitch ovulates, and so is not an exact enough method for timing of the bitch for mating or artificial insemination (AI). The technique is, though, useful in that a smear will show whether the bitch is still in prooestrus or is already in metoestrus.

Measurements of peripheral plasma LH levels may be the most exact method for predicting ovulation in the bitch. LH assays are available, but, because the LH peak only lasts for 1–2 days in the bitch, blood samples would have to be taken daily or every second day during prooestrus, which makes the method impractical and expensive. The method that best combines practical and economic aspects with the requirement for exactness is measurement of the peripheral plasma progesterone concentration. The level of progesterone is basal (<0.5 nmol/l) until the end of prooestrus, when the follicles change from producing oestradiol to producing progesterone shortly before the LH peak. The bitch is unique in this long preovulatory progesterone production. When the LH peaks the progesterone level usually is 6–9 nmol/l. Ovulation occurs 1–2 days later at a progesterone level of 12–24 nmol/l. Progesterone then rapidly rises to a maximum of around 150 nmol/l over about a week, and then slowly decreases during the ensuing 2–3 months. Because canine ova are released as primary oocytes and need 2–5 days to mature, the optimal time for mating or AI would be 2–5 days after ovulation, during the fertilization period, when the progesterone level is 30–60 nmol/l. It should, however, be remembered that plasma levels of progesterone fluctuate considerably during the day (up to 20–40%), but not in a regular diurnal fashion (Linde Forsberg *et al.*, 2008b). Thus, even though the values obtained by a validated RIA (radioimmunoassay) or EIA (enzyme

immunoassay) are very exact, they should be interpreted with this daily variation in mind.

Sperm transport within the female genitalia and fertilization

The male dog deposits the spermatozoa in the bitch's cranial vagina, but because of the copulatory tie and the large volume of the third fraction of the ejaculate, the spermatozoa are forced through the cervical canal into the uterine lumen, and then further through the utero-tubal junction into the oviducts, where fertilization ultimately takes place. Of the several hundred million spermatozoa that are deposited at mating maybe only a thousand will finally reach the oviducts. Active contractions of the vagina and uterus partake in this transport, and spermatozoa are found in the oviducts only minutes after being deposited in the bitch's genital tract (Tsutsui *et al.*, 1988). The main sperm storage sites in the bitch are the crypts of the uterine glands and the utero-tubal junctions (Rijsselaere *et al.*, 2004; England *et al.*, 2006). Temporary attachment of the spermatozoa to the oviductal epithelium is thought to be an integral part of the capacitation process, and ensures the slow release over time of a sufficient population of spermatozoa during the long fertilization period of the bitch. When the spermatozoon has succeeded first to attach to and then to penetrate through the zona pellucida of the ovum into the perivitelline space, it elicits a blockage of the zona that prevents polyspermy, the equatorial segment of the sperm head binds to the plasma membrane of the oocyte and the two cells fuse.

Whelping rate

In species such as the canids with originally only one oestrus cycle per year, a high fertility is paramount. Few data exist for the dog on whelping rates after natural matings, as only the successful matings that result in the birth of a litter of pups are registered with Kennel Clubs. A whelping rate of 85–90% has been reported under optimal conditions in German Shepherd dogs, Golden Retrievers and Labrador Retrievers at a guide dogs for the blind establishment (England and Allen, 1989) and in a large colony of research Beagles (Daurio *et al.*, 1987). Whelping rates among private kennels and hobby breeders may be considerably lower because of breed differences in fertility and varying skills among the breeders. The Chow Chow, for instance, is known to have a low fertility; a whelping rate by natural mating of only 53% over an 8 year period has been reported (Wikström and Linde Forsberg, 2006). Other breeds are more fertile: in Drevers, 78.6% of bitches mated in a 12 year period whelped (Gavrilovic *et al.*, 2008), in Swedish and Finnish Lapphunds whelping rates were 91.3% and 96.6%, respectively (Berglundh *et al.*, 2010), and in Dachshunds the whelping rate was found to be 91% (Lindfors, 2011). Another breed known for its high fertility is the Greyhound, and Pretzer *et al.* (2006) obtained a whelping rate of 87.5% after AI using frozen semen.

Pseudopregnancy

As much as 40% of non-pregnant bitches experience a condition during the luteal phase called pseudopregnancy, a syndrome which to varying degrees mimics the signs of pregnancy, including behavioural changes and/or mammary gland enlargement and milk production. Pseudopregnancy is believed to have been an evolutionary advantage in the wild dog, because it made it possible for other females in the group to produce milk and take over the nursing of the pups if something should happen to the mother. The cause of pseudopregnancy is considered to involve increased prolactin secretion and/or increased sensitivity of various tissues including the mammary gland to prolactin. Prolactin is necessary for luteal function during pregnancy in the dog, but is also secreted during non-pregnant luteal periods, although to a lesser degree.

Pregnancy and parturition

Pregnancy in the dog is dependent on the ovaries for progesterone production during the

entire 9 week period (Sokolowski, 1971).The major luteotrophic hormones in the bitch are LH and prolactin (Concannon *et al.*, 1989).

Apparent gestation length in the bitch averages 63 days, with a variation of from 56 to 72 days if calculated from the day of the first mating to parturition. This surprisingly large variation in the comparatively short canine pregnancy is due to the long behavioural oestrus period of the bitch. Actual gestation length determined endocrinologically is much more constant, with parturition occurring 65 ± 1 day from the preovulatory LH peak, i.e. 63 ± 1 day from the day of ovulation. Gestation length in the dog has been found to vary with breed (Linde Forsberg *et al.*, 2008a; Mir *et al.*, 2011) and with litter size – it is shorter for larger litters, and longer for smaller litters. Gavrilovic *et al.* (2008), Berglundh *et al.* (2010) and Lindfors (2011) all reported that in different breeds of dogs the duration of pregnancy was 0.25 day shorter for each pup more than average for the breed, and 0.25 day longer for each pup less.

Litter size in dogs varies with breed, size and age. It ranges from just one pup in some of the miniature breeds to the record number of 22 in a giant breed. Litter size is smaller in bitches of 1–2 years of age, increases up to 3 to 4 years old, and decreases sharply after 5–6 years (Gavrilovic *et al.*, 2008; Berglundh *et al.*, 2010; Lindfors, 2011). Sverdrup Borge *et al.* (2011), in a study of 10,810 litters of 224 breeds, found that the size of the breed and the age of the bitch were the main factors determining litter size by natural matings. A litter size of only one or two pups predisposes to dystocia because of insufficient uterine stimulation and large pup size – 'the single-pup syndrome' (Darvelid and Linde-Forsberg, 1994). This can be seen in dog breeds of all sizes. Breeders of the miniature breeds tend to accept small litters, but should be encouraged to breed for litter sizes of at least 3 to 4 pups to avoid this complication.

Based on a number of surveys, puppy losses up to weaning age appear to range between 5% and 30% and average around 12% (Linde-Forsberg and Forsberg, 1989, 1993; Lindfors, 2011). Primiparous bitches have higher pup mortality (Gavrilovic *et al.*, 2008; Berglundh *et al.*, 2010). More than 65% of pup mortality occurs at parturition and during the first week of life; few neonatal deaths occur after 3 weeks of age. The possible genetic background of fetal and neonatal deaths has not been investigated in the dog.

Parturition

Stress produced by the reduction of the nutritional supply via the placenta to the fetus stimulates the fetal hypothalamic–pituitary–adrenal axis; this results in the release of adrenocorticosteroid hormone and is thought to be the trigger for parturition. An increase in fetal and maternal cortisol is believed to stimulate the release of prostaglandin $F_{2\alpha}$, which is luteolytic, from the feto-placental tissue, resulting in a decline in plasma progesterone concentration. Increased levels of cortisol and of the prostaglandin $F_{2\alpha}$ metabolite have been measured in the prepartum bitch. Withdrawal of the progesterone blockade of pregnancy is a prerequisite for the normal course of canine parturition, and bitches given long-acting progesterone during pregnancy fail to deliver. In correlation with the gradual decrease in plasma progesterone concentration during the last 7 days before whelping there is a progressive qualitative change in uterine electrical activity, and a significant increase in uterine activity occurs during the last 24 h before parturition with the final fall in plasma progesterone concentration. In the dog, oestrogens have not been seen to increase before parturition as they do in many other species. Oestrogens sensitize the myometrium to oxytocin, which in turn initiates strong contractions in the uterus when it is not under the influence of progesterone. Sensory receptors within the cervix and vagina are stimulated by the distension created by the fetus and the fluid-filled fetal membranes. This afferent stimulation is conveyed to the hypothalamus and results in release of oxytocin. Afferents also participate in a spinal reflex arch with efferent stimulation of the abdominal musculature to produce abdominal straining. Relaxin, which is pregnancy specific, causes the pelvic soft tissues and genital tract to relax, which facilitates fetal passage. In the pregnant bitch, this hormone is produced by the ovary and the placenta, and it rises gradually over the last

two-thirds of pregnancy. Prolactin, the hormone responsible for lactation, begins to increase 3–4 weeks following ovulation and surges dramatically with the abrupt decline in serum progesterone just before parturition.

The final abrupt decrease in progesterone concentration 8–24 h before parturition causes a drop in rectal temperature. This drop is individual but also seems, to a certain extent, to depend on body size, and on the amount of coat. In miniature-breed bitches rectal temperature can fall to 35 °C and in medium-sized bitches to around 36 °C, whereas it seldom falls below 37 °C in bitches of the giant breeds (Linde Forsberg, 2010d). This difference is probably an effect of the surface area/body volume ratio. Several days before parturition the bitch may become restless, seeks seclusion or is excessively attentive, and may refuse all food. She may show nesting behaviour 12–24 h before parturition, concomitant with the increasing frequency and force of uterine contractions, and shivering in an attempt to increase the body temperature by muscular activity.

In primiparous bitches, lactation may be established less than 24 h before parturition, while, after several pregnancies, colostrum can be detected as early as 1 week prepartum.

Parturition is divided into three stages, with the last two stages being repeated for each puppy delivered. The duration of the first stage is normally between 6 and 12 hours. Vaginal relaxation and dilation of the cervix occur during this stage. Intermittent uterine contractions, with no signs of abdominal straining, are present. The bitch appears uncomfortable, and the restless behaviour becomes more intense. Panting, tearing up and rearranging of bedding, shivering and occasional vomiting may be seen. The unapparent uterine contractions increase in both frequency and intensity towards the end of the first stage. During pregnancy, the orientation of the fetuses within the uterus is 50% heading caudally and 50% cranially, but this changes during first-stage labour as the fetus rotates on its long axis, extending its head, neck and limbs to attain normal birth position, resulting in 60% of pups being born in anterior and 40% in posterior presentation (van der Weyden *et al.*, 1981; Linde Forsberg, 2010c,d,e).

The duration of the second stage is usually 3 to 12 h, and in rare cases up to 24 h. At the onset of second-stage labour the rectal temperature rises and quickly returns to normal or slightly above normal. The first fetus engages in the pelvic inlet, and the subsequent intense, expulsive uterine contractions are accompanied by abdominal straining. On entering the birth canal the allantochorionic membrane may rupture and a discharge of some clear fluid may be noted. Covered by the amniotic membrane, the first fetus is usually delivered within 4 h after onset of second-stage labour. In normal labour, the bitch may show weak and infrequent straining for up to 2 h, and at the most 4 h, before giving birth to the first fetus. If the bitch is showing strong, frequent straining without producing a pup, this indicates the presence of some obstruction and she should not be left for more than 20 to 30 min before seeking veterinary advice.

The third stage of parturition – the expulsion of the placenta and shortening of the uterine horns – usually follows within 15 min of the delivery of each fetus. Two or three fetuses may, however, be born before the passage of their placentas occurs. Lochia, i.e. the postpartum discharge of fetal fluids and placental remains, will be seen for up to 3 weeks or more, being most profuse during the first week. Uterine involution is normally completed after 12 to 15 weeks (Al-Bassam *et al.*, 1981).

The total incidence of difficult canine births, dystocia, has not been widely reported, but in a study of 15 breeds was found to vary between 9.1% in the Golden Retriever and 87.5% in the Pekingese (Gill, 2002). In the Drever, 6.25% of bitches had dystocia and 5.36% underwent a caesarean section (Gavrilovic *et al.*, 2008). In Swedish and Finnish Lapphunds, 9.5% and 12.3% experienced dystocia, while 1.6% and 5.3%, respectively, had to have a caesarean section (Berglundh *et al.*, 2010). In the Dachshund, 13.3% of bitches needed veterinary assistance at whelping and 6.7% had to undergo a caesarean section (Lindfors, 2011). In a data set from a Swedish insurance company, for all breeds, the frequency of dystocia was found to be 16%. (Bergström *et al.*, 2006). Many of the achondroplasic breeds have whelping problems, such as the Bulldog breeds, Boston

Terriers and Scottish Terriers. In French Bulldogs 43% of bitches needed a caesarean section (Linde-Forsberg, 2001). Uterine inertia is the most common cause for canine dystocia (Darvelid and Linde-Forsberg, 1994), and some breeds seem to be more prone to develop this disorder, for instance the Boxer, in which 32% of bitches suffer from dystocia (Gill, 2002; Linde Forsberg and Persson, 2002). In the Boston Terrier and Scottish Terrier breeds a significant flattening and narrowing of the pelvis occurs (Eneroth *et al.*, 1999), causing obstructive dystocia. In Boston Terriers, a strong tendency was found for a hereditary influence on pelvic shape from both the mother and the father (Eneroth *et al.*, 2000).

Assisted Reproductive Technologies

Artificial insemination

The first scientific publication on the use of reproductive biotechnology in a mammal is by Abbé Lazzarro Spallanzani in 1784, in which he describes how he performed artificial insemination of a bitch. Although the first artificial insemination in the dog was thus performed more than 220 years ago, it was not until the late 1950s that interest began to focus on this field of research. Harrop (1960) described the first successful AI in a dog, using chilled extended semen. The first litter by frozen–thawed dog semen was reported by Seager in 1969. Since then, interest in canine AI has grown exponentially. With the advent of modern AI technology, breeders not only have the potential to use dogs from all over the world, but can also save deep-frozen semen from valuable dogs to be used in later generations. New knowledge on techniques for semen preparation, oestrus detection and insemination is constantly accumulating.

The keys to obtaining good results by canine AI are proper timing of the insemination, i.e. 2–5 days after ovulation, the use of an adequate number of spermatozoa of good quality, good semen handling and preparation methods, and the use of an intrauterine insemination technique. Whelping rates by intrauterine AI in the dog are significantly better than those obtained by vaginal AI, not only for frozen–thawed semen (by 51%) but also for chilled (by 44%) and fresh semen (by 30%). Litter size using intrauterine AI of frozen–thawed semen is also significantly larger than by vaginal AI. Breed differences in fertility after AI have been described (Linde-Forsberg and Forsberg, 1989, 1993).

In Europe, the recommended number of normal spermatozoa per single AI is 150–200 $\times 10^6$, and it is recommended that two AIs are done per oestrus cycle (Linde-Forsberg *et al.*, 1999; Thomassen *et al.*, 2006). However, in the USA, for instance, 100×10^6 progressively motile spermatozoa (≥50%) and a single AI are commonly considered adequate. Vaginal deposition of fresh as well as frozen–thawed semen appears to require approximately ten times as many spermatozoa to obtain the same whelping rate as intrauterine deposition (Tsutsui *et al.*, 1989; Linde-Forsberg *et al.*, 1999).

Semen to be stored or shipped should always be extended and chilled. The extender helps to protect the spermatozoal membranes from damage caused by changes in temperature and shaking during transport, while also providing energy and stabilizing the pH and osmotic pressure. Furthermore, chilling lowers the metabolic rate, thereby increasing sperm longevity. Good-quality chilled semen may maintain its fertilizing capacity for up to a week or more at 5 °C. It is possible to successfully freeze dog semen that has been collected and then chilled for 2–3 days before being frozen (Verstegen *et al.*, 2005; Hermansson and Linde Forsberg, 2006). It is thus a viable option to have a dog collected nearer to home and ship the chilled semen to a semen bank for freezing.

Dog semen is generally frozen in 0.5 (or 0.25) ml straws, or in pellets. Although both straws and pellets have been found equally good for dog semen cryopreservation, straws are considered more hygienic, and are easier to identify, store and thaw. Extenders used for freezing dog semen usually contain glycerol as cryoprotectant. Rapid thawing at 70 °C for 8 s has been shown to be significantly better than thawing at 37 °C for 30 to 60 s (Rota *et al.*, 1998; Peña and Linde-Forsberg, 2000).

Methods for AI in bitches include: (i) vaginal deposition of semen using a simple plastic

catheter; (ii) transcervical intrauterine deposition (TCI) using either the Scandinavian catheter or a rigid endoscope to visualize the cervix with a dog urinary catheter for the infusion of the semen, and surgical intrauterine deposition; or (iii) intrauterine insemination by laparoscopy (see Linde Forsberg, 2010a). The vast majority of canine inseminations are performed with fresh semen and the semen is usually deposited in the cranial vagina, which is technically quite easy, although results are better after intrauterine deposition (Linde-Forsberg and Forsberg 1989, 1993; Linde-Forsberg, 2000; Linde Forsberg, 2010a,b).

Deposition of frozen semen in the cranial vagina generally results in a poor pregnancy rate (Linde-Forsberg, 1991, 1995, 2000; Linde-Forsberg *et al.*, 1999) although there are some reports of good success (Seager, 1969; Nöthling and Volkmann, 1993). Whelping rates of 80–87.5% have been reported from AIs when frozen–thawed semen of good quality was inseminated at the right time into the uterus of healthy bitches (Linde-Forsberg *et al.*, 1999; Pretzer *et al.*, 2006; Thomassen *et al.*, 2006). Litter size was estimated to be 23.3% (Linde-Forsberg and Forsberg, 1989) and 30.5% (Linde-Forsberg and Forsberg, 1993) smaller in bitches inseminated with frozen compared with fresh semen. Sverdrup Borge *et al.* (2011) compared the litter size at birth between matings and AIs with fresh or frozen semen in 10,810 litters of 224 breeds, and found 0.4 fewer pups per litter by fresh semen and 1.3 pups fewer by frozen semen.

Supply and collection of oocytes

Collection of immature oocytes

To obtain MII mature oocytes and perform fertilization, several approaches are available. The first is to collect an ovary in anoestrus or dioestrus. These are the stages when routine sterilization in dogs is performed in veterinary clinics. The ovaries are dissected *in vitro* and immature oocytes are collected. Those oocytes are blocked at the GV stage in meiosis and are then matured *in vitro* (IVM) for 72 h until MII stage, when they become fertilizable. So far, and despite a number of attempts in several laboratories around the world, the percentage of MII oocytes obtained remains very low (for a review, see Songsasen and Wildt, 2007; Chastant-Maillard *et al.*, 2011). The ultrastructure of MII oocytes obtained this way is not the same as that of oocytes collected *in vivo* (De Lesegno *et al.*, 2008b). One may assume that the poor results obtained are due to the fact that those oocytes come from small follicles (less than 1 mm in diameter), whereas the normal pre-ovulatory diameter is 6–8 mm. Indeed, the rate at which MII oocytes are obtained *in vitro* is correlated with the size of the follicle at harvesting (Songsasen and Wildt, 2005). Furthermore, the cytoplasm of the oocyte is still quite immature (De Lesegno *et al.*, 2008a). A second option is to collect oocytes in pre-ovulatory follicles. The number of oocytes obtained will be limited, but a higher proportion of those oocytes will reach the MII stage after *in vitro* maturation. Puncturing follicles after ovariectomy has been described (Yamada *et al.*, 1993; Reynaud *et al.*, 2009) but the technique of major interest is the so-called Ovum Pick-Up (OPU), in which ultrasound guidance is used to puncture pre-ovulatory follicles. If fully developed, this technique would not call for ovariectomy of the dog and might even be used to collect oocytes over several cycles. In dogs, a preovulatory follicle has a diameter of 6–8 mm. The quality of the ultrasound equipment currently available makes it possible to visualize and puncture those follicles, even though the fatty ovarian bursa makes it difficult to reach the ovary. As far as we know, no research team has yet published a report of non-surgical ovum pick-up in the dog, so the technique requires further development.

Collection of in vivo *matured oocytes*

Because the maturation rates of oocytes obtained *in vitro* remain so low, attempts have been made to obtain MII mature oocytes by collecting them after approximately 3 days of *in vivo* maturation. This requires a precise determination of the time of ovulation, either by recording the increase in pre-ovulatory progesterone or by ultrasound recording of ovulation, followed by flushing of the oviducts either *ex vivo* or *in vivo* following opening of

the abdominal cavity (Lee *et al.*, 2005; Jang *et al.*, 2008). However, as in other OPU techniques, the number of oocytes that can be collected this way is limited because superovulation protocols are not very efficacious.

Cryopreservation of oocytes and gonads (ovaries and testicles)

Cryopreservation of the ovary or of oocytes is one way to preserve the genetic potential of a female of particular interest whether alive or shortly after its death. In endangered wild species, the preservation of the genetic material (gamete or gonad preservation) is also an approach to the overall management of species preservation, particularly in carnivores (Silva *et al.*, 2004).

Oocyte cryopreservation

Oocytes are more difficult to cryopreserve than spermatozoa or embryos (for a review, see Saragusty and Arav, 2011). For a long time, conventional deep-freezing techniques were used with poor results because the oocytes rarely survived the formation of ice crystals within the cell and the destruction of the meiotic spindle. Ultra-rapid freezing, so-called 'vitrification', has led to largely improved results. This is a technique developed 20 years ago for several mammalian species, from mice to man.

In canids, three research teams have reported their experience with vitrification. In Japan, Abe *et al.* (2010) vitrified immature oocytes in their cumuli to test two vitrification media and two kinds of cryotubes. Integrity of the plasma membrane was then assessed by propidium iodide staining; 40–60% of the oocytes showed normal morphology after vitrification, but their ability to resume meiosis was not tested. In Thailand, Turathum *et al.* (2010) studied the vitrification of dog oocytes, their ability to resume meiosis and their ultrastructure. They demonstrated that vitrification is indeed possible but that it reduced the ability of the oocyte to resume meiosis (from 91% to 53%) and that it induced alterations in cytoplasmic organelles (lipid droplets, mitochondria, cortical granules, smooth endoplasmic reticulum). This may be due to the high lipid content of the oocyte in dogs, as in swine, which seems to interfere with vitrification (Zhou and Li, 2009). If this is the case, delipidation techniques might be used – centrifugation, micromanipulation or chemical stimulation of lipolysis (Zhou and Li, 2009). Another team, Boutelle *et al.* (2011), in the USA, reported recently on the vitrification of oocytes from dogs and Mexican grey wolves; they demonstrated that the viability of these oocytes, as tested by propidium iodide, was preserved after vitrification.

These reports seem to open the way for the cryopreservation of female gametes, but considerable work remains to be done after that to mature and fertilize cryopreserved oocytes and eventually to develop embryos.

Ovary cryopreservation

Ovarian cryopreservation is a technique used in several species, particularly humans, to preserve the ovaries in case of cancer chemotherapy (Smitz *et al.*, 2010). However, cryopreservation leads to the loss of most growing follicles. Only small follicles (primordial/primary follicles) survive. In the dog, the only paper published on the subject so far (Ishijima *et al.*, 2006) reported freezing 30 ovarian slices (1–5 mm^3) from ten dogs and demonstrated that the morphology of the ovarian tissue remained normal following vitrification and that the number of follicles found was the same before and after vitrification.

Cryopreservation of testicular tissue

Relatively few studies have been devoted to testicular cryopreservation compared with ovarian cryopreservation. The reason is that, in pubertal males, sperm collection and cryopreservation of spermatozoa is the preferred technique. This technique is well developed and largely used in the dog (see above). Testicle cryopreservation will thus be used mostly to preserve the genetic capital of prepubertal animals, or in case of death by accident. Again, few reports have been published concerning dogs. Dobrinski *et al.* (1999) worked on testicular cells (spermatogonia) frozen and then transplanted to immunodeficient mice that

had been previously neutered chemically. Cells of dog origin were still found 1–15 months after transplantation, but no colonization or active spermatogenesis was observed. Yet this study demonstrated that spermatogonia can survive after cryopreservation and that transplantation of testicular cells may be used for preserving reproductive potential (Dobrinski and Travis, 2007).

Use of cryopreserved tissues (*in vitro* folliculogenesis and transplantation)

Following cryopreservation of ovarian tissue, various options are available, but primarily *in vitro* folliculogenesis and transplantation. Folliculogenesis, that is, the development of a follicle from the primordial to the pre-ovulatory stage, is a lengthy process and its duration varies considerably from species to species. Furthermore, while growing, the oocyte gradually gains a capacity to resume meiosis (nuclear maturation) and then an ability to become fertilizable which can lead to embryo development up to the blastocyst stage (cytoplasmic maturation). Only those oocytes coming from a relatively large follicle will become able to generate an embryo after fertilization. For *in vitro* folliculogenesis and eventual transplantation, these data have to be borne in mind. In dogs, nuclear maturation of the oocyte becomes possible when the size of the follicle exceeds 2 mm (Songsasen and Wildt, 2005). Cytoplasmic maturation is probably assured at a later time, when the follicles are even larger. The difficulty in developing a technique for *in vitro* folliculogenesis is thus to design culture conditions (possibly involving a sequence of several media) that will assure normal growth of the follicles for a sufficiently long time.

In dogs, the first report on *in vitro* folliculogenesis was published more than 10 years ago (Bolamba *et al.*, 1998). Pre-antral follicles and small antral follicles were cultured for a short period (1–3 days), and their oocytes were evaluated. In antral follicles, follicle culture significantly increased the number of oocytes able to resume meiosis. Two recent studies investigated the effects of the diameter of cultured follicles and gonadotrophin concentrations (FSH or FSH + LH; Nagashima *et al.*, 2010; Serafim *et al.*, 2010). Pre-antral follicles measuring 200 to 400 μm at the start of culture were cultured for relatively long periods (18–20 days). The growth in diameter of the follicles and survival – evaluated either by their morphology or using a fluorescent marker (calcein-AM) – were followed for the whole period of culture. The preliminary results are encouraging, as the follicles grew by approximately 60% and an antrum developed in more than 80% of the follicles (Serafim *et al.*, 2010), although considerable variations (10–90%) were observed in the survival rate, as evaluated by intact basal membrane without extrusion of the oocyte. Whereas the presence of FSH appeared to stimulate follicle growth, an overly high concentration of FSH seemed to have negative effects in that it induces follicle atresia (Nagashima *et al.*, 2010; Serafim *et al.*, 2010).

Allografting and xenografting

Transplantation techniques appear to be of major interest for domestic animals and even more so for wild and endangered species (Paris and Schlatt, 2007). Offspring have been obtained in several species following the cryopreservation and transplantation of ovaries. However, the cryopreserved and transplanted ovarian tissue contains mostly small follicles, so that a certain length of time is required for these follicles to reach the antral and pre-ovulatory stages. Moreover, the site of grafting appears to be quite important: clearly, the transplanted tissue has to be revascularized quickly for the follicles to survive.

In the dog, this aspect of transplantation has been investigated quite recently. So far, the best grafting site remains to be determined. Grafting half an ovary into muscle tissue is feasible (Terazono *et al.*, 2011), but few oocytes seem to survive more than 28–31 days after transplantation. In another study, an attempt was made to allograft fresh ovarian tissue into the ovarian bursa (Pullium *et al.*, 2008) using two different techniques: grafting of the whole ovary or the grafting of small fragments (cortical strips) placed into the ovarian bursa. The animals were prepubertal DLA (dog leucocyte antigen) identical and received an immunosuppressant therapy. All six grafted dogs showed

signs of oestrus; four were mated and one of them started a pregnancy which, unfortunately, ended in embryonic resorption. Post-mortem examination of five of the six dogs revealed the presence of fibrous connective tissue which led to occlusion of the oviduct.

Xenografting of fresh ovarian tissue into the renal capsule of SCID (severe combined immunodeficient) mice (Metcalfe *et al.*, 2001) or of vitrified tissues into the ovarian bursa of NOD-SCID (non-obese diabetic-SCID) mice (Ishijima *et al.*, 2006) demonstrated that the follicles may survive and develop after cryopreservation. In these two studies, though, the ovaries remained grafted for only a few weeks, which was not enough time to obtain antral follicles of large diameter. Current studies are designed to stimulate vascularization to preclude the loss of follicles by ischaemia right after grafting (Suzuki *et al.*, 2008).

Semen sexing

Semen sexing is based on the differences in DNA content between the X and the Y chromosomes. This difference is more or less pronounced, depending on the species (2.3–7.5%; for a review, see Johnson, 1995). In the dog, the difference is in the order of 3.7–3.9% (Johnson 1992; Meyers *et al.*, 2008). Following DNA staining, spermatozoa are separated by flow cytometry. Semen sexing is a recent technique which might be of specific interest in dogs for two reasons: the commercial benefit for dog breeders, and the scientific benefit, particularly in the case of sex-linked genetic disease or the development of gene therapies. However, semen sexing requires a high performance cytometer and the number of spermatozoa differentiated is relatively low because 60% of the spermatozoa are either destroyed in the process or are not sexable, and approximately 5 million spermatozoa per hour are being sexed. For semen sexing to be effective, a male with good-quality semen is required so that, after sexing, an intrauterine or intra-oviductal insemination can be effected to optimize the number of sexed spermatozoa available. This technique has been patented by a private company, XY Inc., which has so far published only a summary of its results in a single female Labrador inseminated three times with 18–45 million spermatozoa of fresh semen, leading to the birth of five pups (two males and three females). Although not a proof of an effective sex sorting, this study demonstrates that canine spermatozoa can survive the sexing process and retain their *in vivo* fertilizing capacity.

Oocyte fertilization

Artificial insemination is a fully developed technique and can be used in domestic dogs as well as in wild canids (for a review, see Thomassen and Farstad, 2009). Other techniques of fertilization, though, are not routinely used and are still being developed.

Insemination into the oviduct (intra-tubal insemination)

This type of insemination makes it possible to use semen with few spermatozoa but requires surgery. Few studies have been published so far (Tsutsui *et al.*, 2003; Kim *et al.*, 2007). The objective was to evaluate the minimal number of spermatozoa necessary to achieve pregnancy (from 1×10^6 to 8×10^6 spermatozoa in Tsutsui *et al.*, 2003, and from 4×10^4 to 4×10^6 spermatozoa in Kim *et al.*, 2007). Pups were only obtained with the dose of 4×10^6 so it appears that insemination into the oviduct may be a way to obtain progeny, even though the rate of success (pups per number of corpora lutea) remains poor.

ICSI (intra-cytoplasmic sperm injection)

This technique involves the injection of one spermatozoon into an oocyte to induce fertilization and has generated a revolution in medical assistance to procreation in humans. Indeed, whereas conventional *in vitro* fertilization requires mature lively and mobile spermatozoa, ICSI can be used in cases where fertilization is uncertain (a high rate of polyspermia, or a hard-to-penetrate zona pellucida). The injection of a male gamete may also be effected in rather extreme situations such as:

- injection of spermatozoa only available in very low amount, or of poorly motile spermatozoa, or of apparently normal spermatozoa from poor-quality semen (oligo-astheno-teratozoospermia)
- more generally, in cases of poor-quality semen, when the survival rate after freezing is low, or after sperm sexing
- injection of immature germ cells (spermatids obtained by testicle biopsy).

In the case of the dog, ICSI may be a technique of major interest to preclude the polyspermia that is commonly observed in culture, and also in the use of poorly surviving sperm after freezing (for the time being, cryopreservation of semen is not recommended from dogs whose spermatozoa have a poor survival after freezing). Furthermore, in the case of endangered canid species, if the technique proves efficacious, semen collection or testicle biopsies followed by freezing could be more largely used for the preservation of the genetic capital, particularly in case of accidental death of the animal. In dogs, a single abstract has been published on the subject so far, more than 10 years ago (Fulton *et al.*, 1998). In that study, 38 oocytes were injected and embryos were examined 12 h after ICSI. Decondensed sperm chromatin was observed with the female pronucleus in 16/38 (42%) of the injected oocytes, and two pronuclei were detected in 3/38 (7.8%) of the embryos.

Embryo biotechnologies

In vitro *production of embryos*

The production of embryos *in vitro* requires several steps before transfer to a recipient female or cryopreservation: oocyte maturation, *in vitro* fertilization and embryonic development. As stated above, oocyte maturation is still problematic. The same is true of *in vitro* fertilization. Indeed, only a low proportion of oocytes are fertilized. Despite very high rates of polyspermia, less than 10% of normal embryos are formed (for a review, see Chastant-Maillard *et al.*, 2010). Furthermore, embryonic development stops in culture, very few embryos reach the blastocyst stage and, as far as we know, only a single pregnancy has been obtained (with abortion halfway through gestation) and no pup has been obtained to date.

In vivo *production of embryos*

In the dog, fertilization takes place when the oocyte reaches the MII stage, some 56–60 h following ovulation (Tsutsui, 1989; Reynaud *et al.*, 2006). In the oviduct, the embryos are at the two pronuclei to morula stage 3–10 days after ovulation (Fig. 14.5a). It is then that they can be collected by flushing the oviducts *in vivo* or *ex vivo*. The flushing technique *in vivo* requires some dexterity as the oviduct is ensconced in the ovarian bursa (Fig. 14.5b). Starting 10 days after ovulation, embryos at the blastocyst stage (Fig. 14.5c–d) may be collected from the uterus before implantation (which takes place 18–21 days following ovulation). In any case, the *in vivo* production of canine embryos is limited by physiological constraints because superovulation is not easily obtained in the bitch.

Embryo cryopreservation

The cryopreservation of canine embryos has not been studied thoroughly as embryos are very difficult to obtain *in vitro* and few *in vivo* embryos can be obtained at each cycle. However, a Japanese team (Abe *et al.*, 2011) has just published a major study describing for the first time the cryopreservation of canine embryos by vitrification, and the non-surgical transfer of embryos into the uterus. A total of 474 embryos were collected, then vitrified at various stages (from one cell to blastocyst). At the morula and blastocyst stages, the rate of survival was lower than at earlier stages of embryo development. After the transfer of 77 embryos into the uteri of nine bitches, seven pups were born, representing 9.1% of the embryos transferred.

Preimplantation diagnosis/embryo genotyping

Preimplantation genetic diagnosis has been used in man for some 20 years to try to avoid

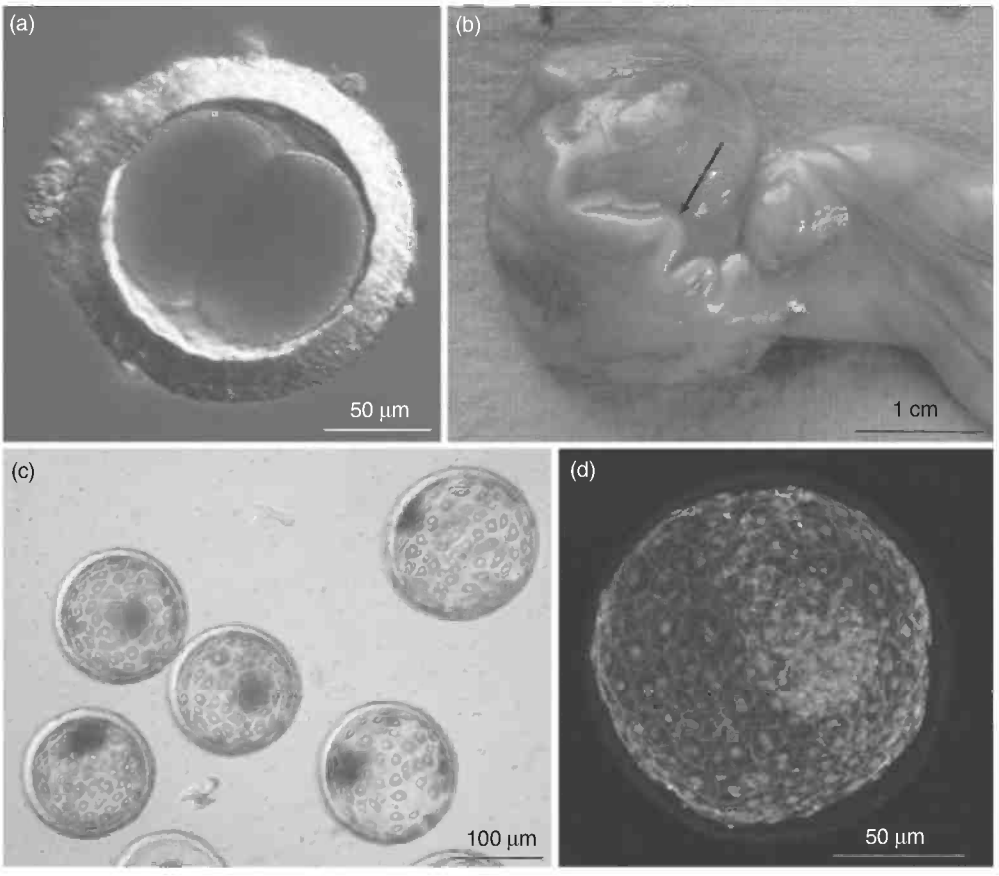

Fig. 14.5. (a) A two-cell canine embryo, collected 124 h after ovulation; (b) canine oviduct in the ovarian bursa during the peri-ovulatory period; (c) canine blastocysts, collected 274 h (11.5 days) after ovulation; (d) actin (cytoskeleton) and DNA (nuclei) staining of a canine blastocyst collected 300 h (12.5 days) after ovulation.

the transmission of serious genetic diseases (Iwarsson *et al.*, 2011). The embryos are micromanipulated *in vitro* to collect a few blastomeres which will then be analysed using FISH (fluorescence *in situ* hybridization) or PCR in a search for gene mutations of interest. In dogs, the genome has been sequenced and more than 500 genetic diseases have been identified; 256 of these diseases may serve as models for human genetic diseases (OMIA, Online Mendelian Inheritance in Animals, available at: http://omia.angis.org.au/home/). These diseases are detected in the young dog or in adults by clinical examination and, if available, by a genetic test. An ability to detect a genetic anomaly at the embryonic stage would offer two advantages: (i) for breeders, it would provide an opportunity to select healthy embryos and to let only non-affected animals come to birth; (ii) for biomedical research, it would offer the possibility of selecting and bringing to life only diseased animals for eventual therapy. Despite its interest and potential application, this technique has not been reported so far in dogs and requires further development.

Cloning/transgenesis

Cloning in domestic carnivores has come into the news media with the announcement, by

an American billionaire, that he would support financially a research team that would clone his dog 'Missy'. The Missiplicity project was thus started and the selected team first managed to clone a cat (Copycat, a kitten presented in February 2002). In contrast, even after several years, no dog cloning was obtained because of the serious physiological constraints of the dog model (as explained above, difficulties in obtaining superovulation, IVM (*in vitro* maturation), IVF (*in vitro* fertilization), IVD (*in vitro* development) and access to biological material (for a review, see Luvoni *et al.*, 2006). Eventually, a Korean research team was the first to report on a canine clone, 'Snuppy', in August 2005 (Lee *et al.*, 2005). Following this canine cloning achievement, the same team cloned some dogs for commercial purposes, although the yield remains quite low (0.4–4%). Commercially, a cloning programme for dogs of private owners was developed by collaboration between an American team (Lou Hawthorne, BioArts International) and the Korean team. The 'BestFriendAgain' programme led to the birth of several clones but this collaboration came to an end in September 2009. The main reasons for this termination were that there is a tiny/niche market for a cloning programme for dogs and that there were numerous bioethical problems.

Now, canine cloning may be used to expand the pool of service dogs (rescue and drug sniffing dogs), for the conservation of endangered species and also for biomedical applications, as the dog is a model for human disease (for review, see Jang *et al.*, 2010).

Canine embryonic stem cells (ESCs)

Despite current expectations in terms of economic and biomedical applications, stem cells have been poorly investigated so far in pet animals. Only a few publications are available on the use of ESC in canids, and all of them are recent (less than 5 years old). A review on the subject has appeared recently (Schneider *et al.*, 2010). Following the dissection of blastocysts, embryonic stem cells were cultured and markers (alkaline phosphatase enzymatic activity, as well as the expression of transcripts for OCT4 and NANOG – both pluripotency-associated embryonic transcription factors) were investigated. The potential of those cells to differentiate into embryoid bodies *in vitro* or into teratomas *in vivo* was studied. For the time being, the results are ambiguous as the cells remained undifferentiated in culture for only a few passages, and only a few cell lines appeared to be capable of inducing teratomas *in vivo* after injection into immunodeficient mice. Within the International Embryo Transfer Society (IETS), a specific group called 'Domestic Animal Biomedical Embryology' (DABE) has been created on this subject.

In another study, an attempt was made to isolate and evaluate multipotent mesenchymal stem cells (MSC) obtained from the amniotic fluid, amnion and matrix of the umbilical cord of the dog (Valentini *et al.*, 2011). The cells collected in that study failed to express all the multipotency markers and their viability declined quite rapidly (between three and seven passages), although the study demonstrated that stem cells can be obtained from another potential source.

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15 Developmental Genetics

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Introduction

Recent progress in mammalian developmental genetics has been significant. However, the vast majority of information has been generated through the use of the mouse model and, to some degree, the use of other mammalian species such as pigs, cattle and, certainly, humans. While in many areas dog genetics has been highly successful, as this book certifies, dog developmental genetics remains poorly investigated and, surprisingly, only a limited embryological description of dog development is currently available (http://php.med.unsw.edu.au/embryology/index.php?title=Dog_Development). Therefore, it might be premature to

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write a chapter entirely devoted to the developmental genetics of the dog. Nevertheless, the high level of similarity in mammalian development and the homology of the genetic systems involved in regulation of the major processes seem to provide sufficient justification for inclusion of this chapter in the book. It should allow the collection of all the available data on the dog and discussion of those data against a general background of mammalian development. It is hoped that this approach could provide a useful basis for future research.

Developmental Stages of the Dog Embryo

Gamete maturation and fertilization, which comprise the first crucial steps in each new developmental cycle in mammals, have been considered in the previous chapter. The major embryological steps and their genetic determination are discussed here. Table 15.1 summarizes the essential events and timing of early embryonic and fetal development in the dog.

The early period of development covers the first 13–14 days after fertilization up until implantation (Fig. 15.1). It is characterized by several crucial events, including cleavage, morula formation and compaction and blastocyst development (Stabenfeldt and Shille, 1977; Concannon, 2000; Concannon *et al.*, 1989, 2001; Renton *et al.*, 1991; Yamada *et al.*, 1993; Reynaud *et al.*, 2005, 2006; Abe *et al.*, 2008). Soon after compaction of the morula at about day 7–8, the conceptus enters the uterus and undergoes blastocyst formation. Tight intercellular junctions develop and this provides a condition for the accumulation of fluid within the central cavity (the blastocoele). The majority of external cells of the blastocyst – called the trophoblast – are concerned with development of extra-embryonic tissues and placenta. A group of cells, located at one end of the embryo (the embryonic pole), form the embryoblast or inner cell mass (ICM). The zona pellucida, so common for mammals at this stage, is an essential feature of the early canine conceptus. A glycohistochemical investigation of lectins and other carbohydrates in the dog's oocytes and in the zona pellucida itself was carried out by Parillo and Verini-Suplizi (1999), who found that the topographical distribution of different carbohydrates including lectins 'is not uniform throughout the zona pellucida, indicating the regionalization of oligosaccharide chains within three concentric bands of the zona matrix: an inner surface close to the oocyte plasma membrane, an intermediate portion and an outer layer in contact with the follicular cells. These results demonstrated variations in the presence and distribution of the carbohydrate residues in the

Table 15.1. Essential events and timing of prenatal development in the dog.[a]

Stage of development	Days after fertilization[b]
Two cells	1–2
Eight cells	3–4.5
Morula compaction	7–8
Blastocyst formation	~9
Free migration between uterine horns	10–11
Hatching from zona pellucida	13–14
Implantation begins	~15
Gastrulation begins	>15
Primitive streak develops	~16
Neural tube forms	~17
Notochord	~17
First somite pair	~18
Head fold	~19
Closing of neural tube	19–20
Vascularized yolk sac	20–21
Beating heart	20–21
Visible limb buds	24–25
Optic and otic vesicles visible	25–26
Well-developed tail and elongating limbs	31–33
Testicular differentiation begins	35–36
Pituitary gland developed	~38
Eyelids close, lids fused, claws on digits	~40
Ossification recognizable	~42
Mammae begin to grow	~42
Teeth begin to grow	53–55
Hair coat developed	~55
Birth	62–65

[a]Compiled from: Barrau *et al.* (1975); Stabenfeldt and Shille (1977); Boeve *et al.* (1989); Concannon, 2000; Concannon *et al.* (1989, 2001); Renton *et al.* (1991); England and Yeager (1993); Harvey *et al.* (1993); Valtonen and Jalkanen (1993); Yamada *et al.* (1993); Meyers-Wallen *et al.* (1994); Sasaki *et al.* (1998); Sasaki and Nishioka (1998); Reynaud *et al.* (2006); Abe *et al.* (2008).
[b]It is generally agreed that the interval between the LH (luteinizing hormone) peak and fertilization is roughly about 3–4 days (for details see Chapter 14).

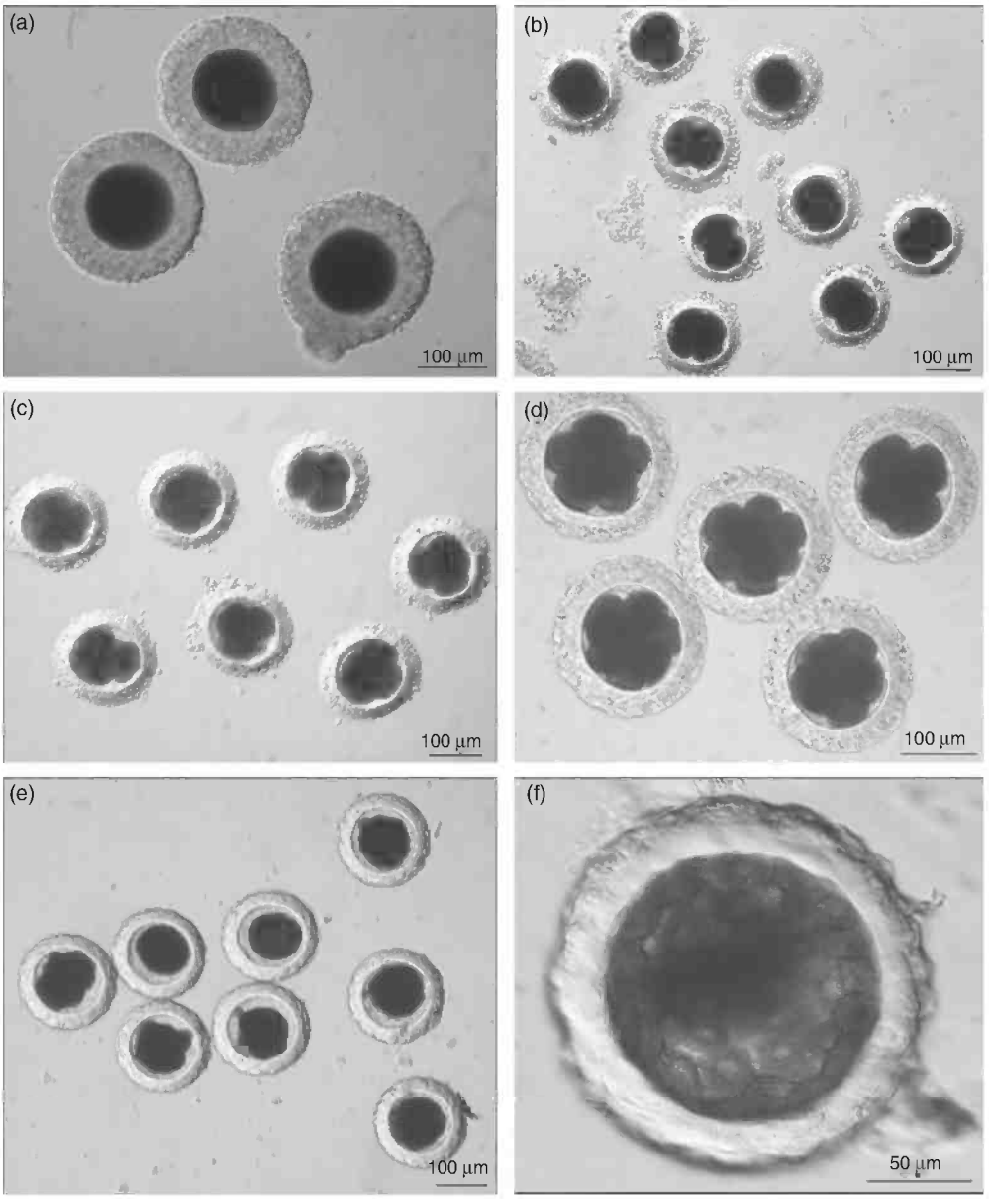

Fig. 15.1. Canine oocytes and embryos observed under light microscopy at different stages after ovulation. Maturation stages were determined by confocal microscopy. (a) Canine oocytes, metaphase II, 82 h (3.5 days) post ovulation. The oocytes have lost their mucified mass, but are still surrounded by the corona radiata. (b) Two-cell canine embryos collected 137 h (5.7 days) after ovulation. (c) Four-cell and eight-cell canine embryos, 153 h (6.4 days) after ovulation. (d) Eight-cell canine embryos collected 174 h (7.2 days) after ovulation still have a few granulosa cells around their zona pellucida. (e) Morula-stage canine embryos collected 254 h (10.6 days) after ovulation. (f) Early blastocyst stage canine embryo, collected 265 h (11 days) after ovulation. Acknowledgement: the authors are very grateful to Karine Reynaud, who created this figure and kindly made it available for the chapter.

canine zona pellucida during different stages of follicular growth'. Parillo and Verini-Suplizi (1999) also observed the presence of vesicles in both the ooplasm and granulosa cells which showed a similar lectin binding pattern to that of the zona pellucida. 'Hatching' from the zona pellucida indicates a preparation for implantation and thus entry into the next stage of development.

During the following period (days 15–34) a number of crucial events happen. These include implantation and development of the placenta, followed by gastrulation, when the ICM differentiates into the three primary germ layers of the embryo – the ectoderm, mesoderm and endoderm, which continue differentiation for some time. During this process, the neural tube opens, the notochord develops and the first somites are formed. Throughout this period, the embryo develops its entire structures, major organs and tissues, and its shape changes dramatically. The essential morphogenetic events that occur at this time include the formation of the head, vertebrae and appendages, the development of the nervous system and blood circulation as well as other major internal organs. By day 34 of gestation, the embryo has developed recognizable taxonomic features.

The final period of development requires another month, during which the canine fetus undergoes extensive growth and final development (Fig. 15.2). The numerous morphological changes that occur, although definite, are not radical. These gradual changes shape the fetus, its major structures and function towards the requirements of postnatal life, including sexual differentiation.

Key general differences in dog development compared with other mammalian species include: the initial long life of the spermatozoa (7 days), the long delay between ovulation and fertilization (2–3.5 days), the time between fertilization and implantation (>15–16 days) and later gastrulation.

Genes Involved in Pre-implantation Development

Expression of maternal genes

While the zygote is usually considered as the starting point of embryonic development, the mature oocyte and its gene expression profile are quintessential for embryogenesis. Direct data relevant to the genetic aspects of canine oocyte maturation are still quite limited (Rodrigues and Rodrigues, 2010). The same is true for the entire developmental process. Fortunately, information collected in other placental mammals can be very helpful in reconstructing the major genetic processes in the developing dog embryo.

As mentioned in Chapter 14, before ovulation, meiosis is arrested in dog oocytes, which remain at the prophase of the first meiotic division, although both transcription and translation are very active and under 'maternal command' (Reynaud *et al.*, 2005). The number of active genes could be similar to that found in murine oocytes, where about 5400 genes and transposable elements are expressed (Evsikov *et al.*, 2006). Numerous, newly synthesized mRNAs are stored and used later during oocyte maturation and up until activation of the embryonic genome, which occurs at the two-cell stage in the mouse (Hamatani *et al.*, 2006) and at the four-cell stage during pig embryo development (Oestrup *et al.*, 2009). Activation of the embryonic genome is also expected to be at the four-cell stage in the dog. Before fertilization, depletion of maternal mRNA intensifies and this continues until activation of the embryonic genome. By this time, nearly 90% of maternal mRNA has been degraded and the majority of mRNA transcripts are exclusively expressed from the oocyte genome (Bettegowda *et al.*, 2008; Fig. 15.3). In the mouse, and probably in other mammals, including the dog, 'housekeeping' genes are under-represented in the oocyte and early embryo transcriptomes. It is likely that the oocyte acts as a 'reprogramming machine' that is necessary for the creation of a totipotent embryo (Evsikov and Marín de Evsikova, 2009b).

Current understanding of the transition towards the mature oocyte and embryonic development is only emerging, and some species-specific deviations are possible. Several genes, some identified recently, guide this process. Among these is *Eif41b*, which is involved in translational repression of maternal mRNAs. In the mouse, an oocyte-specific mammalian form of eukaryotic translation initiation factor (4E), encoded by the novel *Eif41b* gene, may influence the speed of oocyte maturation

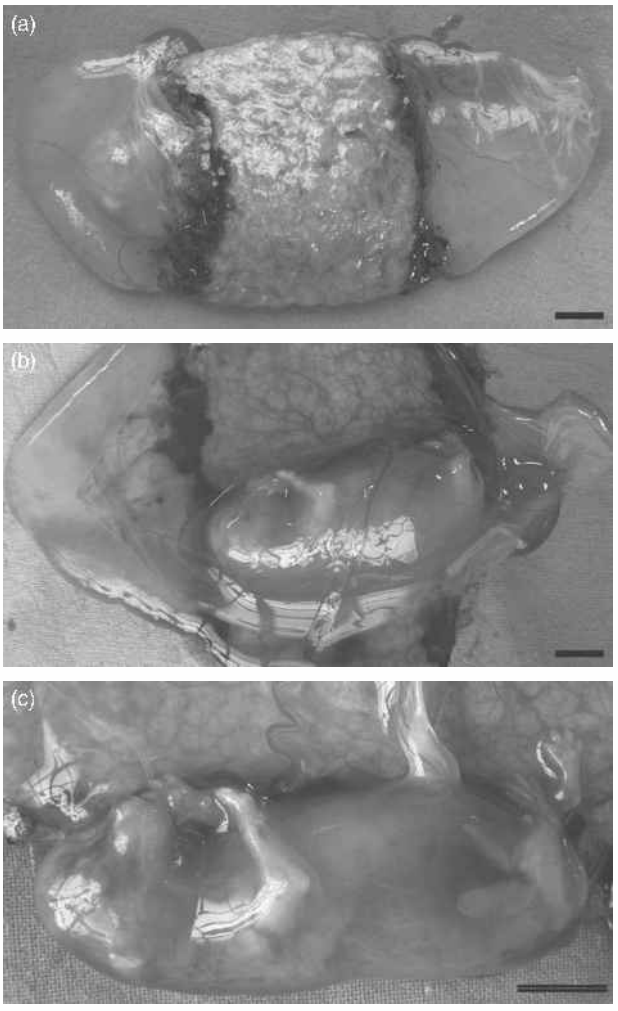

Fig. 15.2. The developing canine embryo at day 40 after mating. This may correspond to approximately 35–38 days after fertilization as bitches are ready for mating a few days before ovulation. (a) Fetus located within the intact fetal membranes and placenta. The placenta is viewed from the maternal side and is classified as a zonary placenta. It forms a complete central girdle in dogs. (b) Fetus located within intact amniotic sac with opened chorion and placenta. The placenta is viewed from the fetal side and major fetal blood vessels can be seen radiating over the surface of the placenta. The yolk sac is visible on the right. On the maternal side, the endometrial blood vessels are bare to their endothelium and come into direct contact with the chorion, forming an endotheliochorial placenta. (c) Fetus with amniotic sac removed. The placental cord is visible at the umbilicus. The limbs are well developed, showing paws and claws. The head surface shows sensory development with eyes, ears, nose and rows of whiskers (vibrissae). Acknowledgement: the authors are very grateful to Karine Reynaud for the original photos.

(Evsikov and Marín de Evsikova, 2009a). Homologues of this gene in other mammals, including the dog, have not been identified as yet. Another example of an oocyte gene is *WEE1B*, which controls the production of the Wee1B protein in the pig. The pig Wee1B protein, which has kinase activity, catalyses the inhibitory phosphorylation of CDC2 protein, which leads to meiotic arrest in oocytes of mammals such as the pig. The inactivation of the *Wee1B* gene, in combination with other factors, leads to the resumption of meiosis

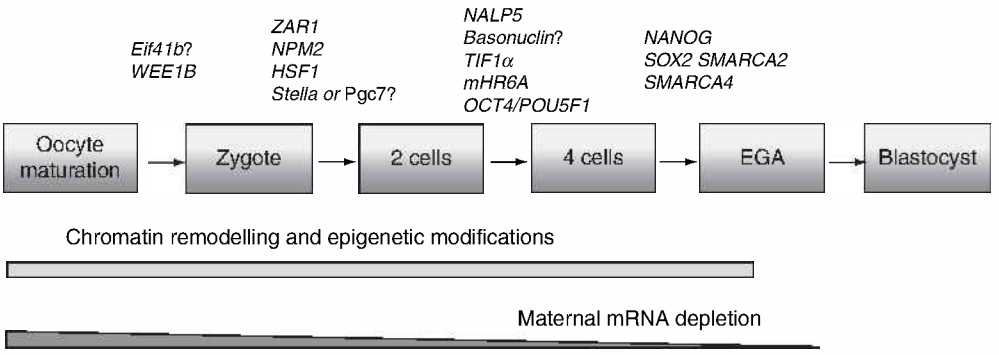

Fig. 15.3. Current knowledge on the genetic regulation of oocyte-to-embryo transition in mammals that is relevant to canine development. Redrawn from Bettegowda *et al.* (2008), with modifications compiled from several sources, including Magnani and Cabot (2008, 2009), Evsikov and Marín de Evsikova (2009b) and Shimaoka *et al.* (2009). Question marks indicate those genes for which activities have not as yet been confirmed in mammalian embryos. EGA, embryonic genome activation.

(Shimaoka *et al.*, 2009). In mature oocytes, the degradation of maternal transcripts becomes more prominent and seems to be nearly completed by the two-cell stage, when the so-called minor zygotic genome activation takes place. In fact, the *ZAR1* (zygote arrest 1) gene is one of the few known oocyte-specific maternal-effect genes essential for the beginning of embryo development (Wu *et al.*, 2003). In the dog, *ZAR1* is located at chromosome 13, consists of four exons and encodes a protein with 104 amino acids (see the *Ensembl* Genome Browser at www.ensemble.org). There are significant differences in the structure and length of this gene among vertebrates, including mammals. Such differences could create some distinctions during the earliest stages of development in mammalian species. It seems surprising that at least some *Zar1*(–/–) mice are viable and look normal. However, *Zar1*(–/–) females are infertile, probably as a result of the arrest of embryonic development in the majority of zygotes at the one-cell stage and also because maternal and paternal genomes remain separate in such zygotes. These *Zar1*(–/–) embryos show a marked reduction in the synthesis of the transcription-requiring complex, with fewer than 20% of them progressing to the two-cell stage, and none developing to the four-cell stage (Wu *et al.*, 2003). The Zar1 protein plays a role in transcription regulation during oocyte maturation and early post-fertilization development (Uzbekova *et al.*, 2006). Direct study of *ZAR1* in the dog might be useful for a better understanding of the early steps of canine development. Cytoplasmic and nuclear determinants of the maternal-to-embryonic transitions are described by Bettegowda *et al.* (2008).

The formation of zona pellucida (ZP) around the oocyte is an essential process. Studies of the zona proteins (at least three major glycoproteins, ZPA, ZPB and ZPC), in the dog have indicated that biosynthesis of the canine zona pellucida requires the integrated participation of both oocytes and granulosa cells. In the juvenile canine ovary, the oocyte is responsible for synthesis of the ZPA protein, for directing synthesis of the ZPB and ZPC proteins by the granulosa cells and for ensuring that transcription of the ZP genes occurs in a sequential manner during folliculogenesis (Blackmore *et al.*, 2004).

The total RNA content in the zygote and in early mammalian blastomeres is commonly much higher than in somatic cells. The oocyte and the following early stages of zygote development are able to synthesize polypeptides in the absence of active transcription. In the dog, as in other mammals, the embryonic genome seems to be inactive until after the four-cell stage, approximately 2–2.5 days after fertilization, when the marked change from maternally to embryonically controlled protein synthesis takes place (Harvey *et al.*, 1993). It is likely

that rather minor differences between mammalian species can be observed in the timing of gene activation and transcription events.

Leptin (a cytokine) and STAT3 (a member of the signal transducer and activation of transcription family of proteins) possibly play a role in early mammalian development, being involved in determination of the animal pole of the mammalian oocyte and in the differentiation of the trophoblast and inner cell mass (Antczak and Van Blercom, 1997). Later, at the morula stage, the 'inner' blastomeres contain little, if any, leptin/STAT3 while the 'outer' blastomeres contain both leptin and STAT3 rich and poor cells (Antczak and Van Blercom, 1997). The first divisions of the mammalian embryo are largely controlled by proteins and transcripts stored during oogenesis and oocyte maturation, and canine embryonic development seems to be no different in that sense. In several mammal species, zygote genome activation (ZGA) occurs after the four-cell stage, while in the dog it appears at the later eight-cell stage (Chastant-Maillard *et al.*, 2009). The nucleoli, which are essential for ribosomal RNA (rRNA) and ribosome production, then develop in order to support protein synthesis. After fertilization, structures resembling the nucleolar remnant exist in the pronuclei and are engaged in the re-establishment of fibrillo-granular nucleoli during the major activation of the embryonic genome (Maddox-Hyttel *et al.*, 2007).

Invertebrates rely on gradients of morphogens in the zygote and early embryo to establish positional information (St Johnston and Nüsslein-Volhard, 1992; Nüsslein-Volhard, 1996). Such gradients are essentially products of maternal gene expression. To what degree similar gradients and elements of the cytoskeleton are important during the earliest stage of mammalian development is still under investigation. In species other than dog, cell polarity has been described at the eight-cell stage of development (Reeve, 1981; Gueth-Hallonet and Maro, 1992). Cell fate, controlled by positional information, seems reversible and provides the developing embryo with a certain degree of flexibility. Although polarity of the post-implantation embryo can be traced back to the eight-cell stage, the role of the oocyte and its organization is not entirely clear (Ciemerych *et al.*, 2000). In dogs, like other mammals, future axis specification probably starts from the early stages of cleavage. This is unlike what is observed in other metazoans and may be related to viviparity (Evsikov and Marin de Evsikova, 2009b). If so, then the gradients that are so important in insects and worms may not be crucial for the very early stages of mammalian development. The establishment of axial polarity during cleavage and blastocyst formation is considered later in this chapter.

