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Analys av MHC klass II och monogena ögonsjukdomar i den skandinaviska vargpopulationen

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Abstract

In the late 1960's all the wolves in Scandinavia went extinct in the wild due to extensive hunting. In 1983 two new wolves immigrated and founded, together with a third immigrant in 1991, the population that exists today. Due to the fact that the population originates from less than a handful of individuals, the genetic variation is small. Between the years 1973 and 2011, 23 wolves of the wolves that have been sampled for blood and tissue were used in this study. A hereditary form of blindness without a known genetic etiology has been reported among captive wolves in Sweden. In an attempt to find the disease-causing gene, the study population of wolves were analyzed for presence of disease-causing mutations in the following monogenic eye diseases; Gr_PRA1, Type-A PRA, Cord1, Prcd-PRA and PLL. The analysis of eye diseases was performed with either real time PCR (TaqMan) or capillary electrophoresis (ABIPrism 3100). To gain further understanding about the genetic complexity and degree of genetic variation in the Scandinavian wolf population, the wolves were also sampled and sequenced for their genetic variation in the MHC class II loci (*DLA-DRB1*, *DLA,DQA1* and *DLA-DQB1*). In the monogenic eye tests all wolves tested normal, but this does not exclude the possibility that individuals in the population carry another mutation causing blindness. In MHC class II, eight haplotypes were found including eight *DRB1*, six *DQA1* and seven *DQB1* alleles. Compared to a common dog breed the Scandinavian wolf population has about half the MHC variation. Because of the lack of genetic variation, license hunting should be abandoned in favor of careful testing of genetic variation, and depending on the result, cautious selection of what animals to euthanize.

Keywords: MHC class II, DLA, PRA, PLL, *Canis lupus*

Sammanfattning

I slutet av sextiotalet utrotades alla vargar i Skandinavien på grund av omfattande jakt. 1983 immigrerade två vargar till Sverige och grundlade tillsammans med ytterligare en immigrant år 1991 den vargpopulation som finns idag. Eftersom populationen etablerades av så få vargar är den genetiska variationen liten. Mellan åren 1973 till 2011 provtogs vargar för blod och vävnad varav 23 användes i denna studie . Vargar i fångenskap i Sverige lider av en ärftlig form av blindhet med okänd genetisk härkomst. I ett försök att finna den orsakande mutationen analyserades den provtagna populationen för sjukdomsorsakande mutationer i följande monogena ögonsjukdomar; Gr_PRA1, Typ-A PRA, Cord1, Prcd-PRA och PLL. Analysen av ögonsjukdomarna genomfördes med antingen realtids-PCR eller kapillärelektrofores. För att få ytterligare kunskap om den genetiska variationen hos vargarna utfördes en sekvensering av MHC klass II loci (*DLA-DRB1, DLA,DQA1* och *DLA-DQB1*). För alla genetiska tester för ögonsjukdomar var vargarna normala. Hos MHC klass II fanns åtta haplotyper med åtta DRB1, sex DQA1 och sju DQB1 alleler. Jämfört med en vanlig hundras har den Skandinaviska vargpopulationen ungefär hälften av MHC variationen. På grund av den begränsade genetiska variationen borde licensjakt överges till förmån för grundliga tester för genetisk variation. Beroende på resultat skall djur noggrant utväljas för avlivning, eftersom det föreligger betydande risker att licensjakt kan resultera i ytterligare minskad genetisk variation.

Nyckelord: MHC klass II, DLA, PRA, PLL, *Canis lupus*

Abbreviations

Bp	Base pair
Cord1	Cone rod dystrophy 1 caused by a mutation in RPGGRIP1
ddH2O	Double distilled water
DLA	Dog leukocyte antigen,
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
DQA1	DQ alpha 1
DQB1	DQ beta 1
DRB1	DR beta 1
GrPRA	Golden Retriever Progressive retinal atrophy
LD	Linkage disequilibrium
mtDNA	Mitochondrial DNA
PCR	Polymerase chain reaction
PDC	Phosphoducin
PLL	Primary lens luxuation
PRA	Progressive Retinal Atrophy
Prcd	Progressive rod cone degeneration
SNP	Single nucleotide polymorphism

Contents

1 Background

The population of wolves (*Canis lupus*) on the Scandinavian Peninsula has gone through an extreme bottleneck causing severe inbreeding. In the late 60's all the wolves went extinct in the wild due to extensive hunting (Wabaken et al. 2001). In 1983 a pair of wolves that immigrated from Finland/Russia founded the population that exists today (Liberg et al. 2005). Three males have immigrated and joined the breeding population in later years, one in 1991 and two in 2008. Breeding in very small populations, such as the Scandinavian wolf population causes inbreeding which in turn reduces fitness (Keller & Waller, 2002). Inbreeding may thereby increase the risk of extinction of the population (Newman and Pilson, 1997). Populations that have experienced severe inbreeding can be genetically improved even by a single immigrant. The male that immigrated in 1991 alleviated the inbreeding depression temporarily, causing quite rapid expansion of the population (Vila et al. 2003).

The domesticated dog (*Canis lupus familiaris*) is a useful model species for studying human genetic diseases because many diseases manifests in similar ways in both species. Some examples of shared genetic diseases include diabetes, atopic dermatitis, autoimmune diseases, epilepsies, and cancers. The wolf is the ancestral species of all modern dog breeds and therefore, the dog may be considered a subspecies to the wolf. Consequently, the wolf is also an important model species since it can be used as a reference out group (Ke et al. 2010).

In captive wolves originating from the now in the wild extinct wolf population of Scandinavia, a hereditary form of blindness has been reported (Laikre et al. 2003). The genetic cause for blindness has not been studied, why it was thought important to investigate the wild Scandinavian wolves for the presence of known disease-causing mutations in a set of common canine genetic eye diseases. The blindness in the captive wolf manifests itself by cataracts, retinal dysplasia and abnormal blood vessels of the retina (Laikre et al. 2003).

Major histocompatibility complex (MHC) class II molecules play an important role in the immune system of animals. Many loci in the Mhc region are characterized by an extensive level of polymorphism and high amino acid diversity. In many species it has been shown that the observed homozygosity is lower than expected under neutrality. This is consistent with different forms of diversifying selection (e.g. overdominance and frequency dependent selection) acting on the Mhc region (Hedrick 1999). For example, it has been suggested that individuals being heterozygous at the MHC class I and II loci would have a better protection against a wider range of pathogens since they will have a greater ability of presenting a larger repertoire of peptide antigens. This selection model is referred to as overdominant selection (heterozygous advantage) and was first proposed by Doherty and Zinkernagel in 1975. Frequency-dependent selection (minority advantage) is another type of diversifying selection that would also maintain an extensive level of polymorphism at MHC loci. In the frequency-dependent model, the maintenance of alleles in the population is driven by host-pathogen interactions (Takahata and Nei, 1990). Because of the crucial importance for MHC class II molecules in defense against pathogens, the second objective of this study was to assess the genetic variation at MHC class II loci in Scandinavian wolves. In the dog as well as wolf, MHC genes are called dog leukocyte antigens (DLA).

2 Literature Review

2.1 Domestication of wolves

The wolf (*Canis lupus*) and the dog (*Canis lupus familiaris*) both belong to the Canidae family (which contains 35 species), under the genus Canis. There are six other species of Canids; two types of wolves (the red wolf, *C. rufus* and the Ethiopian or Abyssinian wolf, *C. simensis*), the coyote (the prairie wolf, *C. latrans*) and three sub-species of jackals (the golden jackal, *C. aureus*, the black-backed jackal *C. mesomelus* and the side-striped jackal, *C. adustus*) (Swintonski et al. 1996). There is yet another sub-species of wolf, the Mexican wolf (*C. baileyi*), that became extinct in the wild in the 1980's but was reintroduced to its natural habitat in the 90's. This wolf sub-species is the most endangered of all wolves and the population today originates from only seven individuals (Fredrickson et al. 2007).

Genetically, wolves and dogs are highly similar and have the same number of chromosomes, i.e. 78 chromosomes: 38 pairs of autosomes and two sex chromosomes (Swintonski et al. 1996). Phylogenetic analyses of mitochondrial DNA (mtDNA) indicate that purebred dogs were domesticated from their wild ancestor, the grey wolf (*C. lupus*), some 15000 years ago in southern China (Vilá et al. 1997; Pang et al. 2009). The reason for this conclusion is that the most diversity of mtDNA was found here compared to all other dog populations surveyed (Pang et al. 2009). On the other hand, according to genome-wide SNP and haplotype analyses, the wolf was domesticated in the Middle East (von Holdt et al. 2010). Early after domestication, when the dog was spread worldwide, there was local interbreeding between the wolf and the dog and some dog breeds, like Alaskan Malamute and Samoyed are two modern breeds resulting from such interbreeding. These breeds are also called "ancient breeds" because they were founded over 500

years ago. Dog breeds that appear to have hybridized to the European Grey Wolf in modern days, according to genomic findings in genome-wide association studies, are Miniature Pinscher, Staffordshire Bullterrier, Greyhound and Whippet (von Holdt et al. 2010).

The reason for why there are not more dog breeds today that have been mixed with wolves in modern days, even though the species co-exist, is probably the strict and controlled breeding practices founded in the Victorian era over one hundred and fifty years ago (von Holdt et al. 2010). Dogs have been clustered in 10 groups (as suggested by Fédération Cynologique Internationale, FCI) of related breeds according to their geographic origin, morphology or function in human activities (Kropatsch et al. 2011), and today there are more than 400 identified breeds of dogs (Clutton-Brock, 1999).

2.2 Dogs and wolves as model species

Genetic studies on dogs are performed both for the sake of the animals themselves and because they are important models for human disease (Galibert & André 2008; Karlsson & Lindblad-Toh 2008). Genes of canids share 75% orthology with humans (Kirkness et al. 2004). The structure of the species is unique with extensive Linkage disequilibrium (LD) within breeds, causing a reduced need of number of genetic markers to be used in genetic association studies. LD is when alleles at two or more loci exhibit non-random association. The degree of LD between breeds is very low and resembles that between different human populations. This, along with the shared environment with man, makes the dog a useful model for studying genetic diseases of humans (Lindblad-Toh et al. 2005; Galibert & André 2008).

Within dog breeds, LD is extensive because of the special way the breeds were created with two distinct bottlenecks (Ke et al. 2010). The first bottleneck occurred when the wolf was domesticated, and the other when the breeds were formed. Greater LD within breeds is caused by them being more genetically similar, or even inbred. Lesser LD between breeds is consequently due to the fact of there being less genetic similarity. In dogs, the LD ranges from hundreds of kilobases up to megabases, compared to humans where the LD is only around tens of kilobases (Lindblad-Toh et al. 2005). Ke et al. (2010) has shown that the dog that had the highest LD in the study of 19 dog breeds, Coyotes, Grey wolf and Ethiopian wolf, was the Boxer, with the Beddlington terrier as a close second. The lowest LD was exhibited by the coyote and the grey wolf, where the coyote sam-

ples were taken from New Mexico and the wolf samples from isolated populations in Alaska and Northern Canada. As a result of the much more homogenous genome structure within dog breeds compared with human the number of Single Nucleotide Polymorphisms (SNPs) needed to cover the entire genome in genome-wide association is much smaller for the dog than humans (Lindblad-Toh et al. 2005).

2.3 Diseases and defects

Defects that have been reported in wolves include transitional vertebra, which is an asymmetrical development of the vertebral body, bite defects, cryptorchidism and defects of the pulmonary system. These defects have also been found in dogs, and are in some cases heritable (Räikkönen et al. 2006, Report from SVA, 2010 & 2011). It has been documented that wolves can be affected by at least seven viruses, 14 ectoparasites and 60 endoparasites. Of specific interest is rabies, Parvo virus, Sarcoptic mange (*Sarcoptes scabiei*) and Echinococcus. These pathogens have the potential to decimate the wolf population critically, and secondly, all except for Parvo virus are zoonotic (Kreeger 2003). In Scandinavian wolves, a few cases of chronic joint inflammation have been reported (Report, SVA 2010).

The effects of inbreeding that have been documented are lower first-year litter size and lower probability for adult wolves to enter the breeding population (Bensch et al. 2006). It has been reported that for every tenth the inbreeding coefficient increases, the litter size decreases by 1.2 pups (Sand et al. 2010). In addition, the population has a higher frequency of congenital malformations than more outbred wolf populations such as the Finnish-Russian (Räikkönen et al 2006).

2.4 Progressive Retinal Atrophy

There are many types of inherited retinal degenerations in canids. Almost all of these retinal diseases cause dysplasia or degeneration of the rods and cones of the eye, leading to gradual loss of vision and eventually blindness. Collectively, these conditions are called progressive retinal atrophy (PRA), and are characterized by rod photoreceptor degeneration and deterioration of the cone photoreceptors. The homologous counterpart of PRA in humans is retinitis pigmentosa (Mellersh et al. 2006; Miyadera et al. 2009). In 2009, 15 mutations in 11 genes reported to cause PRA were known in 34 breeds of dogs. Some breeds display multiple mutations leading to very similar PRA phenotypes (Miyadera et al. 2009).

2.4.1 Cord1

One of the PRA diseases in dogs is the recessive cone – rod dystrophy (Cord1) of miniature longhaired dachshunds (MLHD), English springer spaniels, Beagles, French Bulldogs and Labrador retrievers (Miyadera et al. 2009). In this condition, which is implied by the name, there is primary cone degeneration followed by destruction of the rods. Cord1 is located on chromosome 15 (Cfa15) which carries a gene encoding for the retinitis pigmentosa GTPase regulator-interacting protein (*RPGRIP1*). In the affected breeds a nonsense mutation in the *RPGRIP1* is associated with Cord1. Affected dogs have a 44bp insertion of 29 poly A's followed by a duplication (GGA AGC AAC AGG ATG) of the 15 bp immediately upstream the poly A signal. The insertion is located in exon 2 which causes a frameshift and a premature stop codon located early in exon 3 that terminates translation of the mRNA, hence truncating the protein or nonsense mediated decay (nmd) (Mellersh et al. 2006; Miyadera et al. 2009).

2.4.2 Prcd-PRA

Another form of PRA is the late onset, autosomal recessive, rod-cone degeneration (Prcd-PRA) reported in more than 18 dog breeds (Zangerl et al. 2006). Early signs of the disease include pupil dilation, night blindness and increased reflectivity of the eye (Petersen Jones, 2005). The Prcd gene has to date an unknown function and is located on canine chromosome 9. The disease is caused by a G to A transition at nucleotide 5 (of exon 1) of the coding sequence that results in a change of cysteine into tyrosine in the second amino acid (Zangerl et al. 2006).

2.4.3 Gr_PRA1

Gr_PRA1 is another type of PRA is a late onset disease (diagnosis at ~6 years of age) found in Golden Retrievers. Gr_PRA1 is caused by a frameshift mutation in the solute carrier anion exchanger gene *SLC4A3* on chromosome 37. The mutation is thought to be caused by a cytosine insertion in exon 16 which is predicted to cause a premature stop codon in exon 18. The premature stop is considered to either yield a truncated protein or cause degradation of the mRNA (Downs et al. 2011).

2.4.4 Type-A PRA

A mutation in the phosphoducin (*PDC*) gene causes an autosomal recessive form of PRA (Type-A PRA). The mutation gives rise to a disease that is initialized by loss of night sight followed by loss of sight at daytime caused by abnormal devel-

opment of photoreceptors (Zhang et al. 1998). Phosphoducin is a phosphoprotein found in the cytosol of the photoreceptor cells of the retina and pineal gland (Abe et al. 1994). The disease is caused by a C to G transversion at nucleotide 244 leading to an arginine to glycine substitution at codon 82 (Zhang et al. 1998).

2.5 PLL

Primary lens luxuation (PLL) is a displacement of the lens of the eye, ectopia lentis, and has many possible etiologies. The disorder is very painful, and although not a type of PRA it leads to blindness and is common in many breeds of terriers, but has been reported in 85 different breeds of dogs (Sargan et al. 2007). One form is caused by a mutation in the gene *ADAMTS17* that belongs to a family of genes encoding for metalloproteases that modifies extracellular proteins. The disease has similar genetic etiology as the human disease Weill-Marchesani syndrome (WMS) (Ahram et al. 2009). PLL has been recognized by veterinary medicine for over 75 years and it has several different, both genetic and environmental, causes. At 3-8 years of age luxation occurs (Curtis 1980) but ultrastructural abnormalities can be seen as early as 20 months of age (Curtis 1983). The responsible mutation is located in the splice donor site of exon 10 and is a G to A transition that causes destruction of the splice donor recognition site. Mutation of the transcript of *ADAMTS17* causes it to lack exon 10 and to give rise to a frameshift and a resulting downstream premature stop codon (Farias 2010).

2.6 MHC

The major histocompatibility complex (MHC) is a genomic region with genes involved in the adaptive immune system. MHC codes for three classes of proteins where class I and II molecules are antigen presenting glycoproteins. Class I molecules are present on all somatic cells but class II molecules are only found on professional antigen presenting cells such as macrophages, B-lymphocytes and dendritic cells (Trowsdale 1995, Wagner 1999, Berggren & Seddon 2005). Presentation of antigens to T-cells leads to, among other processes, elimination of infected cells.

Class I and II molecules exhibit extensive polymorphism. It is thought that a large variation of the class I and II genes provides better protection against pathogens since they will have a greater probability of presenting a wider range of antigens (Hedrick 1999). An heterozygous individual would therefore have a higher fitness

than homozygous individuals (heterozygous advantage) (Hedrick 1999). The MHC class II genes in canids include *DLA-DRB1*, *DLA-DQB1*, *DLA-DRA*, and *DLA-DQA1*. Exon 2 of the *DLA-DRB1, DLA-DQB1* and *DLA-DQA1* genes encodes the antigen-binding pocket and display most polymorphisms, whereas *DLA-DRA* has no variation (Wagner et al. 1999).

In wolves from Ethiopia (*C. simensis)* MHC class II haplotypes of exon 2 were tested for their susceptibility for rabies and the effect of rabies vaccination. Kennedy et al. (2011) hypothesized that the MHC indeed might influence the response to vaccination. It was shown that one haplotype was associated with a lower response. This would be interesting to define in the Scandinavian wolf population since one concern with moving wolves from Russia to Sweden is that they will transfer rabies into the country. It would be practical to test the prospective wolf haplotypes and with the result decide whether they are suitable for movement or not.