Activation of the embryonic genome

The first wave of embryonic genome activation in the mouse, the so-called minor activation, occurs primarily in a male pronucleus up until the two-cell stage, and results in the synthesis of only a few specific polypeptides. The second wave, starting from the two-cell stage, leads to massive changes in the gene expression pattern. Data relevant to murine development show that the most significant activation of the embryonic genome takes place at both the two-cell and (particularly) at the four-cell stages. Many hundreds of other genes remain continually active during this period as well. In canine embryos the transition to more intensive transcription is probably shifted towards the four-cell stage. Transcriptional dynamics of some porcine embryonic genes at very early stages provide useful information. For instance, the *ZP3* and *ZP4* genes that code for the major components of the mucoprotein layer of the zona pellucida have very high levels of expression in the germinal vesicle oocyte and progressively decline at the four-cell and blastocyst stages. A similar pattern, slightly shifted towards the blastocyst stage, was observed for *DNMT* genes that are responsible for the DNA methyltransferase involved in the regulation of transcription and genomic imprinting (Ko *et al.*, 2005; Jeong *et al.*, 2009). Another porcine gene, that for prothymosin alpha, which is involved in chromatin remodelling, among other functions, peaks at the four-cell stage and then steadily declines. Expression of the dihydrolipoamide dehydrogenease gene increases from the oocyte to the four-cell and blastocyst stages, thereby reducing lipid and protein peroxidation. Published data also point out that the *SMARCA2* and *SMARCA4* genes, which are

active at early stages of development, play essential roles in controlling the expression of other genes during early mammalian embryogenesis (Magnani and Cabot, 2009).

Significant activation of transcription is an essential prerequisite for the following intensification of translation. *RPL23*, one among 80 genes controlling ribosomal proteins, activates in the porcine and probably in other mammalian embryos at the blastocyst stage before a major increase in translation (Whitworth *et al.*, 2005). Bjerregaard *et al.* (2004) demonstrated in the pre-implantation embryo nucleolus-related gene expression leading to synthesis of several proteins involved in rRNA transcription (upstream binding factor, *UBF-1*; topoisomerase I, *TOP-1*; RNA polymerase I, *POLR1*; and RNA Pol I-associated factor PAF53, *POLR1E*) and processing (fibrillarin, *FBL*; nucleophosmin, *NPM1*; and nucleolin, *NCL*). The first significant activation of the genes was observed at the four-cell stage and it then increased significantly towards the blastocyst stage. Another marker that has been used to characterize genome activation is elongation initiation factor 1A mRNA (*eIF1A*). Magnani and Cabot (2008) observed activation of *eIF1A* at the two-cell stage in porcine embryos. As previously stated, activation of the embryonic genome occurs at the four-to-eight cell stage, while the dominant role of embryonic genes is established only after gastrulation (de Vries *et al.*, 2008).

Reprogramming, methylation pattern and genomic imprinting

During the first 24 h or so after fertilization the mammalian oocyte and sperm undergo natural reprogramming that gives rise to a totipotent zygote (de Vries *et al.*, 2008). Genomic reprogramming is a complex process that involves numerous mechanisms. Protein and mRNA molecules that have accumulated in the oocyte facilitate reprogramming through chromosome remodelling as well as differential utilization and degradation of mRNA. Epigenetic reprogramming occurs on a genome-wide scale that includes DNA demethylation and remodelling of histones. The mechanisms of genome-wide erasure of DNA methylation, which involve modifications to 5-methylcytosine and DNA repair, are being unravelled (Feng *et al.*, 2010). Epigenetic reprogramming has important roles in imprinting, as well as in the acquisition of totipotency and pluripotency, the control of transposons and epigenetic inheritance across generations. Erasure of DNA methylation from chromatin very early during development creates critically important conditions for the next cycle of life. Incomplete epigenetic reprogramming is common for embryos generated by nuclear transfer and contributes to the low efficiency of the cloning procedure (Dean *et al.*, 2001).

The following developmental stages lead to the occurrence of pluripotent cell types with narrowed potential. Gene-expression programmes operating in the pluripotent cells steadily become more defined, production of core transcription factors begins and the expression of pluripotency-associated genes commences. At least three genes, *OCT4, NANOG* and *SOX 2*, which code for transcription factors, have been identified and are responsible for the activation of other genes essential for the maintenance of pluripotency and the repression of genes required for further differentiation (de Vries *et al.*, 2008).

Genes that are required later in development are repressed by histone marks, which confer short-term, and therefore flexible, epigenetic silencing (Reik, 2007). As soon as demethylation is accomplished, a new wave of DNA methylation begins and this leads to stable and long-term epigenetic silencing of certain genetic elements such as transposons, imprinted genes and pluripotency-associated genes. Whether such DNA methylation and epigenetic silencing marks play a key role in determining cell and lineage commitment still remains an open question (Reik, 2007). While practically nothing is known about reprogramming during canine development, there are no indications that the major features discovered in other mammalian species should be significantly different in the dog.

Gametic or genomic imprinting is a developmental phenomenon typical for eutherian mammals and is based on differential expression of maternal and paternal alleles in certain genes. These genes are essential for the regulation of embryonic and placental growth. In genes such as *IGF2*, only the paternal allele is expressed (maternal imprinting). On the contrary, in genes

like *H19*, only the maternal allele is expressed (paternal imprinting). Imprint acquisition occurs before fertilization and imprint propagation extends up until the morula–blastocyst stage (Shemer *et al.*, 1996). In *H19*, the 2 kb region is methylated on the paternal allele during spermatogenesis. The maternal allele has a different methylation pattern (Davis *et al.*, 1999). In pigs *H19* is exclusively expressed from the maternal allele in all major organs in a similar manner to that observed in other species. In contrast, the majority of *IGF2* transcripts are expressed paternally from promoters 2–4 (Li *et al.*, 2008).

The molecular mechanisms of gametic imprinting are still under investigation. It seems possible that the methylation pattern typical for imprinted genes establishes gradually during early development (Shemer *et al.*, 1996). The regulatory elements that control genomic imprinting have differential epigenetic marking in oogenesis and spermatogenesis, which results in parental allele-specific expression of imprinted genes during development and after birth (Feil, 2009). Both DNA and histone methylation are essential for imprinting. The latest data also show that DNA methylation is involved in the acquisition and/or maintenance of histone methylation at imprinting control regions (Henckel *et al.*, 2009; Feng *et al.*, 2010).

The developmental function of gametic imprinting is also not quite clear, but an explanation proposed by Moore and Haig (1991) is widely accepted. This is based on the concept of genetic conflict arising during pregnancy between maternally and paternally inherited genes. Thus, it is likely that gametic imprinting evolved in mammals to regulate intrauterine growth and to increase the safety of embryonic development. Lack of maternally or paternally derived alleles or abnormal expression of such alleles in a zygote may lead to embryonic mortality and impose strict requirements on the stability of imprinting signals. According to the database of imprinted genes (Catalogue of Parent of Origin Effects: Imprinted Genes and Related Effects, at: http://igc.otago.ac.nz/Search.html) there is only one imprinted gene (*IGF2R*) so far identified in the dog (O'Sullivan *et al.*, 2007). In comparison, in the pig more than 60 imprinted genes have been characterized. The total number of imprinted genes in the mammalian genome is likely to be between 100 and 200.

As shown recently, the chromatin regions that retain nucleosomes in sperm are likely to be protected from DNA methylation in the early embryo. This may indicate a connection between the presence of nucleosomes on the paternal genome and the establishment of gene regulation in the embryo (Vavouri and Lehner, 2011).

Gene expression during blastocyst formation and the TE–ICM split

At the morula stage of development, activation of a few selected genes is critical for the synthesis of morphogenetically important proteins such as actin and actin-associated proteins such as alpha-fodrin, vinculin and E-cadherin (Reima *et al.*, 1993). These molecules are distributed evenly in blastomeres during early cleavage, but then gradually accumulate towards the blastocyst stage in the regions of intercellular contact (Reima *et al.*, 1993). This seems to be essential for the development of tight gap junctions related to blastocoel formation and is particularly relevant to the outer cell layer.

As already outlined, formation of the blastocyst, including the blastocoel, ICM and trophoblast ectoderm (or trophectoderm, TE) within the zona pellucida, occurs 9–10 days after fertilization in canine development. Despite the simplicity of the blastocyst structure, the mechanisms of its formation are still elusive. Three models, mosaic, positional and polarization, have been suggested and extensively studied (Johnson, 2009). The ICM differentiates into the epiblast and the primitive endoderm. The epiblast gives rise to the embryo itself and also to some extra-embryonic tissues. The TE is responsible for development of the remaining extra-embryonic tissues and plays a critical role during implantation and formation of the trophoblast layers of the placenta.

In the dog, as in other mammals, the TE makes up the majority of the external cells of the blastocyst. The TE is critical for fetal and maternal contact, known as the trophoblast. Differentiation of the trophoblast begins in mammals probably as early as the morula stage and ultimately results in functionally diverse cells (Roberts *et al.*, 2004). It also has been

known since the early 1980s that TE cells are characterized by the preferential inactivation of the paternal X chromosome (Harper *et al.*, 1982). In the mouse, the *Rex3* gene appears to be capable of contributing to this process, being preferentially expressed from the maternal X chromosome in blastocysts. During later stages of embryonic development, and in adult tissues, either X chromosome can express the gene. Genomic imprinting during the pre-implantation period, as well as in the following stages, does not appear to be directly affected by *Rex3*. Such an expression pattern might somehow be related to preferential paternal X chromosome inactivation (Williams *et al.*, 2002). The cause of TE differentiation, and how this might be related to the preferential X chromosome inactivation, as well as what might be the leading factor, still need further research.

As in many other cases, much of the work on trophoblast gene expression has been done using the murine model (reviewed by Marikawa and Alarcón, 2009). Roberts *et al.* (2004) consider that the first step in trophoblast differentiation is the downregulation of *OCT4*, which normally acts as a negative regulator of genes required for further differentiation (de Vries *et al.*, 2008). *OCT4* acts in the pluriplotent ICM to silence genes related to differentiation, but, once this restraint is removed, the genes, as discussed below, can come under the control of transcriptional activators. Knofler *et al.* (2001) reviewed the other regulatory factors involved in trophoblast development and differentiation. Of these, the T-box gene *Eomes*, which is considered to be among the earliest trophoblast-determining factors in the pre-implantation embryo, is required for trophoblast differentiation (Russ *et al.*, 2000). Both Eomes and the homeodomain protein CDX2 are absent in the ICM, but are present in the TE (Beck *et al.*, 1995). *Cdx2* and *Eomes* murine knockout embryos fail to implant and only develop to the blastocyst stage (Chawengsaksophak *et al.*, 1997; Russ *et al.*, 2000).

The activation of early pluripotential stem cell genes appears to be strongly conserved across species. In the mouse, *Cdx2*, which encodes a caudal-related homeodomain protein, is a key regulator of the TE lineage (Rossant and Tam, 2009). The mouse gene *Tead4*, which produces transcription enhancer, is tentatively considered as an upstream factor relevant to *Cdx2*. *Eomes*, on the contrary, codes for a downstream-located factor (Rossant and Tam, 2009). Cdx2 and Eomes proteins are restricted to outer layer cells. The two genes required for specifying pluripotent cells, *Sox2* (Nichols *et al.*, 1998; Avilion *et al.*, 2003) and *Nanog* (Chambers *et al.*, 2003; Mitsui *et al.*, 2003) are initially expressed in all blastomeres, but progressively become restricted to the ICM cells after blastocyst formation. Activation of the *Tead4* gene (canine *TEAD4* is located at chomosome 27, consists of 14 exons, encoding a protein of 441 amino acids; see *Ensembl* Genome Browser) in the external TE-forming blastocyst cells (Nishioka *et al.*, 2008) seems to be critical for the activation of *Cdx2*, which in turn activates *Eomes* (Fig. 15.4). Canine *Eomes* is at chromosome 23, consists of six exons and encodes a protein of 688 amino acids (see *Ensembl* Genome Browser). This chain of events differs dramatically from those in internal ICM-forming cells, where *Oct4* activates *Nanog* and *Fgf4* genes. These genes have been identified in the dog: *POU5F1* (the *Oct4 equivalent*) is located at chromosome 12, consists of six exons and encodes a protein with 347 amino acids; *NANOG* is located at chromosome 27, consists of four exons and encodes a protein with 298 amino acids; *FGF4* is located at chromosome 18, consists of four exons and encodes a protein with 211 amino acids (see *Ensembl* Genome Browser).

Adjaye *et al.* (2005) identified in developing human blastocysts marker transcripts specific to the ICM (e.g. *OCT4/POU5F1, NANOG, HMGB1* and *DPPA5*) and TE (e.g. *CDX2, ATP1B3, SFN* and *IPL*). The polarity of cells in the blastocyst increases owing to an accumulation of protein kinase 3, polarity protein Par3, and ezrin in the apical domain of blastomeres and apical membrane; other proteins, such as Lg1 and Par1, are exclusively found in the basal portion of blastomeres (Rossant and Tam, 2009). Connexin proteins are differently expressed both temporally and spatially in the pig embryo, where they influence formation of gap junctions in the trophoblast and later control the exponential growth of the trophoblast in pre-implantation pig blastocysts (Fléchon *et al.*, 2004a).

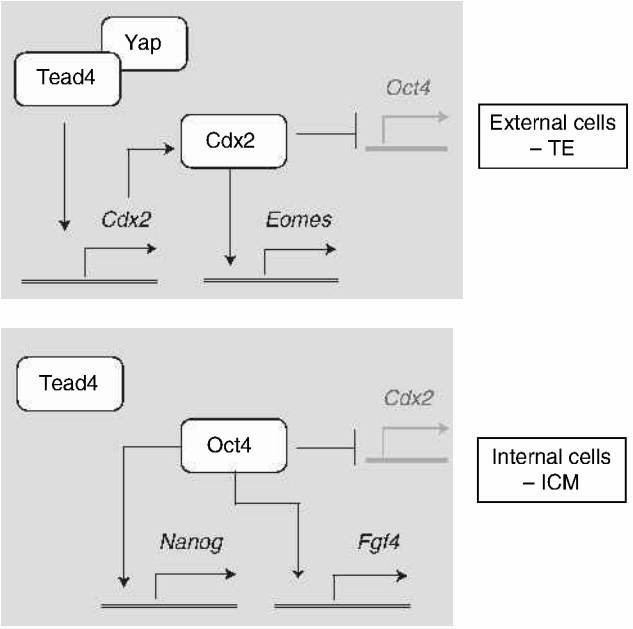

Fig. 15.4. Current views on the genetic regulation of the trophoblast ectoderm (TE)–inner cell mass (ICM) split in mammals. The unfilled curved rectangles represent proteins. Genes are shown above arrows in italic. Active genes are in bold italic, genes with a limited activity are in italic grey (redrawn with modifications from Marikawa and Alarcón, 2009).

Judging by changes found in other mammalian species, one may anticipate an increase in the number of intensively transcribed genes in the early developmental stages of a dog embryo. A comparison of bovine transcriptomes from blastocyst (D7) and conceptus (D14) revealed that ~500 genes were upregulated between these developmental stages and only 26 genes were downregulated (Ushizawa *et al.*, 2004).

Implantation and Maternal Recognition of Pregnancy

Implantation and placental development

There have been some historic morphological studies of the dog embryo implantation process and of the recognition of previous implantation sites (Barrau *et al.*, 1975). In the dog, knobs of trophoblast syncytia are formed and wedged between cells of the uterine luminal epithelium during early implantation (Barrau *et al.*, 1975). The process of invasion continues for about the next 10 days and by then the syncytial trophoblast that covered the tips of the villi has degenerated. Finally haematomas form by focal cell death by either necrosis or apoptosis of fetal and endometrial tissue at the poles of the implantation sites, large pools of extravasated blood accumulate and red blood cells are phagocytized by trophoblastic cells (Barrau *et al.*, 1975). This process makes former implantation and placentation sites visible for as long as even weeks after parturition. The zonary placenta, in which the chorionic villi are restricted to an equatorial band, is typical for the dog (Steven, 1975). A cross section of uterus in the area of zonary placenta is shown in Fig. 15.5. The placenta in the dog as, in most Carnivora, belongs to the endotheliochorial type (Steven, 1975). A detailed description of

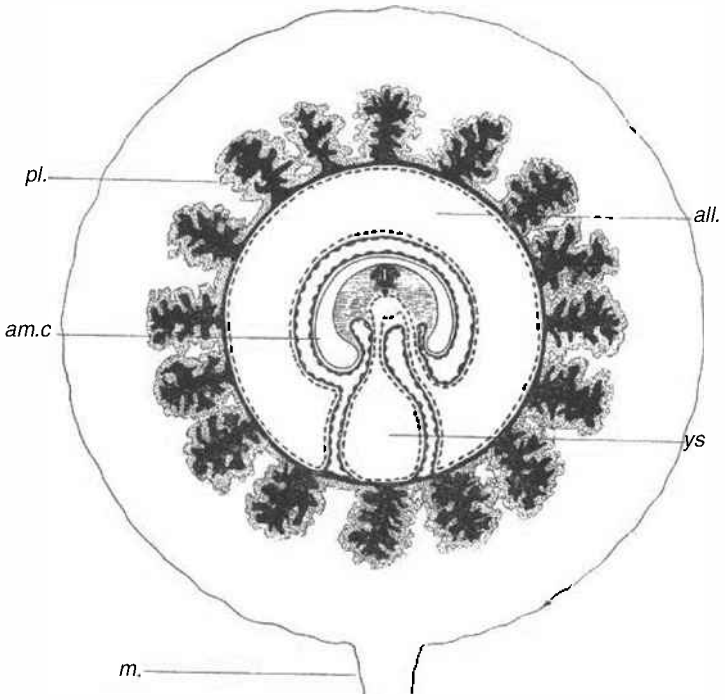

Fig. 15.5. Cross section of the dog uterus in the area of zonary placenta at 20–22 days after fertilization. Key: *all.*, allantois; *am.c.*, amniotic cavity; *m.*, mesometrium; *pl.*, zonary placenta; *ys.*, yolk sac. The yolk sac is small compared with the allantois and of no importance in the later stages of development, though it is essential at first. The embryo itself is shown as a black body surrounded by the amniotic cavity (reprinted from Jenkinson, 1925).

the placentation process in the bitch in the middle part of pregnancy can be found elsewhere (Grether *et al.*, 1998).

Maternal recognition of pregnancy

The maternal recognition of pregnancy and the associated processes of placentation are probably the most variable and divergent signalling mechanisms between species. In species such as the cow, sheep and pig, the recognition process involves a trophoblast-released interferon-tau (trophoblastin) and pregnancy-associated glycoproteins. In primates, a different protein, human chorionic gonadotrophin (hCG) has been identified as the key regulator. In the dog, the regulators have been less well identified (for a review, see Concannon *et al.*, 2001) and may not be directly comparable with the specific signals seen in these other species. Many research studies on this aspect of pregnancy now use murine development models, and some of the data cited here will refer to these studies. The common feature is that, either before, during or following implantation, trophoblast cells secrete signalling molecules that have local uterine endometrial effects as well as maternal endocrine effects on the corpus luteum within the ovary. The development of dog corpora lutea is initially in response to progesterone, which peaks at the time of implantation (Concannon *et al.*, 1989). The corpora lutea formed from the ovulated follicles remain and have an internally programmed lifespan (Meyer, 1994). Both corpora lutea and placentation spots usually disappear in canine females over the 3–4 months after parturition, and before the next pregnancy.

Trophoblast-produced hormones maintain progesterone production by acting directly or indirectly to maintain the corpora lutea. These hormones are also described as 'antiluteolytic' because they act on the endometrium to prevent uterine release of luteolytic prostaglandin F2 alpha (PGF) as well. Uterine stromal and myometrial cells are always progesterone receptor (PR) positive and may respond to progesterone by producing paracrine factors that regulate proliferation and/or differentiation functions of the endometrial epithelia during pregnancy (Spencer and Bazer, 2004).

Recent canine research has identified uterine expression of one of the homeobox transcription factors, protein Hoxa10, as a key component for endometrial receptivity to blastocyst implantation (Guo *et al.*, 2009). Endometrial expression of the transcription factor CCAAT enhancer-binding protein beta (C/EBPbeta) has been identified as a marker of uterine receptivity and is expressed in several species at the site of implantation (Kannan *et al.*, 2010). The uterine stromal cells at the site of the implanted embryo proliferate and undergo differentiation to form the 'decidual' tissue. C/EBPbeta acts as a mediator of the actions of oestrogen and progesterone during decidualization (Bagchi *et al.*, 2006), and is a critical regulator of steroid-induced mitotic expansion of uterine stromal cells during decidualization (Wang *et al.*, 2010). Decidualization results in the differentiation of fibroblast-like mesenchymal cells in the endometrium, leading to the formation of morphologically and biochemically distinct decidual cells that express prolactin and insulin-like growth factor binding protein 1.

Molecular signals affecting implantation and placentation

In various studies and animal models, the uterine expression and secretion of two IL-6 family cytokines, leukaemia inhibitory factor (LIF) and interleukin-11 (IL-11), are key components of implantation and decidualization (Hu *et al.*, 2007). LIF also has a role in uterine epithelial surface pinopod formation and in the loss of polarity that occurs in the receptive luminal epithelial cells (Yoshinaga, 2010). Furthermore, a cell surface Ca^{2+}-dependent adhesion molecule has been shown to be necessary for initial blastocyst adhesion in dogs (Guo *et al.*, 2010).

The dog, like many other mammalian species, is capable of multiple simultaneous pregnancies. Transuterine migration typically occurs in the dog bicornuate uterus and is used for the initial spatial distribution of embryos; it is not affected by the number of ovulated oocytes that occur in the right and left ovaries (Tsutsui *et al.*, 2002). The transuterine migration mechanism of embryos may also be different between dogs and other mammals. Several animal models now suggest that the distribution of embryo implantation within the uterus is also separately regulated by specific factors. For example, in mice, spacing regulators include the distribution of uterine glands (Hondo *et al.*, 2007) and a corresponding *Hoxa10* expression (Guo *et al.*, 2009). Another identified embryo spacing molecular signal is lysophosphatidic acid (LPA). This is a phospholipid derivative that acts through uterine cyclooxygenase-2 (COX-2). COX-2 in turn generates prostaglandins E(2) and I(2), which are closely related to the spatial positioning of embryo implantation (Ye *et al.*, 2005).

The expression of relaxin, a member of the insulin-like superfamily, has an important role in early dog pregnancy. Relaxin is first detected soon after implantation, and reaches peak level at 6–8 weeks of gestation (Steinetz *et al.*, 1987). It is also considered to be a marker of pregnancy in the dog (Steinetz *et al.*, 1989). Detailed investigation of canine preprorelaxin advanced the understanding of the protein and of its gene structure, and it was found that syncytiotrophoblast cells are the source of relaxin in the canine placenta (Klonisch *et al.*, 1999). Interestingly, the concentration of the acute-phase reactant protein fibrinogen increases soon after implantation in dogs at or just before the increase in relaxin, approximately starting from the day 22–23 of gestation (Concannon *et al.*, 1996). Serum C-reactive protein (CRP) concentration also increases significantly at about the same time, reaching peak activity after 30 days of gestation (Eckersall *et al.*, 1993).

The dog shows differences in endometrial hormonal control and sensitivity compared with humans. Oestrogens are significantly lower,

and progestogens stimulate the synthesis and release of growth hormone (GH), which together stimulate mammary growth (Johnson, 1989). Furthermore, even the non-pregnant bitch has both luteinizing hormone receptor and follicle stimulating hormone receptor expression in the lower urinary tract, suggesting that gonadotrophins have a role in the physiology of the lower urinary tract function in the dog as well (Ponglowhapan *et al.*, 2007).

Following implantation, trophoblast development appears to be regulated by several transcription factors. In mice, basic helix-loop-helix (bHLH) proteins such as Mash2 (ASCL2) are crucial in the specification of trophoblast lineage and particularly in sphongiotrophoblast development, and the gene is subject to genomic imprinting (Guillemot *et al.*, 1995). It is also known that developmental restrictions of *Mash2* (*ASCL2*) correlate with potential activation of the Notch2 signalling pathway (Nakayama *et al.*, 1997). Another transcription factor encoded by the *Hxt* gene is also expressed in the trophoblast and is considered to have a positive role in promoting the formation of trophoblast giant cells in the mouse (Cross *et al.*, 1995). This family of transcription factors also includes *Hand1*, which is important for trophoblast giant cell formation in the mouse. Mice lacking the *Hand1* gene show defects in the development of these cells (Riley *et al.*, 1998). *Hand1* expression may also be related to the regulation of *Mash2* (Scott *et al.*, 2000).

Various transcription factors that are widely expressed in embryonic, fetal and adult tissues seem to be necessary for placental development, as their deletion is consistently associated with trophoblast abnormalities. Like implantation, placental development varies widely between species though there are a few key common early transcription factors, such as: ETS2 (Yamamoto *et al.*, 1998), AP1 (Schorpp-Kistner *et al.*, 1999; Schreiber *et al.*, 2000) and AP2gamma (Auman *et al.*, 2002; Werling and Schorle, 2002). Schultz *et al.* (1997) also described the genetic determination of integrin trafficking, which regulates adhesion to fibronectin during differentiation of the mouse peri-implantation blastocyst. In addition, the regulation of several metalloproteinases and the corresponding genes may shed additional light on the process of implantation and further trophoblast development (Bass *et al.*, 1997). As the trophoblast forms and matures, it eventually produces a series of substances and hormones including, but not limited to, growth factors, interferons and pregnancy-associated glycoproteins; these will be discussed later.

Angiogenesis

The term angiogenesis refers to the formation of new vascular beds from pre-existing vessels in a multi-step process. Following implantation, there are substantial changes in vascularization of the uterus as well as in the function of the overall canine maternal cardiovascular system. There is evidence in several species that abnormalities of placental angiogenesis can affect both fetal growth and the success of pregnancy. Vascular growth in general is a balance between stimulating and inhibiting factors. The canine amniotic membrane, which surrounds the embryo, is initially avascular and then becomes vascularized by blood vessel formation in the internal allantoic membrane in late pregnancy. A recent study in mice has identified HOXA13 as an initiating factor for placental vascular patterning, acting on the expression of two pro-vascular factors coded by *Tie2* and *Foxf1* (Shaut *et al.*, 2008).

Both trophoblast and decidual natural killer (NK) cells are well-recognized components of the uterine signalling network with a proven ability to produce growth factors and cytokines that modulate endothelial cell responsiveness during pregnancy (Barrientos *et al.*, 2009). NK cells are short-lived terminally differentiated lymphocytes located within the decidualized endometrium. The key secreted factors are vascular endothelial growth factor (VEGF) (Chennazhi and Nayak, 2009) and its receptor FLT1, both of which have a wide tissue distribution in non-pregnant dogs (Uchida *et al.*, 2008). This growth factor has been more widely studied in canine models of tumour development than in angiogenesis within the uterus.

Implantation and uterine vascular growth both require extensive tissue remodelling. A key protein family identified in tissue remodelling

has been the matrix metalloproteinase (MMP) family of enzymes. MMPs are a large family of endopeptidases that both degrade many extracellular matrix proteins and process a number of bioactive molecules. These MMPs have been identified in many different species during placentation and vascular development. A recent study of MMP-2 and MMP-9 in the dog has shown specific patterns of expression in both the uterus and fetal blood vessels (Beceriklisoy *et al.*, 2007). MMP-9 appears to mainly remodel uterine glands. MMP-2 in the uterus was expressed in the endothelium and smooth muscles of blood vessels and the myometrium of pregnant and non-pregnant bitches. During placentation, MMP-2 is expressed mainly in fetal blood vessels and trophoblastic cells. Both of these MMPs have been identified as being expressed less in spontaneous canine abortions (Kanca *et al.*, 2011).

Genes Involved in Post-implantation Development

Genetic control of gastrulation

The exact details of gastrulation in the dog, as mentioned earlier, remain unknown and we can only reconstruct the order of events using knowledge from other mammalian species. On day 14–15 after fertilization, at the stage preceding gastrulation (pre-streak), a heightened rate of cell proliferation in the posterior section of the epiblast in the canine conceptus is expected. Expression of the *brachyury* (*T*) gene and migration of these cells (precursors of the primitive streak) begin (Fléchon *et al.*, 2004b). This gene belongs to the T-box family, which contributes greatly to tissue specification, morphogenesis and organogenesis (Müller and Herrmann, 1997). Two other key genes (*SOX17* and *NODAL*) also make an important contribution to the earliest stages of development (Hassoun *et al.*, 2009). The *brachyury* gene codes for a homeobox protein, whose very low expression in mammalian embryos can be detected as early as the blastocyst stage, and interacts with several genes, including the *goosecoid* (*GSC*) gene. The intensity of porcine *GSC* expression, for instance, becomes more pronounced in differentiating mesodermal cells that ingress from the epiblast via Hensen's node (van de Pavert *et al.*, 2001). In the dog embryo, similar events could be expected at 16–17 days of gestation. This process finally leads to formation of the mesoderm and embryonic endoderm. Overexpression of *goosecoid* may repress the *brachyury* gene and affect normal development (Boucher *et al.*, 2000). The activity of another gene, *OCT4*, in the mammals that have been studied is confined to the ICM and takes place at the hatched blastocyst stage. Following separation of the hypoblast, and formation of the embryonic disc, this marker of pluripotency is selectively observed in the epiblast. Progressive differentiation of the germ layers and somatic tissues leads to silencing of this gene, with the exception of the primordial germ cells (Vejlsted *et al.*, 2006).

In mammals, as shown for the pig, the migratory cells converge at the midline of the posterior part of the epiblast, which creates a thickened longitudinal band already mentioned and known as the primitive streak (Patten, 1948; van de Pavert *et al.*, 2001). At the pre-streak stage, which precedes gastrulation and migration of extra-embryonic mesoderm, the embryonic disc becomes polarized (Fléchon *et al.*, 2004b). The early primitive streak is characterized by both a highly pseudostratified epithelium with an almost continuous but unusually thick basement membrane, and the expression of *brachyury*, which is crucial for notochord development in all chordates examined; at least 44 notochord-expressed genes are its transcriptional targets (Capellini *et al.*, 2008). Expression of the *NODAL* gene is essential for axial patterning during early mammalian gastrulation, as well as for induction of the dorso-anterior and ventral mesoderm (Jones *et al.*, 1995). As gastrulation proceeds, the primitive streak extends anteriorly and at its distal end Hensen's node is developed; this is composed of a mass of epithelium-like cells without cilia (Blum *et al.*, 2007). Expression of the *goosecoid* gene is typical for these cells, which are the origin of the notochord. Later in development, this gene is expressed in the neural tube (Filosa *et al.*, 1997).

The next step in development is the so-called 'head process', which gives rise to

the notochord. The notochord is a rod-shaped structure which extends along the embryo and represents the initial axial skeleton; it plays an important role in induction of the neural plate, chondrogenesis and somite formation (Gomercic *et al.*, 1991). Development of the notochord in the canine embryo has not been studied. The only available data show that notochordal cells may persist into adulthood in some canine breeds, thus preventing intervertebral disc degeneration (Aguiar *et al.*, 1999). Clearly, activation of the nuclear genes responsible for basic morphogenetic rearrangements is a requisite for notochord formation and development. Glycoproteins comprise a core of the notochord, which has its cells encased in a sheath of collagen fibres. Two genes controlling notochord formation encode laminin b1 and laminin g1, and are essential for building the scaffold on which individual cells organize the rod-like structure typical of the notochord (Parsons *et al.*, 2002). Higher production of the integrin subunits that regulate interactions with collagens and laminin is known for notochordal cells (Chen *et al.*, 2006). In vertebrates, the notochord is replaced during development by the vertebral column. The notochord grows anteriorly from Hensens's node below the embryonic disc and is composed of cells derived from a certain kind of differentiating mesodermal cell that ingresses from the epiblast. Three genes mentioned earlier, *SOX17, NODAL* and *brachyury* (*T*) are involved in the early development of the axial structure during gastrulation (Hassoun *et al.*, 2009). According to Zorn and Wells (2009), the Nodal signalling pathway is necessary and sufficient for initiation of endoderm and mesoderm development, and is required for proper gastrulation and axial patterning. Nodal ligands are members of the TGFβ family of secreted growth factors. *NOTO* is another gene that is required for the formation of the caudal part of the notochord, as well as for ciliogenesis in the posterior notochord. The available data also show that *Noto* acts during murine development as a transcription factor upstream of *Foxj1* and *Rfx3*. According to Beckers *et al.* (2007), this genetic cascade is important for the expression of multiple proteins required for the formation and function of cilia. Later, these processes influence dorsal and ventral axis specification, and neural tube and spinal cord patterning.

As already noted, activation of the nuclear genes responsible for basic morphogenetic rearrangements is the prerequisite for notochord formation and development. The *T* gene, which was first described as the *brachyury* mutation in mice 80 years ago, is an important participant in the events required for differentiation of the notochord and formation of mesoderm during posterior development. The T protein is located in the cell nuclei and acts as a tissue-specific transcription factor (Kispert *et al.*, 1995). Similar observations have been made in domestic dogs. Bobtailed phenotypes are known for many breeds, but the causative mutation has been identified in the Pembroke Welsh Corgis, a short-tailed breed. This missense mutation (C189G) was identified in the *T* gene (Chr 1q23) and is located in a highly conserved region of the T-box domain. It alters the ability of the T protein to bind to its DNA target. Analysis of offspring from several independent bobtail × bobtail crosses indicates that the homozygous embryos are lethal (Haworth *et al.*, 2001). Recently, 17 additional breeds with this mutation were identified, suggesting a common origin (Hytönen *et al.*, 2009). Further investigation of 23 other dog breeds, in which natural bobtailed animals were known, showed that the C189G mutation was present in 17 of them. No dogs homozygous for this mutation were ever found in such breeds, and this is very much in tune with the initial discovery of a similar mutation in the mouse in the late 1920s. Normal embryonic development in the homozygotes is not possible after gastrulation. Interestingly, there were six breeds that did not carry the mutation despite having the short-tail phenotype. Most likely other genetic factors cause the phenotype in these breeds.

Cloning and sequencing of *T* gene led to the discovery of the T-box gene family, which is characterized by a conserved sequence called T-box (Bollag *et al.*, 1994). This ancient family of transcription factors, which underwent duplication around 400 million years ago, is common to all vertebrates (Ruvinsky and Silver, 1997). There are indications that several murine T-box genes are essential for the formation of different mesodermal cell subpopulations, and

that one of the T-box genes is essential for the development of early endoderm during gastrulation (Papaioannou, 1997). Involvement of the T-box genes *Tbx2–Tbx5* in vertebrate limb specification and development has also been demonstrated (Gibson-Brown *et al.*, 1998). Formation of the notochord leads to several key ontogenetic events, including induction of the neural tube and then the central nervous system. A putative morphogen, Shh, secreted by the floor plate and notochord, specifies the fate of multiple cell types in the ventral aspect of the vertebrate nervous system as well as in motor neurons. Shh, in turn, induces expression of the oncogene *Gli-1*, which affects later development of the dorsal midbrain and hindbrain (Hynes *et al.*, 1997). Expression of the *SHH* gene is also important for establishment of the ventral pole of the embryonic dorso–ventral axis (Echelard *et al.*, 1993). Unlike the notochord cells, other emerging mesodermal cells spread out more or less uniformly and give rise to numerous organs and structures.

Establishment of axial identity

The early blastocyst and even possibly the late morula have some degree of polarization which later may influence axial identity. Several genes that significantly contribute to the emerging polarity have been identified so far. Genes encoding ezrin, PAR family proteins and CDX2 are probably the key regulators of the process. Other proteins, such as CDC42, E-cadherin, β-catenin and Hippo are strongly involved in the process, and laminin and integrins play some role (Johnson, 2009). Development of the primitive streak and the notochord is the convincing demonstration that both anterior–posterior (AP) and dorso–ventral (DV) axes are strictly determined.

It has been known since 1924 that in vertebrates the Spemann organizer, which forms at mid-blastula stage, plays a crucial role as signalling centre for the DV axis specification. The Spemann organizer blocks the action of BMP4 (bone morphogenetic protein) by secreting several proteins, such as Noggin, Chordin, Nodal-related and Cerberus. Wnt signalling is strongly involved in the formation of the organizer. The signal transduction cascade is a complex system of interaction of several proteins, which prevent the degradation of β-catenin, which is essential for the following gene activation (Sokol, 1999). Extra-embryonic cells, known as the anterior visceral endoderm (AVE), which migrate from the distal to a more proximal region of the embryo, specify the AP body axis (Migeotte *et al.*, 2010). The AVE secretes inhibitors of the Wnt and Nodal pathways. Other essential regulators of cell migration are Rac proteins, which play a role in AVE migration. *Rac1* mutant murine embryos fail to specify an AP axis. AVE cells extend long lamellar protrusions that span several cell diameters and are polarized in the direction of cell movement. This represents a critical step in the establishment of the mammalian body (Migeotte *et al.*, 2010). *Cdx2* seems to be significantly involved in the integration of the pathways controlling embryonic axial elongation and AP patterning (Chawengsaksophak *et al.*, 2004). A number of other players in the Wnt signalling pathway have been discovered over the years, including *Axin*, mutations in which affect the development of axial skeleton and the tail in particular (Zeng *et al.*, 1997; Fagotto *et al.*, 1999).

The left–right (LR) axis may look as if it is an automatic consequence from the AP and DV axes, as it is perpendicular to both (Levin, 2004). However, the cause of LR asymmetry in vertebrates, and mammals in particular, is a complicated question. Levin (2004) compiled a long list of genes which may affect the symmetry. More recent findings show that, in the developing mouse embryo, leftward fluid flow on the ventral side of Hensen's node determines LR asymmetry. Morphological analyses of the node cilia demonstrated that the cilia stand not perpendicular to the node surface, but tilted posteriorly (Nonaka *et al.*, 2005) – a morphological asymmetry that can produce leftward flow. A genetic cause of left/right asymmetries of the internal organs in vertebrates is steadily becoming clearer. Gros *et al.* (2009) considered two possibilities. The initial asymmetric cell rearrangements in chick embryos create a leftward movement of cells around Hensen's node. This is relevant to the expression of *Shh* and *Fgf8* (fibroblast growth factor 8). The alternative is a passive effect of cell movements. It has also been shown that a Nodal-BMP signalling cascade drives left-right heart morphogenesis by

regulating the speed and direction of cardiomyocyte movement. (Medeiros de Campos-Baptista *et al.*, 2008). Interplay between two TGFβ ligands, GDF1 and Nodal, together with the inhibitors Lefty and Cerl2, provide the signals for the establishment of laterality. The protein APOBEC2, by blocking TGFβ signalling, is also involved in regulating left-right specification (Vonica *et al.*, 2011).

The three germ layers and their derivates

By the end of gastrulation, three germ layers are established: endoderm, mesoderm and ectoderm. Molecular mechanisms driving this highly complex combination of processes began to emerge relatively recently. Zorn and Wells (2009) published one of the first reviews covering the entire process of endoderm development and organ formation. Here we can highlight only the major regulatory systems influencing the variety of genes and processes involved in endoderm morphogenesis and the formation of certain organs. The Nodal signalling pathway is necessary and sufficient to initiate ectoderm and mesoderm development, and itself is influenced by the canonic WNT/β-catenin pathway (Zorn and Wells, 2009). High-level Nodal signalling supports endoderm development and lower activity specifies mesoderm identity. The activity of the Nodal pathway is controlled by an auto-regulatory loop. Several genes in different vertebrates that are involved in the pathway, such as Nodal, have conserved Foxh1 DNA-binding sites in their first introns, sustaining the high activity essential for endoderm development. Conversely, in developing ectoderm, a negative feedback of Nodal activity is caused via the transcriptional target Lefty (Shen, 2007). Soon after gastrulation, the endoderm germ layer forms a primitive gut tube, which leads to organ specification, then to the formation of organ buds and, finally, to more specialized cell lineages (Zorn and Wells, 2009).

Developmental events in the mesoderm and ectoderm progress simultaneously, but independently, with significant interactions between derivates from the germ layers. As is well known, many organs have cellular components originating from different germ layers. Certain genes play a key role in the earliest stages of germ-layer development. For instance, the *Eed* gene, initially identified in mice, is critical for embryonic ectoderm development (Sharan *et al.*, 1991), as deletion of this gene prevents formation of ectoderm. Highly homologous genes have been found in other mammals, including the dog. The murine *brachyury* (*T*) gene is crucial for mesoderm development. Mice homozygous for mutant alleles of the *T* gene do not generate enough mesoderm, and show severe disruption in morphogenesis of mesoderm-derived structures, in particular the notochord (Wilkinson *et al.*, 1990). In mice, one of the T-box genes, *Tbx6*, is implicated in the development of paraxial mesoderm (Chapman *et al.*, 1996). *Tbx6* transcripts are first detected in the gastrulation-stage embryo in the primitive streak and in newly recruited paraxial mesoderm.

FOXI3 was identified as a regulator of ectodermal development in the dog. This gene encodes a previously uncharacterized member of the forkhead box transcription factor family (FOXI3), which is specifically expressed in developing hair and teeth. The Mexican Hairless Dog and Peruvian Hairless Dog, as well as the Chinese Crested Dog, are characterized by missing hair and teeth, a phenotype termed canine ectodermal dysplasia (Drögemüller *et al.*, 2008).

Development of segment identity and *HOX* genes

Segmentation observed in different groups of animals, and particularly in vertebrates, has deep evolutionary roots. Segments with common origin remain relatively separate during development, causing diversification and specialization. This evolutionary developmental strategy has been commonly used for the creation of morphological structures or groups of cells with distinct features. For instance, the development of two major structures, the ectodermal neural tube and the paraxial mesoderm, depends on segmentation. The first is critical for development of the hindbrain, the head process and the spinal cord. The second is

essential in the generation of somites, which give rise to the axial skeleton and skeletal muscles. While direct embryological observations are very limited in the dog, a comparative approach allows the assumption that the first somites can be seen in the middle of the closing neural groove and that their number increases anteriorly. The genetic and cellular processes driving segmentation depend on the expression patterns of *HOX* genes (Alexander *et al.*, 2009).

The homeotic genes, which encode helix-turn helix transcription factors, were first described in *Drosophila* as the primary determinants of segment identity. They all contain a conservative 180 bp DNA sequence motif named the homeobox, which has already been mentioned. Comparative analysis of the *Drosophila* homeotic gene complex, called *HOM-C*, and the mammalian homeobox genes, called the *HOX* complex, demonstrates a striking case of evolutionary conservation. The *HOX* gene family determines a set of transcription factors crucial for the development of axial identity in a wide range of animal species (Maconochie *et al.*, 1996). Figure 15.6 shows the remarkable similarity and collinearity existing in the molecular anatomy of the insect and mammalian *HOX* complexes. The main difference is the number of complexes per genome. In insects, there is only one, while mammals and other higher vertebrates have four separate chromosome clusters (Alexander *et al.*, 2009). There are 39 *HOX* genes in mammalian genomes, which belong to 13 paralogous groups. The *HOX* genes are expressed in segmental fashion in the developing somites and central nervous system, and each *HOX* gene acts from a particular anterior limit in a posterior direction. The anterior and posterior limits are distinct for different *Hox* genes (Fig. 15.6). A hallmark of *HOX* genes is a correlation between their linear arrangement along the chromosome and the timing and AP limits of their expression during development (Alexander *et al.*, 2009). *HOX* genes determine AP positional identity within the paraxial and lateral mesoderm, neuroectoderm, neural crest and endoderm.

Thus, the vertebrate body plan is, at least partially, a result of the interactions of *HOX* genes that provide cells with the essential positional and functional information. Signals from the *HOX* genes force embryonic cells to migrate to the appropriate destination and generate certain structures. Major signalling pathways such as the fibroblast growth factor (Fgf), Wnt and retinoic acid (RA) pathways play important roles in affecting expression of different *HOX* genes in different developmental conditions. The expression of RA and its protein binding ability, as well as its other functions in development of the mammalian conceptus, have been described (Yelich *et al.*, 1997). RA can affect the expression of *HOX* genes, and there is a 5′ to 3′ gradient in responsiveness of the genes to retinoids (Marshall *et al.*, 1996). RA acts via its receptors, which comprise two families, RAR and RXR, which are members of the ligand-activated nuclear receptor superfamily. These receptors interact to form complexes which, in turn, regulate target gene binding to retinoic acid response elements (RAREs). These RAREs are found in the 5′ regulatory regions of the murine *Hox* genes and in those of other mammals. *HOX* genes have a profound influence on the whole array of developmental process and the establishment of segment identity.

Pattern formation

Early pattern formation occurs following implantation and is similar for all vertebrates. Two essential patterning processes are gastrulation – forming the trilaminar embryo, and axis formation – developing the notochord. These processes, which were described earlier, establish embryo patterns for intermediate structures that are transformed during later embryonic development – the overt differentiation of tissues and organs described as organogenesis. During the late embryonic period, musculoskeletal patterning also occurs in the body, head and limbs. A few examples of embryo patterning in musculoskeletal, neural and renal development are given below, and common mechanisms used throughout the embryo are identified.

Limb patterning begins with establishing position on the trunk, and limb identity, by T-box genes (Rodriguez-Esteban *et al.*, 1999).

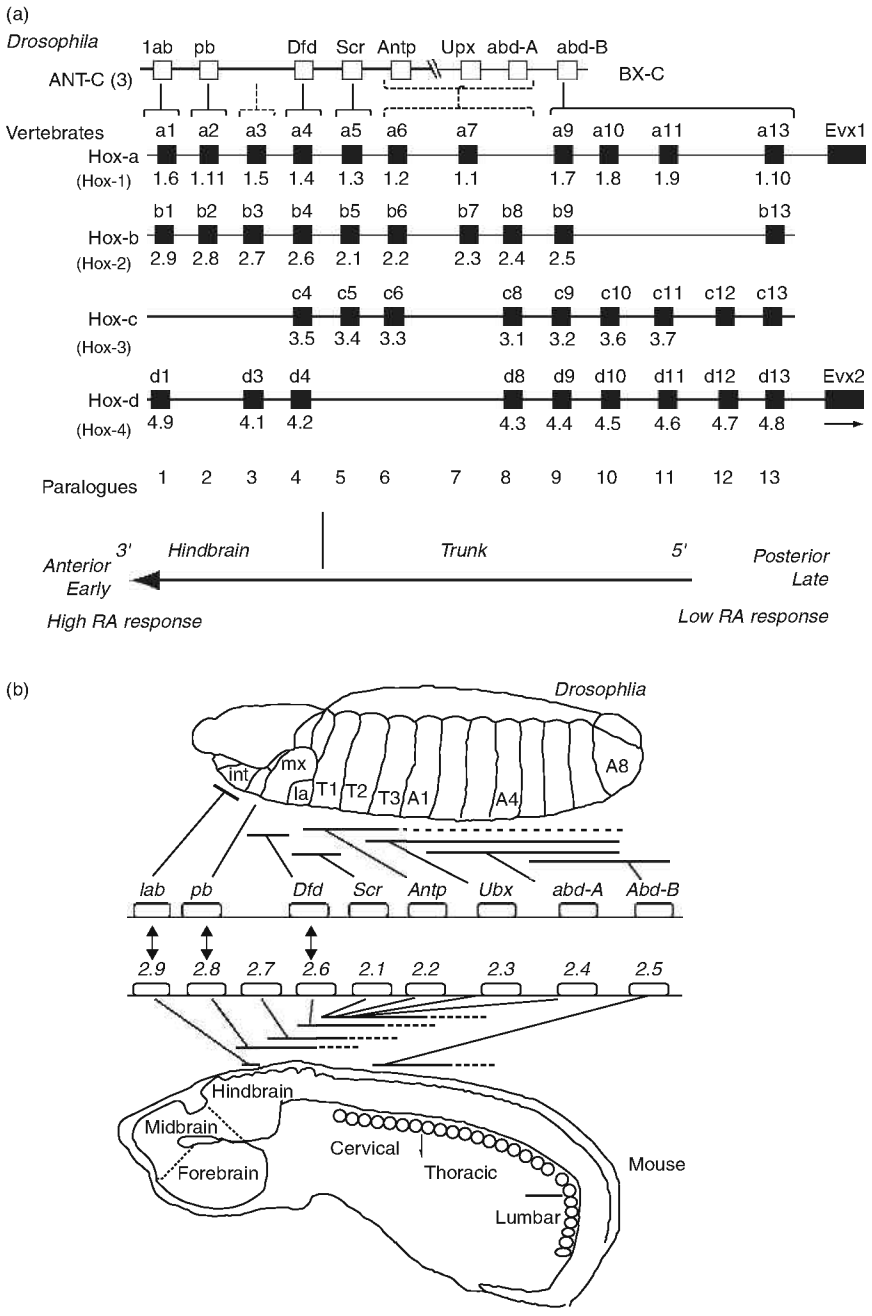

Fig. 15.6. The homeotic complexes of *Drosophila* (*HOM-C*) and the mouse (*Hox*). (a) Alignment of the four mouse *Hox* complexes with that of the *HOM-C* gene complex (*ANT-C* and *BX-C* clusters) from *Drosophila*. The vertical shaded boxes indicate related genes. The 13 paralogous groups are noted at the bottom of the alignment. The collinear properties of both the *Hox* complexes with respect to timing of expression, anteroposterior (A–P) level and retinoic acid (RA) response are also noted at the bottom. From Maconochie *et al.* (1996), with the author's permission. (b) Summary of *Drosophila HOM-C* and mouse *Hox-2* (*Hox-b*) expression patterns. The upper part of the figure is a diagram of a 10 h *Drosophila*

It has been shown that murine *Tbx5* and *Tbx4* expression is primarily restricted to the developing forelimb and hindlimb buds, respectively (Agarwal *et al.*, 2003). These two genes appear to have been divergently selected in vertebrate evolution to play a role in the differential specification of forelimb (pectoral) versus hindlimb (pelvic) identity (Gibson-Brown *et al.*, 1998). Mutations in the human *TBX3* gene cause the ulnar-mammary syndrome, which is characterized by posterior limb deficiencies or duplications, mammary gland dysfunction and genital abnormalities. It has been suggested that *TBX3* and *TBX5* evolved from a common ancestral gene and that each has acquired specific, yet complementary, roles in patterning the mammalian upper limb (Bamshad *et al.*, 1997). *Tbx4* and *Tbx5* are essential regulators of limb outgrowth whose roles seem to be tightly linked to the activity of signalling proteins that are required for initial limb outgrowth – the fibroblast growth factors Fgf4 and Fgf8 (Boulet *et al.*, 2004). Forelimb and hindlimb bud musculoskeleton patterning occurs by similar mechanisms; both are structurally mesoderm (somatic) proliferating cells within a covering ectoderm. The ectoderm at the limb tip is thickened by *Wnt7a*, forming an apical ectodermal ridge (AER) that expresses FGF-encoding genes (*Fgf8, Fgf4, Fgf9, Fgf17*) stimulating underlying mesoderm proliferation and establishing the limb proximodistal axis (reviewed by Towers and Tickle, 2009). Parker *et al.* (2009) made an exciting discovery relevant to *FGF4* and chondrodysplasia in the dog. To quote them:

> Retrotransposition of processed mRNAs is a common source of novel sequence acquired during the evolution of genomes. Although the vast majority of retroposed gene copies, or retrogenes, rapidly accumulate debilitating mutations that disrupt the reading frame, a small percentage become new genes that encode functional proteins. By using a multi-breed association analysis in the domestic dog, the authors demonstrate that expression of a recently acquired retrogene encoding fibroblast growth factor 4 (fgf4) is strongly associated with chondrodysplasia, a short-legged phenotype that defines at least 19 dog breeds including dachshund, corgi, and basset hound. These results illustrate the important role of a single evolutionary event in constraining and directing phenotypic diversity in the domestic dog.

The ventral mesoderm forms a region known as 'the zone of polarizing activity' (ZPA), which secretes sonic hedgehog (Shh), competing with BMP4 and establishing the DV axis (Kicheva and Briscoe, 2010). Abnormalities such as canine preaxial polydactyly (PPD), a developmental trait that restores the missing digit lost during canine evolution, is due to a ZPA-related change in an intronic sequence of *LMBR1* gene (Park *et al.*, 2008).

Differentiation of mesoderm-derived musculoskeletal tissues (cartilage, bone and muscle) is the same throughout the embryo and is regulated by tissue-specific pathways. Chondrogenesis of the mesoderm forms cartilage template structures in the position of, and replaced by, future bone formation, which is described as endochondral ossification (Goldring *et al.*, 2006). In the adult, this cartilage remains only on the articular surfaces of the bony skeleton. Chrondrogenesis transcription factors determined by the *Znf219, Sox9* and *Runx2* genes interact with secreted factors (Indian hedgehog, parathyroid hormone-related peptide, FGFs) to determine whether the differentiated chondrocytes remain within cartilage elements in articular joints or undergo hypertrophic maturation before ossification (Cheng and Genever, 2010). Endochondral ossification occurs in all bones except the cranial vault and scapula. Death of chondrocytes releases VEGF, stimulating vascular growth and the deposition of osteoclasts that further erode cartilage and osteoblasts, which ossify the previous cartilage template region (Mackie *et al.*, 2008). The transcription factors

Fig. 15.6. Continued.

embryo with projections of the expression patterns of different genes from the *HOM-C* complex to particular body segments. The lower part of the figure is a diagram of a 12-day-old mouse embryo with projections of expression patterns of different genes from the *Hox-2* complex to particular body segments (from McGinnis and Krumlauf, 1992, with the authors' permission).

coded by *Runx2* and *Runx3* are essential for chondrocyte maturation, while *Runx2* and *Osterix* are essential for osteoblast differentiation. Osteogenesis is inhibited by Wnt signalling pathway antagonists, including genes like *DKK1, SOST* and *SFRP1* (Fujita and Janz, 2007). Mutations in the canine collagen genes that form bone matrix can lead to abnormal bone formation, such as osteogenesis imperfecta (Campbell *et al.*, 2001). The processes of chondrogenesis and osteogenesis are repeated again at the periphery or at bone ends during postnatal skeletal growth, until fusion of the epiphyseal plates.

Myogenesis forms skeletal muscle from somite myotome-derived mesoderm. Muscle regulatory transcription factors like MyoD, Myf6 and Pax7 control differentiation (Ropka-Molik *et al.*, 2011). The first two of these are bHLH transcription factors that initiate the formation of muscle fibres and regulate transcription of muscle-specific genes. MyoD needs to form a dimer to be active and is maintained in an inactive state by binding of the inhibitor Id. *Pax7*, as a member of the paired-box transcription factors, is also required for muscle growth, and for both renewal and maintenance of muscle stem cells. Studies have shown that the *Lbx1h* gene is also involved in the regulation of muscle precursor cell migration, and is necessary for the acquisition of the dorsal identities of forelimb muscles (Schafer and Braun, 1999). There are several mutations in the dog that cause abnormalities similar to those observed in humans. An autosomal recessive myotonia congenita due to a mutation in canine *CIC-1* (skeletal muscle voltage-dependent chloride channel) is the result of replacement of a threonine residue in the D5 transmembrane segment with methionine (Rhodes *et al.,* 1999; Bhalerao *et al.*, 2002) A canine X-linked muscular dystrophy, homologous to human Duchenne muscular dystrophy, also occurs and provides evidence of the genes involved in muscle development in dogs (Lanfossi *et al.*, 1999). An alternative to dystrophy caused by mutations on the myostatin gene (*MSTN*), and called double muscling, was described in several mammalian species. Recently a very interesting case was investigated in the dog: a new mutation in *MSTN* found in the Whippet that results in a double-muscled phenotype known as the 'bully' whippet. Individuals with this phenotype carry two copies of a two-base-pair deletion in the third exon of *MSTN*, leading to a premature stop codon at amino acid 313. Individuals carrying only one copy of the mutation are, on average, more muscular than wild-type individuals, and are significantly faster than individuals carrying the wild-type genotype in competitive racing events. These results highlight the utility of performance-enhancing polymorphisms, and mark the first time that a mutation in *MSTN* has been quantitatively linked to increased athletic performance (Mosher *et al.*, 2007; Fig. 22.2).

Patterning of the ectoderm-derived neural tube, forming the central nervous system, is initially centralized by the notochord secreted Shh and dorsalized secreted bone morphogenic protein (BMP) as well as the Wnt signalling (Ulloa and Martí, 2010). Factors affecting the spread of Shh include Shh receptor Patched 1 (Ptc1), Hedgehog interacting protein (Hhip1), and the proteins Cdo, Boc and Gas1 (Ribes and Briscoe, 2009). The rostrocaudal pattern is mediated by the homeobox (*Hox)* gene family, differentially expressed along the neural tube and within the neural crest (Mallo *et al.*, 2010). At about the same time, two genes, *Otx2*, expressed in the forebrain and midbrain, and *Gbx2,* expressed in the anterior hindbrain, play an essential and interactive role in the positioning of the mid/hindbrain junction. This junction acts as an organizer, directing development of the midbrain and anterior hindbrain (Millet *et al.*, 1999).

Patterning of the intermediate mesoderm-derived kidney occurs through a series of epithelial to mesenchymal inductive interactions, with patterning signals by Wnts, BMPs, FGFs, Shh, the Ret/glial cell-derived neurotrophic factor and Notch pathways. Overt renal differentiation requires the specific transcription factors Odd1, Eya1, Pax2, Lim1 and Wt-1 (Reidy and Rosenblum, 2009). Developmental and adult canine renal diseases include abnormalities affecting the glomeruli, tubules, interstitium, pelvis and vasculature, and renal tumours (Yhee *et al.*, 2010).

PAX genes code nuclear transcription factors and contain the so-called 'paired domain', a conserved amino acid motif with

DNA-binding activity. These genes are the key regulators of development in organs and structures such as the kidney, eye, ear, nose, limb muscles, vertebral column and brain. Vertebrate *PAX* genes are involved in pattern formation, possibly by determining the time and place of organ initiation or morphogenesis (Dahl *et al.*, 1997). Murine *Pax-1,* for instance, is a mediator of notochord signals during the DV specification of vertebrae (Koseki *et al.*, 1993). The *Pax-3* gene may mediate activation of *MyoD* and *Myf-5*, the myogenic regulatory factors, in response to muscle-inducing signals from either axial tissues or overlying ectoderm, and may act as a regulator of somitic myogenesis (Maroto *et al.*, 1997).

Neoteny

The enormous variability and morphogenic plasticity of the dog still remains a fundamental question. A specific type of selection during domestication is certainly the cornerstone of any explanation for this exciting phenomenon (see Chapter 2). It was observed long ago that adult dogs show numerous traits typical of wolf puppies, which include morphological and behaviouristic features. Since then, the idea that neoteny was involved in the spectacular developmental changes in dogs has been discussed. Coppinger and Schneider (1995) extensively discussed this issue. A recent study of transcriptional neoteny in the human versus the chimpanzee brain provided a significant insight into human evolution (Somel *et al.*, 2009) and shed light on the probable changes that might occur during dog domestication. It was discovered that mRNA expression in the prefrontal cortex of humans and chimpanzees is likely to determine human-specific neotenic changes. The brain transcriptome is dramatically remodelled during postnatal development and developmental changes in the human brain are indeed delayed relative to other primates. This delay is not uniform across the human transcriptome, but affects a specific subset of genes that play a potential role in neural development (Somel *et al.*, 2009). Something similar is probably occurring in the dog.

Retardation of some developmental processes and the acceleration of others seems to be the essence of dramatic differences between the dog breeds. This hardly can be achieved without certain changes in gene activities during development, which might be a result of modification in the activity of a few highly influential genes involved in the production of key hormones and other biologically active molecules (Belyaev, 1979; Trut, 1999). The long-term experiment with fox domestication described in Chapter 2 provides unique opportunities to study such potential changes in gene activity during development (Trut *et al.*, 2004). One might think too that contrasting dog breeds could also provide an excellent model for further investigations in the field.

Sex Differentiation

The major steps in gonad differentiation

Normal sexual development in mammals requires a series of steps, which occur under genetic control. Three major steps are usually recognized: initial sex determination during fertilization, gonadal sex and phenotypic sex.

The earliest stages of gonadal development in mammals occur similarly in XX and XY embryos. Primordial germ cells, which differentiate relatively late in mammals, migrate into the gonads of either presumptive sex indiscriminately, and may function even across a species barrier (McLaren, 1998, 1999). Assuming that gonadal development in the dog does not strongly deviate from that in the mouse and in other mammals, one may expect that a few dozen germ cells, originating from the proximal region of the embryonic ectoderm, start their journey inside the embryo along with the invaginating hindgut. Expression of the *Bmp4* gene in the murine trophectoderm layer, which is in closest contact with the epiblast, influences the differentiation of both the primordial germ cells and the allantois (Lawson *et al.*, 1999). Due to ongoing proliferation, a significant number of germ cells reach the genital ridge, which consists of a thin layer of mesenchymal cells located between the coelomic epithelium and the mesonephros. Two genes, *Sf1* and *Wt1*, are particularly important in the development of murine genital ridge (McLaren, 1998).

These two genes continue to be active during the following early sex differentiation in developing males. Four different cell lines comprise the genital ridge: primordial germ cells, somatic steroidogenic cells, supporting cells and connective tissue. The fate of each lineage depends on the sexual determination of the embryo in which they develop, and their structure, function and pattern of genetic activity are quite different in testes and ovaries.

It has been known for a long time that sex determination in mammals depends on the presence or absence of the Y chromosome. Embryos without a Y chromosome develop as females and those with a Y chromosome develop as males. The discovery of the *SRY* gene was the breakthrough in the molecular understanding of sex determination and differentiation in the mouse and human (Goodfellow and Lovell-Badge, 1993) which paved the way for other mammals. Morphological differences in XY embryos develop before those in XX embryos. The chromosomal constitution determines the migration of cells into gonads and the final differentiation into a testis or an ovary (Hunter, 1995). In the dog, testicular differentiation has been observed at 35–36 days of gestation (Meyers-Wallen *et al.*, 1991, 1994; Table 15.1). During this process, three major groups of undifferentiated gonadal cells are transformed: supporting cells differentiate into Sertoli cells; primordial germ cells into prospermatogonia and steroidogenic precursors into Leydig cells.

In the course of the dog development, as in other mammals, the onset of *SF1* gene expression coincides with the beginning of the sex differentiation period (Meyers-Wallen, 2005). The *SRY* gene (the sex-determining region on Y chromosome) triggers the male differentiation pathway (see next subsection). Secretion of AMH (anti-Müllerian hormone) by Sertoli cells and the regression of Müllerian ducts in canine embryos begins soon after the start of testis differentiation, i.e. at 35–36 days of gestation (Meyers-Wallen *et al.*, 1991, 1994). A whole chain of developmental events follow, and the phenotype typical for males appears. The development of females does not need the triggering action of the *SRY* gene, which is absent in normal females. Three major cellular components of the ovary develop, including follicular cells, oocytes and the internal theca cells that are responsible for the production of oestrogens. Müllerian ducts are transformed into the oviducts, uterus, cervix and the upper parts of vagina, while the Wolffian ducts disappear. Other morphological and physiological features typical for females are formed during the following days and weeks of embryonic development.

The genes involved in sexual differentiation

All of these developmental transformations are guided by numerous genes, some of which are probably not known as yet. Fortunately, the key genes are identified and some of their interactions are now known. Figure 15.7 illustrates certain aspects of these gene interactions and the developmental pathways.

Testicular development is a key element in establishing male sexual differentiation, and the *SRY* gene located on the Y chromosome is essential. Testes determining role of the *SRY* gene in mammals is widely accepted after the experiments performed in the early 1990s (reviewed by Goodfellow and Lovell-Badge, 1993). However, this is not the only critical factor in sexual differentiation, because in humans and other mammals non-functional testes develop even in the absence of the *SRY* gene. In genetic males, *SRY* induces differentiation of the Sertoli cells (reviewed by McLaren, 1991) and the secretion of anti-Müllerian hormone (AMH or MIS) that follows. AMH, which belongs to the transforming growth factor β family, causes regression of the Müllerian ducts, and promotes development of the Wolffian ducts and the differentiation of Leydig cells secreting the male steroid hormone, testosterone (Behringer, 1995). Testosterone binds to androgen receptors which, in turn, act as transcription factors. The complete coding sequence of cDNA (complementary DNA, 1578 bp) for the canine *SRY* gene has been determined (Meyers-Wallen *et al.*, 1999).