3 Material and methods

3.1 The studied dog population

A summary of all the dogs that were used for controls can be seen in appendix 1. The dogs were chosen for their clinical diagnosis and their availability in the laboratory.

3.2 The studied wolf population

The samples from the wolves were taken either from dead animals after hunting, during autopsy, or from live animals during marking. The samples included in the study, were taken from 23 Scandinavian wolves in Sweden and Finland between the years 1977 until 2011. They were chosen in such a way that the total variation of the present population would be captured. This was possible since only three individuals and two later immigrants founded the current Scandinavian population. In Table 1 all the sampled wolves along with important information, such as identity, type of tissue and gender can be viewed.

Table 1. Table of all the sampled wolves, where one wolf (V14) had an unknown year of death

Id	Identity	Type	Sex	Y. O. D	Comment
V1	D-77-01	Tissue	M	1977	Immigrant, no surviving offspring
V2	D-79-01	Tissue	F	1979	Immigrant, no surviving offspring
V3	D-84-03	Tissue	M	1984	Offspring Nyskoga 1 couple
V4	D-85-01	Tissue	F	1985	Immigrant, Alpha-female Nyskoga 1
V5	D-85-02	Tissue	M	1985	Offspring to Nyskoga 1 couple
V6	D-86-01	Tissue	M	1986	Offspring to Nyskoga 1 couple
V7	D-92-01	Tissue	M	1992	Offspring to Gillhov couple
V8	D-92-02	Tissue	M	1992	Offspring to Gillhov couple
V9	D-92-03	Tissue	M	1992	Offspring to Gillhov couple
V10	D-93-01	Tissue	M	1993	Offspring to Gillhov couple
V11	D-96-01	Tissue	M	1996	Offspring to Gillhov couple
V12	SFT13087	Tissue	M	1997	Shot in Finland
V13	SF230	Tissue	M	1998	Shot in Finland
V14	M-98-03	Blood	F	?	Offspring to Gillhov couple
V15	D-99-02	Tissue	M	1999	Offspring to Gillhov couple
V16	M-02-15	Tissue	M	2003	Immigrant, non reproducing
V17	D-04-13	Tissue	F	2004	Offspring Gillhov couple
V18	D-05-18	Tissue	M	2005	Immigrant, non reproducing
V19	M-07-02	Blood	M	2008	Immigrant, non reproducing
V20	M-09-03	Blood	M	Alive	Immigrant and Alpha male in Galven
V21	M-10-20	Tissue	M	Alive	Immigrant and Alpha male in Kynna 2
V23	G82-10	Blood	F	Alive	Immigrant 2010,
V24	D 91-01	Blood	F	1991	Offspring Nyskoga 2

3.2.1 DNA extraction

DNA was extracted from 200 µl blood and tissue (8mm^3) using a fenol/chloroform-isoamylalcohol protocol (Sambrook et al. 1989) at Grimsö forskningsstation (Örebro, Sweden) and sent by mail to Ultuna (Uppsala). The concentration and quality of the DNA was measured by NanoDrop ND-8000.

3.2.2 PCR

All DNA amplifications were performed using the GeneAmp 9700 (Applied-Biosystems, Foster City, CA USA). For reaction conditions see below.

3.3 Monogenic tests

All the primers and probes that were used in this study are defined in Table 2 and 3, respectively. These specific tests were chosen since they were possible to perform in the time frame of this study.

3.3.1 Cord1

PCR amplification

PCR was performed according to a protocol developed by the author. The reaction was performed with 0.5 µl of 20 ng/µl of DNA in a 10µl reaction. The reaction contained 1µl 10xPCR Buffer without $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 1 µl $MgCl_2$ (25 mM), 0.5 µl of each primer (10 µM,) 0.1 µl of dNTP (25 mM free nucleotides), 6,3 µl ddH2O and 0,1 µl Ampli Taq Gold (5 U/µl). A PCR protocol was used with an initial denaturation temperature of 94°C for 5 min. It was followed by 35 cycles of denaturation at 94°C for 1 min, then 40 seconds of annealing at 60°C and extension 72°C for 40 seconds and then a final extension at 72°C for 7 min.

Table 2. Primer sequences of the performed PCR reactions

	Primers		
Sequence	**Forward**	**Reverse**	**T7**
DRB1	CCGTCCCCACAGCACATTTC	TGTGTCACACACCTCAGCACCA	TAATACGACTCACATATAGGG
DQA1	TAAGGTTCTTTTCTCCCTCT	GGACAGATTCAGTGAAGAGA	TAATACGACTCACATATAGGG
DQB1	CTCACTGGCCCGGCTGTCTC	CACCTCGCCGCTGCAACGTG	TAATACGACTCACATATAGGG
SLC4A3	AGAGCAACCTTGTAACCCGTA	GGAAGAAGGCAATGAGAAAGG	
RPGGRIP1	CCCTTTCCTGGGACTTTAGG	CCCTCTGCCTATGTCTCTGC	
PDC	GTCTATTCCCAGATGAGCATTCAAGA	CCTGCATACATTGTCTACGATATTTACGA	
Prcd	CCTTTCTCCTGCAGACTCTGT	CCAAGGTGCTGAGTAGGAAGAG	
ADAMTS17	CACCAACGCCACCTTCTG	GATGCTCCTTCCTGTGTCAGT	

Data analysis

For the Cord1 mutation the alleles of interest were run by using capillary electrophoresis (ABIPrism 3100 Genetic Analyzer, CA, USA) with a 400HD ROX Size Standard template and the acquired data analyzed by GeneMapper 4,0 (AppliedBiosystems). The samples were run with English springer spaniels as controls, see appendix.

3.3.2 SLC4A3

PCR was performed according to a protocol developed by Louise M Downs (Genetics Department, Animal Health Trust, Newmarket, UK) (Downs et al. 2011). The reaction was performed with 0.5 µl of 20 ng/µl of DNA in a 7.5 µl reaction. The reaction contained 1.5µl 5xHotStarPCR Buffer (dNTPs + $MgSO_4$ (Qiagen, Valencia, CA, USA), 0.5 µl of each primer (10 µM) 0.1 µl of dNTP (25 mM free nucleotides), 4.45 µl ddH2O and 0,1 µl HotStar Polymerase (Qiagen) standard PCR protocol with 25 cycles was used with an initial denaturation temperature of 94°C for 10 min. It was followed by denaturation at 94°C for 1 min, then 60 se-

conds of annealing at 60°C and extension 72°C for 2 min. and then a final polymerase inactivation at 94°C for 10 min.

Data analysis

The *SLC4A3* mutation were analyzed using the ABIPrism 3100 Genetic Analyzer using a 400HD ROX Size Standard and the aquired data analyzed by GeneMapper 4,0 (Applied-Biosystems). The samples were run with Golden retrievers and Field spaniels as controls, see appendix.

3.4 TaqMan

All TaqMan real-time PCRs used in this study were performed in the same way as described for PLL.

Table 3. Probe sequences for TaqMan runs

	Probes	
Sequence	**VIC**	**FAM**
PDC	TTCTTTGTCTCGGTGAATT	TCTTTGTCTCCGTGAATT
Prcd	TGAGCCATGTGCACCAC	TGAGCCATGTACACCAC
ADAMTS17	AAACATGGAGATAAGCAG	ACATGGAGGTAAGCAG

3.4.1 PLL

The allelic discrimination was performed by real-time PCR with custom made SNP genotyping assays (Applied Biosystems) using the TaqMan StepOnePlus. Each 20µL reaction contained 10µl of TaqMan Genotyping mastermix (Applied Biosystems), 0.5µl of Assay Mix, 2µl DNA template and 7.5 µl of ddH_2O.

The PCR started with a denaturation step of 10 min at 95°C, and was followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

The wolf samples were run with miniature bull terriers and Lancarshire heelers as controls, see appendix.

3.4.2 Type-A PRA

The same real time PCR method as for *ADAMTS17* was used. The wolf samples were run with miniature schnauzers as controls, see appendix. The PCR product was tagged with M13 uni(-21) and M13 rev(-29) for sequencing.

Sequencing preparation –ExoSAP IT

To perform sequencing the primers and dNTPs had to be removed and this was performed with ExoSap IT (Affymetrix, Santa Clara, CA, USA). 2µl of ExoSAP IT was added to 5µl of PCR product and this was run on a simple PCR program at 37°C for 15 min, where the primers and dNTPS are removed, and then 85°C for 15 min to inactivate ExoSAP. After this 20µl of ddH2O was added and 2*3µl was transferred to two wells on a new plate. After this 3µl of M13 uni(-21) and rev(-29) was added to half the wells, respectively. 3 µl of a concentrated stock solution (5M) was added to each well and then diluted with addition of 12µl ddH2O.

Sequencing

The prepared samples were sent for sequencing to Uppsala genome center (Rudbeckslaboratoriet, Uppsala University).

3.4.3 Prcd-PRA

The same real time PCR method as for *ADAMTS17* was used. The samples were run with English springer spaniels, Labrador retrievers and Chinese crested as controls.

3.5 DLA

3.5.1 PCR amplification and sequencing

PCR was performed according to a protocol developed by Maria Wilbe (Wilbe et al. 2009), Dept. Animal Breeding and Genetics, SLU. The reaction was performed with about 20 ng of DNA in a 20µl reaction. The reaction contained 2µl 10xPCR Buffer without $MgCl_2$ (Invitrogen), 1.2 µl $MgCl_2$ (25mM), 0,5 µl of each primer (10µM) 0.2 µl of dNTP (20 mM free nucleotides), 13.46 µl ddH2O and 0.14 µl Ampli Taq Gold (5 U/µl). T7 was used for labeling the PCR products. A touchdown PCR protocol was used with an initial temperature of 95°C for 15 min, 14 touchdown cycles of 95°C for 30 seconds, followed by 1 min of annealing starting at 62°C (*DRB1*), 54°C (*DQA1*) and 73°C (*DQB1*). This was followed by 20 cycles of 95°C for 30 seconds, 55°C (*DRB1*), 47°C (*DQA1*) and 66°C (*DQB1*) for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min.

To perform sequencing, the primers and dNTPs had to be removed and this was performed with ExoSap IT (Affymetrix). 2µl of ExoSAP IT was added to 5µl of PCR product and this was run on a simple PCR program at 37°C for 15 min, where the primers and dNTPS are removed, and then 85°C for 15 min to inactivate ExoSAP. After this 20µl of ddH2O was added and 3µl was transferred to a new plate. After this 3µl of T7 (5M) sequencing primer was added to each well and then 12µl of additional ddH2O was added to further dilute the samples. The prepared samples were sent for sequencing to Uppsala genome center (Rudbecklaboratoriet, Uppsala University).

3.5.2 Sequence analysis

The acquired sequences were analyzed using the Macintosh software Match Tools and Match Tools Navigator (Applied-Biosystems). First the sequences of exon 2 were compared to a consensus sequence in Match Tools Navigator, where each polymorphic site was manually viewed and corrected if necessary. After this the corrected sequence was uploaded into Match Tools where it was automatically compared to a reference sequence library and the program provides a call for the matching allele. The nucleotide and peptide sequences were aligned in ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). TranSeq in EMBOSS was used for finding the protein and ORF of the nucleotide sequences (http://www.ebi.ac.uk/Tools/emboss/transeq for the peptide sequence. The haplotypes were assumed by the method of “three locus haplotypes” as described by Kennedy et al. (2002). The haplotype construction was based firstly on observing homozygotes and secondly on the assumption that there is minimal recombination between *DQA1* and *DQB1*.

4 Results

4.1 Gel electrophoresis

To ensure that proper PCR protocols were used for Cord1, *SLC4A3* and DLA all the reactions were run on an agarose gel. A picture of a gel run of Cord 1 can be seen in Figure 1 and a picture of a gel run of DLA can be seen in Figure 2.

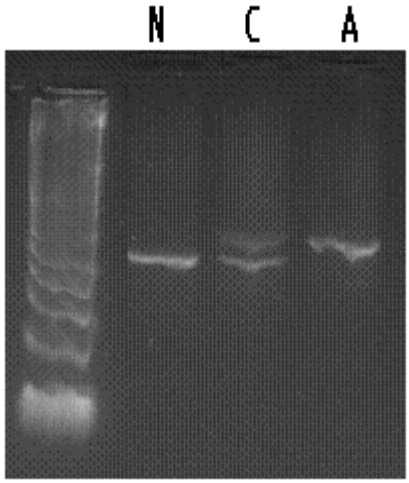


Figure 1. Agarose gel electrophoresis of the Cord1 mutation with samples of a (left to right) Normal (ESS-005), Carrier (ESS-006) and Affected (ESS-014) individual.

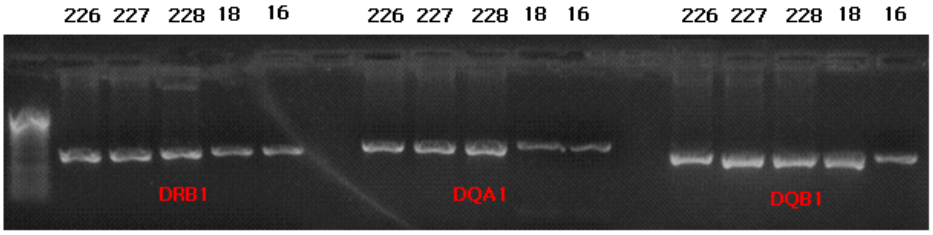


Figure 2. Agarose gel electrophoresis of the *DRB1*, *DQA1* and *DQB1* alleles in three Golden retrievers and two wolves. The *DRB1* allele was run farthest to the left with the three first samples being dogs (GR226, GR227, GR228) and the two last samples being wolves (V16, V18). The same setup was applied for the *DQA1* allele and the *DQB1* allele.

4.2 Genotyping by sequencing of MHC class II

In Table 4 the result of the DLA sequencing of exon 2 of each wolf can be seen along with their corresponding haplotypes. A list of the eight haplotypes and the alleles included is illustrated in Table 6. One wolf, V18, Table 5, was excluded from Table 4 since it was the only one carrying these specific alleles making it impossible to construct haplotypes by the method of three locus haplotypes. Eight different alleles were found at the *DRB1* loci, six at *DQA1* and seven *DQB1*.

Samples V1 and V2 were derived from animals killed in northern Sweden before the now established population existed. V4 is the female that founded the now existing population and V3, V5, V6 are its offspring. V24 is the offspring of two siblings from the Nyskoga 1 couple. All the offspring to the Gillhov couple are descendants of the original Nyskoga 1 couple. V1, V2, V16, V19 and V22 have not (yet) contributed to the genetic variation of the Swedish wolf population. Thus, the Scandinavian wolf population, according to these results, has five assumed DLA class II haplotypes. Additionally, this means that there are only four *DRB1* alleles, three *DQA1* alleles and four *DQB1* alleles in the present Scandinavian wolf population.

*Table 4. The wolves and their corresponding haplotypes organized according to relationship to eachother. Allele DQA1*012011 was previously called DQA1*01201*

ID	Status	DRB1	DQA1	DQB1	DRB1	DQA1	DQB1
V1	Immigrant, no offspring survived	02901	00301	00401	04901	05011	03901
V2	Immigrant, no offspring survived	03101	01101	04001	03101	01101	04001
V4	Immigrant, Alpha-female Nyskoga 1	03101	01101	04001	03101	005011	03901
V5	Offspring Nyskoga 1 couple	03101	01101	04001	03101	01101	04001
V6	Offspring Nyskoga 1 couple	03101	01101	04001	03101	01101	04001
V3	Offspring Nyskoga 1 couple	03101	01101	04001	04901	05011	03901
V24	Offspring Nyskoga 2	03101	005011	03901	04901	05011	03901
V9	Offspring Gillhov couple	04901	05011	03901	04901	05011	03901
V14	Offspring Gillhov couple	04901	05011	03901	04901	05011	03901
V15	Offspring Gillhov couple	04901	05011	03901	04901	05011	03901
V7	Offspring Gillhov couple	04901	05011	03901	04901	05011	03901
V17	Offspring Gillhov couple	04901	05011	03901	04901	05011	03901
V8	Offspring Gillhov couple	04901	05011	03901	04901	05011	03901
V10	Offspring Gillhov couple	03101	01101	04001	04901	05011	03901
V20	Immigrant and Alpha male in Galven	03601	012011	03501	05301	01101	01303
V21	Immigrant and Alpha male in Kynna 2	03601	012011	03501	03601	012011	03501
V16	Immigrant, non reproducing	03601	012011	03501	05401	00301	00401
V22	Immigrant 2010	03601	012011	03501	04901	05011	03901
V19	Immigrant, non reproducing	03101	01101	04001	05301	00301	00401

Table 5. The alleles of the wolf with unique DLA-genotypes.

ID	DRB1	DQA1	DQB1	DRB1	DQA1	DQB1
V18	05601	01001	05601	02002	014012	04401

Table 6. The eight different haplotypes and their respective alleles.

Haplotypes	DRB1	DQA1	DQB1
1	04901	005011	03901
2	03101	01101	04001
3	03101	005011	03901
4	03601	012011	03501
5	05401	00301	00401
6	02901	00301	00401
7	05301	00301	00401
8	05301	01101	01303

The most common genotype found in the study population of Swedish wolves, illustrated in Table 7, was homozygosity of haplotype 1, and was carried by 31,6%. The second most common form was also a homozygous form, but of haplotype 2 carried by 15,8% of the study population. These numbers does not carry any significance though, as the study population includes many offspring due to the lack of sampling of parents.

Table 7. The different genotypes and their frequency in the population

Genotypes	N	Frequency %
1,1	6	31,6
1,2	2	10,5
1,3	1	5,3
1,4	1	5,3
1,6	1	5,3
2,2	3	15,8
2,3	1	5,3
2,7	1	5,3
4,4	1	5,3
4,5	1	5,3
4,8	1	5,3

The frequency of the different *DLA* class II alleles can be seen in Tables 8-10. Two *DRB1* alleles are more common in the population, *DRB1*04901* was found in 42,5% and *DRB1*03101* was found in 30%. For *DQA1*, 005011 (47,5%) and 01101 (27,5%) was the most common and for *DQB1*, 03901 (47,5%) and 04001 (25%) was the most common. Again this was due to the sampling of many offspring.