Several autosomal genes acting downstream of *SRY* have been shown to be involved in the male sex differentiation pathway (Ramkissoon and Goodfellow, 1996; Greenfield, 1998).

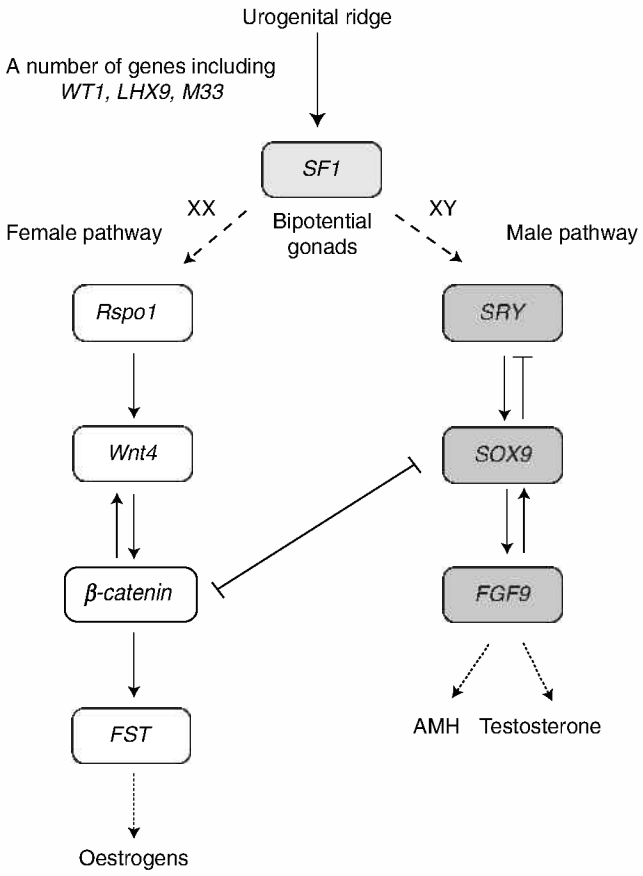

Fig. 15.7. A genetic model for sex determination, controlled by a balance of antagonistic pathways. In XY gonads (dark grey boxes), *SRY* triggers upregulation of *SOX9*, leading to Sertoli cell commitment and testicular differentiation (Sertoli cell differentiation is a result of the establishment of a positive feedback loop between *SOX9* and the secretion of FGF9 (fibroblast growth factor 9; and also PGD_2, prostaglandin D_2, not shown), which act in a paracrine manner to recruit additional Sertoli cells. In XX gonads (white boxes), two independent signalling pathways involving the Rspo1/Wnt4/β-catenin pathway and Foxl2 (via the *FST*; follistatin gene) tilt the balance towards the female side and silence *SOX9* and *FGF9*. Arrows indicate stimulation; T bar indicates inhibition. AMH, anti-Müllerian hormone (modified and redrawn from Edson *et al.*, 2009).

Murine studies reveal that the major role of *SRY* is to promote sufficient expression of the *SOX9* gene, in order to induce Sertoli cell differentiation which drives testis formation (Morais da Silva *et al.*, 1996; Kashimada and Koopman, 2010). Sertoli cell differentiation is a result of the establishment of a positive feedback loop between Sox9 and the secretion of Fgf9 and also PGD_2 (prostaglandin D_2), which act in a paracrine manner to recruit additional Sertoli cells. Arrighi *et al.* (2010) also demonstrated that the canine INSL3-RXFP2 complex plays a paracrine role in the developing testis and possibly as part of an autocrine feedback loop.

In the absence of an *SRY* gene, which is typical for XX embryos, gonads develop into ovaries. Two independent signalling pathways involving the Rspo1 (R-spondin1)/Wnt4/β-catenin pathway and the Foxl2 transcription

factor (the *FST;* follistatin gene) shift development of undifferentiated gonads to ovaries by silencing *Sox9* and *Fgf9* (Edson *et al.*, 2009; Nef and Vassalli, 2009; Piprek, 2009). R-spondin1 has recently been recognized as a key female-determining factor (Nef and Vassalli, 2009). In the potential female embryos, no Leydig cells are formed, no testosterone is produced and the undifferentiated gonads are steadily transformed into ovaries. Quite often, the female developmental programme is considered to be the 'default', while the male programme requires 'switching on' of the *SRY* gene followed by activation of other genes. A comparison of four regulatory regions located upstream of *SRY* shows high conservation between the human, bovine, pig and goat regions. These regions of homology share transcription factor-binding sites that appear to be subject to strong evolutionary pressure for conservation and may, therefore, be important for the correct regulation of *SRY* (Ross *et al.*, 2008). The structure of the *SRY* region, on the contrary, is more variable among placental mammals.

The female uterus, ovary and follicle embryology are less well documented in the dog. During uterine development, as in other species, the mesonephric (Wolffian) duct undergoes regression and the paramesonephric duct proliferates and forms the bicornuate uterus. The paramesonephric ducts are initially derived from coelomic epithelium and have three elements: a canalized epithelial tube, mesenchymal cells surrounding the tube and coelomic epithelial cells. In mice, the LIM homeodomain transcription factor family regulates duct initiation (Kobayashi *et al.*, 2004), while both WNT9b and Pax2 are required for duct elongation, leading the tip proliferation and the duct elongating to reach the developing cloaca (Deutscher and Yao, 2007).

Cycle of the X chromosome

As proposed by Lyon (1961) and now uniformly accepted, one of the X chromosomes in eutherian females undergoes inactivation during early embryonic development. Numerous investigations shed light on different aspects of X chromosome behaviour, including preferential inactivation of the paternal X chromosome in the trophoblast, random inactivation in the ICM and molecular mechanisms of inactivation (Goto and Monk, 1998). This scenario appears to be completely relevant to the cycle of the X chromosome in the dog (Deschênes *et al.*, 1994). Preferential inactivation of the paternal X chromosome in canine XX embryos probably occurs in trophoblast cells at around 11–14 days of gestation and then, soon after, random inactivation of one X chromosome follows in the embryonic disc cells. Thus, females become natural mosaics with one X chromosome randomly inactivated in each somatic cell.

In the dog's post-meiotic oocytes, the X chromosome is active, as in other mammalian species. The paternal X chromosome, on the contrary, enters the zygote inactive but, soon after fertilization, it reactivates. In XX embryos, both X chromosomes are expected to be active until trophoblast differentiation. Then only one X chromosome remains active regardless of the number of X chromosomes in a cell. This is considered to be an essential condition for gene dose compensation. The mechanisms of silencing one X chromosome are complex and have been investigated mainly in the mouse. Several chromatin modifications are necessary in order to form stable facultative chromatin capable of propagating through numerous cell divisions. The so-called *X-inactivation centre* located on the X chromosome contains the *Xist* gene and cis regulatory genetic elements. The *Xist* gene encodes an RNA molecule which plays a key role in inactivation of one X chromosome (Plath *et al.*, 2002). *Xist* is negatively regulated by its antisense transcript, Tsix. It seems, however, that the *Tsix* gene (the reverse spelling of *Xist*) is not the only regulator, and that additional transcription factors are involved in this complex process (Senner and Brockdorff, 2009).

Certain observations made in other mammalian species could be relevant to the dog. Interestingly, porcine *Xist* gene expression may be affected by maternal metabolic state at the time of ovulation (Vinsky *et al.*, 2007). As this study shows, sows that were in a negative metabolic state during the week before ovulation and fertilization not only demonstrated greater than usual post-implantation embryonic mortality,

but the mortality of female embryos was greater than that of male embryos. This was attributed to aberrant *Xist* expression in female embryos, suggesting that maternally influenced epigenetic defects may contribute to sex-biased embryonic loss. In the canine female, X chromosome inactivation also appears to be random, as in other eutherian mammals (Bell *et al.*, 2008).

Anomalies in sexual differentiation in the dog

Detailed descriptions of numerous defects of sexual development in dogs have been given by Meyers-Wallen (1993, 1999); these recognize anomalies of chromosomal, gonadal and phenotypic sex. Three major disorders XXY, XO and XXX, which are based on variations of the normal chromosome set, are described in the dog. The underdeveloped genitalia and sterility associated with the lack of one X chromosome (XO karyotype) observed in the bitch (Meyers-Wallen, 1993) appear to be similar to human Turner syndrome. XXY dogs resemble Klinefelter's syndrome in humans. Chimeras carrying cells with chromosome sets such as XX/XY and XX/XXY demonstrate deviations from normal development (Meyers-Wallen, 1999). This includes true hermaphrodites with both ovarian and testicular tissue.

Recently Meyers-Wallen (2009) reviewed the molecular mechanisms controlling sexual development and molecular methods of identification of causative mutations. The major focus of this review is on XX sex reversal, Persistent Müllerian Duct Syndrome (PMDS) and cryptorchidism. PMDS in the Miniature Schnauzer is caused by a C to T transition in exon 3 of the anti-Müllerian hormone receptor, type II (*AMHR2*) gene, which introduces a DdeI restriction site (Pujar and Meyers-Wallen, 2009).

Gonadal sex abnormalities refer to the situations when chromosomal and gonadal sex are contradictory. Such animals are called sex reversed. In several breeds, such as the American Cocker Spaniel, the English Cocker Spaniel, Chinese Pug, Kerry Blue Terrier, Weimaraner and German Short-haired Pointer, animals with 78,XX chromosome constitution were described who developed varying amounts of testicular tissue (Meyers-Wallen, 1993). A reciprocal translocation was identified in the Yorkshire Terrier. This intersex dog uniformly showed a 78,XY chromosome complement and confirmed that the animal might be a male to female sex-reversed dog. Genomic DNA from this dog probably contained the *SRY* gene. Surprisingly, two different types of X chromosome were observed. The karyotype of this mosaic sex-reversed dog was designated tentatively as 78,XY/78,XYrcp (X; autosome). This is a rare report on a canine intersex dog showing male to female sex reversal (Schelling *et al.*, 2001).

The *SRY* gene, as mentioned above, is crucial for development of the testis, thus directing sexual development towards the male phenotype. In humans and mice, translocation of the *SRY* region from the Y to the X chromosome is responsible for XX reversal males (Cattanach *et al.*, 1982). This is probably not necessarily always the case in the dog. It has been demonstrated, for instance, that the SRY high mobility group (HMG) box was absent in genomic DNA of XX-sex-reversed American Cocker Spaniel and Short-haired Pointer dogs, and the possibility was discussed that a mutant autosomal gene may cause activation of the testis differentiation cascade in the absence of *SRY* (Meyers-Wallen *et al.*, 1995a,b). There have also been cases of canine *SRY*-negative XX sex reversal resulting in a disorder of gonadal development where individuals who have the female karyotype develop testes or ovotestes (Campos *et al.*, 2011).

In a number of situations, chromosal and gonadal sex agree, but the internal and external genitalia are ambiguous or even alternative. These cases are categorized as abnormalities of phenotypic sex and belong to two groups: male or female pseudohermaphrodites (Meyers-Wallen, 1993, 1999). In such abnormal embryos, which have an XY constitution and gonads developing into testes, two groups of events can be recognized. One of them is failure of Müllerian duct regression, which diverts embryos from the typical male developmental pathway, allowing female sexual structures such as oviducts, uterus and vagina to appear. Another is the failure of androgen-dependent masculinization, when Müllerian ducts regress but structures dependent upon androgens (i.e. testosterone) do not respond and masculinization does not occur. There are obviously different

genetic and developmental grounds for this phenomenon. One of them seems to be similar to a syndrome known in humans as testicular feminization, which is caused by an X-linked mutation responsible for defects in the androgen receptor.

Female pseudohermaphrodites with an XX constitution that develop ovaries have also been reported (Meyers-Wallen and Patterson, 1989). Currently there is a push for the reclassification of numerous sex abnormalities and their sometimes confusing terminology. The newly suggested terminology is based on so-called 'gonosomal constellation' and 'gonadal constitution', and may help in a systematic classification of canine intersex cases. This terminology replaces common but confusing definitions like 'true hermaphrodite' and 'pseudohermaphrodite' (Poth *et al.*, 2010).

The most common disorder of sexual development in dogs is cryptorchidism, which accounts for about 13% of males presented to small animal clinics (Dunn *et al.*, 1968; cited by Meyers-Wallen, 1993). Normally, the testes descend into the scrotum during male development. In the dog, this process commences at 42 days of gestation. The testis reaches the internal inguinal ring at the time of birth. The second inguino-scrotal phase commences approximately on the 5th day after birth, and is completed by passage of the testis into the scrotum on the 35th day after the birth (Kawakami *et al.*, 1993). The ligament connecting the testis to the lower abdomen probably plays a critical role in the descent of the testes and is controlled by insulin-like hormone 3 (INSL3), which is produced in testicular Leydig cells. Deletion of *Insl3*, or of the gene *Rxfp2* that codes for its receptor, causes cryptorchidism in mice. INSL3/RXFP2 signalling seems to be important as it targets the β-catenin and Notch pathways during the development of the testes (Kaftanovskaya *et al.*, 2011). More than one reason for cryptorchidism cannot be excluded. For instance, in taxonomically close foxes, cryptorchidism was one of the pleiotropic effects in males homozygous for the autosomal dominant mutation *Star*, which causes black and white spotting. Other known pleiotropic effects in these foxes include heterochromia, deafness and the pathological condition of the vestibular apparatus causing abnormal head-shaking behaviour (Belyaev *et al.*, 1981).

As follows from the above, numerous abnormal outcomes of sexual differentiation highlight the multiplicity of genes involved and complexity of developmental interactions.

Summary

Mammalian developmental genetics has achieved a great deal of progress during the last couple of decades. Most mammalian data have been obtained from murine research, while other mammalian species, including the dog, have not yet made a significant contribution. Fortunately, the similarity of genetic, cellular and morphogenetic processes regulating development allows this emerging knowledge to be spread to other mammalian species. This chapter has also indicated some of the developmental differences between the dog and other mammalian species that are relevant to the early stages of embryonic development. Owing to the enormous variation that has resulted from domestication and breed formation, the dog can provide unique possibilities for the further progression of developmental genetics, which are particularly backed up by genome projects that have already been accomplished. The genetic investigation of dog embryology still has much to deliver, both in demonstrating differences from other mammals and in discovering new facts and principles common to other animals.

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16 Genetics of Morphological Traits in the Domestic Dog

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Introduction

The domestic dog offers an amazing array of phenotypic variation (Fig. 16.1). Fixed within the genomes of diverse dog breeds are powerful, common mutations that control body size, skull shape, coat colour and texture, leg length and a plethora of other morphological features. The vast diversity in phenotype and the large number of nearly independent lines (e.g. over 300 breeds of dog recognized worldwide) coupled with the burgeoning genomic resources have created a unique and powerful system for mapping the genes underlying the key morphological traits common to mammalian body plans.

As an example, the domestic dog exhibits a 40-fold range in body size (Chihuahua versus Great Dane) that rivals that of land mammals as a whole (Evans, 1993). Likewise, leg length varies over a dozen-fold, from 2 inches to 2 feet (Pekingese versus Scottish deerhound or Borzoi), and the range of skull shapes from long and pointy (Collie or Greyhound) to flat and round (Bulldog) and is more extreme than the difference between many mammalian orders (e.g. primates or carnivores).

In assembling the 2002 white paper that ultimately resulted in funding of the dog genome sequence, one goal was to develop the genomic resources and approaches that would allow scientists to understand the genetic variation governing these remarkable traits. The last 5 years have seen strong experimental evidence suggesting that so-called mapping of 'breed fixed' traits is remarkably successful in identifying genomic regions governing a myriad of complex traits. In this chapter, we will summarize the beginning of the morphology

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Fig. 16.1. Domestic dog breeds exhibiting various phenotypic traits. These include, but are not limited to: body size, coat colour and texture, leg length and skull shape, as well as other morphological features. Breeds from left to right clockwise are: Silky Terrier, Schipperke, Vizsla, Cavalier King Charles, Japanese Chin, Saluki, Cavalier King Charles Spaniel, English Cocker Spaniel, Chihuahua, Irish Terrier, Skye Terrier, Chihuahua, Golden Retriever, Wire-haired Dachshund and Samoyed.

studies movement in the dog (for a review, see Ostrander, 2007), and discuss traits that have been successfully mapped and why. We will highlight the difficulties and advantages of working with the canine system and speculate as to why there is so much variation in this one species. Finally, we will summarize the ways in which this information may be helpful for informing human conditions.

The Portuguese Water Dog Story

The first genetic studies of canine skeletal morphology that moved beyond simple descriptive stories were those of Chase and Lark, who began their work by studying the Portuguese Water Dog (PWD) at the University of Utah in the late 1990s. Their studies were conducted under the auspices of a programme called The Georgie Project (http://www.georgieproject.com), which continues to this day. The goal behind The Georgie Project, as it was designed, was to collect both phenotypic and genotypic data on as many living PWDs as possible, and then use that information to determine the number and location of loci controlling a variety of complex traits (Chase *et al.*, 1999). The choice of dog was fortuitous, as the American Kennel Club (AKC) allowed considerable latitude in many morphological features of the breed, in

particular, skeletal size. Unlike other breeds, whose standards allow less than 10% variation, large and small PWDs can differ in size by as much as twofold (American Kennel Club, 1998). In addition, there are only about 10,000 registered PWDs in the USA today, and all can trace their heritage though a 24-generation pedigree with 31 founders (Chase *et al.*, 1999).

Lark and Chase collected not only DNA and pedigree information on the dogs sampled, but also a set of five X-rays from which they distilled a set of 92 metrics, which would ultimately prove useful for trait mapping. Using principal components analysis (PCA), they showed that the first four axes of variation explained about 60% of the variance observed in the 92 metrics. PC1 described overall body size and PC2 demonstrated that the metrics of the head were inversely correlated with those of the pelvis. PC3 showed that the metrics of the skull and limbs were inversely correlated with the skull width and height. Finally, PC4 showed that skull and limb lengths are inversely correlated with the width of the limb and axial skeletons, suggesting an axis that balances speed versus strength.

In a landmark study, published in the *Proceedings of the National Academy of Sciences of the USA* in 2002, Lark and Chase undertook genome-wide scans for skeletal quantitative trait loci (QTLs) using 330 PWDs genotyped across 500 highly polymorphic tetranucleotide-based (Francisco *et al.*, 1996) microsatellites (Chase *et al.*, 2002). Linkage analyses identified several QTLs associated with each PC. By far the strongest results were those associated with PC1, which accounted for almost 45% of variation across the traits; while at least five loci were identified for PC1, of which the most robust was on canine chromosome 15 (CFA15). After examining genotypes from both large and small PWDs, it was clear that the relevant gene on CFA15 was likely to be within a15 Mb region centred at 44.5 Mb.

Mapping Genes for Body Size

Building on the work described above, the fine-mapping of the 15Mb region identified by Chase and colleagues on CFA15 was performed (Sutter *et al.*, 2007). We began this study by analysing large and small PWDs for a set of single nucleotide polymorphism (SNP)-based markers spanning the associated region; the analysis both verified the results and narrowed the region concerned to <4Mb. Using DNA isolated from multiple dogs from each of 14 small breeds, such as the Pekingese, Pomeranian and Toy Poodle, as well as several large breeds, we looked for evidence of loss of heterozygosity in the region that would be consistent with recent selection. We found such a region in small dogs that spanned the *insulin like growth factor 1* gene (*IGF1*) located at approximately 44.5 Mb. The fact that nearly all small breeds had the same haplotype in the region suggests that a single ancient allele of *IGF1* contributes to the miniaturization of most small dog breeds (Fig. 16.2). To test this hypothesis more formally, we analysed hundreds of dogs from over 80 breeds, including tiny, medium and large breeds, and showed that small dogs, regardless of breed, overwhelmingly share a common haplotype termed 'B' across 12 SNPs that span the *IGF1* gene. In comparison, dogs from large breeds segregated a pair of related haplotypes ('F' and 'I'), which are quite divergent from the 'B' haplotype. Amazingly, the B, F and I alleles segregated within the PWDs and were responsible for *within*-breed variation

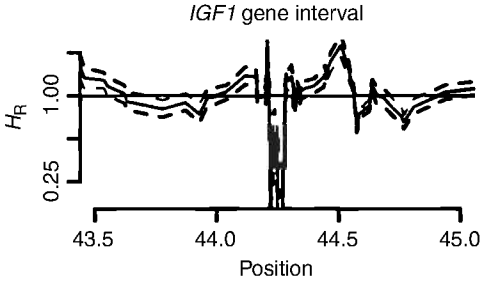

Fig. 16.2. Signatures of recent selection on the *IGF1* locus across 22 small and giant dog breeds. The heterozygosity ratio (H_R) is shown for small versus giant dogs on a sliding 10-SNP (single nucleotide polymorphism) window across *IGF1*. Dashed lines delimit the 95% confidence intervals based on non-parametric bootstrap resampling. The *IGF1* gene interval is indicated above the graphs as a black box drawn to scale. Figure originally published in *Science* 316, 112–115 (Sutter *et al.*, 2007).

in this morphologically diverse breed. This established, for the first time, that a single *IGF1* allele was the major contributor to small skeletal size, and that mutations that create morphological effects can readily cross breed barriers. The exact mutation remains unclear at this time, but is likely to be a SNP at around exon 3. However, what is clear is that a distinct haplotype at the *IGF1* locus characterizes all small dog breeds, is distinct from what is observed in large breeds, and that a single ancient mutation is thus a major contributor to small size in many, if not most, modern breeds.

The above discovery did not preclude the existence of contributions from other genes. Indeed, the initial PWD analysis, as well as an analysis of a large multi-breed data set, identified half a dozen other loci that clearly contribute to body size (Chase *et al.*, 2002; Jones *et al.*, 2008). The work of Jones *et al.* (2008) was distinguished by its use of a very large sample set including 148 breeds. Thus, although the genome scan did not fully cover the entire genome – indeed, it only encompassed 5000 SNPs – it was expansive in that it included several hundred dogs. Interestingly, this report relied on established breed AKC standards. This was important, as it established a precedent for how to do canine morphology studies. It proved that, among pure-bred dogs of a single breed, conformity to the established breed standard for traits such as body size is accurate enough. Individual measurements for each dog in a genome-wide association study (GWAS) is not needed. 'Breed stereotypes', as they have since come to be known, are excellent surrogates.

Work continues today on the identification of the genes and mutations associated with other loci revealed by these and similar studies (Boyko *et al.*, 2010). Studies have also progressed to identifying where the individual haplotypes that predominate in the dog population today originated. So, for instance, Gray *et al.* (2010) have shown that intron 2 of the *IGF1* gene contains a short interspersed nuclear element (SINE) and a single SNP that is found in all small dog breeds and is seen only in a small number of large breeds. By surveying a large sample of grey wolf populations and wild canids, Gray *et al.* (2010) found a total absence of the derived small SNP allele in intron 2, suggesting the mutation concerned arose after (or coincident with) dog domestication. Deep analysis of the genotyping data showed that grey wolf haplotypes from the Middle East have a higher nucleotide diversity than other wolf populations. Because this allele appears to have originated in the Middle East, these data, along with similarities in the haplotypes of Middle Eastern wolves and small domestic dogs, suggest a possible Middle Eastern origin for *IGF1* alleles contributing to small skeletal size.

The absence of both the SINE element and SNP allele in grey wolves argues that the small size mutation in *IGF1* occurred sometime after the major events associated with dog domestication, but, because all small dogs have the same haplotype, the mutations probably arose early in the domestication process. This work of Gray *et al.* (2010), then, was the first to hint at a Middle Eastern origin for at least some dog domestication events.

The CanMap Study

The identification of major genes and variants associated with body size in the domestic dog was exciting to the community and opened the discussion for developing a data set that would, simultaneously, allow researchers to explore a multitude of morphological traits. That data set, described here, is called CanMap and was made public (at http://genome-mirror.bscb.cornell.edu/) earlier in 2010 (Boyko *et al.*, 2010).

Researchers at Cornell University, NHGRI (US National Human Genome Research Institute) and UCLA (University of California Los Angeles) worked together to construct a data set that would allow us to answer questions on genes controlling both simple and complex traits, as well as to tackle the issue of dog domestication. Towards that end we enrolled a set of nearly 915 healthy domestic dogs representing 80 breeds, as well as ten outbred African shelter dogs and 83 wild canids. All dogs were genotyped at NHGRI with the Affymetrix Version 2.0 chip, which allowed us to extract genotype data on 60,968 SNPs. Dogs were selected to represent a breed if they were healthy and unrelated to other

dogs in that breed data set at the grandparent level. This design maximized the number of lineages examined for each breed. By coupling this genomic resource with breed standards and individual measurements, as well as skeletal measurements from museum skull specimens, we identified loci associated with phenotypic variation across 51 traits (Boyko *et al.*, 2010). These included previously studied traits such as body size, back arch, leg length and width, coat length and curl, as well as more subtle traits such as ear position (floppy or straight) and tail position (curl, straight, twisted, curled over the back, etc).

While the individual results were interesting and many have been, or are being, followed up, the most striking result of the study was that the majority of phenotypic variation in dogs, for the traits that we studied, was controlled by a small number of loci (Boyko *et al.*, 2010). We had predicted this, somewhat out of naivety, years earlier when considering the nature of the domestic dog population and how breeds came into existence (Ostrander and Kruglyak, 2000). However, the CanMap data set formally validated that thinking for the first time. In addition, we found that many of the genomic regions that were mapped show signatures of recent selection, with most of the highly differentiated regions being associated with breed-defining traits such as characteristics of the coat, leg length, or body size, i.e. all traits that have been heavily selected by modern dog breeders.

The task now for CanMap researchers and others is to fine map the remaining regions, sequence them using the latest technologies, and find the underlying variants controlling these many interesting phenotypes. For body size, the regions of consideration contain excellent candidate genes, including *HMGA2* on CFA10 and *SMAD2* on CFA7, both of which have been associated with body size in multiple studies (Chase *et al.*, 2002; Jones *et al.*, 2008; Boyko *et al.*, 2010). In addition, *HMGA2* is reported to affect body size in both humans (Weedon *et al.*, 2007) and mice (Zhou *et al.*, 1995). The *STC2* gene on CFA4, a known growth inhibitor in mice (Gagliardi *et al.*, 2005), is another candidate of interest. It would, of course, be interesting to understand how combinations of alleles at multiple loci combine to form the gradient of body size that we observe in domestic dogs today.

Size Sexual Dimorphism in Dogs

While enormous progress has been made in collecting phenotypes for the genetic studies, the phenotype of sexual dimorphism is still to be fully addressed by canine researchers. Size sexual dimorphism occurs in almost all mammals, and Chase *et al.* (2005) reported that size sexual dimorphism existed in PWDs. Their work argued that most of the dimorphism came from interactions between the *IGF1* locus on CFA15 and a locus on the X chromosome that mapped close to the *CHM* marker, although there is no evidence indentifying the X-linked gene itself.

The story is complicated, though, as their data suggest that the haplotype on CFA15 resulting in small size is dominant in females, while in males the haplotype for large size is dominant. Further, females who are homozygous at both the X locus and the large size CFA15 haplotype are, on average, as large as large males. This would explain the presence of a small but persistent number of large females among the breed. However, all females that are heterozygous at the *CHM* marker are comparatively small, regardless of the CFA15 haplotype they carry.

This work is interesting as it provides supporting evidence for what is known as Rensch's rule, which argues that size is frequently correlated with sexual dimorphism (Rensch, 1960). Among large mammals, then, the difference between female and male will be much greater (think elephant or hippopotamus where females are less than half the size of the males) than it will be among small mammals (e.g. mice or rats). While the CFA15 locus is assumed to be *IGF1*, there remains some speculation that a second locus exists in the region. Also, no work has been done to advance knowledge about the locus on X chromosome. In addition, the number of loci that contribute to body size on the X chromosome remains controversial (Boyko *et al.*, 2010).

One obvious question that follows on the heels of this work is whether or not Rensch's

rule is followed in dog breeds. One might predict that the difference between females and males would be greater in St Bernards and Newfoundlands than in Toy Poodles and Pomeranians. Sutter *et al.* (2008) examined this question in a data set that included 13 breed-defining metrics collected on 1155 dogs from 159 breeds. Dogs were only eligible for the study if they were at least 1 year of age. Careful examination of the data revealed no evidence for Rensch's rule among dog breeds, although the study did draw some interesting conclusions. It showed, first, that most dog breeds adhere well to the published AKC standards. Thus, performing mapping studies based on breed stereotypes, as Jones had suggested, did in fact appear to be feasible (Jones *et al.*, 2008). But, perhaps more importantly, an analysis of the data revealed two PCAs, one for overall body size, the same as had been shown by Chase *et al.* (2002), and one for the shape (length versus width) of the skeleton. The fact that the quality of the data set was sufficient to identify these PCAs had strong implications for mapping studies, suggesting that individual measurements are really not needed for studies aimed at mapping the underlying genes controlling morphological traits. This is likely to be particularly true in the case of breed-defining traits, which are of significant interest to many, as they are typically under strong selection by breeders and there is therefore little phenotypic or genotypic heterogeneity.

Mapping Genes for Leg Length and Width

The identification of genes that explained the disproportionately short legs of breeds like the Corgi, Basset Hound and Dachshund has long been an area of interest for many scientists (Pollinger *et al.*, 2005). At least 20 breeds of dog, developed for a variety of reasons, including ratting, and fox and rabbit hunting, have a well-proportioned torso and head, but disproportionately short legs (Fig. 16.3). To be considered chondrodysplastic, breeds must: (i) have a leg to body length ratio of <1.0; (ii) have forelegs that are comparatively heavy and well boned; and (iii) may also have the toes turned out or show bowing in the long bones.

To find the underlying gene we conducted a GWAS study that made use of the CanMap data set (Parker *et al.*, 2009). Specifically, we compared the results from 95 'case' dogs from eight breeds displaying all three chondrodysplastic criteria based on their breed standards, and 702 control dogs from 64 breeds that were not chondrodysplastic. Single marker analysis revealed a strong association result as indicated by an odds ratio (OR) of 33.54 between a single SNP on CFA18 (base position 23,298,242 (CanFam2) and the chondrodysplasia phenotype ($\chi^{2=437}$; P value = 9×10^{-104}). To correct for inflation we also performed independent Mann-Whitney U-tests on the distribution of allele frequencies within the chondrodysplastic and control breeds. Two SNPs on CFA18 retained the strongest association, with P values of 1.15×10^{-5} and 2.74×10^{-5}, respectively. Haplotype analysis was similarly supportive.

A heterozygosity analysis, much as was done for the *IGF1* locus, was carried out using 139 cases, 173 controls and a large number of SNPs, and revealed a region of 125 Kb with excess homozygosity, indicating the presence of a selective sweep. Additional analysis reduced the region to 24 Kb which, upon sequencing, was found to have a perfectly duplicated copy (*fgf4*, a retrogene) of the coding region of the *FGF4* gene. Finally, expression analysis supported the hypothesis that the phenotype was caused by excess *fgf4* expression during a critical time in development, leading to premature closure of the growth plates (Parker *et al.*, 2009). Thus the length of the limbs was shortened while other tissues were not affected.

The work was exciting both because it explained a common phenotype that characterizes many dog breeds, and because it was the first example we were aware of in which an expressed retrogene was responsible for a common mammalian phenotype. A retrogene is a continuous piece of potentially coding DNA that lacks any introns or regulatory machinery. It typically picks up its regulatory signals from the genes around it. While common in insects, an expressed retrogene had yet to be reported that encoded a major

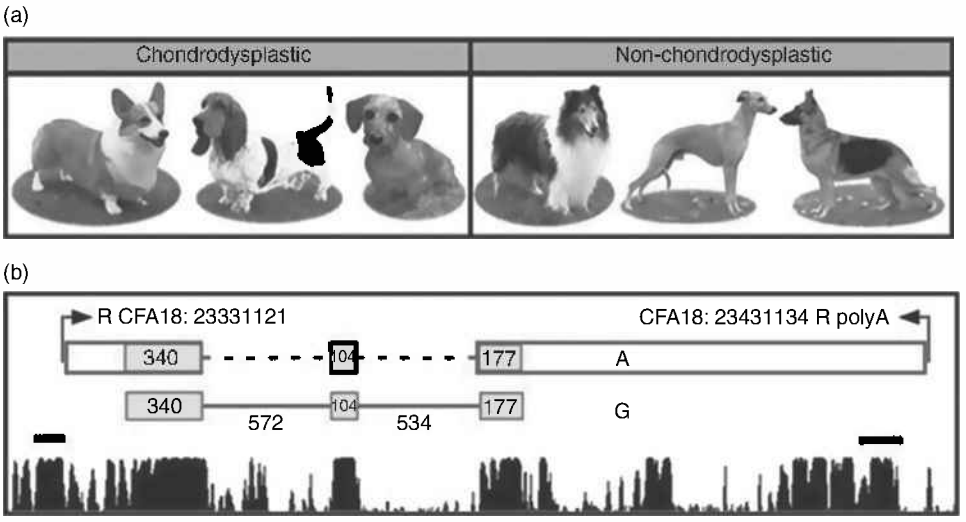

Fig. 16.3. (a) Examples of chondrodysplastic dogs and non-chondrodysplastic dogs. The short-legged Pembroke Welsh Corgi, Basset Hound and Dachshund, versus the Collie, Whippet and German Shepherd, respectively. (b) The gene responsible for chondrodysplasia in dogs (*FGF4*): a comparison of inserts to source *FGF4* gene. The first row on the figure displays the alignment of the mutant allele that contains the insert sequence to the source *FGF4* sequence that lacks the insert. *FGF4* has three coding exons represented by the boxes on the graph, and begins at CFA18 position 51439420 and ends at position 51441146. All three exons are present in the insert, which aligns between positions 51439178 and 51442902. The mutant allele containing the insert includes 242 bases upstream of the start site and 1756 bases downstream of the stop codon followed by a polyA repeat. A 13-base sequence (AAGTCAGACAGAG) derived from the insert site, indicated by the letter R on the figure, is repeated at both ends of the insert. The second line shows the coding sequence of *FGF4* with the size of the exons and introns labelled. Alignment of the mouse promoter and enhancer sequences is indicated by the lines directly above the dog/human/mouse/rat conservation track shown at the bottom of the figure. Coding sequence is predicted based on sequence similarity of translated proteins (figure adapted from *Science* 325, 995–998; Parker *et al.*, 2009).

morphological factor in mammals. As with *IGF1*, all breeds that shared the phenotype, although bred for a variety of purposes, shared a common haplotype around the retrogene, suggesting that the transposition event had occurred only once before the formation of the breeds.

The genetics of leg width in dog breeds has been similarly studied (Quignon *et al.*, 2009). A locus on CFA26 was originally identified in the PWD study associated with PC4. Subsequent mapping in long and short leg breeds with a dense array of SNPs reduced the region from 26 Mb to 500 Kb. The critical region contains two collagen genes, which are likely candidates as they alter bone growth. Extensive sequencing, however, has not yet revealed a causative variation. This study highlights the difficulty that is frequently encountered in canine mapping studies. Long-range haplotype sharing makes it difficult to identify a single causative mutation from the associated markers. As described below, this is a problem inherent in many canine-mapping studies.

Genomics of the Dog and Mapping Complex Traits

As described elsewhere in Chapter 12, the 7.8x sequence and preliminary assembly of the dog genome, published in 2005 (Lindblad-Toh *et al.*, 2005), and the comparison of those data with the previously published 1.5x poodle sequence (Kirkness *et al.*, 2003) were important

for understanding much that we know about the structure of dog breeds today. We know, for instance, that domestication is marked by a major bottleneck, as is the development of individual dog breeds. Most breeds have been around for relatively short periods of time, and many breeds were formed from small numbers of dogs and have been propagated using popular sires (Karlsson and Lindblad-Toh, 2008; Ostrander and Wayne, 2005). As a result, linkage disequilibrium (LD) extends for long distances in the dog genome compared with the human. In a study done by Sutter *et al.* (2004), using five breeds and examining five loci and using the D′ statistic, LD was found to vary from 0.4 Mb in the Golden Retriever to 3.2 Mb in the Akita. A larger analysis, done using genotypes from the 7.8× sequencing data, both verified and extended this result, showing that LD varied enormously across the genome, and that regions of very long LD indeed exist (Lindblad-Toh *et al.*, 2005).

Finally, examination of the CanMap data provided further illumination (Boyko *et al.*, 2010). We found that, while average LD extends over one Mb within every breed we surveyed, across all breeds combined it decays extremely rapidly, suggesting that *identical by descent* (IBD) segments that are shared across many breeds are typically quite short. In addition, nearly every breed featured anywhere from 10–50 runs of homozygosity that were greater than 10 Mb in length. Regions of homozygosity were often unique to a particular breed.

Such results meant that comparatively few SNPs would be needed in initial GWAS studies to find major loci – although in the absence of many breeds sharing a common phenotype for the same reason, finding the causative mutation would be problematic, as was observed in the case of leg width (Quignon *et al.*, 2009).

Genetics of Skull Shape

In addition to body size, the canine skull is perhaps one of the most variable portions of dog anatomy. Skulls can be brachycephalic, as in the Bulldog, which features a flat or pushed-in snout, or they can be dolichocephalic – long and extended as in the Greyhound. Studies of skulls offer particular challenges because there are no AKC standards that can be distilled into simple measurements. Further, the presence of fur and skin folds makes obtaining identical measurements from individuals difficult. In our own experience, only a small set of skull features can reproducibly be measured and then only by skilled technicians. What is much easier is the measurement of archival skulls, such as those housed in many museums. For the CanMap study, we elected to rely on the measurements of skulls collected largely by the Smithsonian Museum. We found that skull shapes were largely dictated by several regions whose genes are not yet known. When we consider individual features of the skull, however, it is possible to further pinpoint loci contributing to specific traits (Fig. 16.4). For snout length, for instance, we used breed-average values for absolute snout length, but we introduced log (body weight) as a covariate in

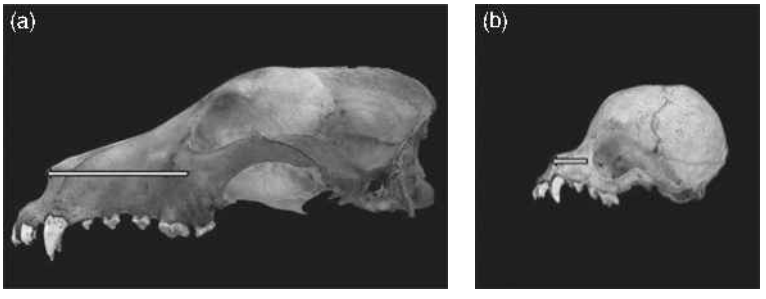

Fig. 16.4. (a) Example of a dolichocephalic skull; notice its length and narrowness. (b) Example of a brachycephalic skull, commonly referred to as flat-faced syndrome; notice the short rostrum and wide skull.

the association model to allow for an allometric correction. This revealed QTLs underlying proportional snout length. Indeed, we found that the strongest signals for proportional snout length are CFA1.59832965 and CF5.32359028. Both loci appear to be important in breeds that are brachycephalic (that is, have short snouts) (Boyko *et al.*, 2010).

Genetics of Coat Colour, Texture and Growth Pattern

The first coat-related trait to be mapped using the GWAS approach was the very interesting ridge that characterizes most, although not all, Rhodesian Ridgebacks (Salmon Hillbertz *et al.*, 2007). This breed was developed in Southern Africa in the early 18th century, when the first European settlers found domesticated dogs in the area; it features the hair on the spine turning forward (Carlson, 1999). The breed increased in popularity in the late 19th century, when big game hunters needed a dog that was strong and intelligent, so as to recognize and avoid predators such as poisonous snakes, but also fast, as they were to be used for lion hunting (Wilcox and Walkowicz, 1995; Carlson, 1999). In addition to the ridgeback fur though, Rhodesian Ridgebacks are also commonly affected by dermoid sinus, a congenital malformation that is similar to a neural-tube defect in humans called dermal sinus. No ridgeless dogs with dermoid sinus have been reported (Salmon Hillbertz *et al.*, 2007).

Using only nine ridgeless and 12 ridged dogs, investigators mapped the locus to a 750 Kb region on CFA18. A critical SNP allowed the investigators to further narrow the region, and it appears that both the ridge and dermoid sinus phenotype are due to a 133.4 kb duplication that goes from nucleotide position 51.40 to 51.53 Mb in the CanFam2 genome assembly (Lindblad-Toh *et al.*, 2005). The duplicated region has four complete genes and is suspected to be the causative mutation. Dogs that are homozygous for the duplication are at a very high risk for the dermoid sinus phenotype, while dogs that are heterozygous are at much lower risk.

While many of the traits discussed so far are due to the presence or absence of simple genetic variants, the Rhodesian Ridgeback story provided insight as to how complex canine genetics can become. Disentangling the causative mutations for related traits is not easy. One additional example of that comes from the work of Cadieu *et al.* (2009), who sought to understand the constitution of canine coats across 80 breeds (Fig. 16.5). Using the CanMap data in combination with breed specific data sets, we found that three major loci control a great deal of the phenotypic variation associated with coat type, including those associated with the growth pattern responsible for the eyebrows and moustache (which are called 'furnishings'), the length of fur and the degree of curl.

The furnishings phenotype is evident in breeds such as the Schnauzer, Poodle and Scottish Terrier. Also, most dogs with the furnishings phenotype also have a wiry coat. An initial GWAS analysis comparing Smooth-coated with Wire-haired Dachshunds resulted in a P value of 3×10^{-27} on CFA13 at nucleotide 11.1 Mb. We replicated this association using the CanMap data set, which included 19 breeds with furnishings and 58 breeds without (P value = 10^{-240}). Fine mapping and direct sequencing allowed us to reduce the region to 238 Kb, and eventually to identify the associated variant, an insertion in the 3′ UTR (untranslated region) of the R-spondin2 gene (*RSPO2*), which segregates perfectly with the phenotype in all breeds tested. Additional data suggested that the mutation affected *RSPO2* expression levels (Cadieu *et al.*, 2009).

Though not previously associated with hair growth, the *RSPO2* gene synergizes with the *Wnt* gene to activate *β-catenin* (Kazanskaya *et al.*, 2004), and *Wnt* signalling is required for the establishment of the hair follicles (Andl *et al.*, 2002; Clevers, 2006). The *Wnt/β-catenin* pathway is important in the development of hair-follicle tumours (Chan *et al.*, 1999), which have been reported to occur most frequently in breeds that have the furnishings phenotype (Meuten, 2002). Tying these results to humans has proven interesting. A recent set of papers demonstrated that a mutation in the *EDAR* gene, which is also in the *Wnt* pathway, is responsible for the relatively coarser hair phenotype that is found among East Asian humans (Mou *et al.*, 2008). This phenotype, of course, has obvious similarity to the canine wire-haired phenotype.

	PHENOTYPE	*FGF5*	*RSPO2*	*KRT71*
(a)	Short	–	–	–
(b)	Wire	–	+	–
(c)	Wire and Curly	–	+	+
(d)	Long	+	–	–
(e)	Long with Furnishings	+	+	–
(f)	Curly	+	–	+
(g)	Curly with Furnishings	+	+	+

Fig. 16.5. Combinations of alleles at three genes create seven different coat phenotypes. Plus (+) and minus signs (–) indicate the presence or absence of the variant (non-ancestral) genotype. A characteristic breed is represented for each of the seven combinations observed in our data set: (a) short hair; (b) wire hair; (c) 'curly-wire' hair; (d) long hair; (e) long, soft hair with furnishings; (f) long, curly hair; and (g) long, curly hair with furnishings. Figure originally published in *Science* 326, 150–153 (Cadieu *et al.*, 2009).

We used the same strategy to identify the major controller of hair length, although this time we had early hints as to what the causative gene would be. A previous study of Welsh Corgis segregating an atypical fluffy long coat had identified mutations in the fibroblast growth factor 5 (*FGF5*) gene as putatively causative (Housley and Venta, 2006). Our GWAS using Long- and Short-haired Dachshunds, as well as analysis of the CanMap data set, localized the long-hair mutation to CFA32 in a region containing the *FGF5* gene with *P* values of 3×10^{-27} and 9×10^{-44}, respectively, for each data set. After extensive sequencing of a 70 Kb selective sweep, the best-associated SNP was one that causes a Cys– > Phe change at a highly conserved site in exon 1 of the *FGF5*. This was the same SNP identified in the earlier Corgi study.

Finally, we found a major locus controlling curl by first comparing PWDs with curly versus wavy hair. This identified a locus on CFA27 with a *P* value of 4×10^{-7}. The CanMap data set corroborated the finding and reduced the locus to a 1 Mb region spanning several keratin genes. Sequencing across a selective sweep covering 32 Kb reduced the number to two genes. Analysis in breeds that were fixed for the curly phenotype pinpointed a single SNP located in exon 2 of the *KRT71* gene that causes an Arg– > Trp change in a conserved region. Genotyping of over 600 dogs at this SNP showed an association between one allele and the curly phenotype with a *P* value of 1×10^{-89} (Cadieu *et al.*, 2009). The identification of *KRT71* as associated with curly fur was not a surprise; the same gene had been identified as causing curly coat in mice (Runkel *et al.*, 2006).

The most interesting aspect of this study is not that we used a single data set to map three genes. Rather, we showed that various combinations of alleles at only three genes explain the fur phenotypes of ~90% of the domestic dog breeds tested. Genotyping 631 dogs in 112

breeds produced seven combinations of genotypes across the three genes. Those combinations explain the seven most common phenotypes: short hair, long hair, curly hair, wiry hair, curly-wire hair, long hair with furnishings and curly hair with furnishings. All the short-haired breeds with straight coats, like the Labrador Retriever or Beagle, carry the ancestral form of each of these three genes. Wire-haired breeds, like the Australian Terrier, have only the variant form of the *RSPO2* gene and the smooth long-haired breeds, like the Golden Retriever, carry only the variant form of *FGF5*. If a dog has the variant forms of both *RSPO2* and *KRT71*, its coat is a curly-wire type, like that of an Airedale terrier, and the face has furnishings. When mutations at both *FGF5* and *KRT71* are observed, the phenotype is long and curly as in an Irish Water Spaniel, but the face is smooth. When the *RSPO2* and *FGF5* mutations are present together, the coat is long and straight and the face has furnishings as in the Bearded Collie and the Maltese. Finally, when all three mutations are present, the coat phenotype is long and curly with furnishings like the Poodle and Bichon Frisé.

The idea that variation at just three genes could explain so much of the fur phenotype highlights the distinction between a true complex trait and a complex phenotype. Body size and skull shape are true quantitative traits. Coat types are complex phenotypes obtained through the combination of multiple single trait mutations. The work on fur, however, is a nice demonstration of how much variation can be derived from mutations at just three loci.

Of note, the work done by Cadieu *et al.* (2009) does not account for all coat phenotypes that segregate in domestic dogs. For instance, the long fur of the Afghan hound is not explained by mutations in the *FGF5* gene. Also, coat-associated traits such as shedding have not yet been addressed in the current studies. Finally, the degree of curl that is observed across breeds is enormous and there are probably modifiers that contribute to that phenotype that are obviously not mapped by our studies.

The issue of coat colour has been tackled in part by Schmutz *et al.* (2002, 2003), Kerns *et al.* (2004), Berryere *et al.* (2005) and Candille *et al.* (2007), and won't be discussed here, but an interesting study by Karlsson *et al.* (2007) which identified the locus for white spotting (*S* locus) in Boxers using a GWAS study of 27,000 markers and less than 20 dogs is worth mention. This study highlighted the power of good experimental design in that it utilized only nine white and nine solid-coloured boxers. The initial region on CFA20 was large, a megabase, but it contained only one gene, the *microphthalmia-associated transcription factor* (*MITF*) gene. Through fine mapping studies and sequencing that included Bull Terriers which segregate the trait, as well as Boxers, the group identified candidate regulatory mutations in the melanocyte-specific promoter of *MITF*. The promoter region is critical for regulation of melanocyte development, migration and survival, and hence the results made excellent sense (Karlsson *et al.*, 2007). In a subsequent study, using 1500 SNPs, Leegwater *et al.* (2007) identified the same locus in the Boxer using a linkage-based approach. It should be noted, however, that these results were not a surprise. In a study a year earlier, in 2006, Rothschild *et al.* (2006), using a candidate gene approach, had demonstrated an association of *MITF* and white spotting in Beagle crosses and Newfoundlands. The data were limited and involved only three families, but the results were significant.

Role of Village Dogs in Understanding Morphology Genes

Many of the world's dogs are not, of course, pure-bred dogs, but rather live in cities and towns as free-living human commensals. Much like the very first domestic dogs, village dogs rely on people and their garbage for food, but are not selectively bred (Coppinger and Coppinger, 2001). In many parts of the world, village dog populations consist of indigenous dogs with high levels of genetic diversity and little if any recent admixture from modern breeds (Irion *et al.*, 2005; Boyko *et al.*, 2009). Whereas morphological variation in pure-bred dogs is largely a consequence of strong artificial selection by humans, variation in most indigenous village dog populations has been shaped by natural selection over millennia.

Very little is currently known about the genetic basis of morphological variation in

village dogs, although it is likely to be more complex than that found in breeds. Boyko *et al.* (2009) have studied population structure in African village dogs. To address questions related to breed origin, admixture and domestication, they sampled 318 village dogs from seven regions of Namibia, Egypt and Uganda, as well as some Puerto Rican street dogs and mixed breed dogs from the USA. They measured genetic diversity by sequencing the mitochondrial D-loop and genotyping 89 microsatellite-based markers. They also analysed breeds that have a theoretical African origin, including Basenjis, Pharaoh Hounds, Rhodesian Ridgebacks and Salukis. They found that African village dogs are a mixture of indigenous dogs that include non-native breed admixed individuals. Some putative African dogs, like the Pharaoh Hound, demonstrate clustering with modern breeds rather than with indigenous African dogs, suggesting that the current forms of these breeds are reconstructions that have occurred in recent times. This matches well with similar findings by Parker *et al.* (2004, 2007) in their cluster analysis of 133 breeds using 96 microsatellite-based markers (see Chapters 1 and 3 for experimental details).

Boyko *et al.* (2010) also looked at the role of known body size loci in the same population of indigenous dogs, and found that *IGF1* explained 17% of the variance in body weight within a population of Egyptian village dogs, versus a third of the variance found in pure-bred dogs. These results may overstate the effect of *IGF1* on village dogs, because it is possible that variation in body size between geographical regions may be almost entirely due to other loci and/or possibly to environmental influences. In addition, weight was used as a surrogate for body size in these studies (Boyko *et al.*, 2009).

Unlike the recent origin of most modern breeds, the long period of time for local adaptation in village dog populations allows for the gradual accumulation of variants of small effect that have an impact on morphological diversity. Likewise, the large effective population size of village dogs means that selection is more likely to result in multiple, partial sweeps underlying a trait rather than large stretches of homozygosity as can be found in pure-bred dogs. Despite these complexities, we believe that village dogs will be highly informative in studies of canine morphological variation. Causal variants underlying morphological variation in pure-bred dogs can be traced evolutionarily by using village dogs to determine the geographical origin, timing and distribution of the variant, and to look for signatures of positive or balancing selection. The relative ease of trait mapping in pure-bred dogs, coupled with the impact of natural selection on village dogs, will develop into a productive partnership which is certain to prove useful in advancing both canine population genomic and QTL mapping, with a long-term goal of understanding the genetic basis of adaptive evolution as well as finding genes that control complex traits.

Implications for Human Conditions

The picture that emerges from what we have described above is one in which a few, large-effect loci have been directionally selected in diverse dog breeds to create a plethora of morphological variation among, but little variation within, dog breeds. In many ways, dogs are the quintessential example of the 'common phenotype/common variant' hypothesis, as many of these alleles are at high frequency across dogs as a whole but are largely fixed for alternative alleles within breeds and, in combination, most traits can be explained with a handful of QTLs acting in concert. This picture stands in sharp contrast to human populations, in which the vast majority of phenotypic variation in traits such as body size and/or disease susceptibility for a score of complex traits cannot be explained even with hundreds of associated loci. A primary reason for this may be the action of natural selection in the two species. In humans, we know that most functional variation, at least at the amino acid level, is largely deleterious from a population genetic point of view (Bustamante *et al.*, 2005; Eyre-Walker and Keightley, 2007; Boyko *et al.*, 2008) and that recent human population growth, coupled with ancient bottlenecks, has largely affected the distribution of these variants (Gutenkunst *et al.*, 2007; Lohmueller *et al.*, 2008). The vast majority of common variations (>5% frequency) that we have queried for association in

humans is likely to be neutral, so we would expect a priori that they have weak effects. This, of course, has been borne out by the estimated odds ratios for most phenotype-associated alleles in humans.

In contrast, dogs have been wilfully selected to carry the various traits we aim to map. Therefore, the geneticist's job has been greatly simplified by the action of selection whereby genetic backgrounds are homogenized and alleles of large effect are preferentially fixed. One could argue that the human traits the community has set out to map have the opposite pattern. Such endeavours often include traits like blood pressure, fasting glucose and cholesterol levels, which relate closely to survival and are presumably under tremendous purifying selection. We would expect these traits to harbour potential functional variation, but that the variation would be likely to be quite rare owing to negative selection. Furthermore, the last 10,000 years have led to dramatic human exponential growth, which will increase the relative abundance of rare genetic variation. As we move towards characterizing more and more of the full spectrum of human genetic variation, we might expect a complementary picture to emerge. Alleles that affect the phenotype under stabilizing selection may begin to explain the diverging pictures of the genetics of complex phenotypes. Such alleles on their own are probably quite rare, but in totality they may be quite common such that every individual may carry several rare alleles, thus offering an explanation for the diverging views on the genetics of complex phenotypes.

Summary

The domestic dog is the most phenotypically diverse mammalian species, with average trait values for characteristics such as body size, skull shape, limb length and head size that range by orders of magnitude across breeds. The advent of fast and inexpensive dense genotyping technologies has allowed the identification of dozens of genomic regions underlying much of this phenotypic diversity and, often, identifying common causal variants shared across individuals and breeds. Furthermore, many of the loci implicated in dog morphology appear to play very similar roles in other systems, including mice and humans. The success of dogs as a model system for identifying genes governing variation in mammalian body plans (and for predicting morphological phenotype from genotype) stands in sharp contrast to the results from humans, where common genetic variants appear to explain a modest amount of the variation among individuals in height, weight and body mass index. We have reviewed recent results from our work and that of others in identifying QTLs and genes governing variation in dog body size, shape and proportion. These data highlight the way in which the 'common disease/common variant' hypothesis may readily explain variation in dogs.

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17 Canine Olfactory Genetics

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Introduction

Olfaction is one of the senses that were developed by animals during evolution to inform themselves on the external world in order to find food, escape predators and dangers, and look for sexual mates. The olfaction process consists of the detection and identification of odorant molecules in the environment. There is a very large panel of odorant molecules and animals have developed a complex repertoire of dedicated olfactory receptors (ORs) to capture this information. Olfaction is well developed in the dog, as in the wolf, its ancestor. In addition, many dog breeds have been created and selected over centuries as hunting dogs, particularly scent dogs that use their olfactory skills to track game. Nowadays, some dogs with exquisite olfactory detection capabilities are specifically trained to detect particular odours, such as those emanating from explosives, drugs or truffles. Some breeds are also used for their olfactory capabilities in looking for individuals after earthquakes or other catastrophes and, more recently, to detect cancers in some patients.

Olfaction as a whole relies on several steps: the perception, discrimination and identification of the odorant signals. Discrimination is the ability to distinguish different signals in a chemically complex environment. Identification is the recognition of these signals by comparing them with already memorized information. The first step of olfaction occurs in the nasal cavity. There, the odorant molecules are captured by ORs. Downstream signalling converts a chemical signal into an electric signal that is transmitted to different parts of the brain. In the dog, the genetics of olfaction has been mainly focused on the study of the OR gene repertoire.

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Olfactory Systems

In mammals, we can distinguish two olfactory systems composed of several structures that are anatomically separated: the main system, which consists of the olfactory mucosa and the olfactory bulb, and the accessory system, which consists of the vomeronasal organ and the accessory olfactory bulb. The olfactory mucosa, which is found in the nasal cavity, is the tissue where interactions between the odorant molecules and the ORs occur. Indeed, this mucosa has a highly specialized epithelium – the olfactory epithelium or olfactory neuroepithelium – which contains neurons; the surface of this epithelium are covered with mucus. The nasal cavity contains the respiratory epithelium, which does not contain any neurons. The two epithelia are distinguished by their colour and their thickness, pink and thin for the respiratory epithelium and brown and thick for the olfactory epithelium. In the dog the two epithelia may have regions where they overlap. The nasal cavity also contains large spongy bones that are wound on themselves and are called the nasal concha or turbinates. There are three in each nostril, one inferior (ventral or ethmoidal), one middle and one superior (or dorsal). These increase the surface of the respiratory and olfactory epithelia, and they consist of four endoturbinates (I–IV): I is in the dorsal turbinate, II is in the middle turbinate and III and IV are in the ventral turbinate (see Fig. 17.1). A study of the comparative functional structure of the olfactory mucosa in the dog and the sheep revealed that it is better structurally refined in the dog, that the olfactory epithelium presents a greater thickness owing to an increase of the number of olfactory cells (Kavoi *et al.*, 2010). The size of the olfactory mucosa is variable depending on the dog breed: for example, it is 200 cm^2 in the German Shepherd and 67 cm^2 in the Cocker Spaniel.

The neuroepithelium is a pseudostratified epithelium made up of three cell types: the support cells, the basal cells and the olfactory neurons. These neurons represent 60–80% of the cells found in the epithelium. They are bipolar neurons composed of a dendrite, a cellular body and an axon. The dendrite ends in the epithelium surface as a bud, from which several cilia emerge. These cilia increase the surface of interaction with the odorant molecules, and it is at their surface that the ORs are expressed. The axon extends towards the olfactory bulb and carries the transmission of the olfactory message from the nasal cavity. The olfactory bulb is the first and unique synaptic relay of the

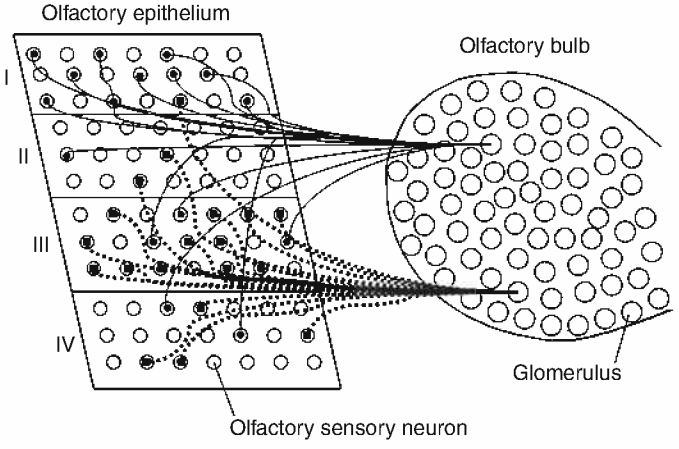

Fig. 17.1. Connections between the olfactory epithelium and the olfactory bulb. The olfactory epithelium is divided between four endoturbinates, which are labelled I to IV. Olfactory sensory neurons (OSNs) are not equally distributed between the four endoturbinates and are only one olfactory receptor (OR) each. The axons of OSNs expressing the same OR converge to the same glomerulus in the olfactory bulb.
The differently expressed ORs are represented by black square and circle (inspired by Mombaerts, 2001).

olfactory signal to the brain. The axons of the olfactory neuron are grouped first into 10–100 fibrils, which constitute the olfactory fibres, and then into the olfactory nerve. This nerve goes through the cribriform plate and reaches the olfactory bulb. The bulb is organized into concentric layers. At its periphery, there are glomeruli, which are spherical structures where axons of neurons expressing the same OR are grouped (Mombaerts *et al.*, 1996; Mombaerts, 2001) (Fig 17.1). The glomeruli make synapses with the apical dendrites of the olfactory bulb cells. The axons of the olfactory bulb cells form the lateral olfactory tract that reaches the cortex.

The accessory olfactory system is responsible for the detection of particular odorant molecules: the pheromones. The distinction between the main and accessory systems is not as strict as was first thought when it was suggested, but not proven, that olfaction might play a role in the detection of pheromones. Pheromones allow chemical communication between individuals of the same species and principally provide sexual and social clues. The vomeronasal organ, which is situated at the base of the nasal septum and is thus separated from the olfactory epithelium, is also composed of three cell types similar to the ones found in the olfactory epithelium. The neurons of the organ express pheromone receptors called vomeronasal receptors (VRs). There are two types of VRs: V1Rs, which are expressed in the apical layer of the vomeronasal organ epithelium; and V2Rs, which are expressed in the basal layer. The axons of the neurons are grouped to form the vomeronasal nerve, which goes to the accessory olfactory bulb. Secondary neurons of this bulb will relay information directly to the tonsil, without being processed by the cortex, i.e. the information is not treated consciously.

Other organs, although not yet identified in dogs, have also been identified in mammals (such as the mouse or rat) that also express chemical receptors. An example is Masera's organ in the nasal septum; even if this expresses only a fraction of the OR repertoire (see Kaluza *et al.* (2004) and Tian and Ma (2004) for studies in the mouse), it would be extremely sensitive to a wide variety of odorant stimuli (see Marshall and Maruniak (1986) and Grosmaitre *et al.* (2007) for studies in the rat). The Grueneberg ganglion also expresses V2Rs and trace amine-associated receptors (TAAR) in mice during the prenatal stages (Fleischer *et al.*, 2007). Indeed, this last organ could be implicated in the detection of stimuli during mother–infant interactions.

The Different Types of Odorant Receptors

ORs were the first identified receptors implicated in olfaction (Buck and Axel, 1991). Since then, other receptors have been identified: the VRs (V1Rs and V2Rs), TAARs and formyl peptide receptor-like proteins (FPRs). VRs were identified using cDNA (complementary DNA) extracted from the vomeronasal organ of the mouse and rat (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). The nature of the ligands is not entirely identified, but it has been suggested that the V1Rs detect volatile odorants and V2Rs non-volatile molecules. The size of the VR repertoire varies even if the vomeronasal organ is functional: rodents have more than 100 functional V1R genes whereas the dog only has eight functional V1Rs (Young *et al.*, 2005). The V2Rs are found only in the form of pseudogenes in the human, chimpanzee, macaque, cow and, also, in the dog (Quignon *et al.*, 2006; Young and Trask, 2007). Thus, the function of the V2Rs in these species is questionable. TAARs are expressed in some olfactory neurons scattered in the olfactory epithelium and in some neurons of the Grueneberg ganglion (Fleischer *et al.*, 2007). The number of genes varies a lot depending of the species: there are 15 in the mouse, 17 in the rat, two in the dog and six in humans (Liberles and Buck, 2006). Concerning FPRs, two studies have shown that they are expressed in the vomeronasal organ of the mouse (Liberles *et al.*, 2009; Rivière *et al.*, 2009). These receptors were first described in the immune system and may play a role in the stimulation of chemotaxis towards infection sites (Migeotte *et al.*, 2006). The expression profiles of the FPRs are very similar to those of the V1Rs and V2Rs, i.e. in some scattered neurons of the vomeronasal organ.