Table 8. The frequency of the different DRB1 alleles within the study population

DRB1	N (40)	Frequency %
04901	17	42,5
03101	12	30
03601	5	12,5
05401	1	2,5
02901	1	5
05301	2	2,5
05601	1	2,5
02002	1	2,5

Table 9. The frequency of the different DQA1 alleles within the study population

DQA1	N (40)	Frequency %
005011	19	47,5
01101	11	27,5
012011	5	12,5
00301	3	7,5
01001	1	2,5
014012	1	2,5

Table 10. The frequency of the different DQB1 alleles within the study population

DQB1	N (40)	Frequency %
03901	19	47,5
04001	10	25
03501	5	12,5
00401	3	7,5
01303	1	2,5
05601	1	2,5
04401	1	2,5

A pedigree for the Scandinavian wolf population based on the study population (Liberg et al. 2005) and their haplotypes were constructed and are shown in Figure 3. In the 1980's two immigrants founded the population that exists today, out of this couple only one (the female) is sampled, V4. These two individuals were carrying three haplotypes out of four possible (four chromosomes). On these three haplotypes they carried with them two different *DRB1* alleles, two *DQA1* alleles and two *DQB1* alleles. The third immigrant (not sampled) that entered the Scandinavian population in the 80's did not carry any new alleles. Two male immigrants

entered the reproductive population in 2008 (V20, V21) and carried two new haplotypes that were introduced, giving the population well needed addition of two new alleles at every DLA class II locus. Two other males (V16, V19) immigrated into Sweden in the first decade of the 21st century but did not get a chance to reproduce resulting in a loss of valuable alleles. These two carried with them two new *DRB1* alleles. In 2010, a female wolf immigrated. This was the first female since the founder couple to enter Sweden without being quickly killed. This female did unfortunately not carry any new DLA alleles.

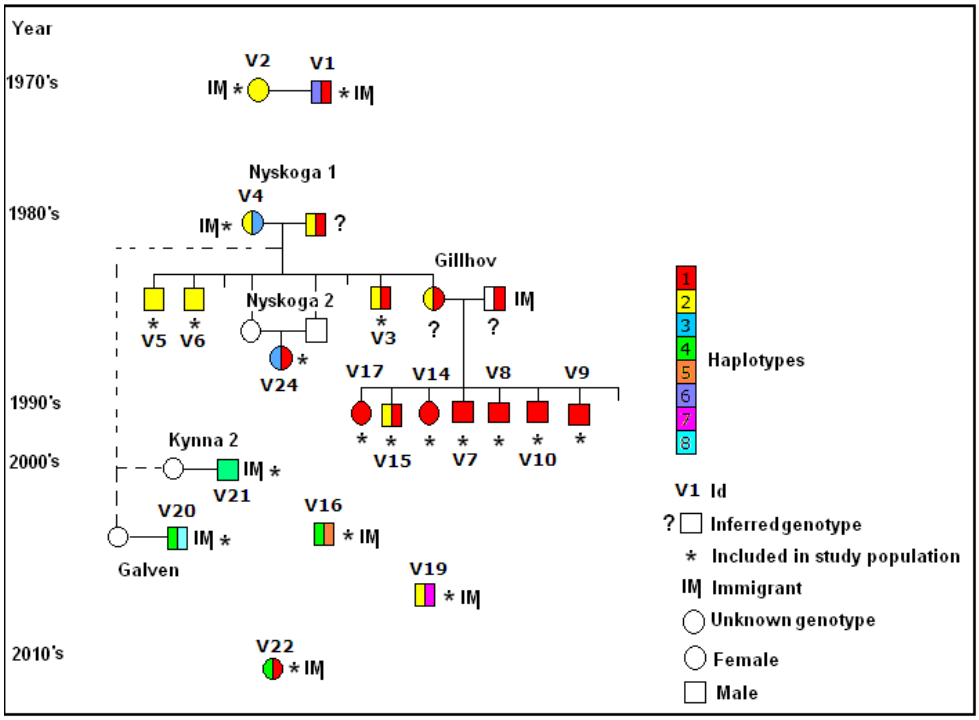


Figure 3. A pedigree of the wolves sampled for MHC class II analysis.

All the DLA alleles found in this study has previously been reported in wolves and most of them have previously also been found in dogs, only five have not. A summary of which alleles that are exclusive to wolves is shown in Table 11.

Table 11. The DLA-alleles found in this study compared to their existence in dogs (Seddon & Ellegren 2002, Seddon & Ellegren 2004 Kennedy et al. 2001, Kennedy et al. 2007a, 2007b)

	Found in	
Allele	**Dog**	**Grey Wolf**
DRB1*02002	*	*
DRB1*02901	*	*
DRB1*03101		*
DRB1*03601		*
DRB1*04901	*	*
DRB1*05301	*	*
DRB1*05401	*	*
DRB1*05601	*	*
DQA1*005011	*	*
DQA1*00301	*	*
DQA1*01001	*	*
DQA1*01101		*
DQA1*012011	*	*
DQA1*014012		*
DQB1*00401	*	*
DQB1*01303	*	*
DQB1*03501	*	*
DQB1*03901	*	*
DQB1*04001		*
DQB1*04401	*	*
DQB1*05601	*	*

An alignment of the amino acid sequences and nucleotide sequences for the identified DLA class II alleles in wolves are shown in Figures 4-9.

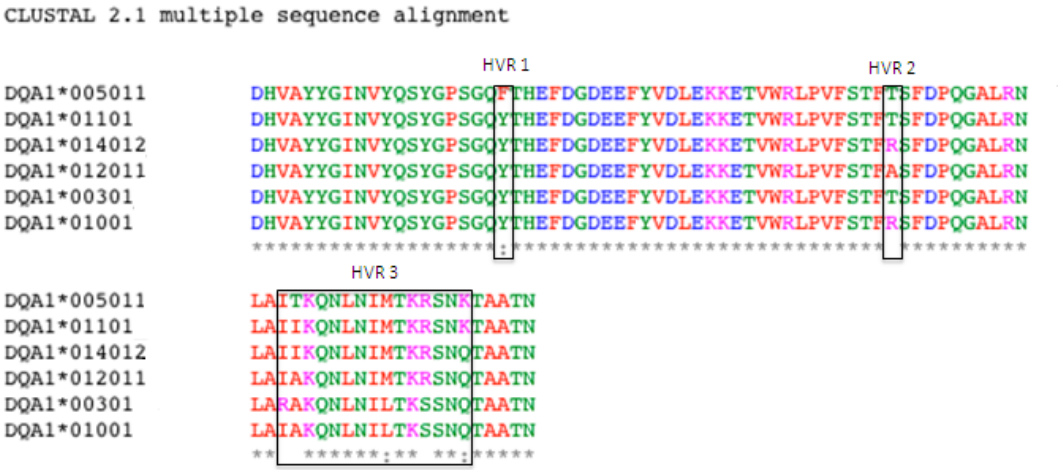


Figure 4. Amino acid alignment of the found DLA-DQA1 alleles

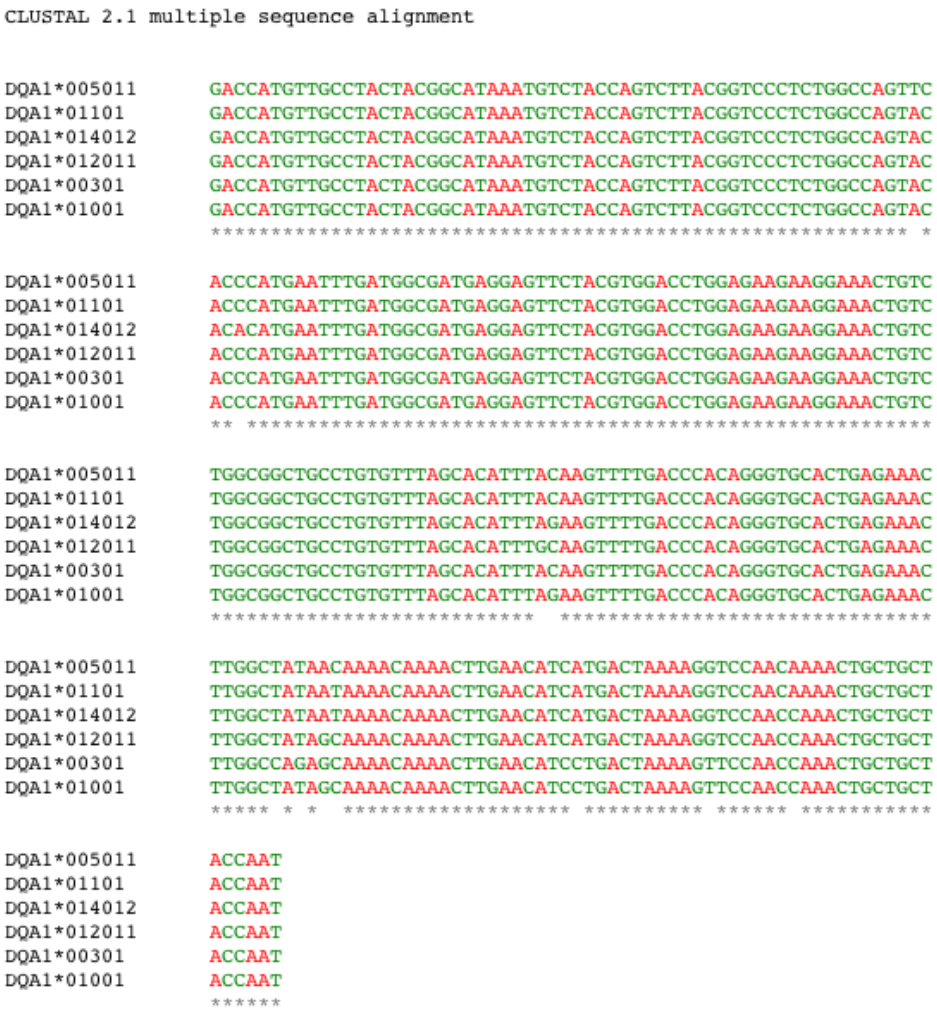


Figure 5. Nucleotide alignment of DQA1.

At the hypervariable region 1 (HVR 1) only *DQA1*005011* differs and has a phenylalanine instead of a tyrosine caused by an A →T transversion. At HVR-2 there are three different amino acids, threonine (hydrophilic), arginine and alanine. In *DQA1*012011* an A→G transition causes a change into alanine. In *DQA1*014012* and **01001* a C→G transition causes the change into arginine. HVR 3 is more polymorphic with five different sites showing polymorphism. The first site is the first amino acid of HVR 3 and has two forms; isoleucine (hydrophobic) or arginine. A C→T transition and two bases further down a G→T transversion in *DQA1*00301* is responsible for the change into arginine. The second amino acid has three forms threonine, isoleucine and alanine caused by an A→T and C→G transition in two adjacent bp. The third site is the ninth amino

acid which has two forms leucine and methionine caused by an A→C transversion in *DQA1*00301* and **01001*. Three amino acids further down the site exhibits two forms; arginine and serine caused by a G→T transversion in *DQA1*00301* and **01001*. The last amino acid of HVR 3 has two forms; lysine and glutamine caused by a C→A transversion in *DQA1*005011* and **01101*.

Figure 6. Amino acid alignment of the identified *DLA-DQB1* alleles

Figure 7. Nucleotide alignment of DQB1.

In HVR 1 of *DQB1* there are two polymorphic sites. The first site has two different amino acids; tyrosine and phenylalanine. The change in amino acid is caused by a T→A transition in *DQB1*04401, *03901* and **00401*. The second polymorphic site in HVR 1 has three different amino acids; phenylalanine, alanine, and glycine caused by a G→T transversion and a C (*DQB1*04401*), G, T polymorphism in two adjacent bp. In HVR 2 there are four polymorphic sites. The first site carries two different amino acids; alanine and threonine, which is caused by a G→A transversion in half of the alleles. The next site is adjacent to the first and also has two different amino acids; arginine and lysine which is caused by a G→T

transversion. The next site after that is also adjoining and has three polymorphisms; aspartic acid, asparagine and tyrosine caused by a G, A (*DQB1*05601*) and T polymorphism. The last amino acid in HVR 2 has a triple polymorphism; histidine, phenylalanine and tyrosine caused by a polymorphism of the triple codon in the second and third position. Three alleles have GCA (*DQB1*03501, *04401, *04401*), three have GTT (*DQB1*05601, *01303, *03901*) and one GTA (*DQB1*00401*). HVR 3 has 10 polymorphic sites out of 19 possible, making it complicated to decide what nucleotide mutation that is causing which amino acid change.

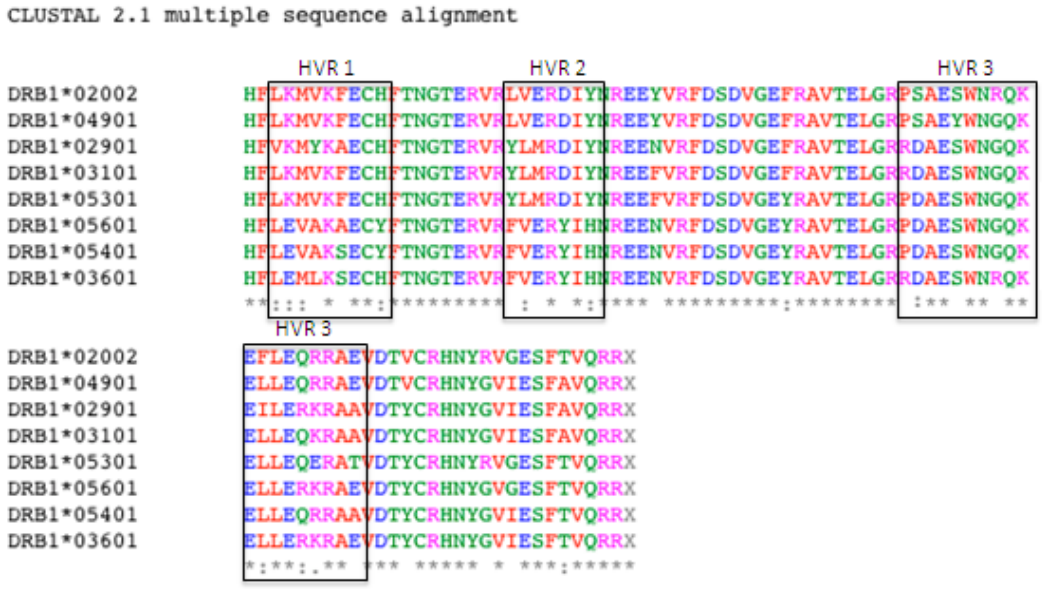


Figure 8. Amino acid alignment of the identified *DLA-DRB1* alleles

Figure 9. Nucleotide alignment of DRB1.

HVR 1 and HVR 2 of *DRB1* has six polymorphic sites, and HVR 3 has eight making it very complicated to decide which base pair substitution that is causing what amino acid change.

4.3 Monogenic tests

All the wolves were tested normal for all the diseases studied in this project. A summary of the results for all wolves can be seen in Table 13. The dogs used as controls can be seen in the appendix along with their breeds and genotypes for the different tests.

Table 13. The results of all the monogenic tests for eye diseases in wolves. Individuals denoted by ? have an unknown genotype for that gene

Id	Identity	Prcd	SLC4A3	RPGGRIP1	ADAMTS17	PDC
V1	D-77-01	Normal	Normal	Normal	Normal	Normal
V2	D-79-01	Normal	Normal	Normal	Normal	Normal
V3	D-84-03	Normal	Normal	?	Normal	Normal
V4	D-85-01	Normal	Normal	Normal	Normal	Normal
V5	D-85-02	Normal	Normal	Normal	Normal	Normal
V6	D-86-01	Normal	Normal	Normal	Normal	Normal
V7	D-92-01	Normal	Normal	Normal	Normal	Normal
V8	D-92-02	Normal	Normal	?	Normal	Normal
V9	D-92-03	Normal	Normal	Normal	Normal	Normal
V10	D-93-01	Normal	?	Normal	Normal	Normal
V11	D-96-01	Normal	Normal	Normal	Normal	Normal
V12	SFT13087	Normal	Normal	?	Normal	Normal
V13	SF230	Normal	Normal	?	Normal	Normal
V14	M-98-03	Normal	Normal	Normal	Normal	Normal
V15	D-99-02	Normal	Normal	Normal	Normal	Normal
V16	M-02-15	Normal	Normal	Normal	?	Normal
V17	D-04-13	Normal	?	Normal	Normal	Normal
V18	D-05-18	Normal	Normal	Normal	Normal	Normal
V19	M-07-02	Normal	Normal	Normal	Normal	Normal
V20	M-09-03	Normal	Normal	Normal	Normal	Normal
V21	M-10-20	Normal	?	Normal	Normal	Normal
V23	G82-10	Normal	?	Normal	Normal	Normal
V24	D 91-01	?	?	Normal	?	Normal

4.3.1 Gr_PRA1

When the genotyping result from the ABIPrism 3100 was analyzed with GeneMapper 4.0 (AppliedBiosystems) the picture in Figure 10 was acquired. The wolf samples were run against three Golden Retrievers used for routine testing and all wolves appeared to be normal at the *SLC4A3* loci.

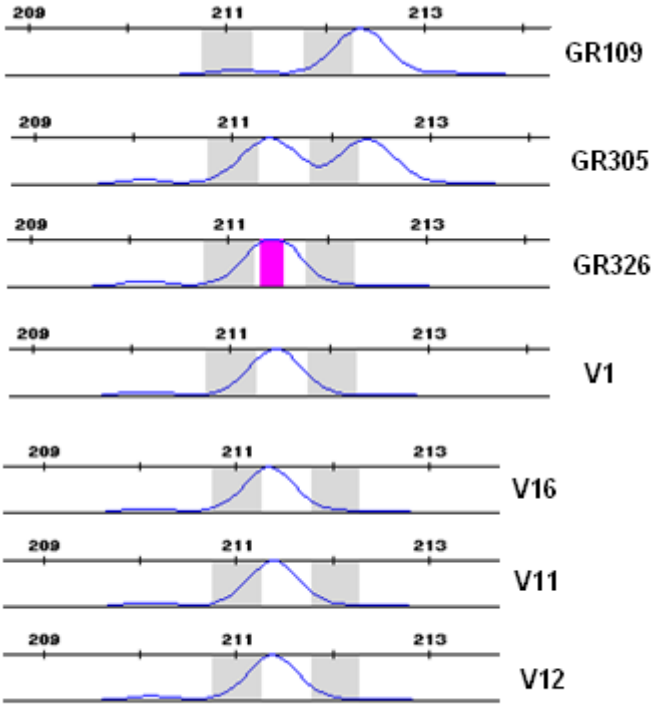


Figure 10. Screenshot from GeneMapper showing the result if the analysis of the SLC4A3 gene for the controls. GR 109 was homozygous affected, GR 305 was a carrier and GR326 was a homozygous normal. The four wolf samples included here were all normal.