The Olfactory Receptors (ORs)

OR gene identification and structure

Until 1991, the OR genes were unknown. Richard Axel and Linda Buck made their discovery based on three assumptions (Buck and Axel, 1991). First, G proteins were known to be implicated in the transduction of the olfactory message, so the ORs should be G protein coupled receptors (GPCRs), and as such should be seven transmembrane proteins. Secondly, the number of OR proteins must be high to detect and discriminate between numerous and diverse odorant molecules. Hence, ORs should be encoded by members of a large family. Finally, only olfactory neurons should express ORs. So Buck and Axel (1991) defined degenerate PCR primers corresponding to conserved amino acid patterns of known GPCRs and amplified mRNA extracted from rat olfactory epithelium cells. This experiment allowed the identification of 18 rat OR genes. Two years later, Raming *et al.* (1993) confirmed that the discovered receptors were able to bind with odorant molecules. Degenerated primers were then used with different species, including the dog (Parmentier *et al.*, 1992; Issel-Tarver and Rine, 1996; Vanderhaeghen *et al.*, 1997; Quignon *et al.*, 2003), to amplify either mRNA from olfactory epithelium or genomic DNA.

In mammals, OR genes are composed of two exons, but only the second one encodes the protein and this has a size of about 1000 nucleotides. The expressed protein has a size of about 300 amino acids, but no signal peptide to direct their intracellular transport has been identified to date. Like all GPCRs, the ORs have seven transmembrane domains (Fig 17.2), but they have a specific amino acid pattern that distinguishes them from other GPCRs: for example the PMYLFGNLS pattern at the beginning of transmembrane domain II (TMII), or MAYDRYVAIC at the end of transmembrane domain IV (TMIV) and at the beginning of intracellular loop 2 (IC2). They also contain variable transmembrane domains (like TMIII, TMIV and TMV) that are implicated in odorant molecule recognition (Katada *et al.*, 2005). Before whole genome sequencing was commonplace, the identification of OR genes using degenerate primers based on the conserved OR patterns was a lengthy and tedious task: DNA needed to be amplified using low stringency parameters, the amplified products cloned and then each clone sequenced independently. Nowadays, the availability of whole genome sequences makes the identification and study of these genes easier. Indeed, in addition to their small size, the genes can be identified using their specific amino acid patterns by a simple mining of the genome using alignment tools such as BLAST, or pattern recognition tools. Identification of the OR repertoire in several species has shown that the OR genes constitute the largest multigenic family known in mammalian genomes.

OR gene repertoires and genomic organization

Data mining using pattern recognition of the unassembled traces of the dog genome led to the identification of 1094 genes, of which 20.3% are pseudogenes containing one or several nonsense or indel (insertion/deletion) mutations (Quignon *et al.*, 2005). This pseudogene rate is much lower than that in the human OR repertoire (about 50%) and could explain in part why dog has a well-developed sense of smell. In comparison, the OR pseudogene rate of other species, such as the mouse or rat, which also have a good sense of smell, is about 20% (Godfrey *et al.*, 2004; Quignon *et al.*, 2005; Zhang *et al.*, 2007a). In addition to this difference in pseudogene composition, the size of the whole repertoire should also be considered. Including pseudogenes, the dog has 1094 OR sequences in the genome, whereas the human has between 600 and 900 (Glusman *et al.*, 2001; Malnic *et al.*, 2004), the mouse has between 1200 and 1400 (Godfrey *et al.*, 2004; Zhang *et al.*, 2007a) and the rat has about 1600 (Quignon *et al.*, 2005; Zhang *et al.*, 2007a) (Table 17.1). These variations in repertoire size, particularly for humans (at 600 to 900), do not reflect any polymorphism, but are to do with the use of different parameters to screen the human genome. In any case, they should not be confounded with the inter-individual variations that

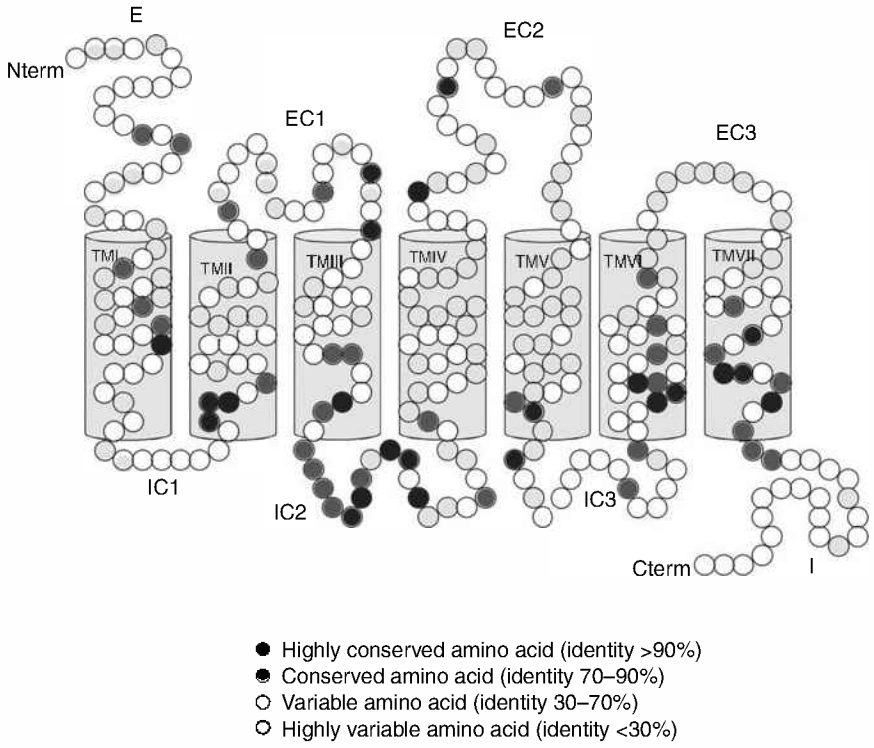

Fig. 17.2. Protein structure of dog olfactory receptor (OR), showing the position of conserved and variable amino acids in the 1009 full-length dog OR proteins (from 791 genes and 218 pseudogenes for which the coding phase was manually restored). E and EC, extracellular domain; I and IC, intracellular domain; TM, transmembrane domain (from Quignon *et al.*, 2005).

Table 17.1. Numbers of olfactory receptor (OR) genes and pseudogenes in four mammalian species. Data were extracted from Godfrey *et al.* (2004) (mouse), Nimura and Nei (2005) (human) and Quignon *et al.* (2005) (dog and rat).

	Human	Dog	Rat	Mouse
Number of OR genes	388	872	1234	913
Number of OR pseudogenes	414	222	311	296
Total number of ORs	802	1094	1545	1209

have been more recently identified through copy number variation (CNV) analysis (Feuk *et al.*, 2006; Freeman *et al.*, 2006). In terms of potentially active OR genes – genes defining an open reading frame (ORF) able to code an OR with all the described patterns – the dog, mouse and rat have 2.5–3.5 times more genes than humans, which relates to the olfactory capacities of these species. The number of potentially active OR genes varies between these four species, but the reasons for these variations are still unknown. One hypothesis could be that the size of the repertoire reflects specific olfactory needs, either the detection of a larger range of odorant, or a higher power of detection of specific odorants.

Localization of the OR repertoire on the canine genome assembly (Lindblad-Toh *et al.*, 2005), using BLAST, radiation hybrid mapping (Quignon *et al.*, 2003) and pattern recognition, showed that the dog OR repertoire is distributed across 49 loci located on 24 out of the 39 chromosome pairs composing the dog karyotype (Quignon *et al.*, 2005) (Fig 17.3).

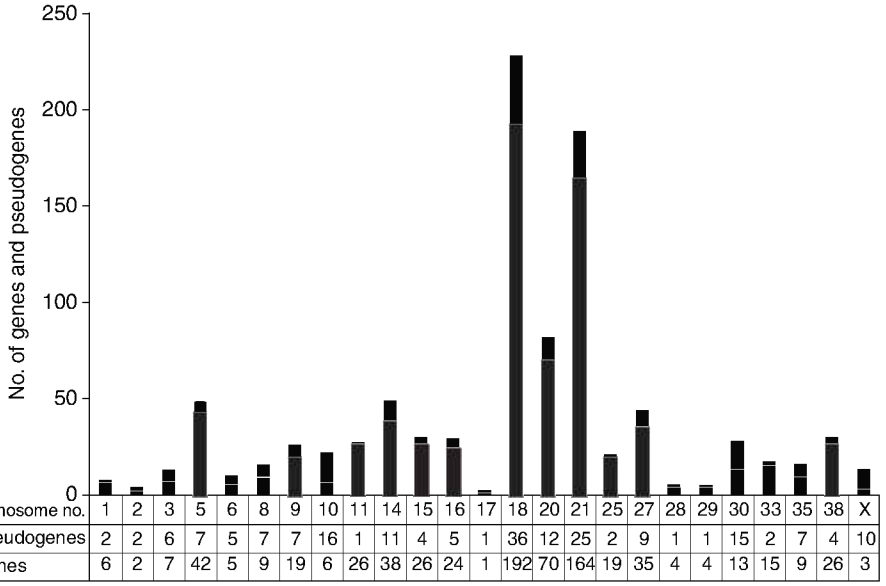

Fig. 17.3. Chromosomal distribution of canine olfactory receptor (OR) genes, showing number of OR genes and pseudogenes for each canine chromosome containing OR sequences (data from Quignon *et al.*, 2005).

This organization in clusters is not specific to the dog as it has also been observed in the human and the mouse (Glusman *et al.*, 2001; Young *et al.*, 2002; Zhang and Firestein, 2002). In order to compare the composition of these clusters, the OR genes were classified into families and subfamilies. This classification was performed using criteria described by Ben-Arie *et al.* (1994). All the proteins of a given species were aligned and the coding phases of the pseudogenes were artificially restored to include them in the alignment, which was then used to calculate the amino acid sequence identity between the OR proteins. An OR family is composed of OR proteins that have at least 40% amino acid identity and subfamily members that have at least 60% identity (Ben-Arie *et al.*, 1994). Humans and dogs have a similar number of subfamilies (300), but nearly half of the human subfamilies are only composed of pseudogenes; this reflects the higher number of pseudogenes in this species rather than diversification of the OR repertoire (Quignon *et al.*, 2005). When looking at the chromosomal localization of OR genes from the same subfamily, the majority are found in only one cluster (i.e. 93% of the canine subfamilies), as has already been shown in the human (Malnic *et al.*, 1999). Orthologous families and subfamilies can be identified between species. With the addition of synteny information, a comparison of clusters between species can be made, as well as a comparison of non-OR genes located between OR clusters. Despite being scattered into several chromosomes in every species, orthologous clusters can easily be found, showing that common ancestors must already have had OR genes on multiple chromosomes, and that local duplication of OR genes would be at the origin of the OR diversity. The evolution of this gene superfamily seems to be concordant with the birth and death model (Sharon *et al.*, 1998), in which new genes are created by successive duplication, followed by divergence and the maintenance of some duplicated genes or the accumulation of deletions in others (Young *et al.*, 2002; Niimura and Nei, 2005).

The amino acid alignment can also be used to construct a phylogram. In this phylogram, in addition to the families and subfamilies of ORs, we can distinctly observe two major branches in which the OR genes were historically called class I and class II. Class II OR genes

were the first OR genes identified by PCR with degenerate primers in terrestrial mammals. Class I OR genes were identified using the same method, but in fishes, and it was thought that class I OR genes were specific to soluble odorants and class II to volatile odorants. The presence of the two classes in an amphibian reinforces this hypothesis (Freitag *et al.*, 1995, 1998), although no experiment was performed to confirm this theory. Whole genome data mining of several species also questions this hypothesis because class I OR genes are found in the human, rat, mouse and dog. Indeed, about 100 genes in the human and about 200 in the dog belong to class I. Interestingly, all the OR genes belonging to this class are localized in only one cluster in the human, mouse, rat and dog. In addition, this cluster does not contain any OR genes from class II. Furthermore, the number of class I genes is quite similar in fishes and in terrestrial mammals, and the number of pseudogenes in class I is smaller than in class II.

OR gene polymorphism

In the cascade of olfactory reactions, ORs are the first elements to be activated, and polymorphisms of the OR genes could at least partly explain inter-individual variations in olfactory sensitivity and capacity. To date, two types of genomic variations leading to OR polymorphism have been reported: SNPs (single nucleotide polymorphisms) and CNVs. Polymorphism in OR was first demonstrated in humans. Two studies performed on the same OR gene cluster were realized on a cohort of unrelated individuals (Gilad *et al.*, 2000; Sharon *et al.*, 2000), and on different human populations (Menashe *et al.*, 2002). These studies revealed a high level of polymorphism in the OR coding regions. Another study focused on SNP variations that might occur in OR pseudogenes, in which ORFs are interrupted by a single mutation when compared with the human reference sequence, and thus might have been acquired recently. When performed with a set of 33 OR pseudogenes, this study revealed a high level of polymorphism between individuals of different origins and a unique set of potentially active OR genes per individual (Menashe *et al.*, 2003). Gilad and Lancet (2003) obtained similar results when they observed significant differences in the size of the intact olfactory repertoire in two human populations.

In the dog, polymorphism of OR genes within and between dog breeds was reported in two studies. In a first (preliminary) study, a small number of OR genes (16) were selected from classes I and II and various families and subfamilies composing the canine OR repertoire. They were analysed in 95 dogs from 20 breeds (Tacher *et al.*, 2005). A total number of 98 SNPs and four indels were detected. All the studied OR genes were polymorphic but at different levels, from two up to 11 SNPs per OR gene. The minor allele frequency (MAF) of these SNPs varied from 0.5% to 50%, with 35 SNPs having a frequency less than 5% in the 95 dogs. More than half of the SNPs induced an amino acid change, with 30 involving a change of amino acid to a different chemical group. These amino acid changes occurred in all parts of the OR protein. Dog OR genes were defined as highly polymorphic, with each analysed gene having multiple alleles, a much higher figure than is found for other coding sequences or even non-coding sequences. Five OR genes had an allele with an interrupted ORF that resulted from a SNP or an indel introducing a premature stop codon. Different subsets of pseudogenes between individuals or breeds were described, as outlined for human populations in the previous paragraph.

In a second extensive study, 109 OR genes were sequenced in a cohort of 48 dogs of six breeds (German Shepherd Dog, Belgian Malinois, Labrador Retriever, English Springer Spaniel, Greyhound and Pekingese) (Robin *et al.*, 2009). These OR genes were selected to be representative of a large number of OR families and subfamilies, and to belong to several clusters with high or low OR gene density or even to isolated OR genes. This study confirmed the high level of polymorphism of dog OR genes, with some 732 mutations detected in all but four of the 109 genes: 710 SNPs, 17 short indels (1–3 nucleotides, or nt) and five longer indels (6–74 nt). Each OR gene contained 1–22 SNPs, and the distribution of the SNPs along the gene was variable. As in the previous study, differences between dog breeds were observed, i.e. the total number of

SNPs identified, as well as the number of OR genes without SNPs, was significantly different between dog breeds. At the whole population level, OR genes tended to be either weakly or highly polymorphic, with some exceptions: some genes were poorly polymorphic or not polymorphic in one breed but highly polymorphic in the five other breeds. As demonstrated in the first study, the MAF varied from 1% to 50% across all breeds, and the frequency within breeds could differ from the frequency across breeds. For example, some alleles were absent in all but one breed where they constituted the major allele. Across all breeds, OR genes were more polymorphic than any other sequenced exon sequences and non-coding DNA (intergenic sequences) (Table 17.2). There was a relationship between the cluster localization (small or large OR genes clusters) and the polymorphism level. Indeed, the least polymorphic OR genes were preferentially localized in small OR gene clusters and the highly polymorphic OR genes in large OR gene clusters. Reciprocally, OR genes in small clusters tended to be less polymorphic than OR genes in large clusters. From the 732 mutations detected, 307 were silent SNPs, 273 were missense SNPs (with 130 that would result in the incorporation of an amino acid of a different chemical group) and 152 led to an interrupted ORF (pseudogene alleles). As described by Tacher *et al.* (2005), amino acid substitutions were distributed along the whole length of the proteins: in the transmembrane, and in the inner and outer domains of the receptor. The K_a/K_s value ratio, which is representative of the strength of selection affecting proteins during evolution, showed an absence of strong selective constraint, resulting in greater diversification of the OR genes. This characteristic was previously observed for a small subset of human and chimpanzee OR genes, and for human TAS2R genes encoding bitter taste receptors (Gilad *et al.*, 2003; Kim *et al.*, 2005). Out of the 109 OR genes analysed, seven were strictly pseudogenes, 86 were intact in all breeds, and 16 genes had both intact and interrupted ORFs (pseudogene alleles). For each of these 16 OR genes, the pseudogene allele frequency varied between breeds. So one OR gene cannot be called either intact or a pseudogene at the whole dog population level without some doubt. This also suggests that pseudogene formation is still an active process, as previously reported for human OR genes (Gilad and Lancet, 2003). This on-going pseudogenization process can be viewed as the counterpart of the acceptance of a large proportion of mutational events, in relation to the K_a/K_s value ratio. This leads to the diversification of the OR gene repertoire and its continuous adaptation to a changing environment. For OR genes that had more than two SNPs, 809 haplotypes were identified, and the mean number of haplotypes per OR gene and per breed varied. A total of 332 breed-specific haplotypes (i.e. 41%) was found. The combination of a small number of haplotypes may result in a haplotype signature for each breed. The extent of linkage disequilibrium (LD), which indicates an association between two polymorphic markers for which pairs of alleles are inherited together, was determined within OR genes. The mean r^2 value calculated for each breed

Table 17.2. Mean N values for olfactory receptor (OR) genes and other sequences in six dog breeds. The N index is based on the number of SNPs (single nuclear polymorphisms) detected through the pairwise comparison of all OR sequences and the occurrence of two alleles of each SNP. Thus, the smallest N value denotes the highest level of polymorphism (Robin *et al.*, 2009).

	Total size (bp)	No. of SNPs	GSD	BM	ESS	GRE	LAB	PEK
109 OR genes	103762	733	926	617	594	778	634	628
Exons	3685	3	29480	29480	9213	5669	10284	8189
Introns	4766	10	2948	2487	1993	2334	2183	2373
Intergenic sequences	18716	97	864	943	848	735	878	863

GSD, German Shepherd Dog; BM, Belgian Malinois; ESS, English Springer Spaniel; GRE, Greyhound; LAB, Labrador Retriever; and PEK, Pekingese.

varied from 0.52 to 0.7, with a mean of 0.33 for the whole population, which is one-tenth of the mean extent of LD previously reported for other dog genes (Lindblad-Toh *et al.*, 2005). In conclusion, this study suggests an ongoing gene conversion process similar to that previously reported in other species, such as humans and primates (Sharon *et al.*, 1999; Newman and Trask, 2003). The LD value calculated within five OR clusters appeared higher for each breed than for the whole set of dogs (between 70% and 94% of SNP pairs, with a D′ statistic value of >0.8 in each breed, compared with 48% for the LD of the whole set). This result is consistent with the analysis of a human OR cluster in different populations (Menashe *et al.*, 2002).

The link between genetic variation in ORs and odour perception was first demonstrated by a correlation between the presence of two SNPs in the human *OR7D4* gene and the quality perception of androstenone (Keller *et al.*, 2007). In addition, Menashe *et al.* (2007) showed an association between sensitivity to isovaleric acid and the genotype of a human segregating pseudogene, *OR11H7P* (Menashe *et al.*, 2007). The canine OR genes have some polymorphisms that are breed specific. Thus, as in the human, this polymorphism could affect the odorant detection capabilities and might in part explain breed olfactory differences. At present, it is not possible to correlate OR genetic diversity with variation in odorant perception in the dog because the ligands of most of the dog ORs are unknown.

More recently, a second form of genetic variation has been reported that corresponds to DNA segments that are CNVs in comparison with a reference genome. Different analyses of the human genome have shown that OR genes are enriched in chromosomic regions containing CNVs (Nozawa *et al.*, 2007; Hasin *et al.*, 2008; Young *et al.*, 2008). Chen *et al.* (2009) established the first dog genome map of CNVs, and demonstrated that the extent of this variation and some of the affected gene classes are similar in canines to those of mice and humans. Notably, there is an over-representation of ORs and immunity-related genes in CNV regions. Another study on the dog genome showed that CNVs span 429 genes that are involved in a wide variety of biological processes, including olfaction (Nicholas *et al.*, 2009). Further analyses of the CNVs could add information on the evolution of OR clusters in the dog genome. In addition, CNV analyses across different breeds could shed some light on breed olfactory performance.

OR gene expression

Until now, no study concerning dog OR gene expression has been performed, mainly because of problems regarding the sampling of olfactory epithelium. However, OR gene expression profiles in olfactory epithelium tissues have been analysed for the mouse, human and rat. The data obtained from these three species show a strong correlation, and it can be assumed that they can be transferred to other mammals, such as the dog.

A study made by Young *et al.* (2003) showed that over one-third of the mouse OR gene repertoire would be expressed in the olfactory epithelium. This result, which was obtained by mRNA cloning and sequencing, indicated that the level of expression varied considerably between OR genes. Further studies were made by microarray hybridizations. Zhang *et al.* (2004) in an analysis of mouse olfactory epithelium mRNA revealed that up to 70% of OR genes are expressed at a detectable level, with no difference between males and females. They also found that OR gene expression is regulated during development, and that the spatial pattern of expression in the olfactory epithelium is reflected in chromosomal organization and is unequally distributed between the dorsal and ventral portions of the turbinates. A small number of OR genes was expressed in non-olfactory tissues (testis, brain, heart, taste and other tissues) as well. However, very few ORs were expressed exclusively in non-olfactory epithelium tissues. Analysis of three human olfactory epithelium RNA samples demonstrated that 76% of predicted OR genes were expressed in the olfactory epithelium and that the repertoire of expressed OR genes varied between the three individuals studied (Zhang *et al.*, 2007b). Surprisingly, this study revealed that 67% of human OR pseudogenes are expressed in the olfactory

epithelium. It also confirmed that some OR genes had a high expression level in non-olfactory tissues. Feldmesser *et al.* (2006) analysed the expression of hundreds of human and mouse OR transcripts using EST (expressed sequence tag) and microarray data in several dozens of human and mouse tissues. This study confirmed that different tissues had specific, relatively small, OR gene subsets that had particularly high expression levels. In another study, whole-rat genome microarrays were used to analyse the transcriptome of the adult rat olfactory epithelium: two-thirds of the probes identified genes expressed at a detectable level in this tissue (Rimbault *et al.*, 2009). Most of the OR genes (65% of the total functional OR repertoire) was expressed and, again, considerable variation in the range of expression levels was observed depending on the OR gene. In addition, OR genes were differently expressed between the four endoturbinates that constitute the olfactory epithelium. As in the mouse, no significant difference was detected between males and females. The transcriptome of the olfactory epithelium of adult rats was compared with those of newborn and aged rats (Fig. 17.4). The vast majority of genes were common to all three age groups but there were marked differences in their expression levels. A number of OR genes were observed to be specifically expressed in adult and older rats or in newborn rats. Even in the absence of knowledge on the ligands that correspond to these specifically expressed ORs in the newborn rat, one could hypothesize that these ORs participate in mother–newborn communication at a stage where the newborns are still blind and deaf.

The first expression experiments of canine OR genes were realized by Northern blotting and showed the expression of some ORs in the testis (Parmentier *et al.*, 1992). A further study using an RNase protection assay demonstrated that the few dog OR genes that are essentially expressed in the testis presented little or no expression in the olfactory mucosa (Vanderhaeghen *et al.*, 1993). RT (reverse transcription)-PCR and sequencing experiments demonstrated that the male germ line of three mammalian species (rat, mouse, dog) was characterized by a specific set of olfactory receptors, which display a pattern of expression suggestive of their potential implication in the control of sperm maturation, migration or fertilization (Vanderhaeghen *et al.*, 1997). These hypotheses were later confirmed by a study on a human OR, OR1D2, also called hOR17-4, which is implicated in sperm chemotaxis. It was found that spermatozoa migrate and accumulate at the maximal concentration area of the odorant molecule bourgeonal (the most active of a range of molecules tested for

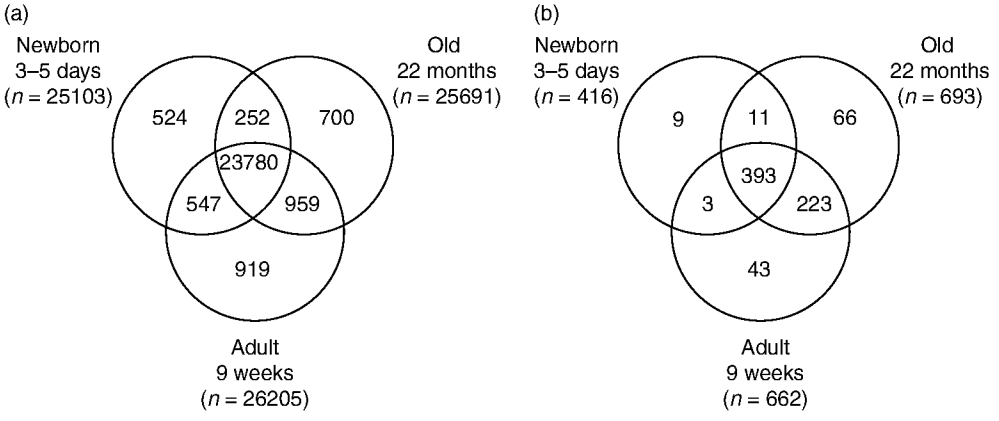

Fig. 17.4. Venn diagrams of transcripts and olfactory receptor (OR) genes expressed in rats. The number of gene transcripts expressed (a) and the number of OR genes expressed (b) is indicated for each age group: newborn, adult and old (from Rimbault *et al.*, 2009).

ability to activate hOR17-4) when they are exposed to crescent gradients of this molecule (Spehr *et al.*, 2003). In the same way, in the mouse, the *Olfr16* gene (or mOR23) enables the spermatozoa to swim along a lyral concentration gradient (Fukuda *et al.*, 2004).

Two important facts emerge from these studies: (i) ORs are essentially, but not exclusively, expressed in the olfactory epithelium; and (ii) the level of expression of the different ORs is very large (several hundred-fold) between the most and the least expressed OR. So far, we unfortunately do not completely appreciate the consequences of these two facts. For example, why are some ORs highly expressed and others barely detectable? Can these levels of expression be tuned in response to the environment or not? Would the same differences exist across dog breeds with different olfactory performances?

Regulation of OR gene expression

The super family of OR genes is not the only one in the genome to be organized in clusters. The Hox genes, globins or even immunoglobulins also have a genomic organization in clusters. For the Hox and globin genes, this organization has a direct impact on the regulation of the expression of these genes. For immunoglobulins, DNA rearrangement called V(D)J (somatic recombination) allows the expression of only one immunoglobulin in one lymphocyte. In the olfactory epithelium, only one OR is expressed in one neuron – even only one allele (Chess *et al.*, 1994). Thus, regulation needs to occur at three levels: (i) the choice of the OR to be expressed by a particular olfactory neuron; (ii) the prevention of expression of a second OR by the same olfactory neuron; and (iii) allelic exclusion, preventing the expression of a second allele by this very same olfactory neuron. In addition, a given OR gene is expressed by a few thousand olfactory neurons, which are usually scattered within a particular spatial zone of the olfactory epithelium (Strotmann *et al.*, 1992; Ngai *et al.*, 1993; Ressler *et al.*, 1993; Vassar *et al.*, 1993; Iwema *et al.*, 2004; Miyamichi *et al.*, 2005; Rimbault *et al.*, 2009). This inactivation phenomenon, or allelic exclusion, is rare and would only concern OR and immunoglobulin gene families. However, the mechanism of choice of OR and of allelic exclusion has not yet been identified. On the basis of previous studies in other multigenic families, three potential mechanisms have been proposed for the choice and activation of OR genes (Serizawa *et al.*, 2004): (i) DNA recombination, as observed for V(D)J joining; (ii) gene conversion, which transfers a copy of the gene into an expression cassette; and (iii) physical interaction of a locus control region (LCR) with only particular OR gene promoter. So far, none of these mechanisms has been proven to be correct or of importance. Negative feedback regulation by the OR protein has been also proposed as a mechanism for prohibiting the expression of a second OR gene (Reed, 2000; Serizawa *et al.*, 2003). There are, however, exceptions to this rule, such as the co-expression of two ORs in some rat olfactory neurons that has been demonstrated by *in situ* hybridization experiments (Rawson *et al.*, 2000).

The implication of olfaction in the choice of a sexual mate according to its major histocompatibility complex (MHC) has raised more interest with the discovery of OR cluster(s) near the MHC in almost all vertebrates studied in detail. The first report of the MHC influencing mating preference was published over 35 years ago in mice (Yamazaki *et al.*, 1976). It was suggested, but not proven, that olfaction might help female mice to distinguish between males that were similar to themselves and males that were MHC dissimilar, the choice being to mate with the latter. In 2002, a study in humans showed that male odour preference by women was associated with the MHC alleles inherited from their fathers (Jacob *et al.*, 2002). However, the OR genes implicated in this process are still unknown, and it is possible that these OR genes do not belong to the OR cluster located on HSA6 close to the MHC locus. In agreement with this last possibility, one must recall that in the dog genome the DLA (dog leucocyte antigen) locus is on CFA12 while the orthologous OR cluster is on CFA35 (Santos *et al.*, 2010).

Signal transduction cascade and specificity of odorant/OR interaction

The first step in the perception of an odorant is the interaction between the odorant and its receptor/s. This binding leads to a signal transduction cascade that transforms chemical information into electrical information. The binding of the odorant activates the OR protein which, in turn, stimulates an olfaction-specific G protein called Gα(olf). The action of adenylyl cyclase III then leads to the production of cyclic AMP (cAMP) which binds and opens cyclic nucleotide-gated channels (CNG) (Nakamura and Gold, 1987). The resulting calcium influx through the CNG channels leads to the opening of the calcium-gated chloride channels (Restrepo *et al.*, 1990; Kleene and Gesteland, 1991; Kurahashi and Yau, 1993; Lowe and Gold, 1993; Leinders-Zufall *et al.*, 1997). Calcium influx and chloride efflux allow the depolarization of the olfactory neurons membrane. Additionally, depending upon the odorant and the OR to which it binds, the IP3 pathway, which also leads to an increase in intracellular calcium concentration, may be activated either instead of or in addition to the cAMP pathway (Restrepo *et al.*, 1990; Bruch, 1996; Spehr *et al.*, 2002).

Information about OR ligands is still very limited. Two main methods to identify odorant/OR pairs are commonly used. The first approach corresponds to exposure of fresh epithelium explants to an odorant, the isolation of responding neurons by microdissection, RT-PCR amplification of the expressed mRNA, cloning and sequencing (Malnic *et al.*, 1999; Touhara *et al.*, 1999). The second approach depends on the transient expression of one selected OR gene in heterologous cells, such as human HEK293 cells. In this case, transfected cells are exposed to an odorant (Krautwurst *et al.*, 1998; Wetzel *et al.*, 1999; Kajiya *et al.*, 2001; Gaillard *et al.*, 2002; Benbernou *et al.*, 2007). An increase in calcium or cAMP intracellular concentration is generally used to detect the odorant/OR interaction *in vitro*.

In vitro interaction between canine ORs and odorant molecules was described by Benbernou *et al.* (2007). In this study, 38 canine OR genes, belonging to class II family 6, were cloned and transiently expressed in a mammalian cell line that expresses Gα(olf). Cells were exposed to a series of C6–C12 aliphatic aldehydes and odorant/OR interaction was detected through calcium concentration measurements. It was observed that no two aldehydes bound to the same set of ORs and that up to 28 of the ORs recognized octanal, indicating that a very complex combinatorial code, combined with a non-additive receptor code, seems to be the strategy for the perception of many individual odorants and the myriad of odorant mixtures.

Canine Olfaction

Dog olfaction is very sensitive compared with that of some other mammals, such as the human. However, the olfactory capabilities of the dog are variable between breeds, with some breeds specifically selected to use their olfaction – like scent hounds. How can this extraordinary capability and these differences between dog breeds be explained? First, we can consider that a larger size of the olfactory epithelium would contribute to better odorant perception. Dog breeds are known to have variably sized olfactory epithelium. The number of olfactory neurons present in the olfactory epithelium could also be implicated. Secondly, a higher number of OR genes could contribute to higher sensitivity to different odorants than a smaller number. So the OR repertoire size and OR pseudogene fraction could explain in part the variability of odorant detection capabilities between mammals. For example, the human, which is considered to be microsmatic, has 2.5 times fewer potentially active OR genes than the dog, which has more than 800 intact OR genes (Quignon *et al.*, 2005). In addition, the diversity of the OR repertoire can contribute to the range of detected odorants. For example, the rat has a repertoire composed of 1493 genes distributed among 282 subfamilies, which is quite similar to the number of canine OR subfamilies (300) even though the dog has a smaller OR repertoire. Thus, a high number of OR genes does not indicate a high diversity of OR genes. Thirdly, other steps occurring during the olfaction process, such as OR gene expression levels or signal transduction efficiency to the brain areas could also contribute to better odorant perception.

No data are available at present that explain the olfactory capability differences between dog breeds. Canine OR genes are highly polymorphic (Tacher *et al.*, 2005; Robin *et al.*, 2009) but no relationship has yet been established between polymorphisms and olfactory capabilities. However, interestingly enough, the clustering of six dog breeds using the different SNPs found in their OR genes largely reflected dog breed structure (Fig. 17.5). In fact, if the SNP combination of one breed allows the identification of that breed among other breeds, this means that the distribution of OR polymorphism is not random. Moreover, dog breeds have different olfactory capacities, so we can hypothesize that OR polymorphism should play an important role in breed olfaction performance. In addition, studies of two human OR genes have demonstrated that OR gene polymorphism contributes to the variability of odorant perception (Keller *et al.*, 2007; Menashe *et al.*, 2007). So we can assume that OR gene polymorphisms are an important factor implicated in olfactory differences between dog breeds. Also, learning, memory and dog behaviour such as obedience can play a role in an odorant stimulus answer and in the communication of any detection to the human trainer. The involvement of behaviour can be illustrated by the rapid sniffing of rats, which has a functional contribution to odour discrimination performance, as it enables the animal to acquire the stimulus quickly when it is available (Wesson *et al.*, 2009).

Historically, dog olfaction capabilities were specifically used for hunting. There are several

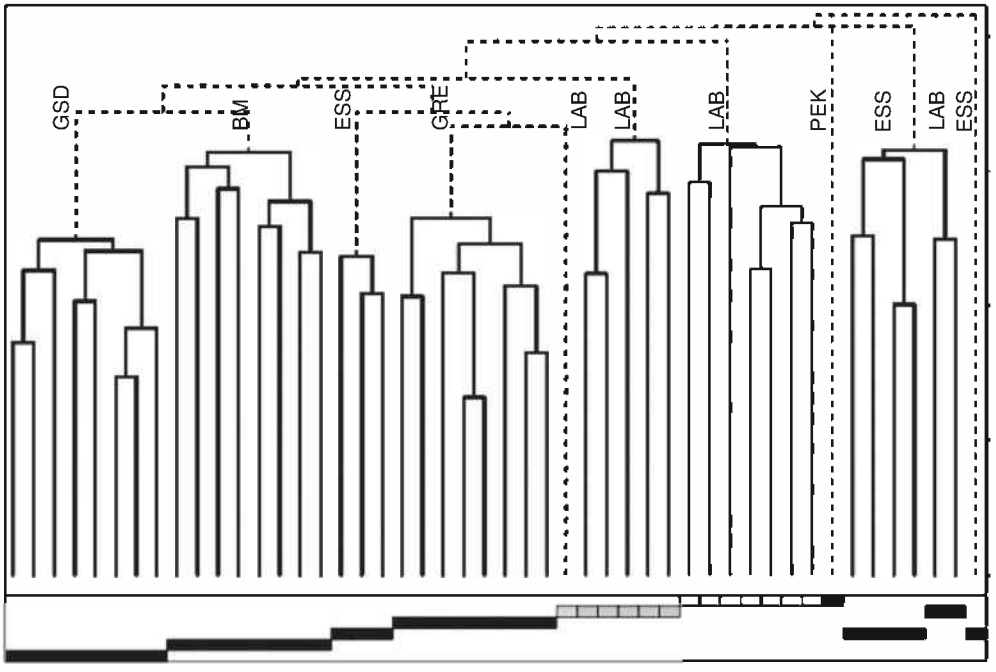

Fig. 17.5. The clustering of dog breeds based on a subset of single nuclear polymorphisms (SNPs). The PLINK and HCLUST programs (Purcell *et al.*, 2007; HCLUST, from http://cran.r-project.org/) were used to cluster OR gene genotypes to determine whether they reflected dog breed structure. When assigning $k = 10$ (ten breeds) and computing a subset of SNPs selected by PLINK (394 SNPs), three breeds (GSD, BM and GRE) clustered perfectly, PEK clustered into two homogeneous groups of seven dogs and one dog, and LAB and ESS clustered partially. Each (main) dotted vertical line represents a cluster; horizontal bars (at the bottom) indicate dogs of the same breed: line 1 Pekingese (PEK), line 2 Labrador Retriever (LAB), line 3 Greyhound (GRE), line 4 English Springer Spaniel (ESS), line 5 Belgian Malinois (BM), line 6 German Shepherd Dog (GSD) (Robin *et al.,* unpublished data).

breeds of hunting dogs developed for various tasks: gun dogs, terriers and hounds, with sight dogs that hunt using their vision acuity (Whippet, Afghan Hound) and scent dogs that hunt using their olfaction (Basset Hound, Beagle). We can hypothesize that the creation of breeds with good olfactory capacities was performed by the selection of major genes implicated in all the steps of odorant identification. More recently, dog olfaction acuity has been used for other purposes, such as drug and explosive detection ('sniffer dogs') (Furton and Myers, 2001) or the retrieval of humans/human remains after an avalanche or earthquake ('cadaver dogs').

Even more amazingly, information has been published on canine scent detection of human malignancies such as melanoma and bladder, lung, and breast cancer, indicating a new diagnostic tool for malignancies. The first melanoma detections by a dog were reported in 1989 (Williams and Pembroke, 1989) and in 2001 (Church and Williams, 2001). Another study demonstrated that the olfactory detection of human bladder cancer was feasible by trained dogs (Willis *et al.*, 2004), and the accuracy of lung and breast cancer detection by trained dogs was also proven (McCulloch *et al.*, 2006). Other studies have shown that a dog was capable of distinguishing different histopathological types and grades of ovarian carcinomas (Horvath *et al.*, 2008) or of detecting prostate cancer with a significant success rate by smelling urine (Cornu *et al.*, 2010). However, only a very small success in the detection of breast and prostate cancers by dogs was reported (Gordon *et al.*, 2008). Other uses of canines can be illustrated by dogs being used to locate live bedbugs and viable bedbug eggs (Pfiester *et al.*, 2008) or to locate live termites and discriminate them from non-termite material (Brooks *et al.*, 2003).

Conclusion

The great sense of olfaction of the dog has been used for a long time, but the deciphering of the mechanisms involved is at its beginning. Given the complexity of the process of perception of an odour and the response that a dog may or may not provide to its handler, it appears essential to divide this whole process into what is relevant to the nose itself and what is relevant to the brain. We now have a good knowledge of the canine OR repertoire and of its genetic polymorphism. Although this high level of polymorphism is probably related to the differences in olfactory capability between breeds, we have no satisfactory explanation yet to go further in this direction. For this, it would be crucial to compare the genetic polymorphism of a set of ORs for which the ligands are known, and the individual dog olfactory performance related to these ligands. In addition, it would be essential to measure and link to the individual performance of a dog the number of olfactory sensory neurons, the level of expression of the same set of ORs and the message transduction efficiency that takes place in the olfactory sensory neurons. But what happens in the nose is only one side of the whole story. What happens in the brain might be even more important. There, two independent problems should be analysed. The first concerns the treatment of the olfactory message and the recognition and memorization of the odorant perception resulting from the interaction of a ligand with its receptor. This first problem has itself an additional level of complexity due to the fact that an odour is generally made of several independent chemical components, and perception of the odour is not necessarily the sum of the individual odorants, but something different – known in perfumery as the accord phenomenon. To what extent this phenomenon exists in the dog, and to what extent it may affect all dogs similarly, or only some dogs, are totally unknown. The second problem concerns dog behaviour, the willingness of the animal to cooperate and to tell its handler that it recognizes the odour. Not being part of the olfaction process per se, this other level of complexity is prone to confuse the results obtained from analysing dog olfactory performances. Thus, clearly, any attempt at analysing dog olfaction performance overall, and why some breed dogs are so gifted, will need to divide these problems into as many questions as possible.

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18 Pedigree Analysis, Genotype Testing and Genetic Counselling

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Introduction

Since domestication, *Canis familiaris* has undergone tremendous diversification such that today we recognize over 400 breeds of dogs throughout the world (Parker and Ostrander, 2005). Individual members of a breed are clearly identifiable as a member of that breed, are distinguishable from other breeds based on phenotype and are now confirmed by genotype (Parker *et al.*, 2004, 2010). For the vast majority of time since domestication, dogs were selected for three functions: hunting, guarding and herding (Parker *et al.*, 2010). The major expansion in the number of different breeds is very recent, having occurred only during the last few centuries. The impetus for this expansion has been man's desire to produce breeds that have characteristics other than those that permit dogs to perform a particular function.

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Beginning in the 19th century, a tremendous growth in dog shows greatly increased the breeding of dogs exclusively for exhibition and competition. Even breeds that were traditionally developed for a particular function are now bred purely for exhibition and competition. The end of the 19th century saw the development of kennel clubs throughout the world that were designed to control the registration of pedigree breeds and their exhibition and competition at dog shows. Today, the majority of pure-bred dog production throughout the world is controlled by one or other of these kennels clubs, and new litters of puppies are only added to their registries provided that both parents are already registered in the breed database.

Origin of mutations

All animals carry several deleterious, perhaps even lethal, mutations in their genomes (Thomson *et al.*, 2010). The canine genome has accumulated many mutations during the time since domestication. Some of these would already have been present in the wolf populations that served as the source of the domesticated dog, others have occurred *de novo*. Not all have been necessarily deleterious. Some have been neutral and therefore have had no consequence for the dog. Some have been exploited by breeders over the centuries to produce the great diversification in breed types. Others have been more detrimental and have resulted in inherited disease in the various dog breeds. Many diseases are associated with the dog in general and not with specific breed conformation (Summers *et al.*, 2010), which suggests that ancestral mutations occurred early in the domestication process (Cruz *et al.*, 2008) and in the development of dog breeds (Ott, 1996). It has been speculated that traits that humans favour in the domestic dog are highly associated with deleterious mutations. That is, through the domestication process, the features that characterize the desirable traits of a dog may be inextricably linked to mutations that impair health (Chase *et al.*, 2009; Akey *et al.*, 2010). As there exists no information in the literature on the prevalence of genetic disease in mixed breeds (Summers *et al.*, 2010), it is not known whether selective breeding has exacerbated the prevalence of genetic diseases within breeds.

In 1965, Scott and Fuller (1965) investigated the inheritance of behaviour, and in doing so uncovered the fact that the dog breeding practices used to establish new breeds – those of line breeding and inbreeding, resulted in an accumulation of deleterious alleles and the expression of recessive disorders. In a recent review, every one of the top 50 breeds evaluated had at least one genetic disorder associated with the conformation demanded by the standard for that breed (Asher *et al.*, 2009). There are now over 1000 reported inherited diseases in the dog (Mellersh, 2008). Finding a cause for these diseases has been made more imperative in the minds of breeders because, as the veterinary field has conquered infectious and parasitic disorders, health issues now more frequently involve disorders that have a genetic basis.

To eliminate disease alleles from a breed population, the key is for breeders to identify stock that may pass on mutant alleles to future generations. Ultimately, the new DNA technologies will provide breeders with the opportunity to better understand the genes carried by their animals. However, even in the absence of specific DNA tests for disease alleles, there are steps that breeders can take to minimize their spread to future generations.

Traits

Selecting for desirable traits (morphology, temperament, etc.) requires knowledge of the genes underlying the expression of those traits. Preserving desirable traits that represent form and function (breeding to the standard) must be achieved within the context of inherited disease, thus the focus of genetic counselling is often to reduce the incidence of such disorders. Similarly, breeders should avoid over exaggerating elements of the breed standard, especially in light of the finding that breeds show at least one inherited disorder associated with the conformation demanded by the standard (Asher *et al.*, 2009).

As discussed in Chapter 5, the Online Mendelian Inheritance in Animals (OMIA, at http://omia.angis.org.au/), as of December 2010, database tabulates more than 540 canine phenotypes purported to have a genetic basis to their expression, while the vast majority represent health disorders, other traits include coat colour, blood groupings, and eye colour. Most traits are polygenic, with only 179 defined as single locus (of which 114 have been characterized at the molecular level). To optimize breeding selection programmes, the genetic contribution to trait expression must be defined, and that relies upon breeders and their willingness to contribute accurate information.

Breeder involvement

There are several considerations when a disorder is found to be recurrent in a breed: accurate diagnosis of the disease; evaluation of the severity of the disease; determination of whether a therapy exists to ameliorate the disease; consideration of personal opinions on the responsibilities of being a 'breeder' of defects; detection of the mode of inheritance of the disease; and assignment of a genetic risk to the disorder (Fisher, 1982; Dodds, 1995). Accurate diagnosis is essential to establish whether the disorder has a genetic basis and therefore can be selected against. In some instances, the severity of the disorder may not be great enough in the minds of breeders to warrant a concerted selection programme against the disorder, even if a genetic basis exists (Hall and Wallace, 1996). In these cases, little effort may be expended if the disorder is thought not to affect the well-being of the dog, if available therapeutics minimize the impact of the disorder on the dog (Dodds, 1995; Asher *et al.*, 2009), or if the condition can be surgically managed. Also, as longevity in dogs increases, questions arise as to whether a disorder that appears in old age should be actively selected against. All of these issues will influence the relative weight that a breeder places on trying to eliminate that particular disorder from a line of dogs – especially if the 'breeder culture' is such that breeders routinely castigate other breeders for either inadvertently producing a defective puppy or for openly discussing the genetic shortcomings of the breed (Fowler *et al.*, 2000). Asher *et al.* (2009) proposed a score for severity of disorder that weights these items and suggested that the score be employed to assist in ethical breeding decisions.

Pedigree Analysis and Modes of Inheritance

Knowledge of just how a particular disease is inherited is absolutely essential to the control of genetic disease. The first indication that a condition might be inherited occurs when a higher than expected incidence of the condition is noted within a breed, a line or a distinct family of dogs, provided that all possible environmental factors have been considered. Once indicated, decisive evidence for or against a genetic causation can be collected by analysing the patterns of occurrence of affected individuals within a closely related family. Visually assessing pedigrees to observe the distribution of the trait through generations is a first step. Simple Mendelian inherited traits exhibit characteristic inheritance patterns; these characteristics of the major forms of inherited disease (autosomal recessive, autosomal dominant, sex-linked recessive and polygenic) are presented in Table 18.1. Pedigrees can then be compared to see whether the observed pattern of appearance of affected individuals fits one of these.

Of the inherited traits so far reported in the dog, as in other diploid eukaryotes, the most common mode of inheritance of disorders is autosomal recessive (Summers *et al.*, 2010). Caution must be applied, however. Whereas particular patterns of affected individuals may be consistent with a particular mode of inheritance, cursory evaluations of pedigree data without statistical support may lead to false conclusions on the mode of inheritance, resulting in improper breeding recommendations. Further, determination based on too small a sample may also lead to erroneous conclusions. For example, population structure within breeds (Calboli *et al.*, 2008) may reduce generalization of the predicted mode of inheritance to the breed as a whole.

Table 18.1. General characteristics of the main modes of inheritance in canine disease of traits with complete penetrance.

Autosomal recessive
- The mutant gene responsible is on one of the 38 pairs of autosomes.
- To be affected dogs must be homozygous for the mutant gene.
- The condition tends to skip a generation until such time as two heterozygous carriers are mated and produce affected offspring.
- Each parent of an affected offspring must be a heterozygous carrier.
- A carrier bred to a carrier will, on average, produce 25% affected, 50% carrier and 25% normal offspring.
- Male and female offspring are equally affected.

Autosomal dominant
- The mutant gene is located on one of the 38 pairs of autosomes.
- The mutant gene is generally present in the heterozygous state and less commonly present in the homozygous state.
- At least one parent of an affected offspring must have the condition (unless the condition shows partial penetrance or there is a new mutation).
- Generally, there is no skipping of generations.
- Assuming that an affected dam or sire is heterozygous, on average 50% of its offspring will be affected.
- Males and females will be equally affected.

Sex-linked (X-linked) recessive
- Approximately 50% of the male offspring of a carrier female will be affected.
- By the same token, 50% of a carrier dam's female offspring will be carriers.
- There is a characteristic pattern of transmission: clinically normal females produce affected sons who in turn produce clinically normal carrier daughters.
- Clinically affected males pass the mutant gene on to all of their daughters, but to none of their sons.
- If both parents are affected, all offspring will be affected.
- An affected male will often have affected relatives on his dam's side, but hardly ever on his sire's side.

Polygenic
- Both dam and sire must contribute one or more alleles to affected offspring, but this contribution need not necessarily be equal.
- There are no predictable ratios in pedigrees because we do not know the number of genes involved.
- Both sexes are affected, but not necessarily in equal numbers.
- The condition will often appear erratic in pedigrees where there are affected dogs.

Where sufficient pedigree information is available, the heritability and mode of inheritance should be assessed. When evaluating the expression of a trait, the genetic and the environmental contributions to the observed phenotype are considered: Phenotype (P) = Genotype (G) + Environment (E). Heritability analyses measure the proportion of phenotypic variation in a population that is due to genetic variation between individuals (Vissher *et al.*, 2008). Defining the heritability allows prediction of the success of specific selection; that is, heritability reflects the extent to which selection for a phenotype will exact change in the underlying genotype. The greater the pedigree size, random mating patterns and accurate phenotyping, the more accurate the heritability estimate for a given trait. Large accurate pedigrees underscore the need for direct breeder involvement.

To assess mode of inheritance in pedigrees in which the disease visually appears to be transmitted through generations, generally two different statistical approaches have been used in simple segregation analyses: the Davie's extension of the singles method of segregation analysis (Nicholas, 1982, 1987) and the direct method of maximum likelihood estimation (Nicholas, 1982). In these analyses, the observed data are analysed to see how well

they fit the expected segregation pattern of a defined Mendelian inheritance pattern (Distl, 2007). For example, three normal offspring to one affected are expected for a condition caused by an autosomal recessive mutation.

Clearly defined phenotypes are essential to the proper categorization of individuals as affected or clear of a disease, and a significant complicating factor to characterizing mode of inheritance centres on the epistatic genetic and environmental affects altering the penetrance and expressivity of a trait. Diversity in the expression of phenotypes can reflect underlying compound gene interaction or environmental modification (see, for example, the complexity associated with human thalassaemias as reviewed by Weatherall, 2000). Penetrance reflects whether an individual with a given genotype will express a trait. Specifically, not all individuals that possess mutated alleles will express the trait owing to epistatic genes, modifiers or environmental background. Complete (100%) penetrance is when all individuals with the disease-causing mutation exhibit expression of the disease. In contrast, incomplete or reduced penetrance is when some individuals fail to express the disease trait even though they possess the mutant genotype. Expressivity refers to the extent of expression of the trait. An example of variable expressivity can be seen in the piebald spotting of dogs: a dog possessing the allele for piebald spotting will have white spotting coat colour patterns, but the extent of white on the dog will vary considerably. Subtle differences in expression may obfuscate correct phenotypic classification, thus confusing heritability and mode of inheritance analyses.

For monogenic traits with complete penetrance, simple segregation analysis is straightforward, especially when specific matings can be arranged. For example, if a condition is thought to result from a single autosomal recessive mutation, specifically planned matings between obligate carriers will be expected to produce 25% affected offspring and a segregation frequency of 0.25. However, in general, specific matings are rarely set up to test a hypothesis of inheritance in dogs. Rather, data are obtained by analysing existing pedigrees in which one or more individuals are affected with the condition. This immediately introduces a bias into the data because there could be carrier/carrier matings in the pedigree that have gone unnoticed simply because, by chance, no affected offspring have been produced. This means that, although all affected offspring of carrier/carrier crosses will be scored, not all of the normal offspring will be scored. Therefore, even if the condition does result from an autosomal recessive mutation, the segregation ratio will be greater than 0.25. Disorders that have late onset further complicate interpretation, as some dogs may not have expressed the disorder and may also be improperly scored.

The investigation of the inheritance of multifocal retinal dysplasia (MRD) in the Golden Retriever (Long and Crispin, 1999) serves as a good example of the statistical methodology available for pedigree analysis. MRD had already been reported to be inherited as a simple autosomal recessive condition in the American Cocker Spaniel (MacMillan and Lipton, 1978) and the English Springer Spaniel (Schmidt *et al.*, 1979), and this investigation sought to confirm a similar mode of inheritance in the Golden Retriever. The researchers reported litter information from 27 different litters, containing a total of 202 offspring. Of these, 56 were affected with MRD (28 males and 28 females) and 146 were clinically unaffected. In addition, affected offspring had been produced in litters where both parents were clinically normal. The equal numbers of affected males and females rules out a sex-linked mode of inheritance, and the fact that affected puppies had been produced from clinically normal parents tends to rule out a dominant mode of transmission. So the data are highly suggestive of an autosomal recessive mode of inheritance. If MRD is due to an autosomal recessive gene, the segregation frequency of affected individuals (p_0) would be 0.25. The probability that the segregation pattern in the litter screening data presented is consistent with an autosomal recessive mode of inheritance was calculated using the Singles Method (Davie, 1979; Nicholas, 1987). In the final analysis, there was no statistical difference between the expected and observed segregation frequency, allowing the authors to conclude that MRD in the Golden Retriever results from an autosomal recessive mutation (Long and Crispin, 1999).

Simple segregation analyses encounter difficulties such that, although the majority of the data fit an expected segregation frequency, within the data set there may be observations that are not compatible with the general theory. These anomalies may be explained by misdiagnosis of phenotype, variable expressivity of the phenotype or the occurrence of phenocopies (a phenotype caused by some sort of environmental influence that mimics the phenotype caused by the gene mutation), which underscore the need for accurate diagnoses. Incomplete penetrance can also confound analyses. A final caveat to simple segregation analysis conclusions lies in the known existence of population substructure within a given breed (Chang *et al.*, 2009). Inheritance predictions established for lines within a breed may not reflect the breed as a whole even if large numbers of dogs are included. The findings would then be subject to a 'decline effect', where significance is lost with regression to the population mean as additional samples/data points are added.

To account for the difficulties encountered in actual populations, complex segregation analyses are employed to mathematically model the inheritance of more complex pedigree data sets and relax preconceived inheritance predictions. Complex segregation analyses, originally based on the Elston–Stewart algorithm, permit inclusion of many and varied terms, including those for major genes, types of data collection, undefined mating types, non-genetic variation (environmental), and penetrance that often accompany retrospectively analysed dog pedigrees (reviewed by Distl, 2007). Many statistical packages exist to assess complexly inherited traits and to more precisely estimate genotype frequencies.

Another useful measure in assessing pedigree structure is the calculation of the inbreeding coefficient – a measure of homozygosity due to inheritance from common ancestors. As noted earlier, inbreeding increases homozygosity, which both fixes desirable traits and enables the expression of recessive genetic disease. Available pedigree programs (such as CompuPed, available at http://www.compuped.com/; Ultimate Dog Breeding Software, available at www.DogBreedingSoftware.com; and Pedigraph, available at http://animalgene.umn.edu/pedigraph/) will calculate the inbreeding coefficient for an individual dog; coefficients should be calculated on as many generations as possible to account for pedigree depth and common distant ancestors (Calboli *et al*, 2008). While inbreeding is a significant concern, a recent study of ten breeds in the UK determined that the average inbreeding coefficient for 88% of the dogs studied was less than 10% (Calboli *et al.*, 2008; Higgins and Nicholas, 2008). However, some less popular breeds, or breeds that experienced severe bottlenecks, or breeds with few founders did have relatively large inbreeding coefficients.

DNA Technology and Mutant Genes in Inherited Diseases

Although a more recent tool than classical breeding selection schemes based upon phenotypic expression, DNA-based testing represents the future. The advent of molecular biological techniques has enabled the identification of genes that are causal in the expression of disorders. These can then be used as the basis for genetic tests to classify individual dogs as carriers for recessive genes or can identify those puppies that will be afflicted with late-onset disorders as adults (van Oost, 1998). This information can then be used judiciously by breeders to propagate the desired traits of certain dogs, while minimizing the spread of known disorders in their breed (Traas *et al.*, 2006). DNA-based tests also exist to aid in the selection of desirable traits such as coat colour. The genes underlying morphological characteristics and behavioural traits are being characterized (as discussed in other chapters of this text) which will, in turn, increase the number of genetic tests available.

DNA tests for disease gene mutations

The first gene demonstrated to be causal for a canine genetic disorder was reported in 1989. The mutation was a single base pair substitution in the canine clotting factor IX gene and

caused haemophilia B in Cairn Terriers (Evans *et al.*, 1989) when in the homozygous state. Carriers of the defective allele could be identified with a genetic test designed to detect that substitution. In the following years, many additional genes have been associated with canine disorders and the causal mutations identified. Genetic tests, based on the DNA sequence data of these genes, have been or are being developed. Any DNA test, to maximize the benefit to breeders and the overall breed population, must be cost-effective, verifiably accurate and easy to administer. The Canine Health Foundation lists 75 available genetic tests for dogs (www.caninehealthfoundation.org). Examples of such tests that are commercially available are listed in Table 18.2.

The development of these tests and the identification of the causal genes are based upon the verification of the mode of inheritance by pedigree analysis to determine that the disorder is in fact genetically based (Famula *et al.*, 2000). For linkage studies, DNA is collected from affected and unaffected dogs of a particular breed, ideally representative of particular lines or families within the breed (Ubbink *et al.*, 1998b); for association studies unrelated case and control dogs are used (Short *et al.*, 2007). The DNA is then evaluated for a unique association between a particular DNA profile and the disorder's phenotype. The screening utilizes molecular markers derived from the canine genome interrogation map (Parker *et al.*, 2010), or single nucleotide polymorphism (SNP) arrays. Candidate genes, which are genes identified as causing similar disorders in humans or other species, are also used as a starting point in the search for the genetic causality of inherited disorders (Sargan, 1995; Parker *et al.*, 2010).

Impediments to DNA test development may include a disorder that visually and statistically implies autosomal recessive yet represents two dominant loci interacting, mitochondrial mutations, or epigenetic modification of the DNA that is transgenerational, so that while appearing Mendelian lacks any alteration of the underlying DNA sequence (Skinner and Guerrero-Bosagna, 2009).

Genotyping using DNA tests

Genotype testing is based either on mutations within the causal gene itself or on linkage to a particular gene. Mutation-based tests, sometimes called 'direct tests', are preferred because the genotyping is 100% accurate. However, linkage-based marker DNA tests, sometimes called 'marker tests', also provide valuable information. Both tests utilize the polymerase chain reaction (PCR), in which the DNA of an individual is duplicated billions of times in a laboratory test tube. Rather than copying an animal's entire DNA, a particular region of DNA is targeted for amplification, which creates substantial quantities of that region of DNA that can then be analysed. This PCR amplification process has the advantage of permitting very small quantities of a dog's DNA to be expanded into useful quantities. It is through this process that the small amount of DNA collected from the cells sloughed off the inside of a dog's cheek or isolated from a modest volume of blood can be used in genotyping, because the vast majority of cells contain all of the animal's DNA (van Oost, 1998). The region targeted for amplification is either the gene that causes the disorder (mutation-based tests), or a region of DNA adjacent to the gene that causes the disorder (linkage-based tests) (Traas *et al.*, 2006).

Mutation-based DNA tests

Mutation-based tests provide accurate genotyping of homozygous affected heterozygous carriers, or homozygous normal animals. In these tests, the DNA representing the particular gene is amplified by PCR and the amplified region is then processed to distinguish the mutant form of the gene from the normal form. In essence, the amplified DNA migrates through a matrix differentially based upon the DNA sequence and exhibits discrete patterns when visualized: the normal gene is distinctly different from the mutant gene. In the case of an autosomal recessive disorder, a homozygous dog genetically free from the mutation will have two copies of the normal gene while the affected dog will have two copies of the mutant

Table 18.2. Available canine DNA tests.[a]

Disorder	Applicable Breed	Testing Laboratories[b]
Arrhythmogenic right ventricular cardiomyopathy	Boxer	NCSU Veterinary Cardiac Genetics Laboratory (North Carolina)
Canine leucocyte adhesion deficiency (CLAD)	Irish Setter, Irish Red and White Setter	Animal Health Trust (UK) Optigen (New York)
Canine multifocal retinopathy (CMR)	Mastiff, Bullmastiff, Great Pyrenees, Dogue De Bordeaux, Coton de Tulear	Optigen
Centro nuclear myopathy (CNM)	Labrador Retriever	Alfort School of Veterinary Medicine (France) Animal Health Trust VetGen (Michigan) Veterinary Diagnostics Center (Ohio)
Cerebellar ataxia	Italian Spinone	Animal Health Trust
Ceroid lipofuscinosis	Border Collie	Animal Health Trust
Cobalamin malabsorption	Giant Schnauzer	PennGen (Pennsylvania)
Collie eye anomaly	Australian Shepherd, Border Collie, Rough and Smooth Collie, Nova Scotia Duck Tolling Retriever, Shetland Sheepdog	Optigen
Cone degeneration	German Shorthaired Pointer	Optigen
Cone–rod dystrophy	Glen of Imaal Terrier	Optigen
Congenital stationary night blindness (CSNB)	Briard	Animal Health Trust HealthGene (Ontario) Optigen
Congenital hypothyroidism with goitre (CHG)	Toy Fox Terrier	HealthGene Michigan State University
Copper toxicosis	Bedlington Terrier	Animal Health Trust VetGen
Cystinuria	Newfoundland	HealthGene Optigen PennGen Veterinary Diagnostics Center VetGen
Degenerative myelopathy	American Eskimo Dog, Bernese Mountain Dog, Boxer, Cardigan Welsh Corgi, Chesapeake Bay Retriever, German Shepherd Dog, Golden Retriever, Great Pyrenees, Kerry Blue Terrier, Pembroke Welsh Corgi, Poodle, Pug, Rhodesian Ridgeback, Shetland Sheepdog, Soft-coated Wheaten Terrier, Wire Fox Terrier	OFA[a] (Missouri)
Dilated cardiomyopathy	Doberman Pinscher	NCSU Veterinary Cardiac Genetics Laboratory
Exercise-induced collapse	Boykin Spaniel, Chesapeake Bay Retriever, Curly Coated Retriever, German Wirehaired Pointer, Labrador Retriever, Pembroke Welsh Corgi	Veterinary Diagnostic Laboratory (Minnesota)
Factor VII Deficiency	Alaskan Klee Kai, Beagle	PennGen

Continued

Table 18.2. Continued.