4.3.2 Cord1

The analysis of the Cord1 mutation of wolves provided Figure 11. The wolf samples were run against three English springer spaniel controls and all wolves appeared to be normal at the Cord1 loci.

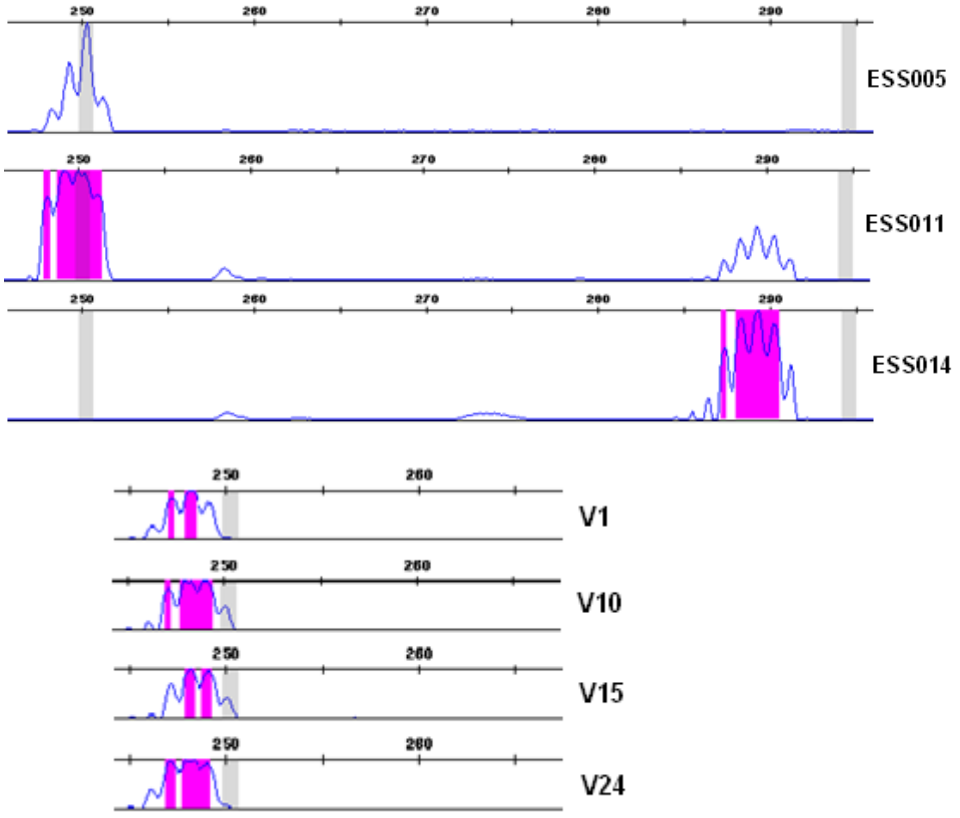


Figure 11. Screenshot from GeneMapper showing the result if the analysis of the Cord1 gene for the controls and a few wolf samples that tested normal.

4.4 TaqMan

4.4.1 Type-A PRA

The wolves were run on a TaqMan genotyping assay for PDC against four miniature schnauzer controls. All the wolves appeared to be normal as can be seen in Figure 12. To ensure that the primers were correct, sequencing was performed on two wolves. The region of the PDC gene containing the mutation of interest can be seen in Figure 13 showing that the correct primers indeed had been used.

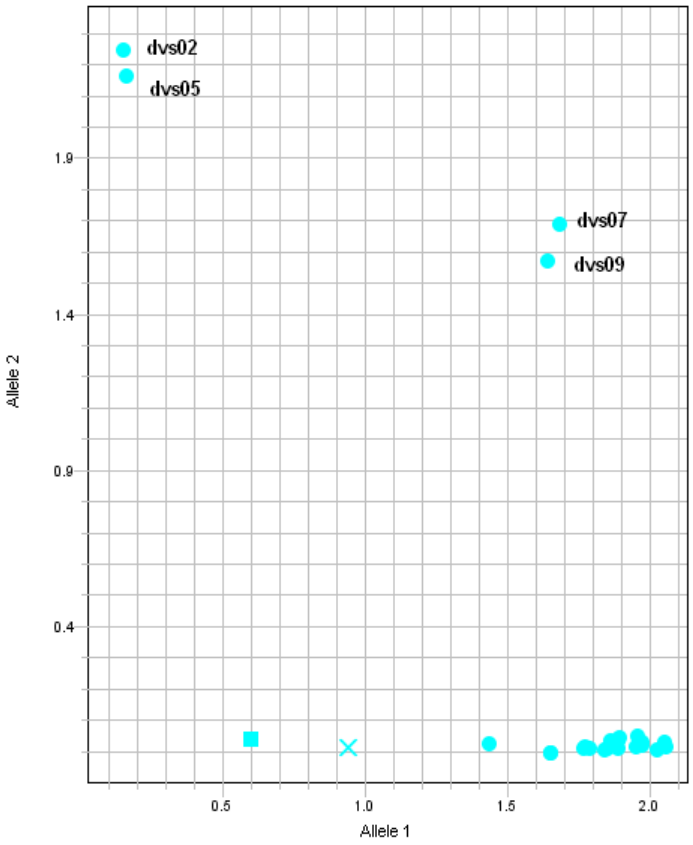
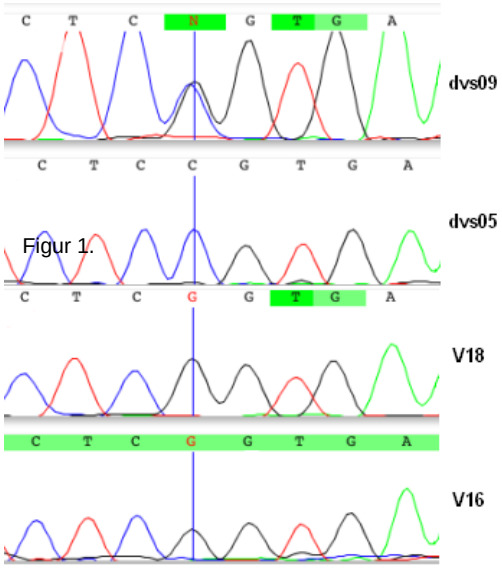


Figure 12. Allelic discrimination plot of TaqMan run of the PDC gene. All wolves (V1-V24) were homozygous normal (Allele1/Allele1). Positive control for carriers were dvs07 and dvs09 and controls for affected were dvs02 and dvs05.



Figur 1.

Figure 13. The region of the PDC sequence containing the nucleotide change (G to C) in a heterozygote (dvs09), a homozygote affected (dvs05) and two homozygous normal wolves (V18, V16).

4.4.2 Prcd-PRA

The wolves were run on a TaqMan genotyping assay for Prcd against three Labrador retriever controls. A synthetic carrier was constructed by mixing equal amounts of DNA templates from the Affected dog L1 and the Normal dog L2. L3was used as a normal control. All the wolves appeared to be normal as can be seen in Figure 14.

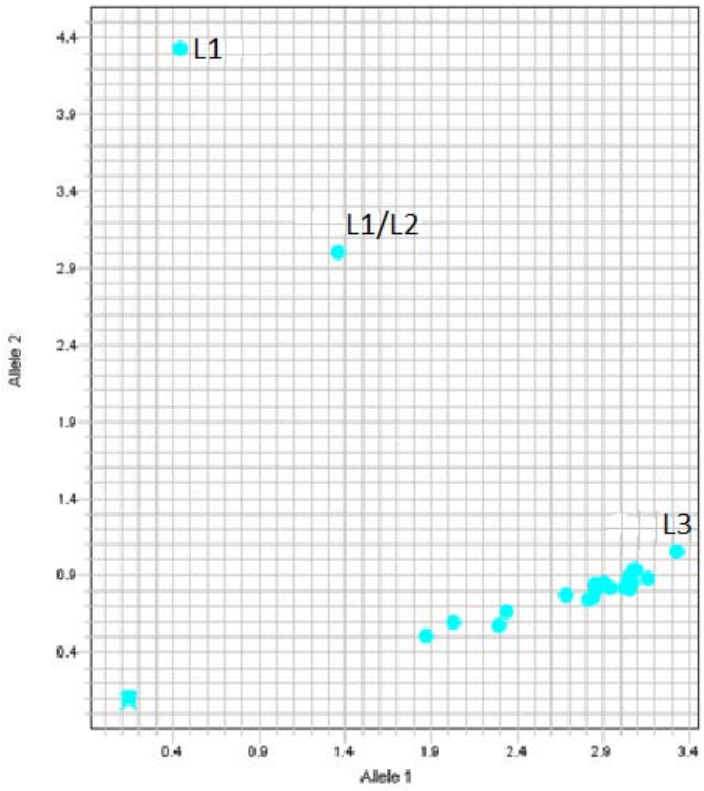
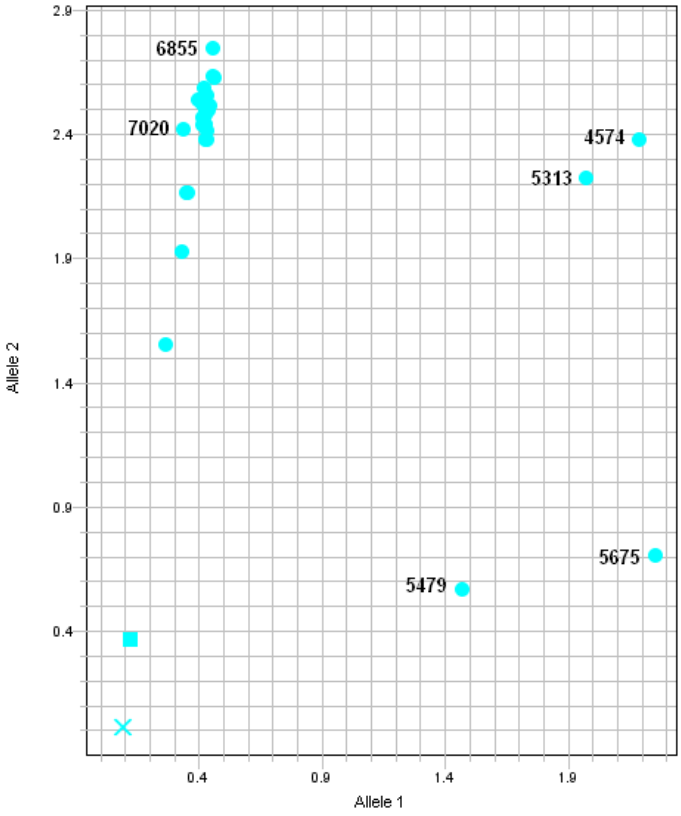