Disorder	Applicable Breed	Testing Laboratories[b]
	Airedale, Alaskan Klee Kai, Beagle, Giant Schnauzer, Scottish Deerhound	VetGen
Factor XI deficiency	Kerry Blue Terrier	PennGen
Fanconi syndrome	Basenji	OFA
Fucosidosis	English Springer Spaniel	Animal Health Trust PennGen
Glanzmann's thrombasthenia	Otterhound, Great Pyrenees	Mary Boudreaux, DVM, PhD (Alabama)
Globoid cell leukodystrophy	Cairn Terrier, West Highland White Terrier	HealthGene Dr David Wenger (Pennsylvania)
Glycogen Storage Disease Type IIIa (GSD IIIa)	Curly Coated Retriever	Michigan State University
GM1 Storage Disease	Portuguese Water Dog	Neurogenetics Laboratory (New York) HealthGene
GM2 Gangliosidosis	Japanese Chin	OFA
Grey collie syndrome (Canine cyclic neutropenia)	Collie	HealthGene VetGen
Hereditary juvenile cataracts	Australian Shepherd, French Bulldog, Staffordshire Bull Terrier	Animal Health Trust
	Boston Terrier	Animal Genetic Testing and Research Lab (Kentucky)
Hereditary nephritis	Samoyed	VetGen
Hyperuricosuria	Dalmatian	UC Davis (California)
Ichthyosis	Norfolk Terrier	Michigan State University
Juvenile dilated cardiomyopathy (JDCM)	Portuguese Water Dog	PennGen
L2HGA – L2 hydroxyglutaric acidurea	Staffordshire Bull Terrier	Animal Health Trust Animal Molecular Genetics Lab (University of Missouri)
Leonberger polyneuropathy (LPN	Leonberger	Veterinary Diagnostics Lab
MDR1 (multiple drug resistance gene)	Australian Shepherd, Border Collie, Collie, Shetland Sheepdog	HealthGene WSU Veterinary Clinical Pharmacology Laboratory (Washington State)
MPS IIIB (mucopolysaccharido-sis type III)	Schipperke	PennGen
MPS VI (mucopolysaccharidosis type VI)	Miniature Pinscher	PennGen
MPS VII (mucopolysaccharidosis type VII)	German Shepherd	PennGen
Musladin–Lueke syndrome	Beagle	UC Davis
Myotonia congenita	Miniature Schnauzer	HealthGene PennGen
Narcolepsy	Dachshund, Doberman Pinscher, Labrador Retriever	Optigen
NCL-A (cerebellar ataxia)	American Pit Bull Terrier, American Staffordshire Terrier	Optigen
Necrotizing meningoencephalitis (NME)	Pug	UC Davis

Continued

Table 18.2. Continued.

Disorder	Applicable Breed	Testing Laboratories[b]
Neonatal encephalopathy with seizures (NEwS)	Poodle	OFA VetGen
Neuronal ceroid lipofuscinosis (NCL)	American Bulldog, Dachshund, English Setter	Animal Molecular Genetics Lab
	American Bulldog, Tibetan Terrier	OFA
	American Bulldog, English Setter	VetGen
Phosphofructokinase deficiency	English Springer Spaniel	Animal Molecular Genetics Lab
	Cocker Spaniel, English Springer Spaniel, mixed breeds	HealthGene Optigen PennGen Veterinary Diagnostics Center VetGen
Polyneuropathy (NDRG1)	Greyhound	Optigen
Primary hyperparathyroidism	Keeshond	Cornell University (New York)
Primary lens luxation (PLL)	American Eskimo Dog, American Hairless (Rat) Terrier, Australian Cattle Dog, Chinese Crested, Chinese Foo Dog, Jack Russell Terrier, Jagd Terrier, Lakeland Terrier, Lancashire Heeler, Miniature Bull Terrier, Parson Russell Terrier, Rat Terrier, Russell Terrier, Sealyham Terrier, Tenterfield Terrier, Tibetan Terrier, Toy Fox Terrier, Volpino Italiano, Welsh Terrier, hybrid/mixed breeds	OFA
Progressive retinal atrophy	Dachshund, English Springer Spaniel, Irish Red and White Setter	Animal Health Trust
	Dachshund, English Springer Spaniel	Animal Molecular Genetics Lab
	Irish Setter, Irish Red and White Setter	HealthGene
	American Eskimo Dog, Australian Cattle Dog, Bullmastiff, Cardigan Welsh Corgi, Chesapeake Bay Retriever, Chinese Crested, Cocker Spaniel, English Cocker Spaniel, Entlebucher, Finnish Lapphund, Irish Red and White Setter, Irish Setter, Kuvasz, Labrador Retriever, Mastiff, Miniature Schnauzer, Nova Scotia Duck Tolling Retriever, Poodle, Portuguese Water Dog, Samoyed, Siberian Husky, Sloughi, Swedish Lapphund	Optigen
	Cocker Spaniel, English Springer Spaniel	VetGen
Pyruvate dehydrogenase phosphatase deficiency (PDH, PDP-1)	Clumber Spaniel, Sussex Spaniel	Animal Health Trust Animal Molecular Genetics Lab VetGen
Pyruvate kinase deficiency	Basenji	Animal Molecular Genetics Lab

Continued

Table 18.2. Continued.

Disorder	Applicable Breed	Testing Laboratories[b]
	American Eskimo Dog, Basenji, Beagle, Chihuahua, Dachshund, West Highland White Terrier	HealthGene Optigen PennGen Veterinary Diagnostics Center VetGen
Retinal dysplasia (RD/OSD)	Labrador Retriever, Samoyed	Optigen
Thrombopathia	Basset Hound	Mary Boudreaux, DVM, PhD
Trapped neutrophil syndrome (TNS)	Border Collie	Dr Alan Wilton (University of New South Wales)
Von Willebrand's disease	Bernese Mountain Dog, Deutsch Drahthaar, Doberman Pinscher, German Pinscher, Kerry Blue Terrier, Kooikerhondje, Manchester Terrier, Papillon, Pembroke Welsh Corgi, Poodle, Scottish Terrier, Shetland Sheepdog, Stabyhound, all pointer breeds	VetGen

[a]Courtesy of The Orthopedic Foundation for Animals website (www.offa.org, accessed December 2010). (By no means complete.)
[b]Brief details of location given at first mention.

form of the gene. Because the amplified products from the homozygous normal dog are identical, a single form would be seen (Fig. 18.1). The same would be true for the homozygous affected dog, but the form of the amplified product would be distinctly different from that of the genetically clear dog. A heterozygous carrier has both a normal and a mutant gene copy, therefore the amplified DNA from this dog would show two discrete forms, one form representing the normal gene and a separate form representing the mutant gene. Thus, the DNA from carriers is readily distinguishable from the DNA of genetically normal or genetically affected individuals.

Interpretation of mutation-based genotyping tests is relatively straightforward for single gene (monogenic) diseases. An individual has zero, one or two copies of the mutant gene (refer to Fig. 18.1). Such tests can be done on the DNA of puppies as early as it is safe to collect the small amount of tissue or blood that is necessary to complete the test. Breeding a carrier to a genetically normal dog would produce, on average, 50% carrier puppies and 50% genetically normal puppies. With the ability to genetically test using a mutation-based test, puppies will be immediately identifiable as being genetically free from possessing a mutant allele or carrying the mutation. A breeder will not have to rely upon statistical odds that half the puppies will be genetically normal; the breeder will know unequivocally. It is important to note, though, that the application of statistical odds still applies and, on average, such a breeding will produce 50% carriers and 50% genetically normal animals. However, segregation of the gene copies during meiosis and fertilization is an independent event, so within any given litter the percentages will vary considerably from the overall average. The advantage of employing genotype testing is that the breeder will know precisely the genotypes for the disorder under consideration for each of the puppies produced (Holmes, 1998). Early genotyping is important because deleterious versions of genes can rapidly spread throughout a dog breed when popular sires or genetic founders carry mutations (Leroy and Baumung, 2011), especially if the disorder is late onset.

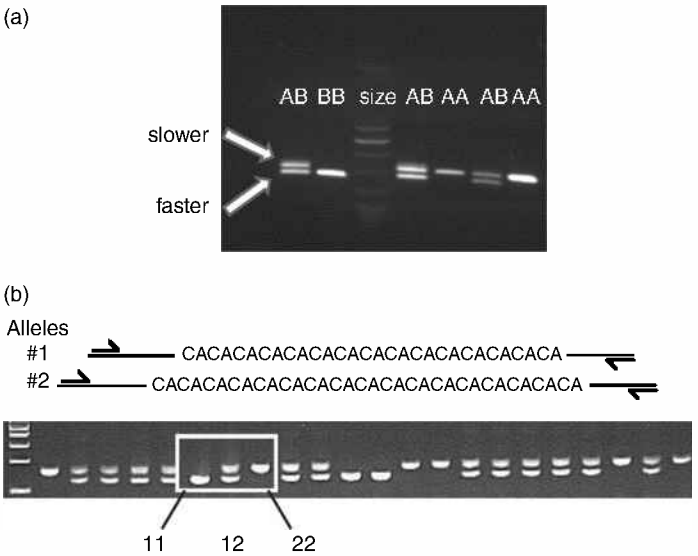

Fig. 18.1. PCR-based genotyping products. (a) An image of PCR-based genotyping products for alleles with a 38 base pair deletion (mutant denoted 'B') and without the deletion (normal, denoted 'A') run on a gel. Smaller DNA products representing the allele with the deletion migrate faster through the gel and are therefore distinguishable from the larger normal alleles. (b) Amplification scheme for alleles that differ in microsatellite repeat numbers used in linkage studies. The PCR-based genotyping relies upon PCR primers flanking the repeat sequence which are denoted by arrowheads. The smaller number of repeats generates a smaller DNA amplification product (identified as allele #1) that migrates through the gel faster than the larger product (identified as allele #2). Individual DNA samples can be genotyped as 11 (homozygous), 12 (heterozygous) or 22 (homozygous).

Genetic testing available for other traits requires greater deliberation. Degenerative myelopathy (DM) is an example of an inherited disorder in which researchers have identified a causal mutation in a major risk factor gene (Awano *et al.*, 2009). Two normal alleles at this risk factor gene appear to assure freedom from the disease, whereas two mutant copies imply significant risk for developing DM, but do not guarantee that the dog will exhibit clinical signs of DM, suggesting there are additional modifier genes that influence DM expression that are yet to be identified. The researchers urge caution in using this test; it should augment breeding decisions, yet overemphasis of the DM test results should be avoided.

Armed with DNA-based information, the breeder can make informed and educated breeding decisions, including recognizing that no single trait defines a breed, and that exerting selection pressure designed solely to eliminate a particular trait, without consideration for other attributes that a particular dog possesses, is not prudent. The advocacy of eliminating all dogs that are carriers from the breeding programme will result in eliminating many other superior qualities (Dodds, 1995; Holmes, 1998; Meyers-Wallen, 2003), and it is imperative to maintain the breed's attributes while selecting against the genetic disorder. By applying genetic testing, the breeder can judiciously use those sires or dams that may carry, or even be affected with, a particular disorder in their breeding schemes. Ideally, if the carrier possesses quality attributes, breed a dog that lacks any mutant allele to the carrier; from the offspring of such matings, the best individuals that are free from carrying the mutant allele can be used as brood stock in subsequent generations. In some cases, if the mutation is prevalent within the breed, and the sire or dam was an affected individual, it will take two generations to eliminate the mutant gene from the brood stock while simultaneously retaining

desirable traits. In that circumstance, an affected sire or dam could be bred to a carrier or unaffected mate to produce carrier offspring and superior carriers could then be bred to produce offspring that when tested do not carry the mutation.

Linkage-based tests

Mutation-based genotype tests, while offering many advantages such as accuracy, tend to be breed specific (van Oost, 1998). Even if a particular disorder affects many breeds, it is possible that each breed will require the development of its own unique mutation-based test, as seen in the different mutations causing von Willebrand's disease in Dutch Kooiker dogs and West Highland Terriers and Dobermanns (Slappendel *et al.*, 1998). In contrast, linkage-based tests are more rapidly developed because of their proximity to the gene and the mutation(s), though they are not as precise in their diagnosis (Curtis *et al.*, 1991; Patterson, 2000). Linkage-based tests may be applicable to many breeds because, although the precise mutation within a particular gene may vary among breeds, the same gene may be mutated. For example, numerous mutations – ranging from single point mutations to insertions to complete or partial gene deletions – in the factor IX gene cause haemophilia B in dogs (Evans *et al.*, 1989; Mauser *et al.*, 1996; Brooks *et al.*, 1997; Gu and Ray, 1997).

Linkage-based tests are developed by screening the DNA of animals with genetic markers that span the entire genome (Curtis *et al.*, 1991; Mellersh, 2008; Parker *et al.*, 2010). These markers can detect inherent polymorphism or genetic variation between individuals within a breed. Their utility lies in their widespread and unique distribution across the genome. If a marker is physically close to the gene that causes the disorder, such that the marker and the disease gene rarely separate during recombination, then that marker can be utilized to detect individuals possessing the mutant gene. Because the marker does not detect the disease gene itself, there is some error due to recombination occurring between the marker and disease gene during the process of forming the reproductive cells (Slappendel *et al.*, 1998). The degree of linkage, reflective of the distance between the disease gene and the marker, is associated with the risk of disease inheritance of the disorder when the particular marker is present. Linkage-based tests require considered interpretation of their results. That is because, just as the name implies, the DNA test is linked to the disorder but does not detect the genetic mutation that is causing it (Holmes, 1998; Ubbink *et al.*, 1998a; Traas *et al.*, 2006). Similar to the mutation-based genotyping tests, there are distinct products that represent the different genotypes derived from the amplification of the DNA. Commercially available linkage-based tests include those for copper toxicosis for Bedlington Terriers (note, this test has further refinements that add a mutation-based genetic test, which is useful in some individuals), lupoid dermatosis for German Shorthair Pointers, and Fanconi syndrome for Basenjis.

The DNA tests utilized to verify dog parentage rely upon markers selected for their inherent polymorphism and reliability in being transmitted from one generation to another, not as indicators of particular genes (van Haeringen, 1998). As such, parentage genotypes do not inform the owner of the presence or absence of disease alleles; they can be thought of as a permanent microchip number that was bestowed by the dog's parents. Similarly, the DNA tests that identify breeds also rely upon markers that are invariant within a breed but vary across and between breeds. Those tests are not intended to be informative of an individual's gene expression profile, but are useful in identifying unique individuals or individuals possessing a DNA sequence common to a particular breed.

Phenotype-based Approaches for Breed Improvement

In the absence of identified genes that underlie the inherited disorder, which was the case until recently, breeders rely upon phenotypes to guide breeding selection decisions. Historically, pedigree assessment was the sole mechanism

to exact improvement. Pedigree information can be invaluable for determining the genetic status of a dog for a given inherited condition, provided that the mode of inheritance is predicted, correct parentage can be relied upon, and accurate clinical diagnoses are available for dogs in the pedigree. Once pedigrees have been studied and evaluated, and a specific disorder's transmission assessed, breeders can predict the likelihood that puppies produced by a specific breeding will exhibit a particular trait, or disorder. This information can then be built into breeding programmes in order to avoid producing affected offspring and, it is hoped, reduce the frequency of mutant alleles within the breed's gene pool. Certainly most breeders would agree that it is desirable to eliminate genetic anomalies through selective breeding.

Pedigree assessment

Traditional pedigree analysis will identify the existence of carriers of recessive alleles (Fig. 18.2). When two clinically normal parents produce one or more offspring affected by a condition known to result from an autosomal recessive disorder, both are identified as obligate carriers. However, it is certainly possible that carriers will go undetected by simply analysing pedigree information. Carrier bitches may go undetected because during their limited breeding life they may not have been mated to a carrier dog, or, if they have, they may, by chance, not have produced an affected offspring, which is certainly possible in breeds that have small litter sizes. Carrier dogs are less likely to go undetected because dogs usually have a much greater mating potential. However, even carrier dogs may go undetected, particularly if the frequency of carrier bitches is low in the population. Late-onset conditions that result from autosomal recessive mutations pose exactly the same problems as mentioned above. Carriers may have been used considerably for breeding before their carrier status becomes known. Again, the increased understanding of the genes involved and the development of DNA tests for the mutations will greatly facilitate breeders' attempts to determine the genotype of individual animals before they are used in a breeding programme.

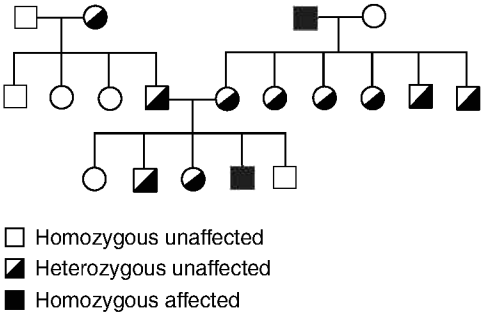

Fig. 18.2. Idealized pedigree for inheritance of an autosomal recessive disorder. The diagram represents the segregation of an autosomal recessive disorder. Clinically affected individuals have two copies of the mutant (black) allele. The key to controlling such disorders through selective breeding is to identify the heterozygous carriers that are clinically unaffected (heterozygous unaffected). Traditional pedigree analysis and, increasingly, DNA testing will help breeders to identify carrier animals and then take note of this when designing breeding programmes.

Test matings

In the past, breeders of livestock have resorted to test matings in order to verify the genetic status of a particular animal when that animal potentially carries a recessive allele. Test matings rely on mating the animal under test to a mate with a known genotype. The ideal way to test mate is to breed the dog in question to a known affected animal. If the test dog is homozygous normal no affected progeny will be produced, if it is a carrier, then, on average, half of the progeny will be affected. Test matings rely on the production of sufficient progeny to ensure that the results can be reliably interpreted. If the test mating involves a known affected, then if six normal offspring are produced the test dog has approximately 98.5% chance of being genetically clear. The mate with the known genotype could also be a carrier. In this case 15 normal progeny will need to be produced in order that the test animal has a 98.5% chance of being homozygous normal.

Although it is possible to test mate both males and females, it is really only feasible to undertake test matings for males because females may not produce sufficient offspring in one litter, necessitating mating the female several times, which spreads the test mating over a long period of time. The other thing to notice about the outcome of test matings is that there can still be doubt about the outcome. So, although there is a 98.5% chance that the test animal is genetically clear when six normal offspring are produced from a mating to an affected mate, there is still a 1.5% chance that the tested animal is a carrier. Thus out of every 200 dogs tested in this way, three carriers will go undetected and slip through as genetically clear. Late-onset disorders do not lend themselves to the test-mating strategy and traits lacking complete penetrance or having variable expressivity also confound the interpretation of its results.

Performing test breedings may provide useful information on the mode of inheritance of a particular trait, but intentionally breeding dogs with the objective of producing disease is not considered ethically responsible. In a scientific venue, test breeding dogs may have merit, but there is still the issue of placing puppies known or suspected of being afflicted with a particular disorder. Further, many breed clubs have codes of ethics that specifically preclude provision of dogs for scientific research, except under limited circumstances. Pedigree analysis generally relies upon the unintentional 'test breedings' done in the dog community: that is, breedings done without the knowledge of the genotypes of the brood stock. Puppies generated from these breedings have provided extremely valuable information on the parental genotypes. Analyses of these pedigrees and of the epidemiological data of the puppies, coupled with deductive reasoning, allow owners and scientists to infer the genotype of a given dog.

Biochemical genotyping

Carriers of recessive disease alleles are generally phenotypically normal and clinical examination cannot distinguish carriers from normals. However, 'genotyping' tests of genetic disorders have been developed by characterization of the relative abundance or activity of a particular protein known to be associated with a disorder (Dodds, 1995). In other words, if a defective protein product was detected, one could conclude that the gene had been mutated (van Oost, 1998). For example, in English Springer Spaniels, a deficiency in the activity of a particular enzyme known as muscle type phosphofructokinase is related to the disease symptoms of acute haemolytic anaemia and jaundice that accompany exercise (Patterson, 2000). Affected homozygotes have only 8–24% of the normal activity values for this enzyme, while heterozygote carriers have 40–60% of normal activity (Vora *et al.*, 1985; Giger *et al.*, 1986). Although affected individuals can be definitively diagnosed by this enzymatic assay, the results cannot unequivocally determine heterozygote carriers, making this tool less than ideal in breeding programmes. Additionally, some disorders affect systems that lack a ready protein-based test. Alternatively, obtaining the tissue to be tested may be traumatic to the dog's health, for example, in disorders affecting the retina (Patterson, 2000). The lack of reliable and/or available protein-based tests has served as the impetus to developing DNA-based genotyping methods.

Breeding values

With unknown genotypic status, estimated breeding values (EBVs) can be employed by assigning a relative genetic merit score for a given phenotypic trait to an individual. A sire's or dam's genetic merit for passing on a particular complex trait can be predicted based upon the depth and breadth of their pedigrees. Each animal can be given a breeding value that relies upon the heritability of the trait and the phenotype; this can be mathematically expressed as $EBV_{individual} = h^2 \times (Phenotype_{individual} - Phenotype_{population\ mean})$. That merit score, an EBV, reflects that individual's genetic capacity to improve the trait within the breed. Frequently, breeders are encouraged to consider the expression of traits within the context of pedigree breadth and depth; EBVs capture that.

The utility of incorporating EBVs into breeding programmes has been shown in many species, particularly in broiler chickens and dairy cattle (Hayes and Goddard, 2010). Recently, a study of syringomyelia in Cavalier King Charles Spaniels demonstrated that, with concerted breeder efforts to reduce the prevalence of the disorder, the average syringomyelia EBV also improved (Lewis *et al.*, 2010), suggesting that the converse would also be true. That is, if the breeders had used syringomyelia EBVs when selecting brood stock, the prevalence of the disease would be reduced, and perhaps at a more rapid pace.

The incorporation of EBVs can preserve breed characteristics while reducing the incidence of inherited disorders over generations (Thomson *et al.*, 2010). While the use of EBVs has most often been applied to complex traits (quantitative or continuous), a recent paper by Lewis *et al.* (2010) demonstrated their utility in binary or qualitative trait selection schemes. Accurate estimation of an individual dog's EBV requires open registry, defined and measurable traits, and maximal participation by breeders. This may prove problematic; for example, breeders in one study desired open communication, but were themselves reluctant to disclose EBVs for their own dogs (van Hagen *et al.*, 2004).

Genetically regulated disorders can be reduced in a breed by using EBVs even in the absence of genetic tests. Many of the phenotypic traits of interest are polygenic in nature, and the contribution from any single gene will be small; determining all the genetic mutations that alter the trait will be difficult. It has been proposed, though, that even complex traits may be governed by a restricted suite of loci exerting significant impact on expression of that trait, and that those genes can be identified (Georges, 1997). The use of EBVs in breeding programmes is especially important, as more traits, both desirable and undesirable, require methodology to assess the composite genetic impact. In other species, one approach has been to use genetic markers that indicate the impact of genes that may be influencing the phenotype of interest. The use of genetic markers has been termed marker assisted selection (MAS). Specifically, identifying genetic markers associated with the expression of a phenotypic trait can be used to predict which dogs will pass on the trait of interest. It is important to note that, to develop robust genetic markers for a given complex trait, divergently selected populations must be bred to permit segregation of the phenotypic trait of interest (Phavaphutanon *et al.*, 2009). In the case of dogs, that would require crossbreeding distinct breeds producing crossbred offspring, and correlating particular markers with particular traits in the crossbred population. While promising, improvement using MAS has not been rapid due to the small number of markers employed (Hayes and Goddard, 2010). The advent of SNP arrays has increased the number of available markers, thereby introducing the concept of combining genomic selection with pedigree, genotypic and phenotypic data for a given population (Higgins and Nicholas, 2008; Thomson *et al.*, 2010). Although proposed for use in guide dog selection (Fuyuno, 2007), it is unlikely, however, that these strategies will replace phenotypic selection. Additional information on MAS appears in the following chapter.

To date, available genetic tests offered for complex disorders in the dog are nominal. One such test is for necrotizing meningoencephalitis (NME) in the Pug. This disorder exhibits variable expressivity with a complex mode of inheritance (Greer *et al.*, 2009). Examination of the dog leucocyte antigen (DLA) region of canine chromosome 12 identified several markers associated with NME creating a 'high risk haplotype' (Greer *et al.*, 2010). Pug dogs homozygous for the NME risk markers have a 12.75 times greater risk of developing NME in their lifetime, whereas dogs homozygous or heterozygous for the normal haplotype are predicted to be at low risk for NME. Although not a diagnostic test, the NME susceptibility markers can be used as an adjunct to other selection criteria.

Breeding Programmes to Address Inherited Diseases

Disorders with a known genotype (DNA tested) or based on phenotypic and clinical diagnosis yield similar suggested breeding programmes to reduce the impact of the disease.

Monogenic dominant diseases

For a condition known to result from a single autosomal dominant mutation, the solution is relatively straightforward because all animals carrying the mutation will be clinically affected (Fig. 18.3). It is simply a question of identifying affected individuals by clinical examination or genetic testing, and selecting against them when developing breeding programmes. For example, von Willebrand's disease in Doberman Pinschers is inherited as an autosomal dominant condition (Riehl *et al.*, 2000), and has a genetic test. Unfortunately, even with autosomal dominant conditions, there can be problems if no genetic test exists. For example, the age of onset of the condition can be crucial. Early-onset conditions that result from an autosomal dominant mutation will be diagnosed in affected animals early in life, before sexual maturity, and these can be selected against. However, if the condition is late onset, affected individuals may not be diagnosed until after they have been used for breeding. Additionally, if the age of onset is late in life, an affected dog may have been used for breeding and died before the age that it would have developed the condition and revealed it as having a dominant mutation. Another complication that could arise is if the dominant mutation shows less than 100% penetrance. If the condition demonstrates incomplete penetrance, some animals carrying the dominant mutation will not become clinically affected and therefore will go unnoticed.

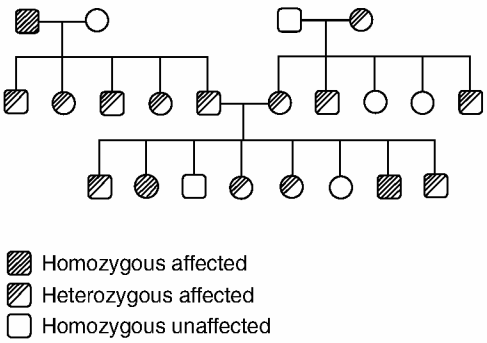

Fig. 18.3. Idealized pedigree for inheritance of an autosomal dominant disorder. The diagram represents the segregation of an autosomal dominant disorder. Provided that there is full penetrance, individuals with one mutant (cross-hatched) allele will be clinically affected. Dominant conditions may express themselves differently if they are homozygous affected (two copies of the dominant mutant allele) rather than heterozygous affected (only one dominant mutant allele and one normal allele). This differential effect is illustrated in the condition known as Ehlers–Danlos syndrome in English Springer Spaniels, in which the homozygous dominant allele seems to be lethal.

Yet, in general, sufficient clinical screening within a breed, particularly of those dogs that form the nucleus of a breed's breeding stock, should easily reduce the frequency of a deleterious dominant gene within a breed. The identification of the genes involved in these diseases and the development of DNA-based tests for the mutation will greatly advance breeders' ability to select against the disease alleles. Additionally, early DNA screening of animals will identify those that carry mutations that cause late-onset conditions or are not fully penetrant.

Genetic diseases that are sex-linked can be similarly handled. In males, the X chromosome is from the dam and the male will have a single copy of genes on the X chromosome. Mutations carried on the X chromosome will be expressed in males, presenting as a dominant condition even if the condition is recessive in the females. On average, half of all male offspring born to a dam heterozygous for the condition will express the disease and all female offspring will be unaffected. A dam producing an affected male necessarily carries the mutation. For example, haemophilia B in dogs, due to a mutation in the X-linked factor IX gene, results in a deficiency of coagulation and clotting, placing an affected dog at risk for severe bleeding complications in response to injury. A mutation-based test to identify the mutation exists (see Table 18.2); therefore, affected males can be bred to homozygous normal females and no affected offspring will be produced.

Mitochondrial inheritance

Diseases caused by mitochondrial mutations are maternally transmitted, but offspring of both sexes will be affected, suggesting a maternal, non-X-linked mode of inheritance. Canine spongiform leukoencephalomyelopathy was the

first mitochondrial disease identified in two breeds arising from spontaneous mutations within the mitochondrial cytochrome *b* gene (Li *et al.*, 2006). Affected puppies developed neurological deficits by 9 weeks of age, reflecting degeneration of the central nervous system. Recently, another mitochondrial mutation has been identified that causes a maternally inherited sensory ataxic neuropathy in the Golden Retriever (Baranowska *et al.*, 2009). That particular report has special significance in that the mutation was traced to a female in the early 1970s but the mutation had been transmitted 'silently' for over 20 years, thus illustrating the potential for dissemination of a deleterious trait.

Complex traits

Reduction in the incidence of a complex condition is certainly possible by selecting for breeding those animals that do not exhibit the condition and removing those more severely affected from the breeding programme. The more heritable the trait and the more rigorous the selection the more likely it will be that the incidence of the disease will fall. Attempts to address the problem of hip dysplasia (discussed elsewhere in this text) in a number of breeds worldwide demonstrate what can be achieved. Many countries and kennel clubs have established hip-screening programmes based on the radiographic evaluation of the phenotype. Radiographs of both hips of an animal are assessed and either a grade or score is given for each hip. For example, in the UK, each hip is assessed on the basis of nine radiographic features of the hip joint (Gibbs, 1997; Willis, 1997). Each of these nine features for each joint is scored by a panel of expert scrutineers and the dog then given an overall hip score. The lower the hip score, the more normal the dog's hips. Similar schemes, although with different evaluation systems, operate in other countries. The benefit of such schemes is that they can be used to select animals for breeding. For example, breeding could be restricted to dogs tested with low scores or, as in the UK, to dogs that have scores lower than the breed mean score (an example of using EBVs). Control programmes based on such selection have shown variable results (Fluckiger *et al.*, 1995; Willis, 1997). However, generally, the results of these studies confirm the benefits of breeding from phenotypically tested dogs with low scores for the reduction of the extent of hip dysplasia (Kaneene *et al.*, 2009).

The best progress was found in Sweden, where disease prevalence decreased during the period of selective breeding in all breeds investigated (Swenson *et al.*, 1997). The study encompassed 83,229 dogs from seven different breeds registered by the Swedish Kennel Club. Since 1984, it has been mandatory in Sweden that the hip score is known and recorded for both the dam and sire if their progeny are to be registered by the Swedish Kennel Club. This measure has led to a dramatic shift to the use, as breeding stock, of dogs that have had their hips evaluated and have low scores. The data presented in Fig. 18.4 represent the prevalence, by year of birth, of hip dysplasia during the period of investigation (1976–1988).

Monogenic recessive diseases

Genetic counselling is less straightforward for conditions caused by an autosomal recessive mutation. The goal would be to reduce the frequency of the disease-causing mutant allele within the breed population. In the absence of a genetic test, identifying affected dogs and selecting against them will certainly reduce the number of affected puppies born, but there will be a less significant reduction in the number of carriers. These carriers will act as a silent reservoir of the recessive mutant allele. The only way to make significant inroads into autosomal recessive conditions is to develop ways of identifying carriers so that they can be taken account of when breeding programmes are developed. As seen in most other species, the majority of canine genetic disorders tend to be either recessive or polygenic in nature (Willis, 2000; Summers *et al.*, 2010). Examples of autosomal recessive disorders include phosphofructokinase deficiency and fucosidosis in Springer Spaniels, narcolepsy in Doberman Pinschers and canine leucocyte adhesion deficiency in Irish Setters (Patterson, 2000).

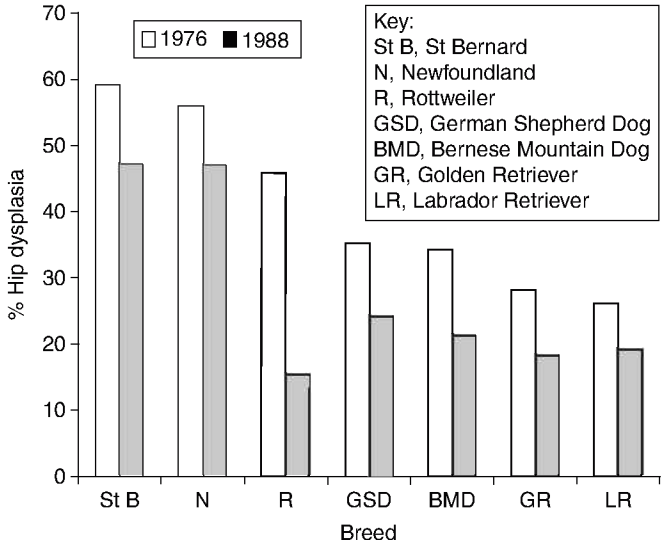

Fig. 18.4. Prevalence, by year of birth, of hip dysplasia (all grades) in seven dog breeds registered with the Swedish Kennel Club. The chart shows the overall prevalence of hip dysplasia in Sweden in 1976 (white bars) and in 1988 (grey bars) for each breed. During this period, it became mandatory for the hip score to be recorded for the dam and sire before their progeny were registered, a measure that led to a dramatic shift towards the use, as breeding stock, of dogs that had their hips evaluated and had low scores. In all breeds analysed there was a significant reduction in the prevalence of hip dysplasia in 1988 compared with 1976 (data taken from Swenson *et al.*, 1997).

Genetic Counselling Based on Predictive Models

The goal of genetic counselling should be to maintain quality attributes within a breed while controlling genetic disease. Prioritization of the traits for selection is imperative in achieving this goal, and this requires concerted efforts by all breeders to prioritize the needs of the breed as a whole rather than to promote personal self-interest. Genetic disorders that are prevalent within a breed require greater time and effort for reduction and elimination. Low-frequency disorders should be managed, through breeding programmes, to prevent dissemination throughout the breed (Leroy and Baumung, 2011).

Canine genetic counsellors need to consider the emotional investment of the owner and breeder. The counsellor should not pass judgement, but merely supply accurate information (Fowler *et al.*, 2000). Companion animals, particularly dogs, have assumed a significant role within the social and familial context, with dogs being highly anthropomorphized (Albert and Bulcroft, 1988). Moreover, owners often view the dog as a self-reflection or part of the family (Hirschman, 1994). Breeders lose objectivity in assessing strengths and weaknesses of a given dog the greater the emotional attachment to that dog. In addition, many breeders have invested years in the development of particular lines within a breed, and may be stigmatized should a dog be diagnosed with a genetic disorder. The role of the genetic counsellor is to provide explanations of test results, quantify the risks associated with a particular breeding, and enumerate the possible outcomes from such a breeding (Fig. 18.5). The need for sensitivity in explaining the results of genetic testing and detailing the implications is essential (Fowler *et al.*, 2000), particularly in light of the knowledge that all animals possess deleterious alleles and of the need to maintain genetic diversity within closed breeding pools.

The overall counselling programme focuses upon the breeder's vision of a desirable phenotype. Except for the genetic tests that

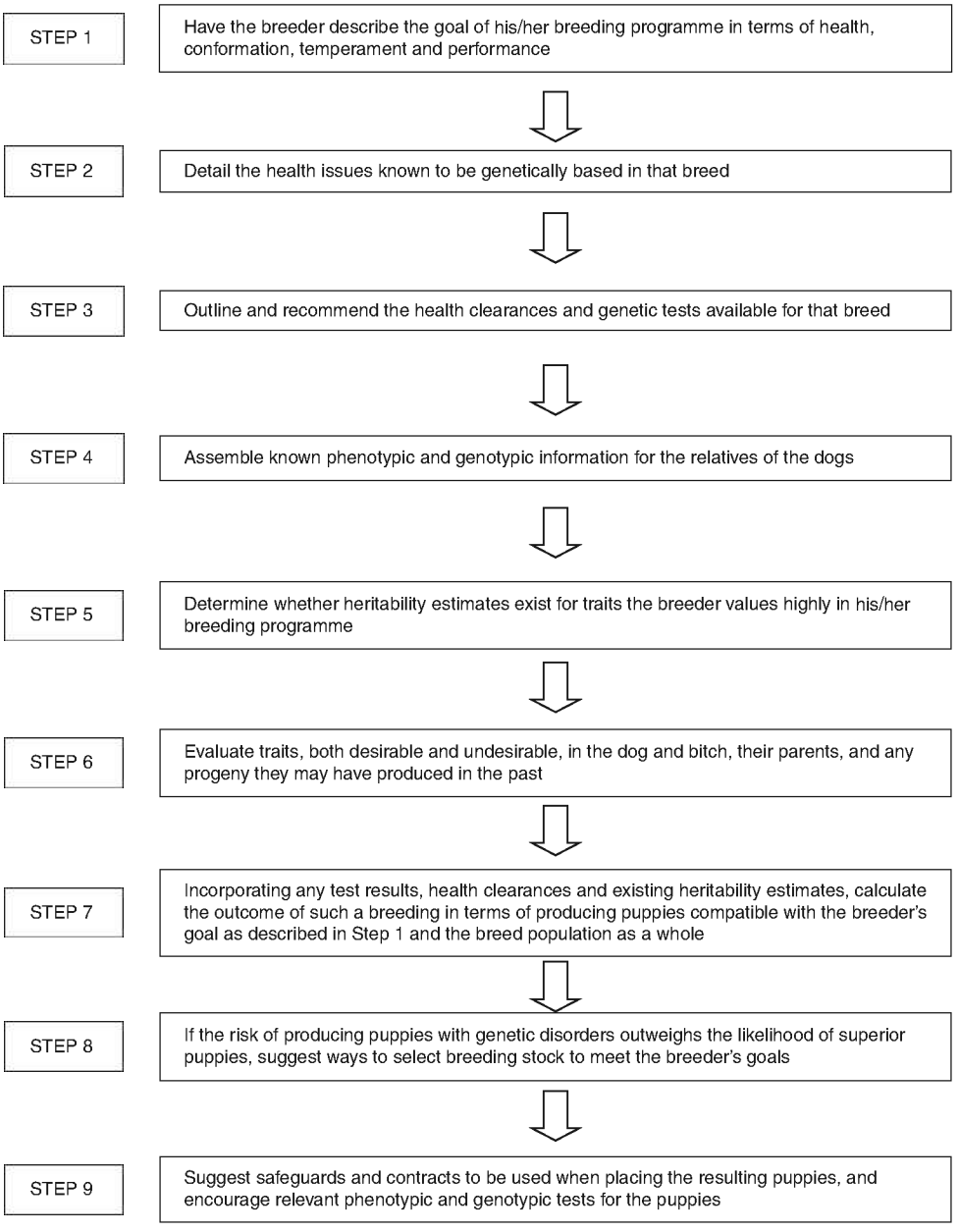

Fig. 18.5. Steps in genetic counselling to follow once a breeder suggests the breeding of a dog and bitch of a given breed.

are available, selection criteria include phenotypic and clinical data. It is important to remember that this represents indirect selection and not the trait itself. For example, selecting upon radiographs to reduce the incidence of hip dysplasia will result in dogs exhibiting better radiographic assessment, and, while that is correlated with reduced dysplasia, the selection is not being exerted upon the clinical signs of dysplasia (Thomson *et al.*, 2010) – although

Malm *et al.* (2010) recently demonstrated that selection based on radiographic data did in fact reduce the clinical consequences in the case of hip dysplasia. There have been few breed-wide genetic counselling schemes based on predicting the probable genotype of an individual dog from knowledge of its pedigree and the phenotype of the dogs within that pedigree, yet there is a clear role for non-DNA based counselling programmes.

Hall and Wallace (1996) used exactly this approach to address the problem of epilepsy in the Keeshond, a condition that results from an autosomal recessive mutation. They were able to analyse the pedigrees of individual dogs sent in by their owners and calculate the probabilities of a mating producing epileptic or carrier dogs, and the inbreeding coefficients of the progeny of such matings. Based on the outcome of these calculations, they have been able to advise the breeder whether or not to proceed with the mating. Since the start of the counselling scheme in 1988, the mean probability that a proposed mating would result in carriers has declined significantly, consistent with a decline in the frequency of the mutation for epilepsy in the Keeshond.

Similarly, van Hagen *et al.* (2004) provided a genetic counselling scheme for breeders of boxers. Breeders were given EBVs for sires and dams for four health disorders and had the option of using those in their mate determination. Although the breeders placed the greatest emphasis on the phenotypic match of the dogs, a third of the breeders did take the EBVs into consideration in their selection of mates. Within the EBVs, greater selection weight appeared to be placed on the two disorders that were late onset and perceived to impact health the most: epilepsy and knee problems. On the whole, however, breeders tended to put their personal interest over the needs of the breed population as a whole (van Hagen *et al.*, 2004).

Genetic Counselling Based on DNA Testing

As previously mentioned, there are in excess of 500 documented canine genetic disorders. The number is expected to grow as additional disorders are characterized as having a genetic basis, and as new, spontaneous mutations arise. Unfortunately, while genotyping exists for some disorders, many hundreds of others lack any type of molecular tool to aid breeders. The existence of DNA testing tends to focus selection upon traits for which a test exists. The Portuguese Water Dog exemplifies that tendency. Gangliosidosis (GM1) was prevalent in Portuguese Water Dogs when a blood protein-based test for the disease became available to breeders in the mid-1980s. Carriers of GM1 were subsequently eliminated from breeding programmes. The incidence of GM1 dramatically declined in Portuguese Water Dogs, while the prevalence of progressive retinal atrophy (PRA) increased; lines of dogs free from GM1 were carriers for PRA (Woodsen, 1999). The breeders had traded one disease for another simply because one disorder had an available test; fortunately for Portuguese Water Dogs, a genetic test for PRA is now available. The prioritization of traits for the entire dog, and the breed population as a whole, needs to be the guiding principle in genetic counselling for breeding programmes, as undue emphasis placed on a single disease or trait is unwise, especially for breeds with small gene pools (Meyers-Wallen, 2003).

Counselling should address the mode of inheritance and any misconceptions that may be associated with carrier status, and ensure the breeder understands the distinction between 'at risk' and 'affected'; this is especially true for the results of testing for variably penetrant disorders (e.g. the DM testing noted earlier). A dog that is DNA tested to be either a carrier or at risk may still be an important contributor to the breed population as a whole if bred judiciously and with DNA testing for all offspring (Traas *et al.*, 2006).

Breeders should be counselled that maintaining genetic diversity within a breed is important, and that although inbreeding has advantages, such as enhancing uniformity within litters, it also has unintended consequences, such as loss of rare alleles, increased homozygosity that enables the expression of recessive disorders, and the reduction of effective population size (Calboli *et al.*, 2008). For the same reasons, breeders should be cautious about using popular sires. This was recently demonstrated in a model simulation of common

breeding practices (Leroy and Baumung, 2011). The use of a popular sire proved to be more effective at dispersing deleterious alleles within a breed than did inbreeding when the allele was recessive and lethal.

The potential role of kennel clubs

Kennel clubs around the world could establish breed-control schemes to eliminate disease alleles from a breed gene pool. The Kennel Club in the UK collaborated with the Irish Setter breed clubs to introduce a breed-control scheme to reduce the incidence of canine leucocyte adhesion deficiency (CLAD) in the breed. The scheme is based on the availability of a mutation-based DNA test that affords 100% reliability in genotyping individual animals with respect to CLAD. The scheme initially required that all Irish Setters be genotyped before they were used for breeding – either by direct DNA testing, or if both parents were DNA tested clear of the mutation. For a 5-year period, carrier/carrier matings were to be avoided, but carriers could be mated to genetically clear dogs; the resultant offspring were required to be DNA tested to identify both the carrier and clear offspring. In 2008, the Kennel Club began to only register Irish Setters shown to be clear by direct DNA testing for CLAD, or hereditarily clear by virtue of having parents proven to be clear of the mutation. Irish Setters that were carriers of CLAD were no longer registered. In this way it is hoped that the mutant allele causing CLAD will be gradually removed from the Irish Setter population without compromising the overall gene pool of the breed. That spectacularly good Irish Setter bitch that just happens to be a carrier was still bred, and the breed would benefit from all of her qualities, but only her genetically clear offspring will be used to continue the pedigree. This scheme will serve as a future model for the treatment of inherited disease in the UK once a mutation-based test becomes available for a breed-specific inherited condition. The only major variable is likely to be the window of time before registration restrictions are made and during which carriers can be mated to clears. In the case of the Irish Setters, the breed clubs involved decided that a 5-year period would be sufficient; for some conditions and breeds it may be decided that a longer period of time will be required.

Many kennel clubs and dog registries currently encourage phenotypic health clearances, such as ophthalmic examinations and hip and elbow evaluations, before breeding. As more genetic tests become available, more kennel clubs are likely to take the stance that the Kennel Club of the UK has taken with respect to requiring the CLAD tests for Irish Setter registration. Additionally, kennel clubs, by offering limited registrations, allow breeders the luxury of time (Willis, 2000). Often, phenotypes of disease, structure or behaviour require time to be expressed. With limited registration, puppies are placed in homes and evaluated at later ages by the breeder; if the breeder considers the dog to be of good quality, the breeder can then permit full registration. This allows the breeder to evaluate the outcome of the breeding without the resulting puppies being bred prematurely – or bred at all – if considered to be of inferior quality or lacking in proper health clearances.

Kennel clubs should consider relaxation of the stud books to admit additional dogs to increase genetic diversity and reduce the negative consequences of inbreeding (McGreevy and Nicholas, 1999; Higgins and Nicholas, 2008). Certain breeds have inadvertently fixed deleterious disease alleles that accompanied selection for breed-specific traits. The Dalmatian is such an example, with all Dalmatians at risk or affected by the autosomal recessive hyperuricosuria condition and a predisposition to form urinary stones. The causal mutation was found in a solute carrier gene (*SLC2A9*); the mutant form of the gene cannot process uric acid for excretion, resulting in the formation of urate stones. Molecular testing has confirmed that the Dalmatian breed is homozygous for the mutant form of *SLC2A9* (Bannasch *et al.*, 2008). Over 30 years ago, in an effort to define stone formation, a Dalmatian was bred to its nearest breed relative that did not form stones (an English Pointer). Twelve generations of backcrossing produced dogs that are >99.98% Dalmatian, heterozygous for the *SLC2A9* gene, and do not produce urinary stones. Kennel clubs should be willing to open stud books to permit such backcrossed dogs to

be used for breeding, and perhaps even recommend controlled outcrossing in certain circumstances (Calboli *et al.*, 2008; Higgins and Nicholas, 2008). The Dalmatian exemplifies the effect of breed selection on the genetic composition of a breed. The selective sweep of genomic selection that resulted in the development of dogs and dog breeds may, if desirable phenotypes are inextricably linked to deleterious genetic variants, require outcrossing (Chase *et al.*, 2009; Akey *et al.*, 2010).

Summary

Any trait of interest to breeders, be it conformational, reproductive or concerning temperament, can be subjected to selective breeding strategies. Seemingly complex traits that control structural or behavioural attributes may be selected for if the traits have sufficient genetic input, as determined by heritability estimates. Unfortunately, the scientific knowledge base on the inheritance of many of these traits is severely limited. However, once heritabilities for the desired traits are ascertained, and the heritabilities are large enough to assure that selection for these traits will produce phenotypic improvement, breeders should be encouraged to incorporate sire and dam selection schemes designed to maximize genetic progress (Famula *et al.*, 2000). Breed clubs can initiate the collection of phenotypic traits, along with pedigrees, to allow heritabilities and EBVs to be assigned to the complex traits, as outlined in the following chapter. Sires and dams can then be ranked as to their predisposition to pass on particular traits, and breeders can utilize that information to select prepotent sires and dams for the traits they deem to be particularly important, while weighing other attributes of the sires and dams. An example of employing EBVs for enhancing desirable traits in dogs is illustrated by guide dog breeding programmes (Humphrey and Warner, 1934; Willis, 1995, Wilsson and Sundgren, 1997; Helmink *et al.*, 2003; Cole *et al.*, 2004).

Minimizing the spread of defects requires the concerted effort of the breeders involved to identify the defect, characterize whether the defect has a genetic basis and, if so, determine the mode of inheritance. It is at that point that breeders can then make some informed decisions on the likelihood of producing an animal with the defect in its pedigree. They need to prioritize the traits of interest (as well as disorders), including the incorporation of a severity index as proposed by Asher *et al.* (2009), consider whether the clinical symptoms expressed are of enough concern to minimize the relative frequency of the mutant allele in the current population, and desire to minimize spread of that allele. Breeders can also generate interest in the creation of a DNA-based test to assist in their breeding selections. Given that the majority of genetic defects are either complex or autosomal recessive, and lack a genetic diagnostic test (Traas *et al.*, 2006; Mellersch, 2008; OMIA), breeders must be counselled as to the benefits and risks of breeding suspected carriers based upon likelihood and probability estimates. The issues become more complex when polygenic traits are considered. When making breeding decisions to minimize the incidence of polygenic conditions, the breeder must consider the 'breadth of the pedigree' and carefully evaluate the phenotypes of not just the sire and dam of the potential mate, but of all the relatives of the breeding pair and, ideally, use EBVs if available.

Implementing selective breeding practices based either on the genetic tests available or upon genetic merit that represents the likelihood that a particular sire or dam will pass on a particular trait (EBVs) (Hall and Wallace, 1996; van Hagen *et al.*, 2004) will result in the improvement of dogs. Molecular diagnostic tools will become available to aid in the selection of brood stock. However, heritability estimates and the relative genetic merit (EBVs) for each trait, and the ranking of prospective parents, will continue to be vital in any selection process. The role of genetic counselling is the interpretation of molecular test results and EBV statistical rankings, then the judicious application of the results to mate selection, preventing undue emphasis on a single test result, encouraging practices that benefit the breed population as a whole, and weighing the overall attributes of a given mate against the faults that may be produced if that animal is bred. Analysis of pedigrees to determine the heritability of particular traits, and the development of genetic tests, will greatly influence the manner in which dogs are bred in the future, hopefully to produce healthy and sound animals.

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19 Genetics of Quantitative Traits and Improvement of the Dog Breeds

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Introduction

Select the best; that is the simplest axiom of animal improvement, and that certainly is the goal of dog breeders: to identify those animals that stand above their cohort and are worthy of producing the next generation. But dog breeding has always seemed to lag behind the well-published success of animal improvement in more economically directed species. Ten years ago, when the 1st edition of this book came out, many authors pointed to the promise of the new tools of molecular biology as a way out of this wilderness. Hence the present review of what challenges remain and the solutions most likely to bear fruit.

It is important to remember, however, the phenotypes that interest dog breeders. Quantitative geneticists rely upon the generally understood explanation of phenotypes (P) as the joint expression of genotypes (G) and the environment (E): the familiar generic model $P = G + E$. Allowing for some oversimplification, the phenotypes that interest dog breeders can be placed into one of two categories: those in which the environmental contribution to expression is almost entirely absent, and those in which non-genetic factors can play a very important role in the eventual expression of phenotype. Continuing our simplification, the former usually involve the expression of only a few loci (often only one), whereas the latter are usually the action of many loci, each with small effect. The distinction between these two general categories has a profound impact as we look for breeding strategies.

For example, a trait that clearly has no environmental impact would be sex. The phenotype that we see in dogs, male or female, is determined exclusively by the inheritance of the *SRY* gene (DiNapoli and Capel, 2008), with no known environmental contribution, i.e. in the manner of the model described above, $P = G$, or nearly so. Accordingly, for those traits with negligible environmental contributions, identifying superior individuals can be relatively straightforward. If we observe the phenotype of an animal, we can predict the genotype with near certainty, especially if we consider the phenotypes of parents and siblings.

With rare exceptions, the traits of interest to most dog enthusiasts and breeders do not follow simple Mendelian monogenic inheritance. Nevertheless, there are some, and geneticists have taken advantage of finding these genes.

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Other chapters of this book have detailed many of these characters, whether cone–rod dystrophy in Wire-haired Dachshunds (Wiik *et al.*, 2008) or degenerative myelopathy in Corgis (Awano *et al.*, 2009). The distinctive element of these characters is that there is a direct link between the unseen genotype of an individual and the realized phenotype; the contribution of the environment is negligible to non-existent. It is in these instances that the promise of genomics and molecular genetics has seen its greatest impact. But since 1994, with the publication of a seminal paper on complex trait dissection (Lander and Schork, 1994), the goal has been to extend the discovery of genes influencing monogenic traits to the discovery of the source of polygenic variation (Goddard and Hayes, 2009).

This simplicity of application is not the case for phenotypes where the environmental contribution can be substantial. In these settings, the phenotype is not a clear indicator of genotype. The discovery of 'good' or 'bad' alleles lacks a clarity of identification because of the obscuring nature of environment in the expression P = G + E. But this complication does not preclude breed improvement. Taking advantage of pedigree information, records on relatives and the use of sophisticated statistical packages (Van Tassell and Van Vleck, 1996; Gilmour *et al.*, 2006), consider, for example, the outline for breed improvement in hip dysplasia (Zhu *et al.*, 2009). This process, beginning with the estimation of heritability (Oberbauer *et al.*, 2006; Silvestre *et al.*, 2007; Hou *et al.*, 2010), and moving to the prediction of breeding values (Zhang *et al.*, 2009), has come to be the gold standard of animal improvement. The heartening element here is that these strategies are finally becoming the tools of professional dog breeders. Applying these techniques to small populations of dogs owned by hobby breeders remains a challenge, but the future continues to show promise of significant and measurable progress.

Traits of Interest

Though each breed of dog has its own idiosyncratic breeding objectives, most characters of substantive interest fall into one of two broad categories: behaviour/temperament and disease. Of the two, much of the attention has focused on disease and the role that inheritance may play in this. Of course, this focus on the study of disease is for the sake of our canine companions (Sargan, 2004; Kennedy *et al.*, 2008; Gough and Thomas, 2010). There are, however, investigators who use the dog as a model for human disease (Ostrander *et al.*, 2008; Shearin and Ostrander, 2010), which, of course, can still lead to therapies for dogs. Quantitative geneticists, at least those interested in animal breeding, have only a handful of questions to address when confronted with a character (phenotype) destined for improvement. First, we ask is the character heritable, is there a similarity between the phenotype of an individual and that of parents and/or siblings? This strength of association is best represented by the quantitative genetic parameter called narrow sense heritability (Lynch and Walsh, 1998). This statistic is also an essential element of our second goal, to identify those individuals of superior genetic merit. As a part of this process, the prediction of genetic merit, quantitative geneticists must also identify important non-genetic contributions to phenotype. That is, identification of (and correction for) these non-genetic contributions to phenotype is essential to identifying animals as suitable candidates for breeding.

A secondary concern for quantitative geneticists is evaluation of the potential for a simple mode of inheritance. Reflexively, quantitative geneticists assume that traits of inheritance are influenced by many loci, each of small effect. For the vast majority of characters, this assumption of complex inheritance is well suited and well justified. However, there are traits that are under the control of one locus, or just a few loci. Moreover, even polygenic traits can be influenced by one or two 'major loci', and thus there is the evaluation of the presence of such major alleles through complex segregation analysis (Kadarmideen and Janss, 2005; Janutta *et al.*, 2006a; Famula *et al.*, 2007).

A final component of today's quantitative geneticist is, having identified inherited characters and their non-genetic components, to locate those loci responsible for the expression of phenotypes of interest. Whether done with SNPs (single nuclear polymorphisms),

microsatellites, candidate loci, or any of dozens of genotyping techniques, the hope of many animal breeders is to use the combined tools of molecular genetics and genetic statistics to locate those areas of the genome associated with the characters we hope to improve. This is the fundamental goal of genomics and constitutes much of the research reviewed in this book. But bringing all this information together, to benefit breeders, remains a challenging problem.

The importance of understanding phenotype

An earlier chapter of this text has covered the genetics of behaviour in detail. Yet there remains a need for a few, brief comments from the perspective of a quantitative geneticist with an eye towards breeding. I mean here a concern for breeding in contrast to a discussion of the broader theory of quantitative genetics, which has been recently reviewed from the aspect of quantitative trait locus (QTL) mapping and genetic architecture (Mackay *et al.*, 2009).

The obvious element to consider here is the complex nature of most behavioural characters. A recent review article outlined the challenges associated with behavioural quantitative genetics (Spady and Ostrander, 2008). As we review recent research, we see that most of the work in this area has encompassed the discovery of QTLs or the evaluation of candidate loci in relation to particular behaviours. For example, genes that might influence aggressive behaviours have been uncovered (Våge *et al.*, 2010), while other candidate loci have receded from consideration (Van Den Berg *et al.*, 2008). Although these two studies were conducted in different breeds, they both illustrate the challenges presented in the evaluation of behavioural genetics; that is, the transformation of a complex behaviour like aggression into a quantifiable objective candidate for genetic analysis.

The interest of dog breeders is most easily classified into a desire to eliminate bad behaviours, or, conversely, to enhance and support desirable behaviours. An example of the former would be the extensive research into undesirable compulsive behaviours and impulsive behaviours. A genetic basis for this suite of disorders is simple to observe, if only through a glance at breed differences (Saetre *et al.*, 2006; Crowell-Davis, 2007; Duffy *et al.*, 2008). Noting behavioural differences across breeds is the simplest way to identify genetic variation (Falconer and Mackay, 1981; Lynch and Walsh, 1998; Bourdon and Bourbon, 2000). This simple discovery can be expanded upon through the estimation of heritability (Saetre *et al.*, 2006; Liinamo *et al.*, 2007) and later by the discovery of individual genes (Dodman *et al.*, 2010). In that sense, behavioural genetics in the dog is no different from the study of body conformation and other continuous polygenic traits (Kharlamova *et al.*, 2007). The challenge in genetic analysis is validation of the behavioural phenotype measured and the impact of non-genetic (environmental) components on the measured behaviour (Martin and Bateson, 1993).

This issue, as would be expected, has been raised in earlier reviews (Spady and Ostrander, 2008). Proper phenotyping remains the principal challenge to any breeding programme directed at behavioural traits. Three basic methods have been considered: test batteries, owner-directed surveys and observational studies (Wilsson and Sundgren, 1997a,b; Jones and Gosling, 2005). Each of these tools has its strengths and weaknesses (Jones and Gosling, 2005). What is more challenging is to envision how any of them can be translated into a phenotyping strategy for breeders. Research behaviourists and professional geneticists can surely benefit from these various tools, though – teasing out environmental contributions, quantifying repeatability and inheritance (Overall and Dunham, 2002; Strandberg *et al.*, 2005; Overall *et al.*, 2006). Such techniques can even assist in the identification of genes through candidate or whole genome association studies (Hejjas *et al.*, 2007a). But getting this information into the hands of breeders, in a workable fashion for everyday breeding decisions, will remain a substantive obstacle.

As we are seeing, the literature is becoming quite rich in its discovery of important genetic indicators associated with interesting behaviours and temperaments (Hejjas *et al.*, 2007b; Jones *et al.*, 2008; Takeuchi *et al.*, 2009a,b), and this list will surely grow in the next 10 years. But what of the application of

these discoveries to the needs of breeders? How to leverage this information to the benefit of the breeder is likely to remain a challenge. Until a repository of data can be established that allows breeders to choose parents from some trustworthy information source, we may be no further along in 10 years from where we are today. The hope is that the discovery of genetic associations between SNPs and behavioural characters can simplify this decision making. However, until we can better quantify behaviours, and at the same time better identify those components of 'nurture', our hunt to better understand 'nature' will proceed in small steps, if at all (Guo, 2000b). This is the ironic nature of quantitative genetics in the age of genomics: in order to better understand genetic contributions we must begin with a better assessment of phenotype (P), including a strong grasp on the component of environment (E). That is, to better know G, we must also expand our efforts to understand the relationships between P and E. Even with such information, the presence of multiple variants and their stochastic interactions with the environment are likely to complicate this process for years to come (Hunter *et al.*, 2008).

As discussed here for behaviour traits, earlier chapters of this text have focused much attention on the genetic elements of a variety of canine diseases. A common thread of that discussion is the need for accurate and repeatable diagnoses. Elucidation of the genetic mechanisms of a disease requires that dogs classified as 'affected' all suffer from the same ailment; failing that, the hunt for any specific mechanism is elusive at best. It is not surprising that disease has often taken the front seat in dog research. Few settings are more frustrating than enduring the illness of a beloved companion, especially if preventive breeding could have been an option. Accordingly, many investigators have undertaken the search for the genetic causes of common diseases, and on occasion they have met with success. Some of the more noteworthy success stories have included the discovery of genes for retinal atrophy, narcolepsy, copper toxicosis and Collie eye anomaly (Haywood, 2006; Aguirre and Acland, 2007; Parker *et al.*, 2007; Mignot, 2010). A common characteristic of these success stories can be found in the simplicity and objectivity of diagnosis and the concomitant good fortune that these ailments are monogenic in origin.

Of course, we also hope to be able to confront more complex disease traits, not unlike the massive search under way in humans (Hirschhorn and Daly, 2005; Hindorff *et al.*, 2009). A recent canine example is found in the study of osteoarthritis (Clements *et al.*, 2010). This work had hoped to find common genetic origins for traits associated with osteoarthritis, those being hip dysplasia, elbow dysplasia and cranial cruciate ligament rupture. Basing their conjecture on previous work in quantitative genetics (Maki *et al.*, 2000; Todhunter *et al.*, 2003) the hope was to find genes through a candidate gene approach in 20 different loci. An advantage in this work is the objectivity of making a diagnosis of disease (Wood *et al.*, 2002), for one of the principal challenges in this process of 'gene hunting' is the measurement of phenotype. In the case of osteoarthritis, this hurdle appears to have been cleared, leaving behind the expected challenges of isolating important genetic and environmental contributions. Yet the work was largely unsuccessful in meeting its stated goals (Clements *et al.*, 2010).

Much, nearly all, of the work done in canine disease genetics is done with the assistance and cooperation of breed clubs and large-scale databases (Janutta *et al.*, 2006b; Parker *et al.*, 2006; Chase *et al.*, 2009). Rarely has this work been conducted in an experimental colony setting, where investigators can control and record various environmental impacts. Such has been a common criticism of much of quantitative genetics research in economically important animals (Kempthorne, 1978, 1997). Nevertheless, in spite of these experimental weaknesses, much genetic progress has been made (Ginja *et al.*, 2009). However, in the search for important genetic indicators and effectors of complex disease, this weakness, being explicit in the identification of cause and effect, remains a formidable obstacle (Guo, 2000a,b).

For example, consider the recent work based on a breed survey of 147 breeds (with 2800 dogs) and 1500 markers (Chase *et al.*, 2009). One of the characters presented was age of death, in which markers on CFA7 (canine

chromosome 7) and CFA15 were found to be in association with longevity. Most investigators would agree that this identification of genes is approximate at best (as did the authors), for, although age at death may be accurately determined, the many non-genetic characters that influence longevity could not be recorded or included in the analysis. To some, it may even be counter-intuitive that our ability to genetically dissect complex traits rests as much on our understanding the non-genetic influences on phenotype as it does on our understanding of genes and their actions (Guo, 2000b).

To illustrate the challenges of demonstrating genetic change in an inherited disease, I will single out epilepsy. While not a problem universal to all dog breeds, this does illustrate the challenges that breeders may face in the realization of animal improvement (Raw and Gaskell, 1985; Berendt, 2008; Berendt *et al.*, 2008). Traits like canine epilepsy are not easily researched (Oberbauer *et al.*, 2003, 2010), for here we are confronted at the outset by the challenge of phenotyping individuals correctly. Unless healthcare professionals can diagnose individuals accurately, and ensure that animals are placed into categories with the same ailment, the elucidation of any genetic causes will remain fraught with error, yielding little in identifiable progress (Helbig *et al.*, 2008). Can we be sure that animals classified as 'yes' (subject to seizures) are indeed suffering from the same ailment? All subsequent genetic analyses, whether the prediction of breeding values or the quest to identify loci associated with disease, hinge on the belief that all diseased dogs are expressing the same genes; what we glibly refer to as a 'trait'. At the same time, it is essential that we have the date of the first seizure, along with other explanatory, non-genetic, variables. This has not always been the case in studies of canine epilepsy, thus confounding opportunities to reduce the incidence of this disease through selection. Moreover, it is a disease that may take 4 or 5 years to present, depending upon contributing environmental non-genetic factors. This leaves the disappointing possibility that a dog may have been used for breeding, only to find, years later, that the animal suffered seizures. Thus, though the desire to identify at-risk dogs early in life is great, the challenges to do so are similarly sizeable (Ware, 2006; Hunter *et al.*, 2008).

Whether behaviour or disease, improvement is reliant upon fully understanding the biology of the traits of interest. Ensuring that phenotypes are reliably measured, and that non-genetic contributions are properly recorded and accounted for in any later analysis, are the keys to successful breed improvement. As we shall see, they are also the key to unlocking the genes that underlie those characters of dogs that we wish to improve.

Quantitative Traits, Breed Improvement and Genomics

Variation, intensity and accuracy, these components of statistics and genetics represent the foundation of animal improvement. Of the three, breeders have largely focused on the improvement of accuracy, the identification of animals of superior genetic merit. Since the close of World War II, animal breeders have sought better ways to use phenotypes, in combination with pedigree information, to create statistics correlated with genetic merit. Beginning with the selection index, investigators have found the ideal means of manipulating quantitative phenotypes, corrected for known environmental (non-genetic) contributions, in such a way that we can maximize the speed and efficiency of manifesting genetic change. Of course, the linchpin to this progress is the collection of objective, informative phenotypes, while at the same time recording all manner of useful related information (e.g. sex of the animal, age at recording, season of measurement, diet, rearing environment and/or a host of other informative characters). Such measurements have made improvement possible, while simultaneously monitoring the change in a population's phenotypic average.

In fact, as we consider the current status of breed improvement, we note that the great majority of this proceeds purely on phenotypic selection. For, while most breeders do not have access to vast databases with information on relatives, they do have access to the phenotypes of the animals directly under their care, and this phenotypic information is certainly sufficient to make breeding decisions. We can see this in traits as straightforward as the improvement in racing performance in Irish

greyhounds (Täubert *et al.*, 2007) or changes in the behaviour of German shepherd dogs (Ruefenacht *et al.*, 2002). The genetic change manifested in these examples is the result of phenotypic selection, a time-tested, if inefficient, tool of animal improvement. In a similar vein, several retrospective studies have demonstrated a noted improvement in hip and elbow dysplasia (Malm *et al.*, 2008; Kaneene *et al.*, 2009). These studies note that, although the rate of improvement is slower than desirable, such improvement is indeed detectable and noteworthy. Taking note of this slow rate of improvement in joint disorders, the British Veterinary Association and the (UK) Kennel Club are beginning the process of moving from phenotypic selection to the use of pedigree information and the prediction of estimated breeding values (Lewis *et al.*, 2010). However, the dawning world of genomics hopes to replace this process with a potentially more accurate, less expensive alternative.

The hope is that rather than collect phenotypes, record pedigrees and accumulate related non-genetic information, we will be able to rely only upon a swab of DNA taken from candidates for selection. This sample, taken at any time in an animal's life trajectory, often well in advance of the expression of any phenotype of interest, would form the basis of an objective discrimination among potential parents. Removing the expense of phenotype collection and data storage, indeed the elimination of phenotypes altogether, is exceedingly attractive to hobby breeders, for these individuals are never likely to have accumulated enough phenotypic information to take advantage of advanced statistical techniques.

In the absence of molecular genetic decision aids, several service dog agencies are now making use of those statistical tools long exploited by livestock breeders. For example, Guide Dogs for the Blind (in San Rafael, California) now use the linear model techniques originally intended for livestock to rank candidates for breeding (Henderson, 1975b). This non-profit organization produces over 1000 puppies a year, using roughly 30 sires and 100 dams in a given calendar year. The decisions on which dogs become breeders make use of an extensive database of pedigree and phenotypic information. Obviously, this scale of production is not possible among hobby breeders, but these service organizations are nevertheless quite capable of taking advantage of the tools developed for other animal improvement purposes (Wirth and Rein, 2008).

In the specific case of Guide Dogs for the Blind (GDB), the organization has addressed several behavioural and animal health traits in the construction of its selection indices (Bourdon and Bourbon, 2000). As an example, the majority of dogs used in this organization are Labrador Retrievers, and a malady common to these animals is tricuspid valve dysplasia (TVD) (Asher *et al.*, 2009; Meurs, 2010). Accordingly, the health-care professionals at GDB evaluate all of their potential service graduates for this disorder, and over recent years have noticed an increase in incidence of the disease. This observation has led to using the information in the selection of breeding stock. Having previously estimated the heritability of TVD in this population (Famula *et al.*, 2002), the prediction of breeding values is relatively straightforward, for animals both with and without recorded TVD phenotypes (Henderson, 1975a,b, 1977; Mrode and Thompson, 2005). Figure 19.1 shows the genetic trend for TVD (Blair and Pollak, 1984; Boichard *et al.*, 1995); the average estimated breeding value of affected animals is scored as a one and that of unaffected animals as zero, hence smaller is better. Since the use of these predictions began in earnest in 2008, we can see a steady decline in the genetic risk of this disorder in this small population of service dogs.

In this example, the nature of the population is small and closed, and this is an additional topic likely to concern dog breeders over the next few years: an increasing concern over populations (breeds) of small size (Oliehoek *et al.*, 2009). Regardless of the use of traditional selection schemes, or even the use of molecular-based tools, most dog breeders are well aware of the challenges faced by membership of a rare breed. Herein, goals include not only elements of breed improvement but also the minimization of disease risk (exacerbated by increased inbreeding), while simultaneously attempting to maintain breed identity and genetic diversity. Indeed, recent investigation has demonstrated an association

Fig. 19.1. The genetic trend in tricuspid valve dysplasia by year of birth against average estimated breeding value in the colony of dogs at Guide Dogs for the Blind at San Rafael, California. Affected dogs are scored 1 and normal dogs scored 0.

between susceptibility to infections and the accumulation of inbreeding (Spielman *et al.*, 2004; Ross Gillespie *et al.*, 2007). In addition, others are asking if a decrease in the diversity of the major histocompatibility complex (MHC) can be associated with a decrease in viability (Radwan *et al.*, 2010). Although presently unclear, such concerns are never far from the mind of small breed enthusiasts.

Additionally, though outlined in sheep, animal geneticists have found that founders play an important role in the eventual development and degree of inbreeding depression (Casellas *et al.*, 2009). Perhaps using genomics can help us understand and mitigate this decline (Allendorf *et al.*, 2010). But so too can traditional breeding methods, through the use of mathematical techniques to maximize genetic progress while minimizing the accumulation of inbreeding (Meuwissen, 1997). Figure 19.2 is a plot of the average inbreeding coefficient by years of birth for the colony at Guide Dogs for the Blind and should serve as an illustration that, with wise mating decisions, the accumulation of inbreeding can be managed.

As we have outlined, breed improvement for most characters is difficult (Zhu *et al.*, 2009). Because P does not provide an unobstructed view of G (indeed, any more than knowing G leads to a prediction of P), we must find strategies to identify animals with a desired genotype to be parents of the next generation. In traditional animal breeding, this has focused on the accumulation of phenotypes and pedigrees and the subsequent analysis of this information with suitable statistical tools. As we have seen, in many settings, this strategy has proven easily implemented and successful. Of course, this comes at a cost.

The accumulation of information in databases is expensive and time-consuming. Such a strategy is not feasible for hobbyists and those with only a limited amount of statistical expertise. Breed clubs, the larger ones anyway, might consider the creation of repositories of communal data, but this rarely sounds like a sustainable path to a bright genetic future. That is why most breed clubs have turned to a genomic alternative. Here, the hope is that the collection of phenotypes and pedigrees could be supplanted by a series of DNA tests. In that setting, a direct observation of G could be made without the need for P. Breeding decisions could be made more accurately and, indeed, sooner. There would be no need for databases, computers, statistics or communal data sharing. One swab of the gums, a little laboratory magic, and breeding decisions could be made efficiently and, more importantly, with pleasingly predictable results. Of course, if this was possible, cattle, poultry and swine breeders would be using these strategies already, for the economic advantages are obvious. But these commercially oriented breeders

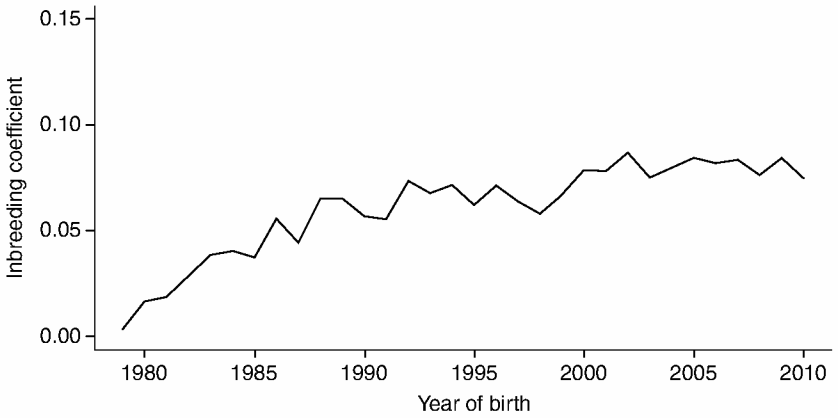

Fig. 19.2. The average inbreeding coefficient by year of birth in the colony of dogs at Guide Dogs for the Blind at San Rafael, California.

are not yet there. Why? Is it possible this strategy can work in dogs?

As we know, and have already seen in earlier chapters of this book, there is no shortage of research into *finding* the genes associated with important traits (Karlsson and Lindblad-Toh, 2008). Genes have been found for all manner of disorders (Benson *et al.*, 2003; Lingaas *et al.*, 2003; Kramer *et al.*, 2004; Capt *et al.*, 2005), and the hunt for others in complex traits continues (Hindorff *et al.*, 2009). But comparatively little has been written on *how to use* this information. For the case of simply inherited traits, where environmental variance is small and the trait is determined by few inherited components, a selection decision can be simple and straightforward. Selecting against such traits does not require elaborate thinking: it needs testing for the offending allele and deciding upon matings accordingly, matings that limit the risk of creating diseased progeny. But what of complex traits? How does one use genomic information to assess the quality of parents?

The earliest work on incorporating genetic information into the statistical decision-making tools of animal breeding date back over 40 years (Smith, 1967). This method, eventually named marker assisted selection (MAS), can follow one of two basic outlines. The first assumes that one locus (or more) with a direct effect on the trait under selection has been identified. In such a straightforward setting, we can genotype the candidates for selection and incorporate this information into our selection decisions. As designed, this method is therefore an enhancement of the usual selection index calculations that incorporates marker information with an animal's own phenotypic information with that of its relatives (Mrode and Thompson, 2005). The intent of such methods is to increase the accuracy of the traditional selection index, while simultaneously shortening the generation interval (Phavaphutanon *et al.*, 2009).

The second strategy is based, as the name MAS suggests, on the genetic information of a 'marker' linked to a locus with an impact on the trait. Here we take advantage of linkage disequilibrium (LD) between the marker and the QTL. Obviously this strategy, which relies on linkage, is less desirable than the first one, but the underlying utility of MAS should be easy to grasp. With the discovery of microsatellites, and now SNPs, the framework of MAS has been extended to the incorporation of genetic information from across the entire genome (Meuwissen *et al.*, 2001). Now, instead of concentrating on a few linked loci, we make use of LD across the genome between SNPs and trait loci to create a more informative selection decision aid.

This strategy, called genomic selection, has the potential to eliminate the need for phenotypes altogether in the computation of

selection indices. The general concept is relatively straightforward. We begin with a collection of dogs, relatives or not, although a sample of unrelated animals simplifies the computations (Goddard and Hayes, 2007; VanRaden, 2008; Goddard, 2009). Ideally, these phenotypes are well understood and can be recorded objectively and accurately. From this same sample of dogs, we record other non-genetic information that is necessary to fully characterize the phenotypes. This non-genetic (i.e. environmental) information is no less critical than the recording of phenotypes, for we need this to properly separate the genotypic from the environmental influences on phenotype. Finally, we collect an amount of tissue sufficient to extract DNA from this sample of dogs, so as to genotype them across the segregating SNPs, a number likely to be in the thousands. This we can call our 'reference population', because from this sample of dogs we will be able to relate environmentally corrected phenotypes directly to the genotypes of each SNP to estimate the contribution of each location to the phenotype of interest. The association between trait and marker can be direct or can be based upon linkage. In either case, we will know what genes contribute to phenotype and can estimate exactly what that contribution is (Meuwissen *et al.*, 2001).

With that accomplished, one can then collect DNA from a separate, new set of individuals and evaluate the expected phenotype from the estimates of SNP effects. From these expected phenotypes, computed and not observed, we can then proceed to a decision on which animals are desirable and which are not. That is, no phenotypes are necessary to aid in the making of a selection decision. Dogs can be selected on this predicted value, free of their own phenotypes or those of relatives. At least, that is the theory. How well this strategy performs, particularly over an extended period of time, is cause for concern and, furthermore, because the associations may be based upon LD, these effects will need to be monitored and re-estimated from time to time (Meuwissen *et al.*, 2001). However, livestock geneticists are all pointed in this direction, even if it is, as yet, unclear how accurately it may work (Schaeffer, 2006; Goddard, 2009; Hayes *et al.*, 2009; VanRaden *et al.*, 2009). What we can assess at this point rests on the basic assumptions of quantitative genetics and polygenic traits, that is, that the effects of most of these genes are small relative to the phenotypic variance (Lynch and Walsh, 1998). As such, it is going to take a relatively large number of animals to accurately establish the important loci in the 'reference population' (Goddard and Hayes, 2009). Depending upon the heritability of the traits of interest, achieving accuracies in genomic selection on a par with those of traditional phenotypic-based strategies can measure in the hundreds, if not thousands, of animals (Goddard and Hayes, 2009). Still, should genes of large effect be segregating, that number could be reduced (Goddard, 2009). Regardless of the exact number, the conclusions are sobering. Genomic selection methods are not likely to come easily, although the hope of eliminating the cost of phenotyping or the need to wait years before a late-onset disease is expressed is well placed. The reality, however, suggests that our focus on phenotypes must remain at the forefront of our thinking. Collecting, assessing and evaluating the root cause of phenotypic variation, whether genetic or environmental, will always remain the key to substantive breed improvement.

Conclusions

This brief discussion of quantitative genetics and breed improvement outlines both the present path to success and an assessment of how progress may look in the future. Selection on single phenotypes remains the key for most improvement strategies in dogs. The search for increased accuracy takes us to the collection of reliable phenotypic data, along with an evaluation of non-genetic influences on phenotype. However, more organizations are beginning to consider the use of the statistical tools available to increase the accuracy of selection decisions through the incorporation of phenotypes on relatives. But, with the continuing discovery of important loci, the desire to incorporate this genomic information into breeding decisions will grow. Implementing this desire will not be simple as we search for efficient strategies to take advantage of our increasing knowledge of the mechanisms of inheritance.

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20 Complex Traits in the Dog

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Introduction

The genetic analysis of complex traits is a rapidly changing field of investigation. It represents the intersection of molecular and computational technology driving the emergence of personalized medicine (Ginsburg and Willard, 2009). As the costs of relevant genomic technologies drop, the feasibility of applying them to individuals grows and the results will begin to have an impact on how we view animal health and biology. It seems certain that as a result animal medicine will change dramatically over the next decade, with a concomitant impact on human health.

Today, the dog is emerging as a model system (Cadieu and Ostrander, 2007; Shearin and Ostrander, 2010) for such studies and the approach to the animal is patterned on the humanity, privacy and respect for client privileges that are associated with human medicine. The importance of the dog model is based on the many health problems that are common to both dogs and humans; and the fact that man's best friend shares our environment and is exposed to many of the same environmental cues that may trigger complex disease processes, such as autoimmune disease and cancer.

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This chapter on the genetic analysis of complex traits is more a perspective than a review of the field. Most examples are taken from our research on the Portuguese Water Dog, the breed which we have studied in detail, primarily because of its population structure (Chase *et al.*, 1999) and because of the excellent collaborative attitude of owners and breeders (Davis, 2007). However, most of what we have learned from this breed can be generalized to other breeds, and many examples of this are included in the excellent review by Parker *et al.* (2010b).

At present, there are few absolutes in the analysis of the genetics of complex traits (quantitative genetics) and progress in the field is certainly raising more questions than it is yielding answers. This is characteristic of a field in transition, and what we hope to present here is a feeling for where we are now (mostly by example), and a view of what the future may bring. With this in mind, we have divided the chapter into two main sections. The first, Modelling Complex Traits, discusses the modelling of the genetic approach to complex traits using morphological traits, which comprise a well-defined set of phenotypes. The second, Health-related Traits, explores how this approach can be used to analyse complex diseases and disease risk.

Modelling Complex Traits

Complex phenotypes are regulated by multiple genes (i.e. they are polygenic) interacting with the environment (Mackay *et al.*, 2009). The expression of the phenotype varies with the genotype of individual dogs and the environmental influences to which each animal is exposed. As a result, phenotypes found in a population (or breed) tend to vary in a continuous manner over a large range. Examples for size, laxity of the hip joint and behaviour are presented in Fig. 20.1. For each phenotype, populations differ, although individual values may overlap between populations: females are, on average smaller than males, although some females are almost as large as the largest males (Fig. 20.1a; Chase *et al.*, 2005); left hip joints are more lax on average than those on the right, although some are as tight as the best right joints (Fig. 20.1b; Chase *et al.*, 2004); hybrid foxes derived from crossing tame with aggressive parents are on average midway in behaviour between the parental behaviours, although there are individuals as tame or as aggressive as either of the parents (Fig. 20.1c; Kukekova *et al.*, 2008).

Most of the traits in the dog that are immediately evident and most dramatic are complex. Differences in size and shape (conformation), coat and appearance, behaviour (e.g. temperament, trainability and obedience) are all examples of complex traits of individuals. Other complex traits with which breeders are familiar, but which are less obvious to owners, are differences in the size of litters, and the survival of puppies (newborn robustness), their boldness (soft versus hard) and their inquisitiveness (curiosity). Complex traits such as physical performance, longevity or frequency of certain diseases (risk) manifest themselves at the population level with different mean values associated with different breeds (Jones *et al.*, 2008; Chase *et al.*, 2009). Finally, most of the observations that veterinarians use to form a diagnosis (radiographic, physiological and biochemical data) present as complex traits – e.g. hip laxity, heart rate and/or murmur, levels of serum biomarkers (such as albumin or creatinine).

Complex traits and genetics

Complex, or quantitative, traits involve interactions between genes themselves and between genes and the environment (Mackay *et al.*, 2009). During the past two decades, the parallel progress in computational tools (software and hardware) and in DNA analysis has led to ever-increasing sophisticated analysis of the genome and, in particular, to comparisons between the genomes of individuals. As a result, it has become possible to minutely measure similarity or dissimilarity between the genomes of individuals and to compare genetic similarity with phenotypic similarity (Manolio *et al.*, 2008; Hayes and Goddard, 2010). (It is this ability that holds the promise for personalized medicine.) By quantitatively comparing the similarity between genomes with the similarity of the accompanying phenotype or trait we can estimate the genetic component regulating the trait – i.e. the heritability (Hayes and

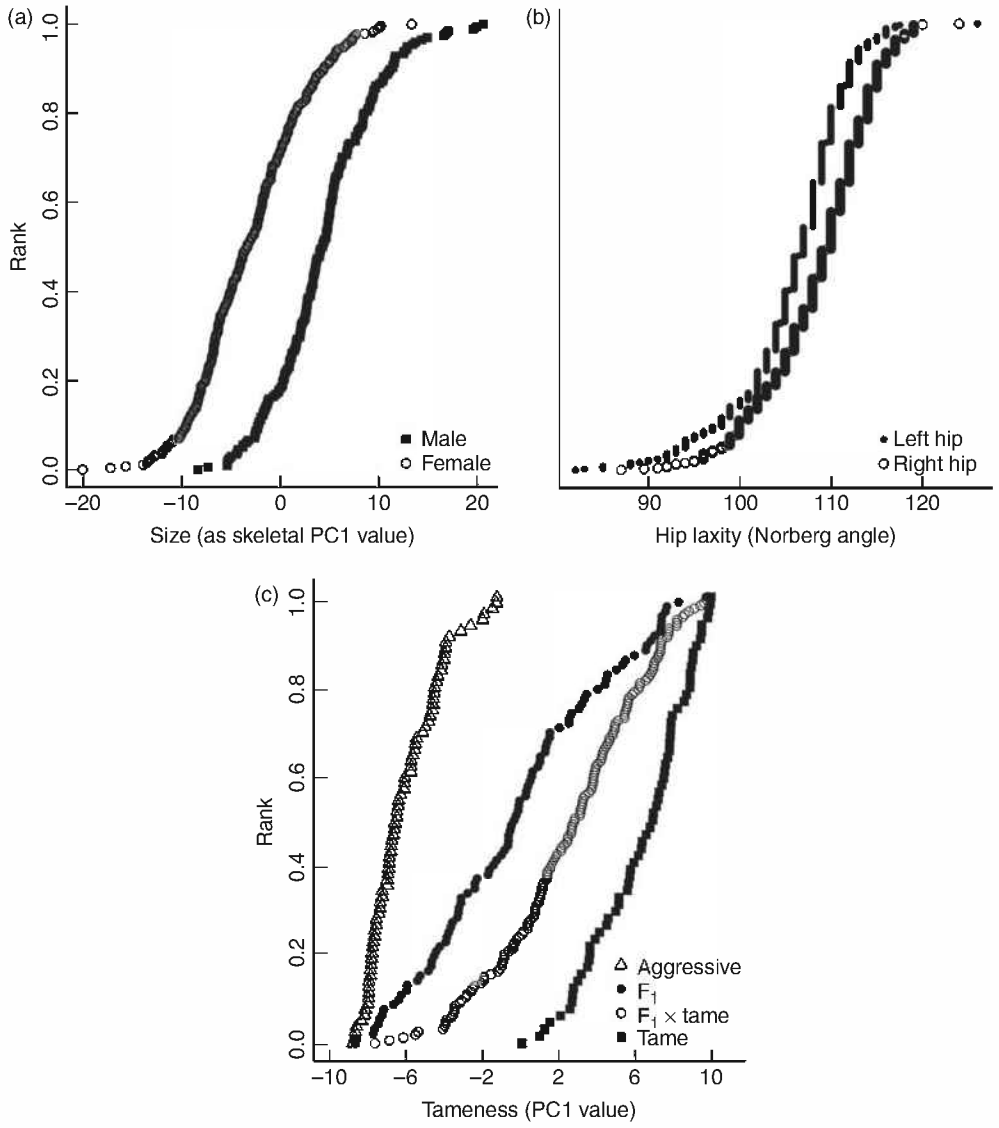

Fig. 20.1. Examples of complex phenotypes. (a) Principal component 1 (PC1) of skeletal variation as a measure of sexual size dimorphism in Portuguese Water (PW) Dogs (Chase *et al.,* 2005). Differences in the skeletal size (PC1) of male and female Portuguese Water Dogs were reconstructed from bone metrics using principal components analysis (PCA). Each point on the graph is the PC1 value for the skeleton of an individual dog. Values are ranked in order of increasing values of PC1 and normalized to a population (male + female) mean value of zero. Radiographs of individual dogs (skull, limbs and pelvis) were used to obtain ~100 metrics defining the skeleton. These were assembled into a matrix comprising all of the values (traits) for all of the dogs in the population. This matrix was used to carry out a PC analysis of skeletal variation in the population. The first principal component, in which the variation in all of the metrics was positively correlated, accounted for about 50% of the skeletal variation. The second component, PC2 (not shown), comprised length metrics inversely correlated to width (an aspect of shape) and accounted for about 15% of the skeletal variation. Other PCs, accounting for lesser amounts of skeletal variation, represent different independent aspects of shape. (b) Norberg angle values (*x*-axis) for right and left coxo-femoral joints of 286 PW dogs ranked according to increasing

Goddard, 2010). When two genomes in a population are similar or dissimilar and the corresponding phenotypes are respectively similar or dissimilar, the trait has a large genetic component. When genomes are similar but phenotypes are not (or vice versa) the trait has a small or vanishing genetic component. Average genetic similarities can be estimated from accurate and, if possible deep, pedigrees (Chase *et al.*, 1999) or, more precisely, by comparing genome sequences (Woods *et al.*, 2006). Because of the ever-declining cost of the measuring aspects of DNA sequence, estimating genetic similarity has become economic. In contrast, defining phenotypes and measuring them quantitatively remains a major challenge. It should be remembered that, in order to measure heritability in a population, both the phenotype and the genotypic information (alleles) underlying that phenotype must be varying (segregating). A region of the genome may regulate the expression of a phenotype, but if the individuals in the population have the same genotype the contribution of that region will not be measurable. Regions of the genome that contribute to the variation of the phenotype (loci or quantitative trait loci – QTLs) are identified by association with the phenotype; this is essentially the same similarity analysis (Lu *et al.*, 2003), but now confined to DNA sequence markers defining the region. A genome-wide association study, or GWAS (So *et al.*, 2010), compares the similarity of all regions of the genome with phenotypic variation in order to identify loci associated with the phenotype. Heritable phenotypes may be controlled by many loci that each have small effects (and therefore are difficult to identify) or by loci among which are some that control a large part of the trait variation (e.g. 10–25%) and which are easier to identify (So *et al.*, 2010). Perhaps the prototypic large QTL is a necessary component of a genetic network that exerts an all-or-none (100%) effect on expression of the phenotype associated with the function or failure of a gene in that region. It is important to note that such simple recessives are, more often than not, part of a larger network or complex of genes controlling the phenotype (e.g. coat colour is controlled by many genes, but in breeds with brown and black coats the *TYRP1* brown coat alleles are recessive to black (Schmutz and Berryere, 2007)).

Morphology as a model system for the analysis of complex traits

Size, shape and appearance of pure-bred dogs varies from breed to breed and, depending on the stringency of the breed standard, may vary significantly within a breed (Sutter *et al.*, 2008). This plethora of sizes, shapes and coats, coupled with the ability to measure variation quantitatively, has made morphology the prototypic phenotype for the analysis of complex traits. Morphological standards are often not well defined: for example, a simple standard, such as height at the withers can be achieved by variation

Fig. 20.1. Continued.

value of the Norberg Angle (Chase *et al.*, 2004). (c) PC1 as a measure of aggressive/friendly behaviour in fox populations reconstructed from behavioural characters using PCA (Kukekova *et al.*, 2008). Four populations were analysed: aggressive and tame fox populations, an F_1 population produced by crossing aggressive and tame foxes and a backcross population produced by crossing the F_1 with tame foxes. Foxes were videotaped during their interactions with investigators and their reactions deconstructed into ~100 different components, all of which could be scored in a binary fashion as present or absent. The components included physical appearance (e.g. position of ears), reactions to attempts of the investigator to touch the fox (e.g. attack versus seeking to be touched), position in cage (e.g. coming towards or avoiding the investigator), etc. The individual component values (presence or absence of each trait) for individual foxes in all four populations comprised the matrix for PC analysis. PC1 explained ~30% of the variation in behaviour and consisted of an inverse correlation between friendly (e.g. wag tail) versus aggressive (e.g. trying to bite) actions. As for Fig. 20.1a, the population was normalized to a mean value of zero that included all populations (where aggressive is the most negative and friendly the most positive). Each fox is represented by a single point and foxes are ranked according to increasing value of PC1.

in the humerus, the radius and ulna, or the metatarsal bones. The standard does not specify a particular length for each of these. Thus, the same standard can be achieved by varying the metrics of different bones. Similarly, the 'stop' (the angle between the cranium and the snout) is often not specified or the width or length of the pelvis. As a result, a great deal of skeletal variation can occur within a breed as well as between breeds. This may be expressed as differences in both size and shape. Although coat length, as well as colour, may be simply defined within a breed, great variation is encountered between breeds; within a breed, patterning of both colour and coat length, or furnishings (eyebrows, beards and whiskers, as well as feathering on the limbs), may vary considerably. Finally, the curl of the coat can vary both between and within breeds. All of these have been used as models for the genetic analysis of complex traits (Chase *et al.*, 2002; Fondon and Garner, 2007; Cadieu *et al.*, 2009; Boyko *et al.*, 2010).

Deconstruction/reconstruction of complex phenotypes

Because of the great variation in morphology and appearance within and between dog breeds, studies of these phenotypes have served as prototypes for the genetic analysis of complex canine traits. For simple genetic traits, a striking change in appearance can be singled out and its genetic basis identified by comparison between breeds that have the trait of interest and others that do not. In this way, the genetic basis for the distinctive appearance of the Rhodesian Ridgeback has been traced to a single difference in DNA sequence by comparing the genomes of this breed with breeds that lack the distinctive fold (Salmon Hillbertz *et al.*, 2007). In other cases, a number of independent phenotypic components can be identified (deconstruction by inspection) as in the case of coat appearance. In this example, a number of characteristic differences in the appearance of the coat distinguishes breeds: (i) coat length differs between breeds and distinguishes some subdivisions within breeds (e.g. Dachshunds), and genetic factors regulating coat length have been identified (Housley and Venta, 2006; Cadieu *et al.*, 2009); (ii) furnishings, or differences in the length of hair in certain regions of the body (beards, eyebrows, feathers on limbs), are regulated by the presence or absence of *Rspo2* (Cadieu *et al.*, 2009); (iii) straight or curled hair is regulated by a number of keratin genes (Cadieu *et al.*, 2009). Hence, deconstruction of coat morphology can be accomplished by identifying three traits which, when reconstructed into different groupings, account for the variants in coat phenotypes that characterize differences between most dog breeds. Moreover, changes in some of these traits are responsible for conformations that result in extreme deviations from the breed standard, e.g. improper coat in the Portuguese Water Dog (Parker *et al.*, 2010a).

Other phenotypes are yet more complex. Understanding the genetic basis for the phenotype, therefore, requires a procedure for simplifying the phenotype (deconstruction) and for reassembling the parts (reconstruction) into smaller components that together create an understandable gestalt. This is perhaps most evident with the size and shape of individual dogs. The large range of variation in size and shape between breeds, and often within a single breed, together with the fact that the adult skeleton of the dog remains relatively unchanged after 2 years of age, has made the size and shape of the dog an excellent model system for the analysis of more complex phenotypes that require deconstruction (Sutter *et al.*, 2008). Variation in these phenotypes is determined by differences in skeletal architecture which, in turn, is determined by variation in the metrics of hundreds of individual bones.

Analysis of the canid skeleton immediately reveals levels of complexity. The length and width of bones involve different metrics corresponding to different growth zones (Standring, 2009). Subsections of the skeleton such as the forelimbs and hind limbs, or the pelvis, present additional complexities, in that the dimensions of different bones determine simple functional aspects and hence may limit the behaviour of the animal (Hildebrand and Goslow, 1998). These subsections interact through joint surfaces, ligaments and muscles/tendons that connect them, and that involve variation in attachment points which change lever arms through which force is constrained to act (Biewener, 1990). This can

result in functional variation involving speed and power and, often, involves trade-offs in which limb robustness is inversely correlated with energy-efficient speed (Kemp *et al.*, 2005). Thus, the long, slender, fragile limbs of the Greyhound can produce speeds that the Pitbull cannot achieve, although the short, thick limbs of the latter can produce forces that would break the limbs of a pursuit hound.

Phenotypic variation within a population (breed)

This higher level of complexity, involving variation in the metrics of many different bones, has been quantified by reconstructing independent components of variation from the measurements of individual bones using principal component analysis (PCA; Jolliffe, 2002). The PCA technique has been applied to variation in dogs as well as in foxes (*Vulpes vulpes*), the ancestral lineage that separated from the dog/wolf sister lineages 10 million years ago. Many of the components of variation appear to be the same or very similar in the fox and the dog (Kharlamova *et al.*, 2007).

PCA redefines clusters of correlated traits into independent linear combinations. Each morphological PC is a different, independent, component of the variation in size or shape, and accounts for some fraction of the overall skeletal variation. The method of extracting PCs (Jolliffe, 2002) produces a ranking in decreasing order according to the amount of variation that they represent (i.e. the amount of variation represented by PC1>PC2>PC3, etc., the sum of which accounts for the total variation). Individual PCs involve different constellations of traits, and each trait contributes a fraction of the variation. The pattern of these contributions (loadings) provides clues to the biological meaning of the PC (Chase *et al.*, 2002). Hence, PC1 represents size, in that the inputs from all of the skeletal measurements (traits) are positively correlated; that is, all of the bones of a small dog (e.g. Chihuahua) are smaller than the corresponding bones in a large dog (such as an Irish Wolfhound). Other PCs represent components of shape, the most prominent of which (PC2) is the inverse correlation between length and width. Figure 20.1a presents values for male and female skeletal PC1 as a quantitative measure of size, estimated from metrics taken from radiographs of the skull, pelvis and limbs of Portuguese Water Dogs (Carrier *et al.*, 2005; Chase *et al.*, 2005).

The PCA technique presents a powerful tool for genetic analysis, in that each animal has a value for each component of variation (PC1, PC2, etc.). Correspondingly, each PC is a quantitative phenotype subject to genetic analysis. This can be seen in an experiment where this form of analysis was applied to behaviour (Kukekova *et al.*, 2011). For Fig. 20.1c, PC analysis was used to reconstruct a behavioural axis of variation between aggressive and tame foxes. Parental tame and aggressive foxes had been selected in an experiment to reproduce the domestication of the dog (Trut, 1999, 2001). The heretofore subjective evaluation of the domestication gestalt was deconstructed into a large number of traits that could be scored from videotaped encounters between investigator and animal (for detail, see Kukekova *et al.*, 2008); these were reconstructed using PC analysis into independent components of variation, the first component of which was characterized by a number of aggressive traits that were inversely correlated with a number of friendly traits. Thus, PC1 became a quantitative estimation of domesticated behaviour. Heritability of this complex phenotype (gestalt) was demonstrated by crossing aggressive with tame foxes to produce an F_1 population whose mean PC1 value lay midway between those of the parental means. Backcrossing this F_1 to tame parents produced a population with a PC1 value midway between that of the F_1 and tame populations. Subsequent experiments demonstrated that PC2 distinguished another, independent, aspect of behaviour in these populations, which quantified behaviour along an axis of variation between passive and active behaviours (Kukekova *et al.*, 2011).

Phenotypic and genotypic variation between populations

Many dog populations are genetic isolates (closed breeding systems). This is either because

of geographical isolation associated with human interactions, as in the case of village dogs (Boyko *et al.*, 2010), or due to constraint by the controlled breeding practices (Sutter *et al.*, 2008) that have led to the more than 300 recognized pure-bred dog populations (breeds) available today. These closed breeding systems often arise from few founders and pass through bottlenecks. Moreover, particular loci may be enriched as a result of popular sire effects. As a consequence, gene pools are limited, and some alleles are lost whereas others are enriched. In the case of complex phenotypes, the closed breeding structure results in different breed gene pools that enhance or decrease phenotypic expression.

Selective breeding in order to create and maintain the breed standards that define pure-bred dogs will drive many phenotypes to fixation. That is, these phenotypes, often complex, become invariant (Sutter *et al.*, 2008). Once again, morphology presents extraordinary examples of extreme phenotypic differences between breeds, the most striking example of which is size. Figure 20.2 shows the variation in size (*x* axis) between a large number of dog breeds; size varies over a range of more than 30-fold, with extreme variation fixed within certain breed types such as the Poodle (standard, miniature, teacup), Schnauzer (giant, standard, miniature) or racing hounds (Greyhound, Italian Greyhound, Whippet). These same breeds exemplify the great differences in shape between breeds (PC2, etc.), which in some chondrodysplastic breeds (Dachshund, Corgi) also become extreme (Parker *et al.*, 2009). Differences between genetic isolates that are bred and raised under a variety of conditions in different environments (or between different breeds raised in common environments) drive home the fact that genetic differences between the breeds underlie the differences in these complex phenotypes.

Because breeds are constrained genetic systems with limited gene pools, their genetic profile can be determined and used as a database in different experiments. Two such

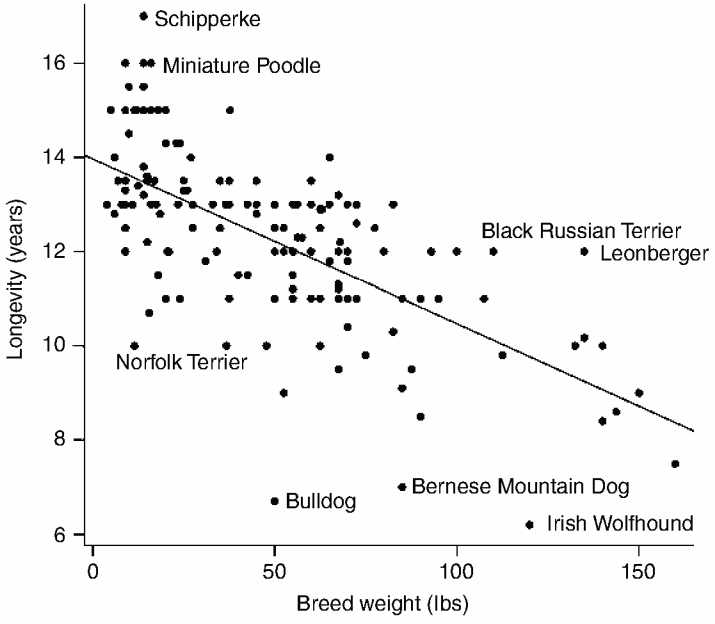

Fig. 20.2. Differences between dog breeds in size and longevity (Jones *et al.*, 2008). Average breed size in pounds is graphed on the *x* axis. Longevity (average age at death) is graphed on the ordinate (*y* axis). Longevity and size are inversely correlated: Breed size varies over a 30-fold range and mean longevity varies by more than twofold. A few of the breeds that differ significantly from the general correlation between weight and size are indicated by name.

databases have been created, one privately by the Masterfood subsidiary of Mars Inc. (Jones *et al.*, 2008) and a public database, 'Canmap', which is available online (Boyko *et al.*, 2010; vonHoldt *et al.*, 2010). In these databases, single nucleotide polymorphism (SNP) markers from several individuals from each breed have been analysed to determine the allele frequency at the particular marker positions (Jones *et al.*, 2008; Boyko *et al.*, 2010). From the databases, it is possible to immediately determine whether a particular marker – and hence region – of the genome (haplotype) has been fixed (driven to homozygosity) in the breed concerned. The ability to use databases that characterize the phenotypic and genotypic variation between breeds has made available extremely powerful methods for determining the genotypic basis of complex phenotypes that differ between breeds.

What can size tell us about the genetic complexity of complex traits?

Whereas some complex traits are expressed as 'all or none' phenotypes (e.g. clinical versus subclinical expression of a disease), size and shape have the advantage that all values can be readily measured and present a continuum of expression. Size, in particular, has been measured within a breed and between breeds. Some breed standards constrain size rather stringently (Wilcox and Walkowicz, 1995), but others are much more relaxed, as in the case of Portuguese Water Dogs, in which the genetics of size was carefully estimated as the first PC of bone metrics obtained from skeletal radiographs (as in Fig. 20.1a). Initially, one major genetic size determinant was identified on canine chromosome 15 (CFA15), a locus associated with *IGF1*, which is known to regulate body weight in mice (Lupu *et al.*, 2001) and humans (Okada *et al.*, 2010). As more dogs were added to the data set, and more radiographic data became available, loci were identified that were associated with other chromosomes (Table 20.1). More recently, techniques have been developed by which phenotypic variation can be associated with genetic loci by comparing phenotypic and genotypic differences between different breeds. This approach has also identified multiple loci associated with size located on different chromosomes (Table 20.1). Several aspects of those data are noteworthy:

1. Despite different sources of phenotypic and genotypic data, the two multi-breed approaches identified the same four loci on chromosomes 7, 9, 10 and 15. In one study (Jones *et al.*, 2008), the X chromosome was not interrogated (so no associations with that chromosome could be identified) and genome coverage on chromosomes 3 and 4 was far less adequate than in the analysis by Boyko *et al.* (2010).
2. The within-breed study identified, and therefore validated, several loci found in the multi-breed analyses (on chromosomes 3, 4, 15 and X).
3. Significant loci identified in both multi-breed analyses on chromosomes 7, 9 and 10 did *not* segregate in the Portuguese Water Dog population (these loci were fixed) and therefore could not be associated with the size phenotype.

This third aspect underscores the fact that identifying associations *within* a breed relies on the segregation of both the phenotype and various haplotypes within the breed. Haplotypes that are fixed or near fixation (identical over the great majority of dogs) will not contribute to the phenotypic variation and hence not be identified. In contrast, phenotypic variation between breeds is most readily associated with genotypic

Table 20.1. Comparison of three genome-wide association studies (GWAS) of size.

Intra-breed segregation in the PW dog[a]	CFA 3, 4, 8, 12, 15, 18, 30, 37, X
Interbreed comparison analysis[b]	CFA 7, 9, 10, 15, 34
Interbreed comparison analysis[c]	CFA 3, 4, 7, 9, 10, 15, X

[a]Size associations measured as GWAS between Portuguese Water (PW) Dog markers and Principal Component 1(PC1) of skeletal metrics. Summary of published and unpublished data of Chase and Lark. Markers are associated with different canine chromosomes (CFA3, 4, etc.).
[b]Data taken from Jones *et al.* (2008). Locus identification using multiple breeds.
[c]Data taken from Boyko *et al.* (2010). Locus identification using multiple breeds.

differences between breeds that result from differences between *fixed* haplotypes. Breeds in which a haplotype is *segregating* do not contribute to multi-breed mapping of that haplotype (Jones *et al.*, 2008). This may, in part, account for the inability of multi-breed analyses to identify several size loci (on chromosomes 8, 12, 30 and 37) that were associated with size in the Portuguese Water Dog.

In summary, the most important take home message from the data in Table 20.1 is that, unlike simple Mendelian traits, complex phenotypes (like size and others discussed below), result from information in multiple genes that can act independently – or may interact – to produce extremely complex regulatory effects.

Morphological gestalts: deconstructing/reconstructing components of shape

A startling aspect of PCA was the separation of independent components of skeletal variation that made functional sense. In their original paper, Chase *et al.* (2002) described a number of components changing the shape of the Portuguese Water Dog along an axis of functional morphology between the generation of energy-efficient speed and the generation of force; in the extreme, these body shapes are exemplified by pursuit hounds – e.g. the Greyhound – on the one hand and the Pitbull or Bulldog on the other. These body shapes included separate components of variation, such as the inverse correlation of bone length versus width (see Table 20.2), or inverse correlation in the size of the cranium versus the post-cranial body. It was possible to demonstrate that a number of independent genetic loci each controlled variation in multiple bones that together constituted aspects of shape. Some of these loci controlled large amounts of variation (e.g. PC2 in Table 20.2), and others very small amounts (e.g. PC21 in Table 20.2). Separate heritable components of variation included: variation within the skull only (snout versus cranium); within the pelvis only (ischium/ilium) (Carrier *et al.*, 2005); between limb/pelvis and skull; between pelvis and limbs. A detailed PC analysis of limb metrics demonstrated independent heritable variation in components involving: lengths versus widths of

Table 20.2. Trait loadings for dog and fox principal components (PCs) 2 and 21 (Kharlamova *et al.*, 2007). The traits are listed to the left as radius (r), metatarsal (m), femur (f), tibia (t) and humerus (h), and length (L), outer diameter (O.D.) and inner diameter I.D. (e.g. radius length: r(L); radius outer diameter r(O.D.); radius inner diameter r(I.D). The percentage variation for each PC heads a column listing the loadings for each trait.

	Dog		Fox	
	PC2	PC21	PC2	PC21
	12.4%	0.2%	14.8%	0.3%
r(L)	–0.30	–0.68	–0.16	–0.59
m(L)	–0.27	–0.16		
f(L)	–0.25		–0.15	
t(L)	–0.26	0.6	–0.15	[0.29]
h(L)	–0.25	[0.2]	–0.12	[0.52]
f(O.D.)	0.18		0.24	
f(I.D.)	0.32		0.40	
h(O.D.)	0.25		0.27	
h(I.D.)	0.38		0.43	
r(O.D.)	0.22		0.25	
r(I.D.)	0.29		0.37	
t(O.D.)	0.14		0.13	
t(I.D.)	0.28		0.37	

multiple limb bones; lengths of forelimbs versus hind limbs (metacarpals versus metatarsals; radius versus tibia) and between lever arms of joints in the fore versus hind limbs.

The fact that single loci can change multiple skeletal structures explains the ability of breeders to rapidly select functional changes in shape, as single loci can regulate changes in constellations of bones. Moreover, the existence of several independent heritable components of variation in shape and function introduces flexibility in the selection process.

Heritable variation can defy stringent selection

Because PCs are independent constellations of traits, they can be very useful in comparative studies to evaluate the degree to which different populations (e.g. breeds) differ with respect to the phenotype being analysed. This was brought out in a morphological analysis of skeletal radiographs of foxes and dogs (Kharlamova *et al.*, 2007), which are lineages separated by 10 million years of evolution including domestication of the wolf and, in the case of purebred dogs, breed selection (Wayne, 1993). Data analysis of differences in morphology (shape) has revealed that often very small components of phenotypic *variation* were maintained despite selective bottlenecks that might be expected to have resulted in loss of variation (*fixation*).

Surprisingly a large number of morphological PCs remain the same for both species. These include major as well as minor PCs, such as the heritable components of limb variation discussed in the previous paragraphs. For example, Table 20.2 presents two independent, heritable, components of shape variation affecting the limb bones of the Portuguese Water Dog and of the fox. PC2 represents variation in length and width (inversely correlated), such that the limb bones of individuals vary along a shape axis between long thin and short thick limbs. This variation accounts for 12–15% of limb variation, and several loci have been identified in the dog that are associated with this change in shape (most notably a locus on CFA12). In contrast, PC21 accounts for a tiny amount of skeletal limb variation (0.2–0.3%) that changes the lengths of the forelimb versus the hind limb bones in both species. In the Portuguese Water Dog, this variation is associated with genetic loci on three chromosomes (CFA24, 27 and 38). These data raise the possibility that very small components of genetically specified *variation* in a complex trait (in this case PC21) may persist over long periods of evolution, and direct attention to the following possible mechanisms: (i) disruptive selection (i.e. selection for and against) operating on very slight increases or decreases in the length of the forelimb relative to the hind limb maintains this variability; (ii) the genes regulating PC21 are closely linked to other major genes that are under opposing selective forces; (iii) PC21 variation in limb morphology is a minor phenotype resulting from the expression of genes (e.g. on CFA24, 27 and 38) that have several other, larger, effects (pleiotropy) that are subject to disruptive selection; and (iv) the genes involved are intrinsically variable (genetic mechanisms giving rise to intrinsic genetic variation in morphology have been described; Fondon and Garner, 2004).

The first three explanations all involve maintenance of variation by a balance between opposing selective forces (disruptive selection). The first possibility suggests the existence of unknown subtle balances between functional morphological effects of limb lengths. The second and third postulate the existence of major selective forces that, through genetic connectivity, exert unintended effects beyond those affecting the target of selection. This latter concept may have important consequences for health-related traits (see next section).

Health-related Traits

Diseases and health-related complex traits

Health-related phenotypes often vary in severity such that the diseased state can be measured quantitatively (e.g. temperature, blood pressure, number of osteophytes on an osteoarthritic joint). An early study of this type of

trait analysed the degree of coxo-femoral subluxation measured from radiographs of the pelvis using the Norberg angle as a quantitative estimate (Chase *et al.*, 2004). This provided a continuous metric ranging from slightly acute to obtuse angles. Heritable variation in the Norberg angle occurs in the Portuguese Water Dog population. A range of values has been observed (Fig. 20.1b; Chase *et al.*, 2004) and, on average, the right hip exhibits less subluxation than the left. GWAS was carried out separately on values obtained from the left and from the right joints. Separate loci were identified on CFA1 that explained a part of the variation in subluxation. An interesting finding was that one locus was significant for the right and the other for the left coxo-femoral joint, suggesting a genetic discrimination between the right and left sides of the dog. Two aspects of the study are characteristic of complex health-related traits: (i) different haplotypes at a locus were associated with different amounts of subluxation variation in the Portuguese Water Dog population; and (ii) the two loci identified each explained only a part (e.g. 10–15%) of the total variation. Subsequent experiments in other laboratories confirmed the asymmetric nature of subluxation (Todhunter *et al.*, 2005); validated the original loci by demonstrating their association with subluxation in other breeds (Phavaphutanon *et al.*, 2009); and identified many additional loci on other chromosomes that together account for a much higher proportion of the observed variation in subluxation (Phavaphutanon *et al.*, 2009; Zhu *et al.*, 2009). In summary, the data on coxo-femoral subluxation demonstrate that the degree to which this phenotype is expressed is regulated by multiple loci – i.e. this is a complex polygenic trait.

Often, diseases are reported as present or absent. However, the predisposition towards, or away from, the disease (risk) may be segregating in a breed. In this case, the phenotype is the frequency of the disease and the identification of loci occurs by associating loci with different frequencies that characterize genotypic subpopulations. An example of this is the frequency of Addison's disease in the Portuguese Water Dog population (~1.5%; Chase *et al.*, 2006). In this population, the disease onset is late and there is a higher incidence in female than in male dogs, consistent with an autoimmune disease. Several loci associated with Addison's disease have been identified in humans (Park *et al.*, 2002). A comparison of the genotypes for these loci between Addisonian and non-Addisonian Portuguese Water Dogs (older dogs that did not develop the disease) demonstrated that a locus on CFA12 in the region of the immune/histocompatability genes was significantly associated with an increase in the frequency of the disease. Another locus was found on CFA27 that appeared to decrease the frequency of the disease. Thus, association analysis identified regions that appear to predispose towards as well as away from development of the disease. That is, they affect disease 'risk'.

State of health at time of death

More often than not, disease phenotypes present an incomplete picture. The extent of the disease process is difficult to determine and many of the internal factors affecting the individual cannot be determined without invasive procedures that in dogs, as in humans, are proscribed for ethical and humanitarian reasons. Autopsy has long been recognized as the gold standard in ascertaining cause of death. A careful review of outcomes from human autopsies led to the conclusion that a major diagnostic error will be revealed in about 25% (Shojania *et al.*, 2003; Roulson *et al.*, 2005). Moreover, half of human autopsies performed produce findings that were not suspected before death (Roulson *et al.*, 2005). So genetics based on disease reporting contains a large background of uncertainty – 'noise' that can reduce the disease signal. Unfortunately, the frequency of human autopsies has declined in recent years, precisely at a time when advances in human genetics have created a demand for accurate reporting of terminal and subclinical disease.

In long-range studies of animal health, the autopsy has proved invaluable for obtaining an all-encompassing picture of organ metrics, tumour characterization and other tissue histopathology which, in turn, leads to a more complete picture of the impact of various life histories on senescence. For example, studies

of this type have: analysed primary and secondary heart tumours in dogs and cats (Aupperle *et al.*, 2007); related periodontal disease to pathological changes in the organs of dogs (Pavlica *et al.*, 2008); and studied the effects of ageing on the feline kidney (Lawler *et al.*, 2006). The ability to undertake extensive post-mortem analysis on animal populations promises to open new avenues for investigating the genetics of disease and senescence in the dog.

Autopsy, in conjunction with clinical history, is customarily limited to confirming a terminal diagnosis or resolving conflicting aspects of a diagnosis. However, by greatly extending the procedure it can be used to present a much broader picture of an animal's 'state of health' at time of death. In a comparison of six breeds, such necropsies revealed multiple disease states within individual dogs, as well as significant differences in the incidence of particular diseases (Table 20.3). The number of dogs evaluated in each breed ranged from 60 to more than 130. In all breeds, each cadaver was carefully analysed by autopsy to determine state of health and cause of death. The diagnoses involved frequent (>10%) degenerative disease syndromes not attributable to an increased incidence of a single, simple, genetic effect. These more frequent terminal diagnoses differed markedly between breeds.

An extensive database is being assembled on results from necropsies of the Portuguese Water Dog (Chase *et al.*, 2011). In a remarkable collaboration with owners and breeders (Davis, 2007), cadavers of Portuguese Water Dogs are packed on ice immediately following death and air shipped to the University of Utah where they undergo a standardized autopsy procedure: metrics (weights and dimensions) of approximately 50 tissues and organs are measured in the gross autopsy procedure and a large number of standardized tissue samples are analysed for histopathology. The genotype of each dog is also determined and before death one or more samples of fasting serum are obtained. At present, results from more than 250 dogs are in the database and a preliminary report on the 'state of health' of the first 150 individuals has been published (Chase *et al.*, 2011). It is estimated that, in order to associate genetic loci with specific pathologies and/or tissue/organ metrics, results from ~500 autopsies will be required. However, the current database has been sufficient to establish heritabilities for a number of tissue and organ metrics, as well as for many histological changes. Thus, the feasibility of using autopsy to identify loci responsible for the disease process (predisposing towards or away from the pathological observations observed) has been established.

Table 20.3. Major causes of natural death in dogs from six different breeds.[a] Only those causes of death that were reasonably frequent and occurred in more than one breed are shown.

	Miniature Schnauzer	Siberian Husky[b]	English Pointer[b]	Labrador Retriever	English Setter	Portuguese Water Dog	All breeds
Total necropsies	76	62	82	80	62	133	362
Spinal disc disease	12	4	1	7	0	0	24
Liver disease	12	3	6	2	3	1	27
Heart disease	11	2	16	3	5	7	44
Renal disease	5	0	2	0	0	12	19
Osteoarthritis	0	14	19	39	9	1	82
Pancreatitis	1	1	0	0	0	13	15
Total sarcomas	14	6	38	8	5	49	120
Fibrosarcoma	1	0	7	2	0	1	11
Lymphosarcoma	5	3	17	4	2	12	43
Haemangiosarcoma		0	3	2	2	32	39
Total carcinomas	6	6	19	8	11	12	62

[a]Data on the five breeds other than the Portuguese Water Dog were furnished by Nestlé Purina. Data on the Portuguese Water Dogs were obtained from the owners (see text).
[b]Multiple causes of death were attributed to several individuals in these breeds.

Almost all of the Portuguese Water Dog cadavers examined had histological changes in multiple tissues, ranging from two to 12 per dog. Associations between subclinical pathologies included those of inflammatory bowel disease with pancreatitis and osteoporosis, and tartar formation and peritonitis with atherosclerosis and amyloidosis. In addition, two specific clusters of histological changes could be correlated with ageing: hyperplasia, adenomas and haemosiderosis constituted one group; inflammation, plasmocytic and lymphocytic infiltration, fibrosis and atrophy another. Heritability was established for variation in weight of the heart, tongue, kidney, brain, oesophagus, some lobes of the lung and several skeletal muscles, as well as the lengths of the intestines and the Achilles tendon. Pathologies observed in the liver, spleen, pancreas, gastrointestinal tract, thyroid and salivary glands, as well as haemangiosarcomas and lymphosarcomas also have a heritable component. A preliminary GWAS, though underpowered, revealed several regions of interest on chromosomes 9, 13, 16, 20, 24 and 38.

A number of interesting correlations were established (Chase *et al.*, 2011): variation in stomach weight with incidence of haemangiosarcoma as well as splenic congestion; kidney weight with adrenal atrophy; spleen weight with kidney amyloidosis; and length of the small intestine with age of death. Such correlations establish the possibility of reconstructing disease gestalts from detailed pathological or organ metric data. Such a reconstruction was highly successful in defining behavioural gestalts from presence or absence of multiple small behavioural reactions (Fig. 20.1c; Kukekova *et al.*, 2011). In summary, autopsy presents a method of deconstructing a state of health into a number of contributory factors from which a much more precise definition of a health or disease state (syndrome) can be reconstructed using PCA.

Breeds are intermediates between generalized and personalized medicine

Because dog breeds are genetic isolates, i.e. closed genetic systems, each breed is a different snapshot of the dog genome in which some genes, or loci, are fixed, whereas others are still varying (segregating) (Lindblad-Toh *et al.*, 2005; vonHoldt *et al.*, 2010). The resulting variation in health phenotypes between breeds has already given rise to canine health care, which is breed focused in that it recognizes breed-specific health phenotypes (Chase *et al.*, 2009). The rapid development of human personalized medicine makes it likely that the next major step in canine health care will focus on developing the genetic basis for these phenotypic differences in order to treat dogs using information based on their genotypes rather than on the particular breed to which they might belong. Knowledge of this type can be applied to any dog, regardless of whether it is pure bred or not. The value of pure-bred dogs at this juncture is that they provide an accessible approach to determine the genetic factors that underlie disease risk, and that these factors could prove invaluable in diagnosis.

Later life has not been a selective priority in the establishment of dog breeds. As a result, many health problems, often the most common, are associated with longevity and appear later in a dog's life. This selective effect of breeding is best illustrated by size. The gene *IGF1* is known to affect size in humans and rodents (Brockman *et al.*, 2000; Okada *et al.*, 2010). It was first associated with size variation in the Portuguese Water Dog, in which the breed standard for size is not stringent and haplotypes for small and large size are segregating (Chase *et al.*, 2002). Subsequently, the Portuguese Water Dog small haplotype was found to be fixed in the majority of smaller dog breeds (Sutter *et al.*, 2007), presumably as a result of an ancient selective sweep, resulting in a strong genetic signal associated with size variation between small and large dog breeds. However, *IGF1* has a much broader health-defining role than its effect on size. It has been found to play a role in the longevity of a number of organisms (Kenyon, 2001; Bartke, 2005), and is part of a key pathway influencing growth that includes the growth hormone gene, *GH1*. This pathway, a complex network of feedback loops, contains many other elements besides *IGF1* and *GH1*, including *IGF2*, various transcription factors, hormones, binding proteins, etc.

Increasingly, various investigations are focusing on the impact of *IGF1* and its affiliated network on human disease and organ-specific growth – e.g. *IGF1* is involved with growth and development of the brain (Bondy *et al.*, 2003; Aberg *et al.*, 2006) and has been shown to have specific effects on pancreatitis (Warzecha *et al.*, 2003; Dembinski *et al.*, 2006). A recent review by Rodriguez *et al.* (2007) summarized much of the evidence implicating the *IGF1* pathway in human disease, and concluded that: 'The constellation of genes in this key pathway contains potential candidates in a number of complex diseases, including growth disorders, metabolic syndromes, diabetes, cardiovascular disease, central nervous system diseases, and in longevity, aging and cancer'.

A striking relationship between size and longevity occurs in dog breeds (Fig. 20.2), such that smaller breeds live longer, on average, than larger breeds (Galis *et al.*, 2007; Jones *et al.*, 2008). In a multiple breed genetic analysis of data such as those shown in Fig. 20.2, Jones *et al.* (2008) found several loci associated with longevity, the most significant of which was a region containing *IGF1* on CFA15. So selecting breeds with different sizes altered the longevity of each breed as well. The data in Fig. 20.2 also underscore the role played by the variation between breeds in their genome pools (the different 'snapshots of the dog genome'). Thus, a number of breeds deviate significantly from the regression, most notably several exceptionally long-lived small breeds (e.g. Schipperke) and some larger breeds (e.g. Black Russian Terrier).

Using breed phenotypes and genotypes to explore the genetic basis for complex diseases

Genes that regulate basic physiological and morphological phenotypes vary between breeds (Parker *et al.*, 2010b), and these may play a major role in the predisposition towards or away from polygenic disease. This belief is rooted in the striking differences between dog breeds in the incidence of complex diseases (Fleischer *et al.*, 2008). The ability to use multiple breeds to associate breed genotypes with breed complex phenotypes has allowed the exploration of pre-existing health-related databases on the assumption that, within a breed, fixed genotypes and phenotypes will not change (Chase *et al.*, 2009). Consequently, phenotypic data already collected on different breeds can be used in conjunction with breed genotypes determined at a later date. Another assumption is that differences between breeds are essentially due to differences in genotype and that effects of environment will be similar enough to be ignored as a first approximation, although this last may not be true for certain working or sporting breeds such as Greyhounds or other herding dogs; or for dogs maintained under quite different geographical or cultural conditions.

Figure 20.3a presents a sample of the type of data that is available. The Veterinary Medicine Database (VMDB, http://www.vmdb.org/vmdb.html) consists of ~1.5 million canine disease reports from 22 veterinary medical schools. This database represents one of the best compilations of disease diagnoses available, despite a possible selective bias between breeds in the frequency of visits to schools of veterinary medicine. VMDB records of visits and diagnoses for dogs admitted to veterinary colleges are categorized according to the breed, gender and age of the animals admitted. Figure 20.3a contrasts expected risk for four different diseases in different breeds. Breeds are ranked according to increasing frequency of disease. Each point represents a breed. It can be seen that, for any disease, risk varies from breed to breed over a ~20-fold range of frequencies and that different diseases occur at quite different levels of risk (more than fivefold). The significant variation in disease incidence between breeds is almost certainly due to underlying differences between the collective genotypes that are responsible for the differences in their anatomy and physiology. By comparing fixed regions of different breed genomes, we can begin to estimate their genetic impact on disease incidence.

This is illustrated by the impact of *IGF1* on three diseases. We have used VMDB data to examine the effect of different *IGF1* haplotypes on CHD (canine hip dysplasia), patella luxation and pancreatitis. Small and large breeds were defined on the basis of the

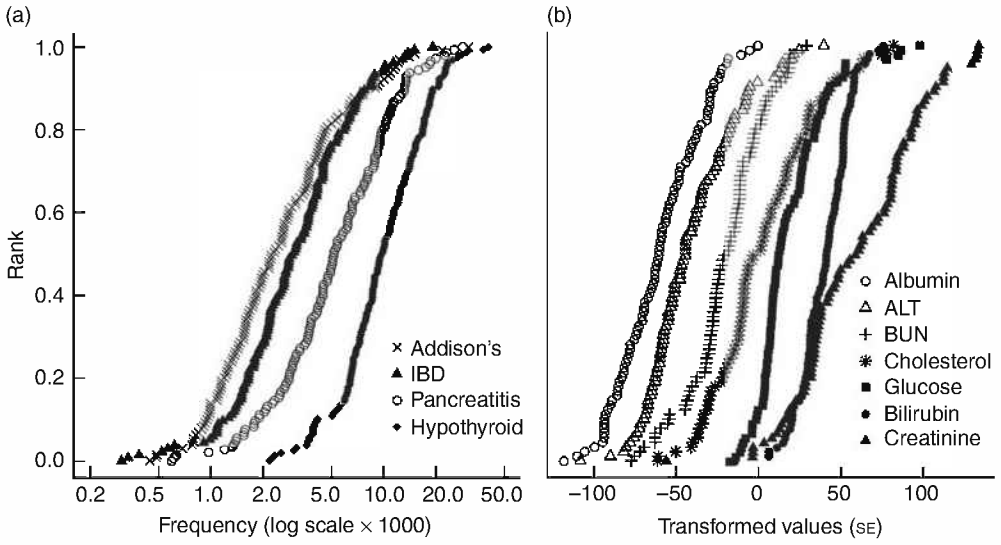

Fig. 20.3. Data from health-related databases showing differences between breeds in disease risk and mean values of serum biomarkers. (a) Variation in the frequency (risk) of four diseases (hypothyroidism, pancreatitis, inflammatory bowel disease (IBD) and Addison's disease) in different breeds. Each point represents a different breed. Breeds are ranked according to increasing frequency of disease. Data from the Veterinary Medicine Database (VMDB; http://www.vmdb.org/vmdb.html). (b) Variation between breeds in values of serum biomarkers. Each point represents a different breed. Breeds are ranked according to increasing value of the biomarker. Data from the ANTECH DIAGNOSTIC Superchem serum profile panel was obtained from Zoasis, and corrected to remove extreme values (diseased dogs). Each breed value was then scaled according to the average breed standard error, and means calculated for each breed and each biomarker. Units on the *x* axis are in numbers of standard errors. Thus, the range of variation between breeds for any one test is highly significant (e.g. >100 SE). Curves for individual biomarkers have been separated on the *x* axis for clarity. Accordingly, breed values can be compared within the range of any particular biomarker, but not between biomarkers. ALT, aspartate aminotransferase; BUN, blood urea nitrogen.

frequency of *IGF1*: >0.85 for the small haplotype associated with size in small dogs; and <0.15 for the large haplotype. Differences in breed frequencies between these two groups are presented in Fig. 20.4, in which breeds of each type are ranked according to increasing frequency of disease found in the breed. These data suggest a prominent role of *IGF1* in determining the predisposition of different genetic isolates (breeds) towards or away from these three diseases.

The importance of firmly establishing a relationship such as the association of *IGF1* with pancreatitis is that it can provide an aspect of risk assessment in any dog (regardless of breed) with respect to the disease in question. In the near future, as more genotypic data are accumulated, increasing the overlap between breeds in the VMDB and CanMap databases, it will become possible to survey the entire genome to identify other regions associated with disease.

Biomarkers and disease risk

Serum biomarker values are complex traits whose variation results from both environmental (diet, exercise, etc.) and genetic influences. Values can change depending on rates of synthesis and secretion into the vascular system as well as on rates of elimination from the serum. They provide insight into the health of an organism by monitoring the concentration or activity of a variety of substances ranging from

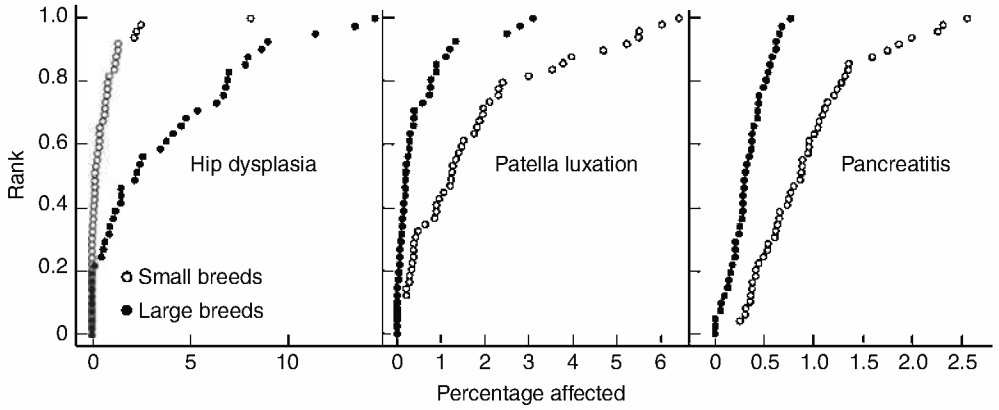

Fig. 20.4. Breed distributions of three diseases associated with *IGF1*. Disease records from 22 veterinary hospitals were obtained from the Veterinary Medicine Database (VMDB; http://www.vmdb.org/vmdb.html). In all, 129 breeds were used (each had records for more than 100 individuals. Disease frequencies were divided into two groupings, small and large breeds. Small breeds were defined as those having a frequency of >0.85 for the small *IGF1* haplotype (Sutter *et al.*, 2007). Conversely, large breeds were defined as those having a frequency of <0.15 for this same haplotype. Cumulative distributions are shown for each group. Breeds are ranked on the *y* axis according to increasing disease frequency.

simple electrolytes to much more complex molecules such as enzymes, cytokines, etc. (Burtis *et al.*, 2006) and are used routinely as diagnostic aids. As a result, automated clinical biochemistry profiling of biological fluids has become the worldwide medical standard for health screening in all species. Several disease-independent factors can lead to variability in phenotypic expression of clinical biochemistry measures *between* species (Wolford *et al.*, 1986). Among these are anatomy and function (ruminant versus monogastric), taxonomically driven dietary habits (herbivore, omnivore, carnivore), or species-related behaviours (social, solitary). For example, several differences (e.g. alkaline phosphatase) differentiate cats and dogs (Lawler *et al.*, 2006, 2007). *Within* species, many disease-independent physiological and environmental factors, e.g. stress, age, obesity, pregnancy, circadian fluctuations, can also influence serum variables (Kaneko *et al.*, 1980). Finally, several studies have suggested a genetic basis within species for variability in the expression of clinical chemistry metrics (Havlik *et al.*, 1977; Hamsten *et al.*, 1986; Randi *et al.*, 1991; Heller *et al.*, 1993; Snieder *et al.*, 1999; Bathum *et al.*, 2004; Lawler *et al.*, 2006). It seems probable that genes which control the quantitative variation of some biomarkers also will predispose to higher or lower frequencies of specific genetic diseases. GWAS in humans that test for haplotypes associated with plasma concentrations of clinical biomarkers are already discovering new functions for the genome, leading to new hypotheses for understanding the relationship of human biology to disease (Chasman *et al.*, 2009).

Do genes control the variation of biomarkers in healthy dogs? One of the most commonly used diagnostic tests is the fasting serum profile. Healthy dogs vary in their blood profiles and there are significant differences in blood profiles between breeds and between individuals within a breed. Fasting sera obtained from ~250 normal (no clinical symptoms of disease) Portuguese Water Dogs showed significant heritable variation in 12 biomarkers from the ANTECH DIAGNOSTIC Superchem panel of serum chemistries (Zoasis Corp., Irvine, California). Heritable differences included protein, metabolic, lipid and enzyme biomarkers. Although the range of variation did not extend outside the range for 'normal' healthy animals, significant genetic differences in serum biomarker levels are segregating in this breed.

The Zoasis database contains serum biomarker data accumulated by the ANTECH DIAGNOSTIC service on thousands of dogs

categorized by age, spay/neuter status and breed. Figure 20.3b compares average values of biomarkers in different breeds. Each breed is represented by a point (as in Fig. 20.3a). Because different tests are reported in different units, values have been transformed to common units to allow direct comparison of the range of values that characterize each breed. Each test is shown separated from others by an arbitrary value on the *x* axis, for purposes of clarity. Although the values cannot be compared between different tests, differences between breeds in mean values for any biomarker are quite significant, and the range of values differs for different biomarkers (e.g. serum cholesterol or creatinine versus glucose or bilirubin). Thus, there is evidence for genetic control of biomarker variation within and between breeds.

Using differences between breeds, as well as segregation within individual breeds, it will be possible in the near future to associate particular DNA haplotypes with variation in serum biomarkers. If these haplotypes are also implicated in disease risk, the value of the biomarker will be increased with respect to preventive as well as diagnostic medicine, and the genes involved may help to define a mechanism whereby the biomarker can serve as a surrogate for the diseased state.

Where to next?

During the next decade, we can expect that genetic loci that have an impact on disease risk will be identified in increasing numbers. As we have seen, this depends on associating genetic information from DNA sequences with phenotypic information. We can anticipate that the near future will provide multiple examples of simple, independently acting, regulatory genes that affect health of the individual animal, be that animal pure bred or mixed breed. As such, these loci will become valuable diagnostic tools. Already, pure-bred dogs are providing examples of loci that can be expected to have an impact on health. These are regulatory genes selected by breeders in their quest for new breed types. Thus, *Fgf3, Fgf4, Fgf5, Fgf*19, *Oraov*1, *Rspo2*, and *Krt72* join *IGF1* (Parker *et al.*, 2010a) as genes that must have multiple effects on the phenotype over and beyond the morphological change selected. If they affect a disease process in an independent manner, they will become useful as diagnostic tools in all breeds, as well as in mixed breeds. If their detection is compromised by multiple interactions, they may only be useful in those dog breeds where interactive factors are not varying – i.e. gene fixation has reduced network complexity.

The process of developing useful genetic tools for diagnosis or risk assessment will have several stages:

1. Detection and identification using pure-bred dog populations.
2. Validation and evaluation in mixed-breed populations to determine the general usefulness of the gene/locus test in terms of the effects of a background of genetic variation. Mixed breeds will provide an ultimate testing ground for the independent action of such loci because they are available in large numbers and present a kaleidoscope of genotypic variation. So, if the genetic signal defining a disease-risk locus is comparable between mixed-breed and pure-bred populations, it indicates that the locus in question is acting independently of the rest of the genome.
3. If validation is successful, in both pure-bred and mixed-breed populations, determining a treatment (e.g. pharmaceutical or dietary) that will offset the predicted predisposition towards disease.

One obvious step forward would be the collection of a high-quality genomic DNA sample from all animals treated at university veterinary hospitals (such an effort is already under way at Cornell; see Castelhano *et al.*, 2009; Piatt, 2010). Analysis of such samples would be an important addendum to databases such as the VMDB. For particular loci that are already implicated in a disease process – for example *IGF1* – it would be reasonable to include genotyping as part of animal medical records. This information would then feed back into a better understanding of the genetic basis of the disease process.

Progress on the genetic basis of disease phenotypes will require organization of much better phenotypic databases than currently exist. The amount and quality of phenotypic

information available will determine how useful genotypic data will be. Currently, the ability to analyse an individual dog's genotype is accessible at an ever-decreasing cost (an advance that owes its origin to advances in human personalized medicine). It seems reasonable that within a very few years an individual dog's genome will be sequenced at a cost of a few hundred dollars. Clearly, the bottleneck in discovering loci that affect disease risk will not be genotyping, given that genotypic databases are already expanding rapidly. Phenotypic databases present a greater challenge. Whereas a genotype is a fixed entity on which we can all agree (a DNA sequence), disease- or health-related phenotypes are much less well defined. Most diagnosed diseases present a variety of symptoms. More often than not, only a fraction of the available assays are carried out and many of the symptomatic descriptions are subjective. This does not necessarily reduce the accuracy of a diagnosis, but makes compilation of a detailed database difficult. Moreover, disease reporting, as in the VMDB described above, is often coded in broad as opposed to more specific categories, according to available reported information (e.g. cancer, versus lymphosarcoma, or lymphosarcoma of the liver). Databases such as the autopsy project described above may provide the prototypes for better formulation of disease gestalts. However, any improvement in phenotypic databases will be reflected in greater benefits from the application of available genetic data.

Differences between observed disease phenotypes can result from different aspects of life history (in particular diet and exercise) that are important factors in understanding the expression of disease phenotypes. If useful life history data become available, this factor can be removed from the genetic analysis (where it constitutes noise), thereby improving our ability to detect a genetic component. Moreover, the interaction between life history information and genotype will allow us to better understand the mechanism of action of genes that determine risk. Quantifying such data is difficult and must be compatible with incorporation into a phenotypic database. One possibility, currently under investigation, is to develop surrogate metrics for diet and exercise based on information inherent in the animal itself. For example, Lieberman (1996) has shown that bone metrics from the skull can be used as an indicator of exercise history, and dietary histories may be estimated using isotopic discrimination techniques applied to hair, bone or dentition samples (Layman *et al.*, 2007; Newsome *et al.*, 2007; Inger and Bearhop, 2008).

Using genetic information for healthier dogs

Assuming that much of the aforementioned will be accomplished, and that genes, or well-defined haplotypes, will be identified for factors predisposing to the complex disease syndromes that develop during a dogs lifespan – to what extent will this information be useful? Clearly it will be important for understanding the disease process and for consequently developing aspects of pharmaceutical as well as dietary and lifestyle-based intervention to ameliorate or even prevent the disease. For this aspect, the accumulation of life history data will become as important as genetic information. An often-overlooked benefit is the development of pharmaceutical intervention. This will be far less expensive than the cost for the development of human pharmaceuticals. Eventually, this aspect of canine medicine may pave the way for parallel development of human medications; comparing the genetic basis of the disease process in dogs and humans will allow short cuts in developing human pharmaceuticals based on what is learned in the dog.

It is important to understand that this type of genetic data (DNA sequence data) is available from the moment the dog is born. Such a genetic blueprint heralds the future health of an animal as it will unfold during its life. Thus it becomes an unbelievably powerful prognostic tool for naturopathic or pharmaceutical intervention during early life to prevent or ameliorate development of disease. Clearly this can be done using knowledge of the impact of diet during post-natal growth. Similarly, development and application of orthopaedic restraints or supports may alter the prognosis for various types of orthopaedic disease. Early knowledge of the probability of developing a disease in later life is invaluable.

Will this type of genetic information be valuable in breeding strategies? Unfortunately, only marginally, as most complex diseases involve interactions with many other genetic components. Understanding the degree of complexity in such interactions will be a long time in coming and, more often than not, attempts to breed away from a particular genetic component will have plural effects (pleiotropy, see above) many of which may be unwanted. In those instances where a gene is acting independently, breeding based on genetic information may meet with success, but even then unexpected effects due to genetic linkage may result in 'throwing the baby out with the bathwater'. Often, gene expression can be modified by interactions with the rest of the genome, leading to unwanted results. A striking example of this may be seen in Labradoodle litters, where the expression of a gene responsible for furnishings (*Rspo2*) can give rise to desired coats, or to quite different litter mates with adorable, but unsought, wisps of hair growing out from the most unexpected parts of their anatomy. For most purposes, as with complex traits in plants, breeding by selecting on the phenotype remains much more efficacious than breeding by genetic design. Even in cases where the phenotype is drastic or terminal (e.g. Addison's disease), the development of preventive measures, based on genotype and knowledge of mechanism, will probably be more effective than attempting to breed away from the responsible genetic cause.

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Our research is an example of public awareness of scientific objectives in which [mostly] non-scientists collaborate closely with scientists to improve the health of the dogs they love. We are greatly indebted to the very many Portuguese Water Dog owners and breeders, whose love for their individual dogs and for their breed led them to participate first in the radiographic project and subsequently in our autopsy project. Their participation in this final act of saying goodbye will remain a monument to what people can do for the dogs they love.

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21 The Canine Model in Medical Genetics

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Introduction

The advantages of domestic animals, including dogs, as models of human genetic diseases have been recognized for decades (see for example: Mulvihill, 1972; Patterson, 1974; Patterson *et al.*, 1982) and predate the discovery of the first disease-causing DNA mutation in a dog (Evans *et al.*, 1989). The tools available to study the genetics of disease in both humans and dogs have changed a great deal since then, largely as a result of the advent of genome sequencing. The first drafts of the human genome sequence in 2001 gave us the first sequence of a mammal, and this has since been used as the model genome for many different mammals. The dog genome was not far behind, with the release of the first dog genome sequence assembly in July 2004. This gave the canine geneticist an incredible resource for genetics research in the dog, and also allowed unparalleled opportunities for using the dog as a model for medical genetics in other mammals. Not only did the availability of dog and human genome sequences drastically reduce the time needed to discover the genes and mutations underlying canine genetic diseases; it also made it possible to accurately match dog genetic disorders with the equivalent human disorders because the locations and DNA sequences of the genes could be compared between the species in the context of all genes in both species. In other words, it became possible to identify the disease gene that was under study in the dog that was the true equivalent to the gene causing that disease in humans, and not just another gene with a similar DNA sequence (i.e. to distinguish orthologous genes – true equivalents – from paralogous genes –

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similar genes with related sequences in another location of the genome). This comparative genomics approach is, of course, also possible with laboratory animal models such as the mouse, rat and zebra fish, whose genomes were sequenced early. However, the similarities in size and physiology of the dog and human often make the dog a much more appropriate medical model than traditional laboratory animal species. For example, in the fields of experimental cardiac procedures and surgery, the dog has been a preferred animal model for many years because of the transferability of lessons learned and skills developed owing to similarities in size, development, shared environment, diet and medical knowledge. The medical knowledge of canine diseases, including the treatment and care of disease-affected animals, is second only to that in people because of the advances in the veterinary care of our much loved pets, and, perhaps of most importance, genetic diseases in dogs are naturally occurring, and include genetically complex diseases. These situations assist in making the dog a useful model, particularly for translational research, because the advances in treatment of canine diseases can be readily adapted for use in human patients.

The current breeding structure of dog populations contributes to the usefulness of the dog as a genetic model. The domestication of the dog in small numbers from the wolf (see Vilà and Leonard, Chapter 1) is estimated to have occurred about 25,000 years ago and produced a population with limited genetic diversity. Recent breeding practices imposed by the desires of the breeders for different dog traits have resulted in nearly 400 dog breeds that encompass broad spectra of physical and behavioural phenotypes (see Parker, Chapter 3). Each breed is genetically isolated and some breeds were initiated from only a few progenitors. This, together with the somewhat common practice of using a limited number of animals for mating within a breed (popular sires), and mating animals of varying degrees of relatedness (inbreeding), has led to the increased incidence of one or more genetic diseases within many breeds. Most present-day breeds have specific predispositions to inherited traits and diseases, e.g. deafness is common among Dalmatians (Strain, 1996). However, genetic diseases with similar characteristics can be found in a number of different breeds. Some of these are likely to have arisen independently, and may be due to different defects in the same gene or to defects in different genes that give similar phenotypes. However, within a breed, it is more likely that dogs with the same disease phenotype have the same underlying disease-associated alleles. This large array of genetic diseases available for study means the dog can be a model for a large number of human genetic diseases.

Technically every variant identified that causes a genetic disease in dogs could be used as a model for human medicine. Studies of the aetiology of diseases, the development of symptoms and the trial of treatments could all benefit from study in a large animal. There are now more than 100 genetic diseases or traits in dogs in which the underlying DNA variation has been identified, and each is a potential model for human disease. However, the purpose of this chapter is not to create a catalogue of those diseases, which is already available elsewhere (see Nicholas, Chapter 5; Sargan, 2004; Lenffer *et al.*, 2006; Boyko, 2011; IDID (Inherited diseases in dogs: Database), 2011; OMIA (Online Mendelian Inheritance in Animals), 2011). The reasons why the dog makes a good model have also been the subject of several reviews and commentaries (e.g. Cyranoski, 2010; Parker *et al.*, 2010; Shearin and Ostrander, 2010). There are several different levels at which the dog model can specifically assist in human medicine, including:

- the identification of disease genes
- the analysis of gene function and disease pathogenesis
- the treatment of disease.

In this chapter, we will discuss all three of these aspects of canine models as they concern an example of a set of genes associated with disease in humans and dogs (Batten's disease or NCL), and then proceed to expand on the most salient features and examples of each these three aspects for other canine genetic diseases as models in medical genetics.

Batten's disease is a group of lysosomal storage diseases referred to as neuronal ceroid lipofuscinoses (NCLs), which result in nerve degeneration due to the accumulation of aggregates in the lysosomes, resulting in early death in children. DNA variants in different genes are associated with different forms of the disease, often differentiated by the age of onset and the

compounds that aggregate. DNA variations in at least ten different genes are known to cause NCLs in humans (Mole *et al.*, 2011), and mutations in six of these, as well as in two additional genes, have been shown to cause NCL in different dog breeds. A *PPT1* defect causes CLN1, an early-onset NCL in Dachshunds (Sanders *et al.*, 2010), while a *TPP1* defect in the same breed causes CLN2, a juvenile-onset NCL (Awano *et al.*, 2006b). There is a *CLN5* defect in NCL-affected Border Collies (Melville *et al.*, 2005), a *CLN6* mutation in affected Australian Shepherds (Katz *et al.*, 2011), a mutation in *CLN8* in English Setters with NCL (Katz *et al.*, 2005), and a *CTSD* mutation in Bulldogs suffering from NCL (Awano *et al.*, 2006a). An *ARSG* defect in American Staffordshire terriers (Abitbol *et al.*, 2010), and an *ATP13A2* defect in Tibetan Terriers (Farias *et al.*, 2011) show all the characteristics of NCL disease in dogs, although mutations in these genes have not been associated with NCL in humans, and they could be responsible for rare forms of human NCL in which the gene responsible has yet to be identified. NCLs occur in other breeds of dogs, but disease-associated mutations have yet to be identified, as in Polish Owczarek Nizinny dogs (Narfström *et al.*, 2007), Australian cattle dogs (Wood *et al.*, 1987) and Miniature Schnauzers (Palmer *et al.*, 1997).

Dog models of NCL have been used to examine the biochemical and histological changes in the brain during the early stages of the disease, at a time when the equivalent samples are difficult to obtain from human patients. Where it is difficult to obtain tissue from affected children, characteristics of the cellular biology of disease have been examined in the dog model: for example, mitochondrial function testing of the liver (Siakotos *et al.*, 1998) and the use of cell extracts to identify enzymes that have ability to degrade the lysosomal aggregates (Elleder *et al.*, 1995). Much of the early work on animal models of NCL was performed by Bob Jolly, who demonstrated that: 'Analogous diseases [to NCLs] in animals can be expected to reflect the same spectrum of biochemical changes, and they warrant in-depth study to help understand the pathogenesis and heterogeneity of the group' (Jolly, 1995).

Before the recent explosion in molecular biology research and the identification of the genetic basis of dog NCLs, some use was made of dog models to trial therapies, for example, the efficacy of bone marrow transplantation to treat NCL (Deeg *et al.*, 1990) and that of antioxidant therapy (Santavuori and Westermarck, 1984). Although canine NCLs are also potential models for trials of gene therapy or stem cell transplantation into the brain to delay the onset of neurological degeneration, most of the clinical trials for these types of potential NCL therapy have been performed directly on human patients without the extensive use of large animal models (Hobert and Dawson, 2006; Mole *et al.*, 2011).

Identification of Disease Genes

The dog has several advantages that can make it easier to identify the genetic basis for a disease than it is in humans. Some diseases that are complex and/or rare in humans can be monogenic and common in one or more dog breeds. Because of their relatively large family sizes, dogs are particularly useful for traditional pedigree-based disease gene mapping to identify new candidate genes for rare human disorders. The extensive pedigree records kept by each breed make an ideal resource to map a disease that is common within a breed (e.g. NCL in Border Collies; Melville *et al.*, 2005), and the ability to generate multi-generation pedigrees in a breeding colony in a short period of time makes it possible to map rare diseases and to manipulate genetic background to reduce the complexity of some diseases of variable expression (e.g. Collie eye anomaly; Lowe *et al.*, 2003; Parker *et al.*, 2007). Jónasdóttir *et al.* (2000) illustrated the power of this strategy by producing a pedigree for hereditary renal cancer syndrome (cystadenocarcinoma and nodular dermatofibrosis, RCND) in dogs, overcoming the situation of the rarity of informative high-risk cancer families in humans. Starting with crosses to a single affected German Shepherd they created a large pedigree and mapped the tumour suppressor gene to canine chromosome 5 (CFA5), and discovered that the disease was caused by a mutation in the folliculin gene (*FLCN*), which is the homologue to the human Birt–Hogg–Dubé syndrome gene (Lingaas *et al.*, 2003).

Another advantage of dogs is that the genetic history of dog breeds has created associations (linkage disequilibrium, LD) between genes that are adjacent to each other that are much stronger than in human populations. LD is the basis for mapping genes by association to known genetic markers and it can be used to scan the whole genome in genome-wide association studies (GWAS). Karlsson *et al.* (2007) performed GWAS to map the genes responsible for two simply inherited dog traits using only 20 dogs (including cases and control dogs) and 27,000 single nucleotide polymorphisms (SNPs) spread across the genome. An equivalent study in humans would usually require many more cases because of disease heterogeneity, as well as many more SNP markers, so dog models can be useful in identifying novel genes involved in disease that are difficult to identify in humans. Examples of diseases where the disease-associated genes were identified first in dogs (rather than in humans or mice, for example) include narcolepsy (Lin *et al.*, 1999), copper toxicosis (van de Sluis *et al.*, 2002; Stuehler *et al.*, 2004), ichthyosis (Boyko, 2011) and progressive retinal atrophy (Zangerl *et al.*, 2006). The identification of these genes in dog studies led to the discovery of the genetic basis for cases of these diseases in humans.

Great progress is currently being made in mapping complex diseases in dogs (see Lark and Chase, Chapter 20), including cancer, diabetes, immune disorders, behavioural disorders and cardiac disease. Causal variants contributing to complex disorders in certain breeds are likely to be identified, and the expectation is that these studies will begin to identify pathways of interacting genes involved in disease development, as well as gene interactions that could contribute to our understanding of the biological basis of the diseases. The dog model is also being used to identify genes involved in behavioural traits (see Yokoyama and Hamilton, Chapter 13; Spady and Ostrander, 2008; Cyranoski, 2010). For example, Dodman *et al.* (2010) have mapped the compulsive behaviour of flank sucking in Dobermans – which has parallels with human obsessive–compulsive disorder – to a region that contains the *CDH2* gene. Autoimmune disorders often have a complex genetic basis, with strong interactions with the environment that make the genetic basis difficult to identify in humans. The close association of dogs with people means that they share some of the environmental factors, suggesting that naturally occurring autoimmune disorders in dogs may be useful in identifying the genes involved in those diseases in humans. Potential examples of this include the several loci involved in systemic lupus erythematosus (SLE) in dogs that have been identified by GWAS (Wilbe *et al.*, 2010), as well as two loci that regulate late-onset Addison's disease in Portuguese Water Dogs, which is very similar to the human disease (Chase *et al.*, 2006).

Analysis of Gene Function and Disease Pathogenesis

Mapping complex diseases in dogs could also identify pathways involved in disease aetiology, and be used for directly testing gene and pathway involvement in human disease. Take the extreme example of the complex genetic trait of height, which is a quantitative trait resulting from the action and interaction of many genes. Genetic studies in humans have located about 50 loci that explain about 5% of the variation in height in the human population (Yang *et al.*, 2010), but in dogs a small number of genes has been shown to control around 50% of the variation (Boyko *et al.*, 2010). Because the dog has a small number of genes with strong effect, it may be easier to identify some of them, and they could then be further investigated to understand their function and their influence on growth in humans.

Similarly, for complex traits, such as diabetes mellitus (DM), which is clinically similar to type I DM in humans, some breeds of dogs are at increased risk compared with other breeds and with mixed-breed dogs (Hess *et al.*, 2000; Guptill *et al.*, 2003). A reasonable hypothesis is that predisposed breeds have a high allele frequency for at least one predisposing allele, which makes the predisposing locus easier to detect in that breed, with the possibility that the results from the predisposed breed may be relevant to dogs of multiple breeds (e.g. Short *et al.*, 2007). Knowledge of a single gene involved in a complex disease can also be used

to identify a pathway that when disturbed leads to the disease. Other genes for components of this pathway make good candidates for additional genes involved in the disease. In this way, small advances in the understanding of complex diseases in the dog can lead to a fruitful new direction in investigation in human research.

The reduced genetic heterogeneity in dog breeds compared with human populations also makes investigations into gene expression levels and pathway analysis a viable approach for detecting genes involved in complex traits. Expression levels of most genes can be measured using microarrays, which can examine all of the dog genes simultaneously. Recent advances in high-throughput sequencing can now be applied to dog samples by massive sequencing of mRNA isolated from tissues relevant to the disease process (referred to as RNA-seq). Quantification of mRNA abundance by RNA-seq allows much greater power in quantification of gene expression levels and in identifying alternatively spliced mRNA transcripts for the same gene (e.g. Trapnell *et al.*, 2010) so as to better characterize the aetiology of disease processes.

Examples of the direct analysis of biochemical pathways in complex disease in dogs include: neurochemical studies in narcoleptic dogs, which showed that there was an imbalance between monoaminergic and cholinergic systems, and that orexin neurotransmission deficiency was causing narcolepsy (Lin *et al.*, 1999); and the use of a dog model of von Willebrand's disease to study intracellular trafficking of von Willebrand's Factors (Haberichter *et al.*, 2005). All of these results from the study of dogs – from the mapping of predisposing allele loci to direct analysis of biochemical pathways – can contribute to the understanding of the pathophysiology of disease in humans.

Treatment of Disease

The ability to perform trials of clinically relevant disease treatments has probably been the biggest contribution of the dog model to human medicine. While the potential for use of the dog as a model for treatment of NCLs has perhaps not been used to its best advantage, extensive use has been made of other diseases in the dog. Not only has the efficacy of dietary supplements and pharmacological treatments for many conditions (Hoffman and Dressman, 2001) been tested in dog models, but the usefulness, proof of principle, safety and optimal conditions for gene therapy treatments have often been first investigated in dog models of human disease before transferring the technology to human medicine. Amelioration of the clinical consequences of genetic diseases can be achieved by restoring or enhancing the defective function, or by blocking the unwanted expression of a gene rendered out of control. Restoration of function can be achieved by enzyme replacement from an exogenous source. It can also be achieved by gene or cellular therapy, which involves replacing the defective gene in a fraction of the somatic cells so that a sufficient amount of the gene product is available to perform the function of the defective product. In both cases, the dog has advantages for treatment trials over the mouse, particularly in consideration of the discrepancy in size between human and mouse, which can create large differences in responses to treatment (Galibert *et al.*, 2001). More specifically, research in dogs to test potential treatments for use in human patients has been extremely useful for haemophilia, several forms of mucopolysaccharidoses, muscular dystrophy, immune deficiencies, metabolic disorders and genetic eye defects, to name a few. See Table 21.1 for a list of examples.

Supplements and pharmacological treatments

The physiology and pathophysiology and even the classification and symptomatology of canine diseases are better documented than in other non-human species, even other primates, because pet dogs are commonly in frequent contact with veterinarians. Drugs targeting specific biochemical pathways are being tested in dog models and their use has begun to be implemented in human clinical trials (Hoffman and Dressman, 2001). Examples include

Table 21.1. Examples of genetic diseases in canine breeds that have been used as models for human diseases by disease class.

Disease class Disease name – Gene (where known)	Dog breeds	Use	References
Blood cell disorders			
CLAD, leucocyte adhesion deficiency Type I – *ITGB2*	Irish setters	Bone marrow transplant, gene therapy	Bauer *et al.* (2008, 2009)
Erythrocyte pyruvate kinase deficiency – *PKLR*	Basenji, Beagle, West Highland White Terrier	Bone marrow transplant, lymphocyte infusion	Zaucha *et al.* (2001); Takatu *et al.* (2003); Bauer *et al.* (2009)
Brain chemistry disorders			
Narcolepsy – *HCRTR2*	Doberman Pinscher, Labrador Retriever	Pathophysiology and clinical management, pharmacological studies and improved treatment, enzyme replacement	Faraco *et al.* (1999); Fujiki *et al.* (2003); Schatzberg *et al.* (2004); Tonokura *et al.* (2007); Chen *et al.* (2009)
Carbohydrate metabolism disorders			
Glycogen storage disease Ia, von Gierke disease – *G6PC*	Maltese Dog	Gene therapy	Beaty *et al.* (2002); Chou and Mansfield (2007); Koeberl *et al.* (2009)
Endocrine diseases			
Type 1 diabetes	Cavalier King Charles Spaniel	Deconstructing complex disease	Short *et al.* (2007)
Eye disorders			
Glaucoma – *ANGPTL7*	Beagle	Gene expression	Kuchtey *et al.* (2008)
Eye disorders (retinal)			
Retinitis pigmentosa – *RPE65*	Briard	Gene therapy	Acland *et al.* (2005); Narfström *et al.* (2005); Stieger *et al.* (2009)
Achromatopsia – *CNGB3*	Alaskan Malamute, German Short-haired Pointer	Gene therapy with enhanced cell targeting	Komáromy *et al.* (2010)
Rod–cone dysplasia 1 – *PDE6*B	Irish setter	Drug testing, gene therapy	Pearce-Kelling *et al.* (2001); Stieger *et al.* (2009)
Haemophilia			
Haemophilia B – coagulation factor IX – *F9*	Airedale Terrier, American Pitbull Terrier mix, German Wire-haired Pointer, Labrador Retriever, Lhasa Apso	Transfusion therapy, gene therapy, cross-supplementing therapy	Brooks *et al.* (1997); Monahan *et al.* (1998); Herzog *et al.* (2001); Hasbrouck and High (2008); Nichols *et al.* (2009); Haurigot *et al.* (2010)
Haemophilia A – coagulation factor VIII – *F8*	German Shepherd	Enzyme replacement, gene therapy, cross-supplementing therapy	Pemberton (2004); Nichols *et al.* (2009); Sabatino *et al.* (2009)

Continued

Table 21.1. Continued.

Disease class Disease name – Gene (where known)	Dog breeds	Use	References
Factor VII deficiency – *F7*	Beagle	Evaluation of treatment, recombinant enzyme replacement	Ferguson *et al.* (1991); Ghosh *et al.* (2007); Margaritis *et al.* (2009); Nichols *et al.* (2009)
von Willebrand's disease – *VWF*	Scottish Terrier	Replacement therapy, gene therapy, intracellular trafficking, cell biology/ gene function	Haberichter *et al.* (2005); De Meyer *et al.* (2006); Nichols *et al.* (2009)
Heart diseases			
Arrhythmogenic right ventricular cardiomyopathy (ARVC) – *STRN*	Boxer	Potential therapy	Meurs *et al.* (2010)
Juvenile dilated cardiomyopathy (JDCM) – unidentified gene on CFA8	Portuguese Water Dog	Disease gene discovery, dietary supplement, potential gene therapy	Alroy *et al.* (2000); Werner *et al.* (2008); Sleeper *et al.* (2009, 2010)
Immune deficiencies			
X-linked severe combined immune deficiency – *IL2RG*	Basset Hound, Cardigan Welsh Corgi	Bone marrow transplant, gene therapy	Hartnett *et al.* (2002); Goldschmidt *et al.* (2006); Ting-De Ravin *et al.*, 2006; Suter *et al.* (2007)
Hermansky-Pudlak syndrome Type 2 and cyclic neutropenia – *AP3B1*	Collie (Grey collie syndrome)	Enzyme replacement, gene therapy	Yanay *et al.* (2006); Bauer *et al.* (2009)
Glanzmann thrombasthenia – *ITGA2B*	Great Pyrenees	Bone marrow transplant, platelet-directed gene therapy	Wilcox *et al.* (2000, 2008); Niemeyer *et al.* (2003); Bauer *et al.* (2009)
Lysosomal storage diseases			
Fucosidosis – *FUCA1*	English Springer Spaniel	Bone marrow transplant, RV (retroviral) vector-transduced allogeneic autologous bone marrow	Taylor *et al.* (1992); Occhiodoro and Anson (1996); Ferrara *et al.* (1997)
Globoid cell leucodystrophy, Krabbe's disease – *GALC*	Cairn Terrier, West Highland Terrier	Gene therapy, monitoring demyelination progression *in vivo*	McGowan *et al.* (2000); Haskins (2009)
Mucopolysaccharidosis I, Hurler Syndrome, α-L-iduronidase deficiency – *IDUA*	Plott Hound	Bone marrow transplant, enzyme replacement, gene therapy, recombinant cell implantation, immune tolerance avoidance	Kakkis *et al.* (1996); Lutzko *et al.* (1999); Barsoum *et al.* (2003); Traas *et al.* (2007); Gagliardi and Bunnell (2009); Haskins (2009)

Mucopolysaccharidosis VII, Sly syndrome, β-glucuronidase deficiency – *GUSB*	German Shepherd	Bone marrow transplant, haematopoietic stem cell therapy, *ex vivo* gene therapy, neural cell transplant	Haskins *et al.* (1991); Sammarco *et al.* (2000); Martin *et al.* (2006); Walton and Wolfe (2007, 2008); Herati *et al.* (2008); Gagliardi and Bunnell (2009)
Metal transport disorder			
Copper toxicosis – *COMMD1*	Bedlington Terrier	Understanding copper homeostasis	van De Sluis *et al.* (2002); de Bie *et al.* (2007); Vonk *et al.* (2008)
Muscular disorders			
Duchenne muscular dystrophy – *DMD*	Beagle, Cavalier King Charles Spaniel, Golden Retriever	Gene therapy, anti-sense RNA induced exon skipping, stem cell therapy	Howell *et al.* (1997, 1998); McClorey *et al.* (2006); Sampaolesi *et al.* (2006); Yokota *et al.* (2009); Walmsley *et al.* (2010)
Renal diseases			
Hereditary nephropathy X-linked – *COL4A5*	Samoyed	Gene therapy	Cox *et al.* (2003); Harvey *et al.* (2003); Tsai *et al.* (2007)
Hyperuricosuria and hyperuricaemia – *SLC2A9*	Dalmatian	Organ transplant, understanding urate transport	Bannasch *et al.* (2008)
Skin/ectoderm diseases			
Hypohydrotic ectodermal dysplasia – *EDA*	German Shepherd	Enzyme replacement	Casal *et al.* (2007); Mauldin *et al.* (2009)
Tumours			
Glioblastoma multiforme (GBM)	Boxer	Treatment with transgene expression in brain	Candolfi *et al.* (2007)
Cushing's disease from ACTH (adrenocorticotrophin)-producing pituitary adenoma	Various	Adenoma control via effecting receptor expression	de Bruin *et al.* (2008)
General			
Bone marrow transplant	Mixed breed	Cell targets for gene transfer	Suter *et al.* (2004)

investigations of narcolepsy in the dog which have opened new arenas for the study of this condition in human patients (see Galibert *et al.*, 2001). Dog models have allowed pharmacological studies to improve treatments for narcoleptic patients (Nishino and Mignot, 1997; Lin *et al.*, 1999; Nishino *et al.*, 2000) and are being used to study the hypocretin system as a target for new therapeutic approaches (Dauvilliers and Tafti, 2006). Transitional cell carcinoma (TCC) is a malignancy of the bladder that is common in several dog breeds (Glickman *et al.*, 2004) and shares similarities with human TCC. Drug trials for the treatment of canine TCC have had some success (Mohammed *et al.*, 2002), confirming the usefulness of canine TCC as a therapeutic model.

Enzyme replacement

Enzyme replacement is another area of treatment that has often been tested in the dog model. Studies in dogs with mucopolysaccharidosis (MPS) Type 1, a lysosomal storage disease, have shown that repeated delivery of recombinant lysosomal proteins via injection into the cerebrospinal fluid (CSF) enables widespread distribution of the recombinant protein within the brain, leading to a reduction in the pathology of this lysosomal storage disorder (Hemsley and Hopwood, 2009). Postnatal administration of recombinant ectodysplasin A protein to dogs with X-linked hypohydrotic ectodermal dysplasia, caused by an *EDA* gene mutation as in humans with this disease, ameliorated multiple clinically relevant deficiencies, including dental abnormalities, sweat gland formation and function, and defects in respiratory function (Casal *et al.*, 2007; Mauldin *et al.*, 2009). Hypocretin replacement therapy for narcolepsy is another example that has been studied in the dog model (Schatzberg *et al.*, 2004).

RNA treatment

RNA can be used to knock down gene expression or alter RNA splicing to correct genetic defects (McClorey *et al.*, 2006). Yokota *et al.* (2009) used RNA to induce exon skipping in dystrophin in dogs with muscular dystrophy (DMD) to remove exons from mRNA that contained mutations causing the frameshifts that resulted in complete absence of a functional protein. The dystrophin protein produced by this artificially induced exon skipping, while shorter than the normal protein, can maintain enough function to ameliorate the effects of the disease. Dogs were chosen for the research because they accurately mimic the physiological effects of human DMD, so the approach could be assessed to see if it could restore enough of the dystrophin function to halt the progression of the disease. The mouse model is not useful for these experiments as the effect of the disease in mice is only mild muscle deterioration.

Bone marrow transplantation

Heterologous bone marrow transplantation (BMT) in humans has been performed for decades, with dogs playing a major role as a preclinical model for developing successful protocols (see Diaconescu and Storb, 2005). BMT provides both normal bone marrow and bone marrow-derived cells, which release normal lysosomal enzyme continuously; the treatment has demonstrated clinical benefit in a variety of disorders, including lysosomal storage disorders such as mucopolysaccharidosis VII (Martin *et al.*, 2006). While more recent studies have used bone marrow cells as a target tissue for gene delivery to the body, advances in this approach use cell populations enriched in haematopoietic stem cells, as exemplified in bone marrow cell transplantation to treat canine X-linked severe combined immunodeficiency (Hartnett *et al.*, 2002). Transplantation of stem cells can also be used to provide healthy cells in other disease-affected tissues, such as the central nervous system (CNS), but some tissues, such as muscle, which is affected in muscular dystrophy, have proven difficult to repopulate. However, vessel-associated stem cells called mesoangioblasts have been successfully transplanted and expressed dystrophin in the DMD-affected Golden Retriever, allowing recovery of muscle use (Sampaolesi *et al.*, 2006).

Gene therapy

The dog has been widely used as a model for gene therapy because the similar size of dogs and humans makes dosage determination, reactivity and response easier to evaluate. Dogs have been used to assess basic methods such as type of delivery, vectors used, dosages and safety, as well as levels of expression when cloned genes are transferred into host cells *in vitro* and *in vivo*. A number of reviews discuss gene therapy studies that have used different vehicles for introducing the gene, including retroviral, adenoviral, lentiviral and adeno-associated viral vectors, hydrodynamic injection of plasmid DNA and the transfer of stem cells (Monahan and White, 2002; Chuah *et al.*, 2004; Lozier, 2004; Nathwani *et al.*, 2004; High, 2005; Casal and Haskins, 2006; Lillicrap *et al.*, 2006; Ponder, 2006). The efficacy of gene therapy to replace defective genes has been investigated for a number of diseases in the dog model. Reviews of several animal models for gene therapy, including the dog, are available in a single volume from the Institute for Laboratory Animal Research (Wolfe, 2009); this includes reviews of lysosomal storage disorders (Haskins, 2009), metabolic disorders (Koeberl *et al.*, 2009), neurological disorders (Gagliardi and Bunnell, 2009), haemophilias (Nichols *et al.*, 2009), immune and blood cell systems (Bauer *et al.*, 2009), muscular dystrophy (Wang *et al.*, 2009), cardiovascular genetic disease (Sleeper *et al.*, 2009) and retinal disorders (Stieger *et al.*, 2009). Several dog models that have been exploited to particular benefit in studying the safety and efficacy of gene transfer are discussed in more detail below.

Haemophilias

There are several dog models for clotting disorders (see Table 21.1), and for haemophilia B, Factor IX deficiency, there are different mutations in different dog breeds (Tsai *et al.*, 2007). As for many genetic diseases, a common approach to developing gene therapies for human haemophilia has been to initially test potential therapeutic approaches in a small animal model and then try the more successful approaches in haemophilic dogs (Rawle and Lillicrap, 2004; High, 2005; Casal and Haskins, 2006; Ponder, 2006). As outlined by Nichols *et al.* (2009), and relevant to many inherited diseases for which gene therapy is a possible treatment modality, the primary benefits of the preclinical testing of gene transfer strategies in (haemophilic) dogs are: estimation of vector dosing for the human liver-based trial; assessment of the functional status of the expressed protein; monitoring of the expression of the functional protein over several years; assessment of the degree of correction of the disease phenotype; testing of scaled-up vector production; and delivery to large amounts of target tissue. These canine gene therapy trials also determined the immune response to the procedure in an outbred animal (High, 2005), and the safety of novel delivery systems (e.g. Haurigot *et al.*, 2010). Dogs have proven a good model for gene therapy preclinical trials because a blood level of normal clotting factor from a gene introduced by gene therapy in a large dog closely approximates the therapeutic level for humans (Øvlisen *et al.*, 2008). Human clinical gene therapy trials have been developed for haemophilia A (Factor VIII deficiency) and haemophilia B (Factor IX deficiency) and von Willebrand's disease (VWD) based directly on preclinical experimental studies in dog models (Murphy and High, 2008; Nichols *et al.*, 2009). Dogs with well-characterized inherited bleeding disorders that mirror human disease have allowed the development of detection assays, the transfer of experimental therapies into clinical practice and the continued preclinical evaluation of promising new therapies (Nichols *et al.*, 2009).

Retinal eye disorders

A number of genetic diseases affecting the retina and resulting in blindness occur in dogs, where the architecture and large size of the canine eye provides an excellent model for assessing the pathobiology and for evaluating treatment approaches (Acland *et al.*, 2001). Gene therapy has been used to successfully restore vision in Briard Dogs with progressive retinal atrophy (PRA), which is a homologue of one form of retinitis pigmentosa (RP) in humans that causes childhood blindness as a result of mutations in *RPE65* (see Mellersh, Chapter 1; Narfström *et al.*, 2003, 2005; Acland *et al.*, 2005).

Gene therapy protocols for defects in photoreceptor cells have been performed in dogs of seven different breeds. Each breed carried defects in one of five genes that have been identified as affecting the function of these cells and resulting in blindness (Stieger *et al.*, 2009). In addition, there are at least two other canine models where the retinal pigment epithelium (RPE) is affected that are in gene therapy trials. Best's disease (vitelliform macular dystrophy) which occurs in the Great Pyrenees and the Coton de Tulear breeds is due to different mutations in the *VMD2* gene in each breed (Guziewicz *et al.*, 2007; Stieger *et al.*, 2009). Achromatopsia in the German Short-haired Pointer and Alaskan Malamute is due to different defects in the *CNGB3* gene and has been successfully treated by gene therapy (Komáromy *et al.*, 2010). This therapy approach required targeting of therapeutic vectors to mutant cones by the use of human red cone opsin promoter to drive expression of the transgene to restore cone function. The dose efficacy and safety data obtained from the successful use of the canine model led to consideration of human trials (e.g. Jacobson *et al.*, 2006). Clinical gene therapy trials for retinal degeneration have been developed directly from experimental studies in these dog models (Kaplan, 2008) and are now ongoing.

Lysosomal storage diseases

Currently, at least 18 Lysosomal Storage Diseases (LSDs) have been described in dogs and cats (Haskins and Giger, 2008), and 13 of these exist in research colonies that are available for gene therapy trials (Haskins, 2009). Multiple approaches, using different vectors, different transgenes, local administration and a combination of therapies, have been used in the dog models of LSDs to assess their use in human therapy. The major hurdles for successful gene therapy for LSDs are the difficulty of obtaining adequate levels of gene product in specific tissues (such as the CNS), maintaining *in vivo* expression and regulating gene expression. Because of the blood–brain barrier, somatic cells that produce the replacement proteins may not provide enzyme protein for the CNS where it is required, as most LSDs have significant CNS lesions. Direct intracranial injection of viral gene vectors has resulted in reduced lysosomal storage and functional improvement. Another approach to treating the CNS has been to provide a high dose of serum enzyme that can cross the blood–brain barrier. Gene therapy experiments in MPS I and MPS VII dogs have shown that this approach can work, as dogs with high serum enzyme activity had more improvement in CNS storage than did those with less activity (Traas *et al.*, 2007; Haskins, 2009). Other LSDs with actively used dog models are fucosidosis, globoid cell leucodystrophy (Krabbe's disease) and glycogen storage disease (Table 21.1; Haskins, 2009).

Other diseases

Gene therapy has been applied to several other genetic disorders in the dog (see Table 21.1). Two promising models of inherited cardiomyopathy include dilated cardiomyopathy in Portuguese Water Dogs (Sleeper *et al.*, 2009, 2010) and arrhythmogenic right ventricular cardiomyopathy in Boxers caused by a mutation in the gene encoding striatin (Meurs *et al.*, 2010). The renal disease, Alport's syndrome, which is caused by a lack of collagen due to mutations in *COL4A5*, has been studied in the Samoyed (Harvey *et al.*, 2003). Dog models also exist for inherited metabolic disorders (reviewed in Koeberl *et al.*, 2009), immune deficiencies and haemopoetic abnormalities (Malech and Hickstein, 2007). The dog has even been used to test cancer treatment, even though the cancer is sporadic and of unknown genetic cause. In this case, the brain tumour glioblastoma multiforme (GBM), which occurs in dogs, can be controlled by expressing therapeutic transgenes (Candolfi *et al.*, 2007).

Conclusion

Without doubt, the contribution of human medical genetics knowledge has been indispensable to advances in the study of canine genetic diseases. The transfer of information from human to dog through comparative genomics has allowed a rapid increase in the rate of identification of genes and gene function without need for experimentation. This has led to

increased progress in understanding the canine genome and the genetics of many dog diseases. However, the hundreds of naturally occurring genetic defects in the dog provide a resource that can be used as models for human medical genetics. A portion of these canine defects have been utilized to test treatment strategies and to understand the biology of the disease process, and have led to significant advances in medical genetics that have been directly beneficial to human patients. Many of these advances were made using purpose-bred animals in the face of the difficulties associated with the resources required to maintain and house a large animal model. However, clinical trials on canine patients represent a promising approach for the future, particularly with respect to complex genetic diseases. Recent advances in genetics and genomics technologies will allow the dissection of the genetic bases of complex diseases in dogs, and the results will also be relevant to human disease. While investigations into pathophysiology may be challenging, if diseases are being studied in the pet population of dogs, progress will be made. The inclusion of other mammalian species in this comparative genomic comparison will provide further insight into the genetics of disease, particularly in the area of control of gene regulation and conservation of non-coding DNA.

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22 Genetic Aspects of Performance in Working Dogs

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Introduction

For thousands of years, the dog has proven itself an invaluable asset to man by serving as hunter, protector, transportation, aide and loyal companion. Dog domestication and breed development have paralleled the changing culture of man. Archaeological evidence has identified the earliest dog remains between 12,000 and 31,000 years ago, with the suggestion that these first dogs were used in the tracking, capture and transport of large 'ice-age' game (Germonpré *et al.*, 2009). Eventually, pastoral societies gave rise to home and flock guard dogs and herding dogs. As civilizations moved to conquer and defend, dogs became weapons of war (Coren, 1994; Thurston, 1996). Contemporary culture has diversified working dogs into breeds inherently developed for specific tasks and an assortment of dogs trained to fulfil modern needs. Today, pointers, retrievers, scent hounds and coursing hounds are common hunting breed groups. Herding and flock guard dogs remain efficient and effective means for moving and protecting flocks of sheep and cattle. Specific breeds, most notably the German Shepherd Dog and the Labrador Retriever, possess desirable breed qualities that make them ideal military, law enforcement and service dogs. Both pure-bred and mixed breeds are used for search and rescue, therapy and dog sledding (Fig. 22.1). The unique diversity of the dogs utilized, whether bred to do a task or chosen based on physical and behavioural traits that allow them to be trained for a particular duty, makes them ideal candidates for the study of performance genetics. Such studies are a means to decipher the genetic complexity of heritable behavioural traits, including herding, pointing, trainability and work ethic, along with the underlying genetics of multifaceted physiological qualities such as scenting ability, speed and endurance. Our aim is to give a detailed account of what encompasses a

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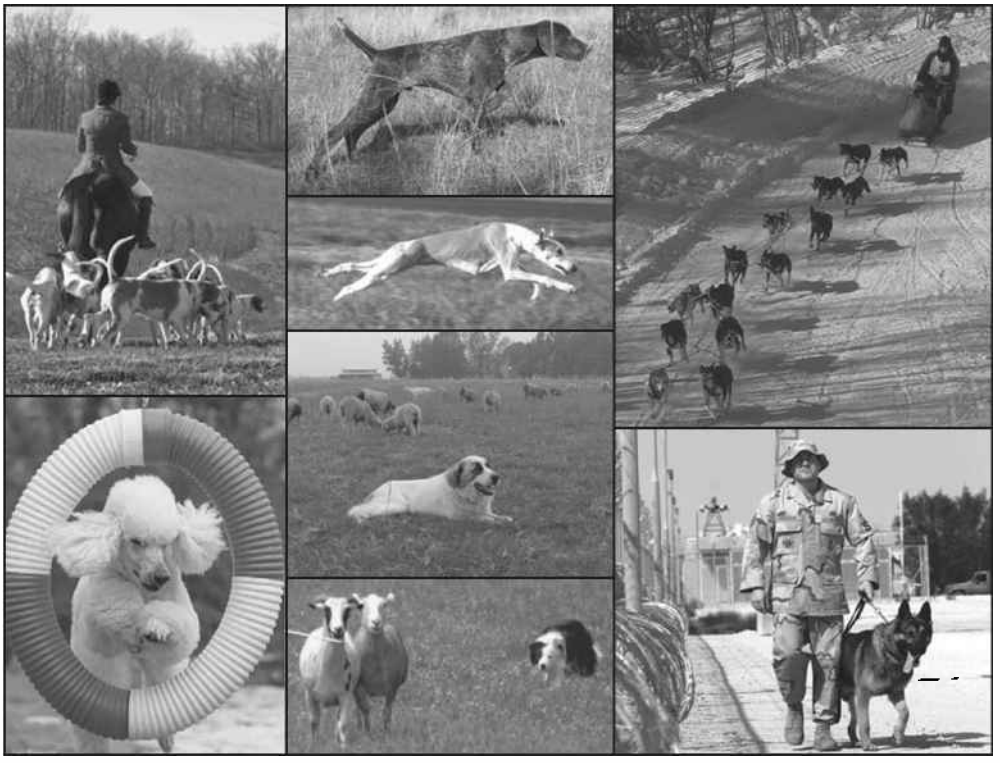

Fig. 22.1. Working dogs of the 21st century perform both ancient and modern duties. Left column: Foxhounds utilize their scenting ability to track prey during colonial-style fox-hunts; a standard poodle navigates a hoop jump during an agility trial. Middle column: A German Short-haired Pointer stands point on sighted game; sighthounds, such as the Whippet, use speed and agility to capture prey; flock guard dogs protect livestock from natural predators such as wolves, coyotes and bears (photo courtesy of Cormo Sheep and Wool Farm); a Border Collie uses body positioning and stare intimidation to move its flock in the desired direction. Right column: Alaskan sled dogs are competitively raced in short (4–30 mile) and long (several 100 miles) distance races; dogs employed by the military are vital for detecting explosives (US Air Force photo/Tech. Sgt Frank Hatcher).

working dog, ranging from the tasks they perform, to physiological factors, and, more recently, to the genetics behind these hard-working canines.

Military Working Dogs

Throughout time, human conflict has brought dogs into war, serving as scouts, weapons and a line of defence. Attila the Hun, the Spanish conquistadors, the Romans and Napoleon all used dogs as a weapon of war during their conquests (Coren, 1994; Lemish, 1996; Thurston, 1996). Other rulers, such as Charles V of Spain, William the Conqueror and Queen Elizabeth I, relied on war dogs to help defend their crowns.

Despite hundreds of years and technological advances in warfare, dogs were continually used throughout World War I, World War II and in Vietnam. In WWI, German, French and Belgian armed services were the primary forces using dogs: over 75,000 mixed and pure-bred dogs were used as either messengers or sentries (Coren, 1994). The Red Cross relied on dogs to find wounded soldiers, carry medical supplies into the field and then transport disabled soldiers out (Lemish, 1996). While many European countries had established military dog programmes, WWII saw the USA accepting

civilian German Shepherd Dogs, Belgian Sheepdogs, Doberman Pinschers, Collies and Giant Schnauzers volunteered by pet owners and breeders to serve in the war effort. The Doberman Pinscher was named the official war dog breed for the US Marine Corps and these dogs were honoured with the 'Always Faithful' statue commemorating their bravery during the liberation of Guam in 1944 (Lemish, 1996; Putney, 2001). While approximately 4000 dogs served in the war in Vietnam and are credited with saving at least 10,000 lives, only a handful returned to the States afterwards. Regretfully, the USA considered these dogs as surplus equipment at the war's end, and forced handlers to leave them behind (Emert, 1985a).

The Iraq and Afghanistan wars have dogs performing many of the same duties today as they had historically (Fig. 22.1), but with a modern twist. For instance, Britain's Special Forces assault teams include German Shepherd Dogs capable of parachuting out of aeroplanes at 25,000 feet, strapped to their handlers and wearing their own oxygen masks. After landing in hostile territory, cameras fixed to their heads allow personnel a visual of the area as they scout for insurgent hideouts (Dunn, 2008).

The USA took another approach in combining dogs and the military when the Defense Advanced Research Projects Agency (DARPA) funded studies on Alaskan sled dogs competing in the gruelling 1100 mile Iditarod race across Alaska (Iditarod, 2010). While there are no designs for Alaskan sled dogs to become the next war dog, they are ideal models for the study of fatigue or, more importantly, their lack of fatigue while under extreme physiological and metabolic demands. It is the sled dog's intriguing ability to maintain a high level of calorific burn for extended periods without utilizing their fat and glycogen reserves which would, in turn, cause fatigue (Robson, 2008). In spite of the fact that man can deliver atomic bombs, launch missiles with pinpoint accuracy, and employ the use of unmanned aerial vehicles, dogs have not lost their military value. Dogs have served as weapons of war for centuries and continue to serve, proving ferocious, courageous and intelligent. They have saved countless lives with their deeds, conquered great lands, and, most importantly, inspired men with their undying loyalty and spirit (Sheppard Software, 2010).

Law Enforcement Dogs

Dogs serve with local and national law enforcement agencies assisting in protection, apprehension of criminals, building searches, missing person searches, explosive and narcotics detection, arson investigations and the confiscation of illegal imports. Within the USA, these tasks place them among the state police, FBI (Federal Bureau of Investigation), DEA (Drug Enforcement Administration), ATF (Bureau of Alcohol, Tobacco, Firearms and Explosives), USDA (US Department of Agriculture) and TSA (Transportation Security Administration) to name just a few of the US agencies employing canine assistance. The International Police Work Dog Association (IPWDA) was established as the Indiana Police Work Association in 1998, and has since expanded to the 800-strong IPWDA, which unites law enforcement agencies worldwide in the training, standardization and improvement of law enforcement working dogs (IPWDA, 2011).

Historically, European countries have used dogs in law enforcement since the 13th century and many had developed official dog programmes by the 20th century. The USA attempted its first police dog programme in 1907, although this ended in failure owing to lack of trainers, dogs and funding. A second attempt in 1956 established a successful training programme in Baltimore, Maryland, and led to the development of canine units across the country (Emert, 1985b). The German Shepherd Dog, Belgian Malinois, Labrador Retriever and Golden Retriever are the most commonly used breeds in law enforcement, and they are selected for their intelligence, strength, aggression, loyalty, agility and trainability. Those used specifically for detection must also possess a strong hunt-and-retrieve drive, endurance and stable temperament. Basic police dog training consists of obedience, agility, attack methods, building searches and tracking.

The length of time that dogs have been used in detection work can be explained by the staggering magnitude by which a dog's sense of

smell surpasses that of a human. Dogs, dependent upon breed, are estimated to be 1000 to 10,000 times more sensitive to smells than a human (Coren, 2004). For instance, a Bloodhound's sense of smell is finer than a German Shepherd Dog's and has been demonstrated to improve with the maturation of the neurological system after 1 year of age (Harvey and Harvey, 2003). Genetic investigation of the canine olfactory system has produced a comprehensive genetic catalogue of 817 novel canine olfactory receptors (ORs). It estimated that the canine genome possesses 1300 OR genes, with roughly 18% of those being pseudogenes; this compares with 1100 human OR genes consisting of approximately 60% pseudogenes (Quignon *et al.*, 2003; Olender *et al.*, 2004). This research has explained the genetic basis for the superior scenting abilities of dogs as opposed to humans. It has also created a foundation for future research investigating the role of specific OR genes. We could speculate that OR gene expression may vary with respect to breed differences in smell sensitivity or the targeting of specific scents used in detection work.

Some air-scenting dogs are specially trained for cadaver and forensic evidence recovery. They are trained to detect odours associated with the five stages of decomposition, which include locating evidence such as teeth, bones and blood. They can efficiently search large areas of terrain and alert on remains above ground, within burial sites and even in water (Komar, 1999; Lasseter *et al.*, 2003). Depending on the area of detection, dogs are taught to alert either passively (sit, lie down, bark) or aggressively (paw at or dig) (Emert, 1985b). The dog's specific detection and alert training are dependent upon whether they are targeting a missing person, illegal drugs or explosives.

The demand for explosives detection dogs in the USA has risen dramatically since the terrorist attack of 11 September 2001 on the Twin Towers. Section 1307 of the Implementing Recommendations of the 9/11 Commission Act of 2007 called for an annual increase of 200 canine explosive dog/handler detection teams from 2008 to 2010. This massive undertaking was charged to the TSA and was specifically implemented for securing high-capacity public transportation areas such as airports and train stations (Berrick, 2008).

While the need for explosives detection dogs has increased, the importance of dogs used in narcotics, illegal imports and arson detection also remains strong. Dogs detecting fire accelerants drastically decrease investigation time by either eliminating accelerant usage or pinpointing the area with accelerant. They have also proven more sensitive than electronic hydrocarbon detector devices (Andersson, 1997). Arson dogs are even employed by private insurance companies to identify fraudulent fire claims, therefore saving these companies and their clients millions of dollars (State Farm Mutual Automobile Insurance Company, 2011).

A unique group of Beagles, known as the Beagle Brigade, has protected American agricultural interests since 1984. Over 60 Beagles serve the USDA at international airports and border crossings by alerting to illegal agricultural products that may harbour pests or diseases. The dogs of the Beagle Brigade have proven to be 90% accurate after 2 years of service and have prevented the entry of roughly 75,000 agricultural goods into the USA over the past 11 years (Cherry and Redding, 1995). While the use of dogs in US law enforcement is highlighted here, dogs are an integral part of national security worldwide.

Many military and law enforcement dogs are chosen for possessing the desirable characteristics of large stature, ferocity, scenting ability and trainability. The high standard of performance required in these dogs induces intense genetic selection of said traits. We can speculate that these groups of dogs may give us insight into the genetic components regulating such behaviours. Thus there is the potential for a unique behavioural study looking at traits such as ferocity compared with mild temperament within breeds as well as across breeds in respect to German Shepherd Dogs and Labrador Retrievers, which are frequently used breeds for both military and law enforcement as well as their use as service dogs for disabled people.

Search and Rescue Dogs

The American Rescue Dog Association (ARDA), founded in 1972, was the first established search dog organization in the USA.

ARDA brought together state rescue dog associations from across the nation to share training techniques, develop uniform standards and create a national alerting system for major emergencies, in the hope of providing competent, well-trained search and rescue personnel and canines (ARDA, 2010). Search and rescue (SAR) dogs utilize three methods to locate an individual based on their unique biological odour signatures: tracking, trailing and air scenting (Coren, 2004). Tracking and trailing are both used to search for a specific person (e.g. a lost child or hiker, a fleeing criminal) and therefore require a scent article from that individual. A tracking dog works on a leash, requires a place of origin and then follows in the missing person's exact footprints, using ground scents from the individual and disturbed vegetation. Trailing relies upon the scent of skin cells that linger in the area near the tracks. These dogs may work on or off leash and can perform even when others have contaminated the tracks (Coren, 2004). However, both tracking and trailing dogs have greater success when other individuals are not present to obscure the primary scent (Jones *et al.*, 2004).

For SAR instances in which the identity and the number of victims are unknown, dogs trained in air scenting are the most proficient. These dogs rely on odours carried by the wind, with no restrictions on point of origin or scent articles (ARDA, 2010). SAR dogs are vital when time is of the essence for finding survivors trapped by natural disasters such as avalanches, hurricanes, tornadoes and earthquakes. To this effect, the Swiss Army started training the first avalanche or snow rescue dogs in the 1930s. Today, a well-trained avalanche dog is the equivalent of 20 human searchers and can cover the same amount of search area in an eighth of the time (Gilmore, 2002). Earthquakes are another example in which SAR dogs are heavily utilized. Canine teams have searched through earthquake debris in Izmit and Duzce in Turkey, in Touliu, Taiwan and, most recently, in Haiti (ARDA, 2010). In a worldwide relief effort, dozens of countries, including the USA, France, China, Russia, Peru, Canada, Spain and Mexico, sent SAR dog teams to Haiti after the devastating earthquake in January 2010 (Viegas, 2010).

Unfortunately, natural disasters are not the only time in which SAR dogs are one of our greatest assets in the search for those still living. ARDA teams assisted in SAR efforts after both the Oklahoma City bombing in 1995 and the Embassy bombing in Nairobi, Kenya in 1998 (ARDA, 2010). An estimated 250–300 SAR dogs were used to locate victims of the World Trade Center and Pentagon attacks on 11 September 2001 (Otto *et al.*, 2004). Owing to the high number of dogs and the severity of the 11 September 2001 disasters, a cohort of these search and rescue dogs have undergone preliminary investigations, and remain in an ongoing study aimed at identifying behavioural and physiological effects resulting from their work at 11 September disaster sites. Preliminary examinations found serum concentrations of globulin, bilirubin and alkaline phosphatase activity to be significantly elevated in deployed dogs as opposed to non-deployed control dogs, suggesting antigen or toxin exposure, although these higher levels were still within normal-range values. There were also no pulmonary abnormalities detected on radiographs and no significant differences in behaviour or medical history identified (Otto *et al.*, 2004). An update released in 2010 reported a higher incidence in radiographic cardiac abnormalities in the deployed dogs and there are plans to continue future surveillance (Otto *et al.*, 2010). SAR dogs may provide an early warning for biological effects to both animals and humans in disaster areas.

Although some groups exclusively use breeds such as the German Shepherd Dog or Labrador Retriever, SAR dogs include a wide variety of both pure-bred and mixed breed dogs. Key components to a successful SAR dog include proven scenting ability, hardiness and versatility to given climates and terrain, a strong retrieval drive, and proper training (ARDA, 2010).

Service Dogs

The Americans with Disabilities Act (US Department of Justice, 2011) defines a service animal as any guide dog, signal dog, or other animal individually trained to provide assistance for an individual with a disability. On a more personal level, service dogs provide

freedom, independence, security and companionship to people with disabilities. The law ensures that service animals are permitted into restaurants, stores, modes of transportation or any privately owned business that serves the public (US Department of Justice, 2011). Twenty-seven countries, divided into the regional chapters of Europe, Asia, North America, Latin American and Australia/New Zealand, are recognized as having well-established assistance dog programmes. Assistance Dogs International (ADI) is a coalition of these organizations established to bring individual groups together to improve service working dogs (ADI, 2011). The most prevalent service dogs are those that guide the blind and hearing impaired. Morris Frank, a blind American, and Dorothy Harrison Eustis, a dog trainer of German Shepherd Dogs, helped to found the first guide dog school in America, The Seeing Eye, Inc. in 1929 (The Seeing Eye, 2011).

Since the 1970s, dogs have been formally trained to alert the hearing impaired to sounds common in the home and work environments, such as ringing telephones, doorbells, a baby's cry, smoke alarms, the presence of other people and even incoming e-mail alerts. Unlike seeing eye dogs, which are primarily German Shepherd Dogs, Labrador Retrievers and Golden Retrievers, or a mix thereof, hearing assistance dogs are commonly obtained from shelters and selected for size (small to medium build), friendliness and high energy (Emert, 1985c; Coren, 2002).

Mobility assistance dogs are specifically trained to fulfil a variety of needs of their wheelchair-bound handlers. These individuals include persons suffering from muscular dystrophy, multiple sclerosis, spinal cord injuries or other conditions that result in ambulatory motor impairment. The dogs can open doors, pick up items, and assist in showering and dressing, the washing and drying of clothes, and grocery shopping. They are also trained for emergency situations in which they are able to pull their owners up from a sitting or lying position, remove them from dangerous situations, and call for help. Assistance dogs not only improve the well-being of their owners, they alleviate some of the financial burdens and time constraints placed on caregivers (Allen and Blascovich, 1996).

In recent years, service dogs have proven themselves valuable to persons afflicted with chronic medical conditions such as epilepsy, diabetes, paralysis, autism and numerous psychological disorders. In the case of epilepsy assistance, several schools now train dogs to respond to either the onset of a seizure (seizure assist) or to detect the seizure prior to occurrence (seizure alert). These dogs are able to remove their owners from dangerous surroundings, provide comfort during the seizure and call for help if necessary (Weisbord and Kachanoff, 2000). Seizure victims with assistance dogs show reduced anxiety, increased self-esteem, and reported a feeling of control and predictability. Both the dogs and the concurrent lifestyle changes seen in their owners may be contributing factors to a reduced seizure frequency found in those persons with trained dogs (Strong *et al.*, 1999).

Autistic children are some of the newest beneficiaries of assistance dogs. The dogs provide both comfort and calming to children in overstimulated situations, such as in stores or doctors' offices, or among large groups of people. They encourage positive social interaction, which helps to improve language and behaviour. The dogs can also be trained to alert on impulsive behaviour or find a child who acts impulsively – often exemplified by running away. While there is a wide range in the severity of autism, assistance dogs have proved their worth to both the families of and the children with autism (Friedman, 2010; Assistance Dogs for Autism, 2011).

A few well-established service dog programmes such as The Seeing Eye (2011) and Guide Dogs of America (2011) have their own large-scale breeding programmes (Leighton, 2010; Maki, 2010). Throughout the years, they have focused on producing high-quality guide dogs in the most efficient and economic manner. This has involved keeping extensive records on each dog's health and performance ability and using said information to selectively breed for the most desirable traits. Responsiveness, trainability, distraction rate and overall programme completion are some of the performance characteristics scored on individual dogs, along with health issues including hip dysplasia and cancer incidence. More recently, genetic research has been applied to

such programmes. One approach is to use a dog's phenotypic performance and the health scores previously mentioned along with breeding population information, including estimated inbreeding values and genetic diversity, to predict an individual's breeding value. The breeding value score can then be used as a quantitative measure to assist in the optimization of the breeding programme, focusing on retaining high-performing dogs and high population genetic diversity, and reducing the frequency of deleterious health issues (Maki, 2010). Programmes like that of The Seeing Eye possess an invaluable DNA repository, including phenotypic ratings and pedigree information, which can be used for genome-wide association studies (GWAS) with respect to the health and performance characteristics selected for in these dogs (Leighton, 2010; Maki, 2010).

Therapy Dogs

Therapy dogs may not fit neatly into the common understanding of a working dog, but they do provide an invaluable service for the people they encounter. The dogs are individually owned dogs that have undergone specific training and certification to provide emotional support for individuals in the care of hospitals, nursing homes, veterans' homes, special needs centres and schools. One of the main purposes of a therapy dog is to improve the emotional well-being of the people that they encounter during their visits (TDInc., 2010). While the patient seems the obvious target to receive such attention, families, friends, nurses and doctors all benefit from therapy dogs. A 2-year survey of facilities incorporating therapy dogs found that their most prominent benefits for the patient were increased physical activity, alertness, verbalization, socialization and a positive mood alteration. Families and staff also found the dogs' visits beneficial because they decreased tension and stress and increased patient/staff interactions. While therapy dogs are thought to have beneficial physiological effects on patients, such as decreasing blood pressure levels, actual studies have yet to be conducted to support these theories (Jones, 1998). As of 2009, over 21,000 therapy dog/handler teams were registered with Therapy Dog International group, which is one of the main therapy dog certification and registration organizations servicing the USA and Canada since 1976 (TDI, 2010).

Hunting Dogs (Pointing, Retrieving, Tracking, Coursing)

As previously mentioned, the earliest known use for dogs was in assistance to obtain food for humans. These dogs, along with modern breeds such as the Greyhound, Whippet, Rhodesian Ridgeback, Afghan Hound, Borzoi, Ibizan Hound, Italian Greyhound, Pharaoh Hound, Saluki, Irish Wolfhound and Scottish Deerhound, were bred and trained for the chase and capture of game. Breeds were developed to specialize in the particular prey they sought and the consequent climate and terrain that they hunted in. For instance, Irish Wolfhounds were renowned in medieval times as a hunter of wolves and large Irish Elk, but also served as a war dog and guardian to both family and estate. The royal dog of Egypt, the Saluki, often hunted in tandem with falcons which located prey such as the gazelle, which the Saluki would then run and capture. The hare was the natural prey for both Greyhounds and Whippets, which are renowned for their speed and agility (see Fig. 22.1 and the American Kennel Club – AKC, 2011). However, not all hunting dogs are a recognized pure breed. The Lurcher, for example, is a mixed-breed dog of predominantly sighthound ancestry. The most common Lurcher cross is that between a Greyhound and a Scottish Deerhound; these are also known as Longdogs or Staghounds. During the Middle Ages, it was illegal for anyone but royalty to own pure-bred hunting dogs or to hunt in the royal forests, so gypsies and peasants owned Lurchers and used them to steal game from royal lands, thereby providing families with much needed food (Celtic Lurchers, 2007).

Coursing dogs, particularly the Whippet, have been a recent highlight in genetic research on performance mechanisms. Mosher *et al.* (2007) identified a mutation in the myostatin gene (*MSTN*) of racing whippets which they quantitatively linked to increased athletic

performance. Dogs carrying only one copy of the two base pair deletion consistently excelled in competition, with faster race times. Heterozygous dogs also exhibited a more muscular phenotype compared with dogs homozygous for the wild genotype. Dogs homozygous for the mutation produced a 'double-muscling' phenotype detrimental to the breed and their performance (Fig. 22.2). Broader investigation found that neither the Greyhound nor the Lurcher, both of which are used for racing, possessed the *MSTN* mutation. Authors speculated that the lack of the *MSTN* mutation in the Greyhound could be due to small sample size or the fact that the mutation is not beneficial to racing Greyhounds and is not, therefore, experiencing selective pressure. The Whippet *MSTN* mutation documented the first performance enhancing polymorphism discovered in a canine athlete (Mosher *et al.*, 2007).

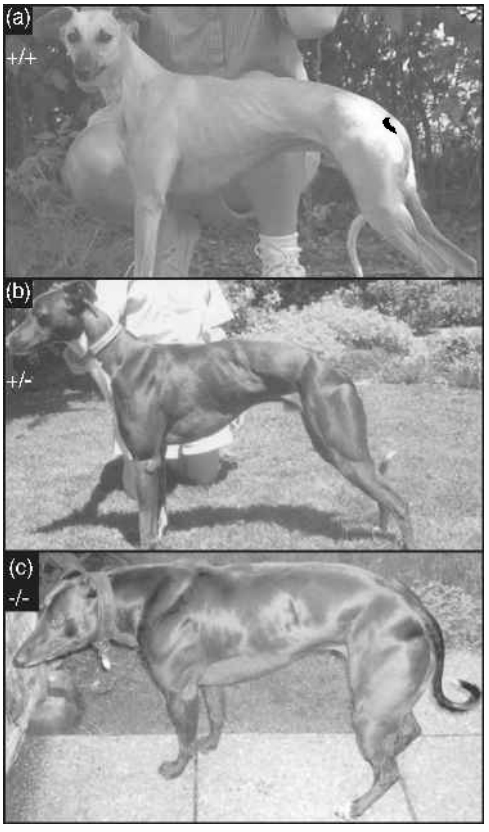

Fig. 22.2. A 2 base-pair deletion in exon 3 of the myostatin (*MSTN*) gene alters muscle composition in whippets. (a) A whippet homozygous (+/+) for the wild type *MSTN* allele. (b) A whippet heterozygous (+/–) for the *MSTN* mutation. (c) A whippet homozygous (–/–) for the *MSTN* mutation showing the 'double-muscling' phenotype.

Certain types of game are more efficiently hunted by dogs capable of tracking or scenting their prey. Scent hounds originated from Celtic mastiffs with superior scenting abilities that were selectively bred to decrease both body size and aggressiveness, while increasing speed and endurance (Wilcox and Walkowicz, 1995). From their early war dog ancestry, scent hounds rose in status as favoured hunting dogs of the British aristocrats throughout the 1800s and early 1900s. Today, the Foxhound (Fig. 22.1), Bloodhound and Coonhound are used not only for traditional foxhunting but also as trackers of large game, specifically the bear. Smaller scent hounds like the Beagle and Basset Hound are frequently used to track and flush out rabbits (AKC, 2011).

Dogs that have been bred to instinctively point, flush or retrieve game in partnership with a hunter using a rifle or shotgun are commonly referred to as gun dogs. Pointing dogs such as the English Pointer have been around since the mid-1600s. They were originally used to locate prey for the purposes of netting fowl or for Greyhounds to be set after hare. Now, they are included among other pointing dogs such as the Gordon Setter, Irish Red and White Setters, Irish Setter, German Short-haired Pointer and German Wire-haired Pointer (Fig. 22.1). A flushing dog is a dog trained to flush out prey, particularly birds, by first finding the prey and then driving it from its hiding spot for capture. The closely related spaniels and cockers are primarily used for flushing purposes. Retrieving dogs include the Labrador Retriever, Golden Retriever, Chesapeake Bay Retriever and the Portuguese and Spanish Water Dogs, to name but a few. Their main service is to retrieve birds or other prey and return them to the hunter without damage. Retrieval is often from water or marshland areas, depending upon the type of fowl hunted. An important attribute of retrievers is to have a 'soft mouth', which pertains to the dogs' willingness to carry game in their mouths without biting it. Many gun dogs are versatile and

therefore capable of performing two or even all three of these tasks on a variety of game (AKC, 2011).

In modern culture, where hunting is not a necessity to most people, many individuals still choose to hunt with dogs. For some, dogs are a practical means of access to the game they pursue. For others, it is the satisfaction and gratification of working a well-trained dog and the sport of hunting. There are still a few individuals who continue the partnership between working dogs and falcons, or between two different styles of hunting dogs, such as pointers and sighthounds. For either hunting or dog-training enthusiasts, there are now dog sporting events for all styles of hunting dogs. Sighthounds can be found at the racetrack competing for the rank of fastest dog or in field trial lure coursing events which assess both skill and drive but are not conditional on speed (CHAMP, 2011). Both scent and gun dogs are tested in a wide variety of field trials (American Field, 2011). Innate skills such as scenting, pointing, retrieving and flushing are tested along with the training aptitude of the dogs to follow their hunters' commands (AKC, 2011).

Breed behavioural traits such as pointing, retrieving and flushing have proven both interesting and challenging to geneticists. While there is a multitude of breeds exhibiting these traits, there has been minimal success in identifying their genetic basis. To date, only pointing behaviour has produced a single quantitative trait locus (QTL) located on canine chromosome 8 (CFA8) at 33344686 bp (further detail is given in the next section on farm dogs) (Jones *et al.*, 2008; Chase *et al.*, 2009). The number of breeds, the shared and divergent ancestry of those breeds, and the range in which a breed exhibits one or more of the traits add to the complexity of identifying causative genes. There is also the confounding effect of a dog's training on phenotype assessment, which increases the difficulty of pinpointing the genetic basis of these behavioural traits.

Farm Dogs (Herding, Guarding)

Flock guard dogs have been present for roughly 6000 years, migrating with Neolithic tribes out of Turkey and Iran to move throughout Africa, Europe and Asia. They were bred for an intimidating size capable of confronting bears and wolves, an independence to be able to work without human direction, hardiness to survive rugged terrain and feed themselves, and a dedication and loyalty to protect their flocks (Fig. 22.1). It was also necessary for guard dogs to possess speed, agility and endurance when fending off predators, and imperative that they not have any tendencies to hunt or chase their flocks. Flock guard dogs are generally introduced to their charges at 8 weeks of age and will live with their flocks full time. Their thick, white coats lend flock guard dogs strategic advantages by providing ample warmth and protection from the harsh climates they live in, allowing them acceptance into the flock, providing easy visual distinction between them and predators, and making them visible to owners at a distance when they are apart from the flock (Wilcox and Walkowicz, 1995).

Herding dogs have been around since the 1570s, and are derived from both Nordic dogs and the ancient dogs of Tibet and East Asia. Although early dogs were developed to work with reindeer, most eventually switched to sheep, with some varieties selected especially for heeling cattle and hogs. Today, there is even an occasional herding dog employed on turkey ranches. The manner in which a herding dog works ranges from the 'huntaway' or heeler to the 'strong-eyed' dog. The heeler dogs drive their flock by circling, barking and nipping at the heels. The 'strong-eyed' dogs generally work in more open terrain with a silent countenance and use their positioning and stare to move the flock in the desired direction (Fig. 22.1). Cattle and hog farmers crossed herding breeds with mastiff or flock guard dogs to create a tougher, more aggressive herding dog capable of handling the more dangerous livestock (Wilcox and Walkowicz, 1995).

Competitive trials are also very popular among herding dogs. Similar to hunting dog field trials, herding dog trials score dogs based on their training response, skill level and the efficiency with which they accomplish their task. The AKC sanctions standardized, non-competitive events for herding and flock guard dogs through which a dog's basic instinct and trainability are measured. This includes the

Instinct Test, which is based solely on natural instinct, therefore requiring no prior training (AKC, 2011). Competitive field trials for hunting and herding breeds help to preserve and develop their innate skills and trainability while demonstrating that breeds can still perform the useful functions for which they were originally bred.

Obedience, rally and agility are competitive events displaying a dog's trainability and, in the case of agility events, they also show a dog's versatile and agile nature. While hunting and herding breeds are common to these events and may excel owing to their working dog nature, AKC competition is open to all pure breed and mixed breed dogs enrolled in either the AKC Canine Partners Program, Purebred Alternative Listing Program, or members of the Foundation Stock Service Program (AKC, 2011). Obedience, rally and agility were designed as competitive events for the average pet owner as opposed to the hunter or farmer employing a working dog. The events are based upon the handler's ability to train and the dog's aptitude to follow direction and perform the desired tasks. They eliminate the need for game or farm animals and minimize the area required to perform (AKC, 2011).

From a research standpoint, hunting and herding dogs are a means to associate specific breed behavioural traits with contributing loci and genes. To this effect, a SNP-based GWAS used 2801 dogs, representing 147 breeds, to identify loci associated with the phenotypic characteristics of pointing, herding, boldness and trainability. The GWAS used across-breed mapping to identify markers near or at fixation in breeds possessing the targeted phenotype. Individual dogs were genotyped using 1536 SNPs, of which 674 were spaced across the 38 chromosomes. Another 862 SNPs were concentrated in regions demonstrating maximum allele frequency variation between breeds. The resultant median distance between markers was 409 kb. Table 22.1 lists ten loci associated with these behaviours and potential candidate genes within those regions (Jones *et al.*, 2008; Chase *et al.*, 2009). This research needs to be examined further to identify causative genetic factors.

Sled Dogs

Sled dogs have provided a means of transportation, protection and companionship in northern snow-dominated climates for many years. They were specifically bred for hauling cargo-laden sleds across the Arctic terrain (Rennick, 1987; Collins, 1991). The origin of sled dogs varies widely depending upon the evidence upon which one relies. Inuit dogs are often regarded as the earliest and purest line of sled dogs, deriving from dogs migrating across the Bering Sea with the Thule people, ancestors of

Table 22.1. A genome-wide SNP (single nucleotide polymorphism) scan was used to associate SNP markers with the performance behaviours of herding, pointing, boldness and trainability in 2801 dogs from 147 breeds (after Jones *et al.*, 2008; Chase *et al.*, 2009).

Trait	Chromosome no.	Position (bp)	Log *P*	Candidate genes
Herding[a]	CFA1	27630805	7.20	*MC2R, C18orf1*
Boldness[b]	CFA1	67693978	4.26	
Herding[b]	CFA4	42765963	4.83	
Boldness[b]	CFA4	40782966	4.15	*DRD1*
Pointing[b]	CFA8	33344686	5.33	*CNIH*
Trainability[b]	CFA10	13396503	3.77	
Herding[b]	CFA15	44229716	4.89	
Boldness[a]	CFA15	44137464	5.05	
Boldness[b]	CFA17	15478350	4.40	
Boldness[a]	CFA22	25446003	6.09	*PCDH9*

[a]Markers were set at a genome-wide threshold significance of 0.001.
[b]Markers were set at a threshold significance of 0.05.

the Inuit people – who include natives from Canada, Denmark (Greenland), Russia (Siberia) and the USA (Alaska) – between 500 and 1100 CE (Montcombroux, 2002). Eventually, the Inuit dog was regarded as belonging to the native cultures of Greenland, Canada and Alaska (ISDI, 2011). The names of Eskimo dog, predecessor to the Alaskan Malamute, and husky became commonly used in reference to Inuit dogs. The Chukchi, an indigenous Siberian people, developed the Siberian Husky (AKC, 2011) – which was known earlier as the Arctic Husky (UKC, 2011) – as a long-distance sled dog approximately 3000 years ago; this dog was later brought into Alaska in the 1900s. We can speculate that the Inuit dog, with its subsequent descendants including the Alaskan Malamute and Alaskan sled dog, along with the Siberian Husky, originated from the same stock of early northern domesticated dogs.

The 'Era of the Sled Dog' extended from the late 1800s to the early 1900s, and encompassed the days of the Alaska Gold Rush and early polar exploration (Wendt, 1999). The Royal Canadian Mounted Police were enforcing the law in northern territories with sled dog patrols as early as 1873 (ISDRA, 1998). Local residents and gold prospectors relied on sled dogs both for their individual transportation and as a valuable freight system for delivering mail, supplies and passengers throughout the north (Wendt, 1999). In 1908, the All Alaska Sweepstakes, the first formally organized sled dog race, provided a distraction from the long dark winters, an excuse for celebration and gambling, and the opportunity for dog drivers to prove the skill of their teams and win prize money (Wendt, 1999). Sled dogs also served as an integral part of polar exploration. Robert Cook, Frederick Peary, Roald Amundsen, Admiral Byrd, Robert Scott and Ernest Shackleton are some of the most notable polar explorers to use sled dogs during their expeditions (Vaughan, 1990). For vehicular use, the sled dog became nearly obsolete in the 1930s as modern modes of transportation became accessible. Dog drivers therefore turned their full attention to the establishment of the sport of sled dog racing, in which the once working class dog evolved into a high performance athlete (Wendt, 1999).

Sled dog racing has diverged over the past century into two vastly different racing styles: sprint (short distance) and distance (long distance) racing. Sled dogs competing in distance racing are selected primarily for their endurance abilities as they cover several 100 miles during multiple days of racing. Teams generally consist of 12–14 dogs averaging 12 mph and carry approximately 250 lb of survival gear, including food, a cook stove, sleeping bag and axe (Iditarod, 2010; Yukon Quest, 2010). The Iditarod Sled Dog Race is one of the most recognized long-distance sled dog races in the world. It commemorates the dedication and indomitable spirit of 22 sled dog teams who relayed serum over 1000 miles from Anchorage to Nome in Alaska during a diphtheria outbreak in January 1925. A statue of Balto, the lead dog of the team, reaching Nome stands in New York City's Central Park to honour the heroic efforts of these sled dogs (Iditarod, 2010). The Yukon Quest Sled Dog Race is another well-known long-distance race following the prospector and mail courier routes of the Gold Rush between Whitehorse, Canada and Fairbanks, Alaska. While the Yukon Quest is slightly shorter than the Iditarod, covering 991 miles, it is famed as the 'most difficult sled dog race in the world' due to the harsh weather, difficult trail – consisting of four mountain ascents over 3400 ft in elevation – and the limited support for mushers and dogs (Yukon Quest, 2010).

The fundamental element of a sprint sled dog is its speed (Fig. 22.1). Top sprint teams average 18–25 mph with optimal snow and trail conditions. Sleds can be as light as 14 lb, requiring only minimum gear such as the dog bag and snow hook. Where distance racing is reminiscent of a marathon, sprint racing is more analogous to track events, with classes defined by the number of dogs on a team and the distance covered. There are five common classes held at sprinting events: the 4-dog, 6-dog, 8-dog, 10-dog and Open or Unlimited class (ten or more dogs). On average, the course distance each class competes over equals a mile for each dog on the team. Therefore, the 6-dog class is held on a 6 mile course. Distance requirements for the Open or Unlimited class range from 12 to 30 miles. Teams are timed and repeat the same course

over 2 or 3 consecutive days. The overall winner is the team with the fastest combined time for each trial (ISDRA, 2011). The Open North American Championship, held in Fairbanks, Alaska, is renowned as the most established sprint sled dog race and has been run annually since 1946 (ADMA, 2011). Sled dog racing was a demonstration sport in both the 1932 Lake Placid, New York and the 1952 Oslo, Norway Olympic Games. Unfortunately, neither debut earned sled dog racing official Olympic status (Hegener, 2010). Despite the lack of Olympic recognition, a World Championship event is held every 2 years in which drivers must compete for rights to represent their country and participate in the championship (IFSS, 2011).

Modern Alaskan sled dogs are a recognized population with northern working dog ancestry that have been selected solely for their performance. They are not confined to a breed standard of size or appearance and therefore are not formally recognized as a breed by the AKC. Consistency in sled dog behaviour and selection for athletic ability have produced dogs of a particular physique. They are known for their quick, efficient gait, pulling strength and endurance. Weight, averaging 55 lb, and density of coat vary depending upon the racing style, geographical location, lineage and cross breeding to pure-bred lines.

The ability of elite sled dogs to excel in performance while under extreme physical and mental stress has gained them public and scientific notoriety. Distance dogs in particular have been the focus of numerous physiological studies. For instance, one study found that repetitive endurance exercise resulted in electrocardiographic changes reflecting cardiac hypertrophy (Constable *et al.*, 2000). Another associated an increased prevalence of gastric lesions in elite distance dogs, as is common in elite human and equine athletes (Davis *et al.*, 2003). Data have also suggested that only modest exercise is required to increase intestinal protein loss while substantial exercise is required to cause alterations in the proximal gastrointestinal tract (Davis *et al.*, 2006). Several studies have evaluated haematological, hormonal and enzymatic levels in association with long-distance training and racing (McKenzie *et al.*, 2005, 2007, 2008, 2009; Durocher *et al.*, 2007; Davis *et al.*, 2008). In particular, enhanced endurance performance has been associated with sled dogs having higher plasma vitamin E concentrations (>40.7 $\mu g\ ml^{-1}$) proving 1.9 times likelier to finish the Iditarod Sled Dog Race and have 1.8 times less of a risk of being withdrawn from the race (Piercy *et al.*, 2001).

Alaskan sled dogs recently proved their uniqueness and importance in the exploration of performance genetics. The two distinct racing populations of Alaskan sled dogs, distance and sprint, are selected respectively for their endurance or speed capabilities. Therefore, the gene coding for angiotensin-converting enzyme (*ACE*), which was previously associated with human endurance, and the myostatin (*MSTN*) gene, previously associated with Whippet speed enhancement, were explored in the sled dog. While neither gene produced genetic variants associated with either endurance or speed, a SNP within the *ACE* gene significantly distinguished between the sprint and distance sled dog populations (Huson *et al.*, 2011).

The northern mixed breed ancestry of Alaskan sled dogs has also provided a rare opportunity to explore the origins of their working dog nature. A genetic investigation into the breed composition of the Alaskan sled dog identified them as a distinct genetic breed developed solely for their athletic prowess in comparison with recognized pure breeds, which are commonly standardized by their physical appearance (Huson *et al.*, 2010). The study also identified domestic breeds such as the Alaskan Malamute, Siberian Husky, Pointer, Saluki and Anatolian Shepherd as lesser component parts of the genetic composition of Alaskan sled dogs. Breed identification and population clustering values were determined by assessing allele frequency patterns and similarities in an unbiased STRUCTURE analysis using a panel of 96 microsatellite-based markers (Parker *et al.*, 2004, 2007; Huson *et al.*, 2010). Differences in both the breeds present and the population clustering values of the component breeds were analysed for population structure within Alaskan sled dogs, and for breed performance enhancement in both distance and sprint dog populations. Clustering analysis distinguished

between the extreme genetic representatives of distance and sprint racing dogs and a third population consisting of genetically similar dogs from both racing styles (Fig. 22.3). Differences in the proportions of major component breeds – based on allele frequency patterns – in the three sled dog clusters are shown in Fig. 22.4. Variance in the breed composition between elite and poor-performing Alaskan sled dogs within each racing population was assessed using phenotypic ratings for speed, endurance and work ethic. The dominant breed component of all Alaskan sled dogs, and that which contributed to enhanced performance of both racing populations and all three phenotypes, was the 'Alaskan sled dog' breed itself. The most influential domestic breed components were the Alaskan Malamute and Siberian Husky, which increased by 11% each in distance dogs exhibiting high endurance. The Saluki demonstrated a positive influence in speed performance of sprint dogs and the Anatolian Shepherd showed a positive enhancement in work ethic within distance dogs (Table 22.2) (Huson *et al.*, 2010). This research sets the stage for the discovery of genes associated with the athletic attributes of speed, endurance and work ethic by capitalizing on the benefits of both mixed breed and pure breeds selected for performance.

Summary

Understanding the genetic aspects of performance in working dogs is a vastly complex area of research. It is common knowledge that the selective breeding of dogs for specific traits is based upon genetic heritability. However, only a small amount of concrete genetic data is currently known with respect to the performance of working dogs. Reliable phenotypic characterization and the genetic complexity of performance traits are the primary difficulties in pinpointing genes and causative mutations. Nevertheless, working dogs are valuable resources for performance attributes, with each new discovery providing an important building block for future research.

Working dogs offer a wide range of behavioural, metabolic and physiological performance attributes to explore. Current research has identified loci associated with the inherent

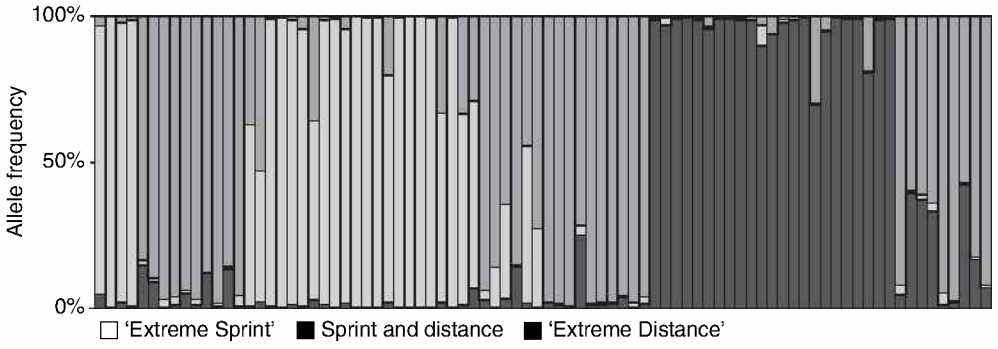

Fig. 22.3. Allele frequency patterns assign sprint and distance sled dogs to three populations in a study of the population structure of 84 unrelated Alaskan sled dogs of even distribution between four sprint racing and four distance racing kennels. The 42 Alaskan sled dogs from the sprint kennels are on the left side of the figure and the 42 Alaskan sled dogs from the distance kennels are on the right side of the figure. Each population is designated by a different shade in the chart. Individuals are categorized based on the percentage of their allelic pattern belonging to each of the populations. One population (light grey) consists only of dogs from sprint racing kennels and is referred to as 'Extreme Sprint'. This population includes the most genetically distinct sprint racing sled dogs as compared with distance racing sled dogs. A second population (dark grey) consists of dogs solely from distance racing kennels, referred to as 'Extreme Distance'. This population includes the most genetically distinct distance racing sled dogs in comparison with sprinting dogs. A third population (mid grey) included a mixture of dogs from both racing styles.

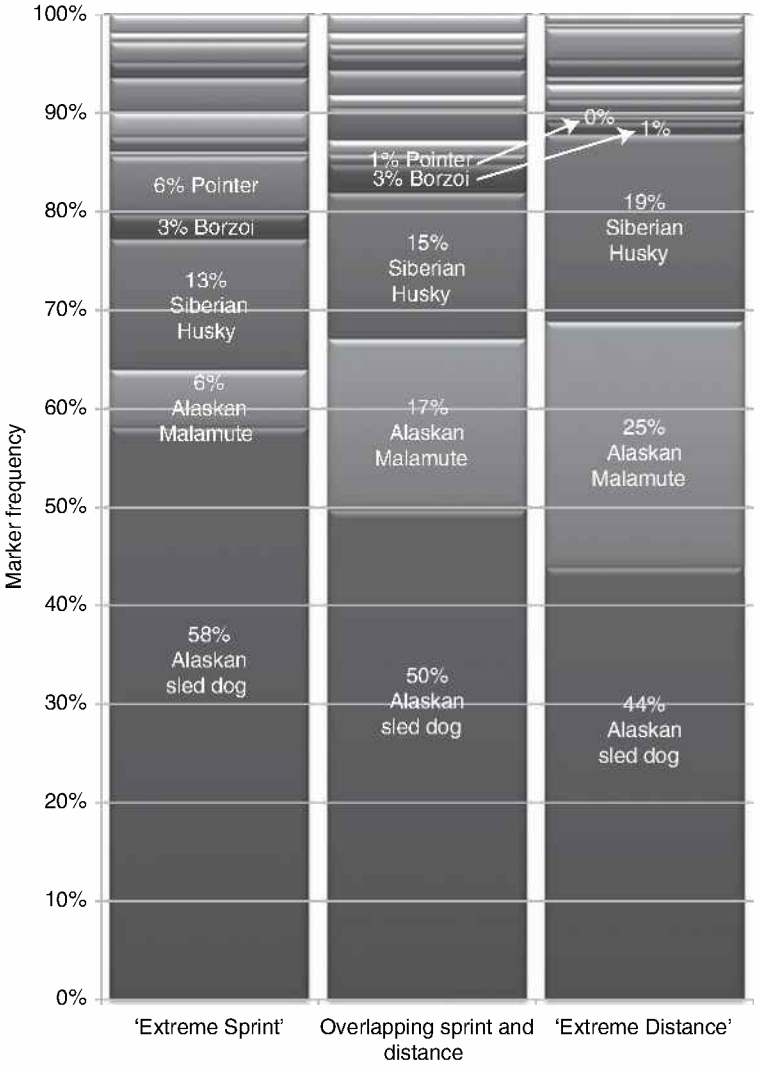

Fig. 22.4. Breed composition of Alaskan sled dogs reflected by three populations based on racing style. Alaskan sled dogs were assigned to three populations as described in Fig. 22.3, based on a clustering analysis of microsatellite-based markers (allele frequency) that are used to establish the breed composition of each group. The three populations represented 'Extreme Sprint', 'Extreme Distance' and a third overlapping population of sprint and distance sled dogs. The percentage of each breed is denoted by a different shade. The left-most group comprises ten individuals representative of the 'Extreme Sprint' sled dogs; the right-most group comprises ten individuals representative of the 'Extreme Distance' sled dogs; the middle group comprises the remaining ten sprint and ten distance sled dogs, which cluster together. There is an overall trend for increased Alaskan sled dog, Pointer and Saluki signature in sprint sled dogs and an increase in Alaskan Malamute and Siberian Husky signature in distance sled dogs.

breed behavioural characteristics of pointing, herding, boldness and trainability (Jones *et al.*, 2008; Chase *et al.*, 2009). Dog genetics has also catalogued 817 novel canine olfactory receptor genes, thus improving our understanding of the complexities of smell sensitivity (Quignon *et al.*, 2003). It has been demonstrated that genetic components of domestic

Table 22.2. The percentage change in Alaskan sled dog breed composition between high- and low-performing individuals (Huson *et al.*, 2010).

Performance phenotype	Racing style	Sled dog	Alaskan Malamute	Siberian Husky	Saluki	Pointer	Weimaraner	Samoyed	Anatolian Shepherd
Speed	Sprint[a]	5%[b]	−6%	−3%	3%[c]	−3%	1%	0%	0%
Speed	Distance[a]	25%[b]	−15%	−10%	−6%	−2%	1%	0%	3%[c]
Endurance	Sprint[a]	26%[b]	−10%	−7%	0%	−9%	−2%	0%	0%
Endurance	Distance[a]	−15%	11%[d]	11%[d]	2%	0%	0%	2%	0%
Work ethic	Sprint[a]	38%[b]	−23%	−17%	−6%	−6%	−6%	2%	0%
Work ethic	Distance[a]	11%[b]	−13%	−13%	0%	0%	0%	0%	6%[e]

[a]The average breed composition of the five most representative dogs within the given race style for each athletic attribute.
[b]There is an overall trend of increased Alaskan sled dog signature in higher performing dogs of all athletic phenotypes.
[c]The Saluki and Anatolian Shepherd show slight elevation for the speed phenotype.
[d]Alaskan Malamute and Siberian Husky show an increase in representation within distance sled dogs for high endurance performance.
[e]The Anatolian Shepherd is increased for the enhancement of the behavioural trait of work ethic in distance sled dogs.

breeds such as the Alaskan Malamute, Siberian Husky, Pointer, Saluki and Anatolian Shepherd are associated with enhanced speed, endurance and work ethic when mixed with Alaskan sled dogs (Huson *et al.*, 2010) and that a specific mutation in the *MSTN* gene may be responsible for increased racing speed in Whippets (Mosher *et al.*, 2007). Much of the current research needs to be examined further to isolate causative genetic factors, while a vast amount of metabolic, physiological and behavioural functions have yet to be explored.

Human health implications are an important factor in the genetic investigation of working dog performance. Novel loci, genes and mutations associated with performance may contribute to the selection of appropriate metabolic systems necessary for the rehabilitation of people suffering physically disabling diseases such as muscular dystrophy, fibromyalgia and chronic fatigue syndrome, or people who have experienced traumatic injuries from car accidents or combat. Likewise, the catalogue of canine OR genes and prospective breed comparisons can potentially lead to a better understanding of differences in human smell sensitivity related to age or pregnancy. Research on sled dog fatigue is an example of how working dogs offer a perspective into the body's ability to handle physical and mental stress (Robson, 2008). Working dogs have also provided an early warning or plausible expectations regarding the effect of situations such as the 11 September 2001 terrorist attacks and military deployments on responders, both human and canine (Otto *et al.*, 2004, 2010). Regardless of the many ways that dog research improves human health, it is the companionship, loyalty, integrity and courage offered unconditionally by dogs that is often cited as the most rewarding aspect of and important task in what they perform, and yet these traits are not likely to lend themselves readily to mapping.

As discussed throughout this section, there is a wide variety of working dogs performing a multitude of tasks in today's world. Tasks such as hunting, herding and guarding have remained basically the same over thousands of years, while new jobs, including search and rescue and therapy work, have been developed only recently. Some working dogs, such as sled dogs and coursing hounds, have transitioned into sporting dogs where their talents are still prized but are not required in modern-day life. That is not to say that many of these dogs do not still 'earn their living', but to recognize that in some cases their 'work' has become a competitive sport for people. Some dogs inherently possess the capability to perform jobs they were specifically bred for, while the mental drive and physical characteristics of other dogs allow them to be trained for a particular duty. The diversity of both working dogs and their tasks is a gateway to understanding the genetic complexities of behavioural and physiological performance.

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23 Genetic Nomenclature

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Introduction

Genetics includes the study of genotypes, phenotypes and the mechanisms of genetic control between them. Genetic terms describe the processes, genes, alleles and traits with which genetic phenomena are described and examined. In this chapter we will concentrate on the discussions of genetic term standardizations and, at the end of the chapter, we will list some terms relevant to genetic processes and concepts in a Genetic Glossary.

A standardized genetic nomenclature is vital for unambiguous concept description, efficient genetic data management and effective communications among not only scientists, but also among canine veterinarians, breeding societies and those individuals who are interested in the subject. This issue becomes even more evident in the post-genomics era, owing to the rapid accumulation of large quantities of genetic data, and the use of computer software to manage such data, which imposes a challenge for the precise definition and interpretation of genetic terms.

For example, the Myostatin gene (*MSTN*) is also known as Growth and Differentiation Factor 8 gene (*GDF8*) (one can also find inappropriate abbreviations such as *GDF-8* in the literature) and is referred to as the 'bully whippet' locus in dogs. While all these names are interchangeably used in the literature, it gets more complicated when one considers paralogous gene duplications across species, which led Rodgers *et al.* (2007) to propose MSTN-1 and MSTN-2 as paralogue names. Unfortunately, this naming scheme does not follow Human Gene Nomenclature Committee (HGNC) guidelines, which would indicate that the relevant genes should be named *MSTN1* and *MSTN2*. (Note that work on the standardization of human gene nomenclature is far more advanced than that in other species.)

From the previous example, we see that there is evidently a need for all researchers to follow a standardized genetic nomenclature in order for them to communicate to each other correctly on the terms they use. When a term is used by someone to describe a genetic phenomenon, the correct use of the term will help to quickly and precisely place the subject under the unambiguously defined scope. More importantly, a standard nomenclature will help to minimize the time one has to spend in differentiating the instances where two terms may

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actually mean the same or different things, which is often a costly process. The need for a standardized genetic nomenclature becomes more pressing when ontologies are employed in biological research for computers to manage the genetic terms. Ontology provides a new way to effectively use, standardize and manage genetics terms. The Gene Ontology (GO) Consortium has provided a good example (The Gene Ontology Consortium, 2000). When genomics information must be transferred across species to perpetuate genetic discoveries, the role of a standardized genetic nomenclature becomes even more important (Bruford, 2010).

The goal of this chapter is to help establish guidelines for nomenclature, with the hope that it will facilitate comparison of results between experiments and, most importantly, prevent confusion.

Locus and Gene Names and Symbols

Locus name and symbol

These guidelines for gene nomenclature are adapted and abbreviated from the Human Gene Nomenclature Committee Guidelines (http://www.genenames.org/guidelines.html).

A gene is defined as: 'A functional hereditary unit that occupies a fixed location on a chromosome, has a specific influence on phenotype, and is capable of mutation to various allelic forms. In the absence of demonstrated function a gene may be characterized by sequence, transcription or homology'. A 'locus', which is not synonymous with a gene, refers to a position in the genome that can be identified by a marker. A 'chromosome region' is defined as a genomic region that has been associated with a particular syndrome or phenotype.

Gene names and symbols will follow the human gene when 1:1 orthology is known. Gene names should be short and specific and convey the character or function of the gene. They will be written using American spelling and contain only Latin letters or a combination of Latin letters and Arabic numerals. The first letter of a gene symbol should be the same as for the gene name. The symbol will consist of upper-case Latin letters and possibly Arabic numerals. Gene symbols must be unique.

The locus name should be in capitalized Latin letters or a combination of Latin letters and Arabic numerals. If the locus name is two or more words, each word should be in capital Latin characters. The locus symbol should consist of as few Latin letters as possible or a combination of Latin letters and Arabic numerals. The characters of a symbol should always be capital Latin characters, and should begin with the initial letter of the name of the locus. If the locus name is two or more words, then the initial letters should be used in the locus symbol. The locus name and symbol should be printed in italics wherever possible; otherwise they should be underlined.

When assigning gene nomenclature, the gene name and symbol should be assigned based on existing HGNC nomenclature where possible (i.e. 1:1 for canine:human orthologues). Ensembl has used the new EPO (Enredo, Pecan, Ortheus) pipeline (Paten *et al.*, 2008) for whole-genome alignment of the dog genome. Initial efforts to provide information about genes predicted during the canine genome sequencing effort assigned standardized nomenclature based on human gene nomenclature for 3613 canine genes (http://uswest.ensembl.org/Canis_familiaris/Info/StatsTable).

There are two categories of novel canine genes: (i) novel genes predicted by bioinformatic gene prediction programs; and (ii) novel canine genes that were studied before the completion of the canine genome. In cases where no known strict 1:1 human orthologue exists, the LOC # or Ensembl ID should be used as a temporary gene symbol. In order to assign a name to a novel gene, it will need to be manually curated and assigned a unique name following HGNC guidelines.

Allele name and symbol

These guidelines for allele nomenclature are adapted from Young (1998) and mouse genome nomenclature guidelines (http://www.informatics.jax.org/mgihome/nomen/gene.shtml), in accordance with HGNC guidelines.

The allele names should be as brief as possible, yet still convey the variation associated with the allele. Alleles do not have to be named, but should be given symbols. If a new allele is similar to one that is already named, it should be named according to the breed, geographical location or population of origin. If new alleles are to be named for a recognized locus, they should conform to nomenclature established for that locus. The first letter of the allele name should be lower case. However, this does not apply when the allele is only a symbol.

An allele symbol should be as brief as possible and consist of Latin letters or a combination of Latin letters and Arabic numerals. Like a gene symbol, an allele symbol should be an abbreviation of the allele name, and should start with the same letter. The allele name and symbol may be identical for a locus detected by biochemical, serological or nucleotide methods. The wild-type allele can be denoted as a + (e.g. $MSTN^{+}$). Neither + nor – symbols should be used in alleles detected by biochemical, serological or nucleotide methods. Null alleles should be designated by the number zero. The initial letter of the symbol of the top dominant allele should be capitalized (e.g. A^{y}/a^{t} for sabled red coat colour at the Agouti locus). All alleles that are codominant should have an initial capital letter (e.g. DEA^{4}/DEA^{6} of the canine blood group; DEA is short for Dog Erythrocyte Antigen). The initial letter of all other alleles should be lower case. A single nucleotide polymorphism (SNP) allele should be designated based on its dbSNP_id, followed by a hyphen and the specific nucleotide (e.g. $MSTN^{rs1234567\text{-}T}$). If the SNP occurs outside an identified gene, the SNP locus can be designated using the dbSNP_id as the locus symbol, and the nucleotide allelic variants are then superscripted as alleles (e.g. $\mathrm{rs1234567}^{T}$).

The allele symbol should always be written with the locus symbol. Specifically, the allele symbol is written as a superscript following the locus symbol. For example, a SNP allele can be designated based on its dbSNP_id, followed by a hyphen and the specific nucleotide, as in $MSTN^{rs1234567\text{-}T}$. The allele symbol should be printed immediately adjacent to the locus symbol, with no gaps. The allele name and symbol should be printed in italics whenever possible, or otherwise be underlined.

Genotype terminology

The genotype of an individual should be shown by printing the relevant locus, gene or allele symbols for the two homologous chromosomes concerned, separated by a slash, e.g. $MSTN^{rs1234567\text{-}T/rs1234567\text{-}C}$. Unlinked loci should be separated by semicolons, e.g. $CD11^{RsaI\text{-}2400/2200}$; $ESR^{PvuII\text{-}5700/4200}$. Linked or syntenic loci should be separated by a space or dash and listed in linkage order (e.g. $POU1F1^{A/G}$–$STCH^{C/G}$–$PRSS7^{A/T}$), or in alphabetical order if the linkage order is not known. For X-linked loci, the hemizygous case should have a /Y following the locus and allele symbol, e.g. $AR^{Eco57I\text{-}1094}$ /Y. Likewise, Y-linked loci should be designated by /X following the locus and allele symbol.

Future Prospects

The Gene Ontology project is already playing a role in robust annotation of mammalian genes in the context of mutations, quantitative trait loci, etc. (Smith *et al.*, 2005). Undoubtedly, a standardized dog genetic nomenclature will more effectively facilitate efficient dog genome annotation and transfer of knowledge from information-rich species such as humans and the mouse, and make it possible for new bioinformatics tools to easily streamline data management and genetic analysis.

Several genome databases, GeneCards, Ensembl and NCBI GeneDB, have played a role in the usage of commonly accepted gene/trait notations. Undoubtedly, existing and new genome databases and tools will further develop and evolve. As such, a standardized genetic nomenclature in dogs will definitely become crucial for information sharing and comparisons between different research groups, across experiments and even across species.

In October 2009, the 'Gene Nomenclature Across Species' Meeting was held in Hinxton, England. The following recommendations from the meeting will be useful to guide the standardization of dog genetic terms: (i) gene nomenclature should reflect homologous relationships across vertebrate species; (ii) consensus naming has already

been implemented in the human, mouse, rat, chicken, zebra fish and *Xenopus*. Effort should be expanded to other vertebrate genomes; (iii) guidelines for the naming of genes across vertebrates should follow rules for the naming of paralogues and be published for sharing; (iv) the formation of novel species-specific gene nomenclature committees should be encouraged; (v) automated naming efforts should initially concentrate on consensus 1:1 orthologues as identified by at least two independent and comprehensive orthology resources; and (vi) there is a need to increase community awareness of standardized gene nomenclature, especially in journals (Bruford, 2010).

In summary, a standardized genetic nomenclature will benefit canine genetics by facilitating communication. Furthermore, it will facilitate information transfer between species.

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Genetic Glossary

Bold words are glossary entries. *Italicized words* are concepts that may be independent glossary entries as well.

Adaptation traits – Adaptation traits contribute to individual fitness and to the evolution of animal genetic resources. By definition, these traits are also important to the ability of the animal genetic resource to be sustained in the production environment.

Additive genetic effect – The effect of an allele on animal performance, independent of the effect of the other allele at a locus; these effects of the two alleles at a locus add up (thus are 'additive'). Alleles at a locus may have other effects (*dominance, epistasis*), so that there are not genes that have just 'additive' effects and other genes with only 'dominance' effects. Additive genetic effects can be inherited; other genetic effects such as dominance and epistasis are the result of allele combinations that are lost between generations. The additive genetic effect that an animal has for a trait is equal to its breeding value.

Allele – One of a pair, or series, of alternative forms of a gene that can occur at a given locus on homologous chromosomes.

Amino acid – Any one of a class of organic compounds containing the amino (NH_2) group and the carboxyl (COOH) group. Amino acids are combined to form proteins.

Ancestor – Any individual from which an animal is descended.

Animal model – A system for *genetic evaluation* that estimates breeding values of individual animals (males, females) at the same time. The system uses production data on all known relatives in calculating a genetic evaluation.

Assortative mating – Assigning animals as mates based on phenotypic or genetic likeness. *Positive assortative mating* is mating animals that are more similar than average. *Negative assortative mating* is mating animals that are less similar than average.

Autosome – Any chromosome that is not a sex chromosome.
Backcross – The cross produced by mating a first-cross animal back to one of its parent lines or breeds.
Breed – Either a subspecific group of domestic livestock with definable and identifiable external characteristics that enable it to be separated by visual appraisal from other similarly defined groups within the same species, or a group for which geographical and/or cultural separation from phenotypically similar groups has led to acceptance of its separate identity.
Breeding value – The mean genetic value of an individual as a parent. This can be estimated as the average superiority of an individual's progeny relative to all other progeny under conditions of random mating.
Categorical trait – Scores are given usually in a few categories up to several categories (e.g. scores of 1–5 for leg movement).
Centromere – Spindle-fibre attachment region of a chromosome.
Chromosome – Microscopically observable linear arrangement of DNA in the nucleus of a cell. Chromosomes carry the genes responsible for the determination and transmission of hereditary characteristics.
Codominant alleles – Alleles, each of which produces an independent effect in *heterozygotes*.
Combining ability – The mean performance of a line when involved in a *crossbreeding* system. General combining ability is the average performance when a breed or line is crossed with two or more other breeds or lines. Specific combining ability is the degree to which the performance of a specific cross deviates from the average general combining ability of two lines.
Composite (synthetic) breed – A hybrid with at least two and typically more breeds in its background. Composites are expected to be bred to their own kind, retaining a level of hybrid vigour normally associated with traditional crossbreeding systems.
Correlation coefficient – A measure of the interdependence of two random variables that ranges in value from –1 to +1, indicating perfect negative correlation at –1, absence of correlation at zero, and perfect positive correlation at +1. It determines the degree to which the movement of two variables is associated. No cause and effect is implied.
Covariance – The degree to which two measurements vary together. A positive covariance is when two measurements tend to increase together. A negative covariance is when one measurement increases and the other tends to decrease.
Crossbreeding – Matings between animals of different breeds or lines.
Crossover – The process during meiosis when chromosomal segments from different members of a *homologous* pair of chromosomes break, and part of one will join a part of the other, so that two gametes that form possess new combinations of *genes*. The frequency of crossover between two *loci* is proportional to the physical distance between them.
Crossover unit – Each unit is equal to a one per cent frequency of crossover *gametes*.
Cytoplasm – The protoplasm outside a cell *nucleus*.
Descendant – An individual descended from other individuals.
DNA – Deoxyribonucleic acid, the chemical material which carries information to code for a gene.
Dominance genetic effects – The effect that an allele has on animal performance, which depends upon the genotype at the locus. For example, the 'a' allele may have a different effect on animal performance in 'aa' animals than in 'Aa' animals. See *Additive genetic effect*.
Dominant – Applied to one member of an allelic pair of genes, which has the ability to express itself wholly or largely at the exclusion of the expression of the other allele. Depending on the location and on the type of chromosomes, there could be *autosomal dominant* or *X-linked dominant* genes.
Environment – The aggregate of all the external conditions and influences affecting the life and development of an organism.
Environmental correlation – When two traits tend to change in association with each other as a result of environmental effects.
Environmental variance – Variation in phenotype which results from variation in environmental effects.
Epistasis – When the gene at one locus affects the expression of the gene at another locus.
Estimated breeding value – A prediction of a breeding value. See *Breeding value*.
Family size – The mean number of offspring per parent that successfully reproduce.
Full sibs – Individuals with the same male and female parents.
Gamete – A sperm or egg cell containing the haploid (1*n*) number of chromosomes.
Gene – A functional hereditary unit that occupies a fixed location on a chromosome, has a specific influence on phenotype, and is capable of mutation to various allelic forms.

Genetic abnormality – A disease or phenotypic disorder that is inherited genetically.

Genetic correlation – When two traits tend to change in the same or opposite directions as a result of genetic effects.

Genetic disorder – see *Genetic abnormality*.

Genetic distance – A measure of gene differences between populations (hence genetic relationships among them) described by some numerical quantity; gene differences are usually referred to as measured by a function of gene frequencies.

Genetic drift – Changes in gene frequency in small breeding populations due to chance fluctuations.

Genetic evaluation –Predictive assessment of conformational characteristics or phenotypic improvement of potential gains to be derived by the use of the individual in question in a breeding programme.

Genetic gain – The amount of increase in performance that is achieved through genetic selection after one generation of selection.

Genetic map – See *Linkage map.*

Genetic marker – A gene or DNA sequence having a known location on a chromosome and associated with a particular gene or trait; a gene phenotypically associated with a particular, easily identified trait and used to identify an individual or cell carrying that gene.

Genetic merit – Inherited performance qualities.

Genetic resistance – Genetically determined resistance to certain infectious agents.

Genetic variance – Variation in phenotype which results from variations in genetic composition among individuals.

Genome – The complete set of *genes* and *non-coding* sequences present in each cell of an organism, or the genes in a complete haploid set of *chromosomes* of a particular organism.

Genotype – The genetic constitution of one or a few *gene*(s) or *locus* (*loci*), or total genetic make-up (genes) of an individual organism.

Genotype–environment interaction – When the difference in performance between two genotypes differs, depending upon the environment in which performance is measured. This may be a change in the magnitude of the difference or a change in rank of the genotypes.

Half sibs – Individuals that share only one common parent.

Haplotype – A set of alleles at a closely linked group of loci, so closely linked that the allelic set behaves almost as one allele in terms of inheritance.

Hardy–Weinberg law – A population is in *genotypic equilibrium* if p and q are the frequencies of *alleles* A and a, respectively, and p^2, $2pq$ and q^2 are the *genotypic frequencies* of AA, Aa and aa under the condition of *random mating*.

Heritability – Degree to which a given trait is controlled by inheritance; the proportion of total phenotypic variation that is attributable to *genetic variation* (in contrast to environment-caused variation).

Heterosis – The degree to which the performance of a crossbred animal is better or worse than the average performance of its parents.

Heterozygote, adj. **heterozygous** – An organism with unlike members of any given pair or series of alleles, which consequently produces unlike gametes.

Homologous chromosomes – Chromosomes which occur in pairs and are similar in size and shape, one having come from the male and one from the female parent.

Homozygote, adj. **homozygous** – An organism whose chromosomes carry identical members of a given pair of genes. The gametes are therefore all alike with respect to this locus.

Inbreeding – Matings among related individuals which results in progeny that have less heterozygosity and hence more *homozygous* gene pairs than the average of the population.

Inbreeding coefficient – A measurement of the increase in *homozygosity*; each unit is equal to a 1% increase in *homozygosity* relative to the average *homozygosity* in the base population.

Inbreeding depression – The decreased performance normally associated with accumulation of *inbreeding*. Many recessive genes result in undesired traits or decreased performance when they are expressed. Inbred animals have more recessive genes in the *homozygous* condition that are expressed and result in reduced performance or undesired traits.

Introgression – A breeding strategy for transferring specific favourable alleles from a donor population to a recipient population. This would, for example, be of great interest for genes responsible for disease resistance, which could be *introgressed* into a susceptible but otherwise economically superior breed.

Karyotype – The appearance of the metaphase chromosomes of an individual or species which shows the comparative size, shape and morphology of the different chromosomes.

Liability – Both internal (e.g. *genetic merit*) and external (e.g. nutrition, disease, exposure) forces that influence the expression of a threshold character (e.g. disease, conception, abnormalities, etc.).

Line breeding – Mating of selected individuals from successive generations to produce animals with a high relationship to one or more selected ancestors. It is a mild form of *inbreeding*.

Linkage – Association of genes physically located on the same *chromosome*. A group of linked genes is called a *linkage group*.

Linkage map – A linear map of an experimental population that shows the position of its known genes and/or genetic markers relative to each other in terms of *recombination frequency*.

Locus, pl. **loci** – A fixed position on a *chromosome* occupied by a given *gene* or one of its *alleles*.

Major gene – A gene that has an easily recognizable and measurable effect on a characteristic.

Marker – Specific and identifiable sequences of the DNA molecule. These markers may or may not be functional genes.

Marker assisted selection (MAS) – Selection for specific alleles using genetic markers.

Maternal heterosis – The advantage of the *crossbred* mother over the average of *pure-bred* mothers.

Mating systems – The rules which describe how selected breeds and/or individuals will be paired at mating.

Meiosis – The process by which the chromosome number of a reproductive cell becomes reduced to half the *diploid* (2*n*) or somatic number. It results in the formation of eggs or sperm.

Migration – Movement of animals, and consequently genes, from one population to another.

Mitochondria – Small bodies in the cytoplasm of most plant and animal cells responsible for energy production.

Mitochondrial inheritance – Inheritance carried by genes in mitochondrial DNA.

Mitosis – Cell division process in which there is first a duplication of *chromosomes*, followed by migration of *chromosomes* to the ends of the spindle and a dividing of the *cytoplasm*, resulting in the formation of two *cells* with the diploid (2*n*) number of *chromosomes*.

Molecular genetics – The branch of genetic studies that deals with hereditary transmission and variation on the molecular level. It deals with the *expression of genes* by studying the *DNA* sequences of *chromosomes*.

Multiple alleles – Three or more alternative forms of a *gene* representing the same locus in a given pair of *chromosomes*.

Mutation – A sudden change in the *genotype* of an organism. The term is most often used in reference to *point mutations* (changes in base sequence within a gene), but can refer to *chromosomal changes*.

Natural selection – Natural processes favouring reproduction by individuals that are better adapted, and tending to eliminate those less adapted to their environment.

Nucleus – Part of a *cell* containing *chromosomes* and surrounded by *cytoplasm*.

Outcrossing – Mating of individuals that are less closely related than the average of the population.

Overdominance – A form of *dominance* where the performance of the *heterozygote* exceeds that of the best *homozygote*.

Partial dominance – A form of *dominance* where the performance of the *heterozygote* is intermediate between that of the two *homozygotes*, but more closely resembles the performance of the *homozygous dominant* type.

Pedigree – Usually refers to *pedigree chart* or what a pedigree chart represents in genetics. It is a document to record the ancestry of an individual. A pedigree can also be used to illustrate the family structure or breeding scheme.

Penetrance – The proportion of the individuals with a particular gene combination that expresses the corresponding *trait*.

Permanent environmental effects – Environmental effects that result in permanent effects on the phenotypic expression of a trait. For example, severe mastitis during lactation may have a permanent effect on milk production and litter weaning weight for an animal in subsequent litters.

Phenotype – Actual exhibit of observable *traits*. Normally, this refers to characteristics of an individual such as size, shape, colour or performance.

Phenotypic correlation – When two traits tend to change in the same or a different direction as a net result of genetic and environmental effects.

Phenotypic value – A performance record; a measure of an animal's performance for a trait.

Phenotypic variation – Variation in phenotype which results from variation in genetic and environmental effects on the individuals.

Pleiotropy – The property of a gene whereby it affects two or more characters, so that, if the gene is segregating, it causes simultaneous variation in the characters it affects.

Polymorphism – Where *DNA* or *genes* have more than two forms or *alleles* in the population.
Population – Entire group of organisms of a kind that interbreed.
Population genetics – The branch of genetics which deals with frequencies of *alleles* in groups of individuals.
Progeny – Offspring or individuals resulting from specific matings.
Progeny test – A test used to help predict an individual's breeding values, involving multiple matings of that individual and evaluation of its offspring.
Protein – Any of a group of complex nitrogenous organic compounds that contain *amino acids* as their basic structural units, occur in all living matter, and are essential for the growth and repair of animal tissue.
Qualitative trait – A trait that can generally be classified into a limited number of categories, and the animal can be said to 'possess' the quality or not. Examples include hair colour, skin colour and ear stature.
Quantitative trait – A trait that is represented by an almost continuous distribution of measurements. Examples include body weight and height.
Quantitative trait locus (QTL) – A *locus* that affects a *quantitative trait*.
Random mating – A mating system in which animals are assigned as breeding pairs at random, without regard to genetic relationship or performance.
Recessive – Applies to one member of an *allelic pair* which lacks the ability to manifest itself when the other, *dominant,* member is present. Depending on the location and on the type of chromosomes, there could be *autosomal recessive* or *X-linked recessive* genes.
Reciprocal cross – A breeding scheme where males of breed A are mated to females of breed B and males of breed B are mated to females of breed A.
Recombination – The observed new combinations of *DNA* segments, or *loci*, or *traits*, which are different from those combinations exhibited by the parents.
Recurrent selection – A method of selection for combining ability or *heterosis*. Selection within one line is based on performance of crossbred progeny from matings with a 'tester' line.
Repeatability – The proportion of total phenotypic variation that is attributable to variations caused by genetic and permanent *environmental effects*. It is a measure of the degree to which early measures of a trait can predict later records of the same trait.
RNA – Ribonucleic acid, involved in the transcription of genetic information from DNA.
Segregation – The separation of paired *alleles* at *loci* during *germ cell* formation.
Selection – Any natural or artificial process favouring the survival and propagation of certain individuals in a population.
Selection criteria – The character(s) upon which selection decisions are based, with the intent of changing the character(s) in the *selection objective*.
Selection differential – The difference in mean performance of the selected group of animals relative to the mean performance of all animals available for *selection*.
Selection index – The combining of measurements from several sources into an estimate of genetic value; when more than one measurement on a trait, and/or measurements of the trait on relatives, and/or measurements of more than one trait are combined into a single estimate of overall genetic value.
Selection intensity – The proportion of animals selected to be parents relative to the total number available for selection. The smaller the proportion selected, the higher the selection intensity.
Selection objective – The character(s) which are intended to be modified by selection.
Sex chromosomes – The X or Y chromosomes.
Sex-influenced – Traits for which the expression depends on the sex of the individual.
Sex-limited – A trait that can be expressed only in one sex, such as milk production.
Sex linked – Genes that are located on the sex (X or Y) *chromosomes*.
Zygote – The cell produced by the union of mature *gametes* (egg and sperm) in reproduction.

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