Figure 14. Allelic discrimination plot of the Prcd gene. All wolves (V1-V22/23) were homozygous unaffected (Allele2/Allele2). Positive controls for affected was L1, for Carrier a L1/L2 mix was used and for a Normal control L3 was used.

4.4.3 PLL

The wolves were run on a TaqMan genotyping assay for *ADAMTS17* against six controls from the breeds Miniature bull terrier and Lancashire heeler. All the wolves appeared to be normal as can be seen in Figure 15.



Figur 15. Allelic discrimination plot of TaqMan run of the ADAMTS17 gene. (V1-V22/23) were homozygous unaffected (Allele 2/Allele2). Positive control for carriers were 4574 and 5313, controls for affected were 5479 and 5675, and controls for Normal were 6855 and 7020.

5 Discussion

5.1 Monogenic tests

Several disease-causing mutations have been found in dogs. As dogs have a common origin with the wolf, it may be possible that some of the disease-causing alleles were already present in the wild ancestor before the divergence. Even though the wolves were normal for all the gene tests employed in this study it does not exclude the possibility that they may carry other mutations responsible for the blindness seen in captive wolves reported by Laikre et al. (1993). We did not expect to find any genetically affected wolves by these eye diseases because it most likely would have affected the longevity of the animals in the wild if they were blind, and thus this would have been reported before. However, we wanted to examine the possibility that there are carriers in the population. Also, only very few of the possible mutations were tested for. A large part of the population probably would have been affected or carriers given that many are closely related (Vila et al. 2003). The wolves in captivity today are presumably not closely related to the now existing Scandinavian population due to that they were captured in the wild before the now existing wild population (Laikre et al 1993). For future research, the wolves in captivity could (or could?) be tested for all genetic eye diseases reported in dogs. If no tests will come out positive there might be a new mutation causing the blindness. This should then be studied which could be valuable not only for the sake of the captive wolves but for human and veterinary medicine as well. If the wolves carry a known mutation more knowledge of this mutation could be gained because the population is so closely related. Closely related individuals tend to express disease mutations in higher frequency.

5.2 MHC polymorphism

Inbreeding reduces the genetic variation in a population, and variation at the MHC loci is essential for effective immunity, and thus inbreeding could be thought to decrease the variation at the MHC loci (Hedrick 1999). In the surveyed population eight MHC haplotypes were found. In 80 dog breeds surveyed by Kennedy et al. (2002) 58 haplotypes were found and 28 haplotypes in North American grey wolves were reported by Kennedy et al. (2007c). In a common dog breed, such as the Golden Retriever, eight DRB1 alleles, nine DQA1 alleles and seven DQB1 alleles have been identified (Kennedy et al. 2002). In this study, eight DRB1 alleles, six DQA1 alleles and seven DQB1 alleles were found in the total study population, which is almost the same amount. In a more inbred dog breed, like the Cocker Spaniel, only five of each DLA allele has been identified. In the Scandinavian reproductive population today, there are five haplotypes with four DRB1 alleles, three DQA1 alleles and four DQB1 alleles which is roughly half of the amount observed in Golden Retrievers. This indicates that the Scandinavian population has approximately half the variation of a normal population, if dog breeds can ever be considered as a normal population (Kennedy et al. 2002).

The wolves that were killed before entering reproduction (V1, V16, V19), carried new DLA alleles. Because these wolves were lost, the Scandinavian population never acquired four possible new DRB1 alleles (*02901, *05401, *05601, and *02002) three DQA1 alleles (*00301, *01001, and *014012) and three DQB1 alleles (*00401, *05601, and *04401). Perhaps a better way than license hunting (although no wolves in this study wolves were killed by license hunting) would be to sample wolves, possibly for analysis of DLA variation, and then from the result of this deduce what wolves should be humanely euthanized. DLA could in this way be used as a sort of marker for genetic variation. This also means that there needs to be a library for reference on existing DLA alleles in the Scandinavian population. Whether MHC diversity is essential for reproductive success or not has been debated since there are some wildlife species with little variation that are viable and yet others that are not (Kennedy et al. 2007c). Given this, one cannot be absolutely certain in concluding that wolves are in need of greater genetic variation at the MHC class II loci.

It is possible that the findings in this study mean that wolves because of the inbreeding have become less resistant to pathogens. For future research it would be very interesting to sample genotypes from wolves that have succumbed to different pathogens to see if they have little MHC variation. These findings could

then be crosschecked against their haplotypes to see what haplotypes cause a susceptibility to different pathogens. In the extension this could be beneficial for human medicine as well.

In April 2011 an offspring to V20 was moved because it was killing reindeers and causing trouble for the farmers in Norrbotten (Naturvårdsverket, 2011a). After analyzing the DLA alleles of V20 we can conclude that this was a good choice from a MHC-perspective, since it carried valuable alleles, where one was unique to the Scandinavian population, DQB1*01303.

The newly immigrated female (V23) carries DLA alleles already present in the Scandinavian population, but is still valuable because of other yet to be defined genetic variation it may carry. Specifically, no new mtDNA has entered the breeding population since the first founder couple, which is why this particular wolf is so valuable. Mitochondrial DNA is only transferred from females. MtDNA encodes for genes responsible for converting chemical energy into ATP, the energy of choice for eukaryotic cells. Accumulation of deleterious genes in mtDNA can therefore be thought as harmful. It can thus be deduced that moving this immigrant from Jämtland to Örebro in march 2011 (Naturvårdsverket, 2011b) was the correct course of action.

The wolf that had entirely different DLA alleles than the rest of the studied population (V18) may originate from a completely different location than the other sampled wolves. It was shot in Finnmark, which is a region including Norway north of Lofoten and Swedish and Finnish Lappland. This wolf would have been very valuable to integrate into the breeding population because of its unique alleles.

The wolves in the present Swedish population are thought to have immigrated from the Finnish/Russian population. When comparing DLA alleles reported in Russian wolves with the alleles from this study the following alleles have not been reported in Russia; *DRB1*02002, DRB1*03101, DRB1*03601, DRB1*04901, DRB1*05401, DRB1*05601, DQA1*01101, DQA1*012011, DQA1*014012, DQB1*03901, DQB1*04001, DQB1*04401,* and *DQB1*0560*1 (Kennedy et al. 2007b). This means that in order to obtain a comprehensive picture of the genetic variation at the MHC class II loci in Scandinavian wolves more Russian wolves, preferably those close to Finland need to be sampled.

According to this study the most polymorphic DLA locus was *DRB1* which further supports the hypothesis that recombination rarely occurs between *DQA1* and *DQB1*. The reason for why *DRB1* is more polymorphic could be that selection favors mutations in *DRB1* rather than recombination of *DQA1* and *DQB1*, and that

it is vital for the reproductive success for the latter not to recombine. The same conclusion was reached by Kennedy et al. (2007c).

After comparison of MHC alleles with the MHC alleles of Ethiopian wolves (Kennedy et al. 2010) it could be concluded that they didn't share any alleles. Because of this neither assumptions nor clues of how the Scandinavian wolf population would respond to rabies vaccination could be made.

As dogs are descendants of wolves it is not surprising that they share so many DLA alleles. It would not be unexpected if all alleles found in wolves will later be found in dogs. Neither would it be startling to discover that wolves indeed carry unique alleles, because not all wolves have contributed to the variation of dogs, and given that recombination occurs. Also, there is a possibility that recent (during the last few hundred years) wolf-dog hybridization is responsible for the shared alleles (Kennedy et al. 2007c, von Holdt et al. 2010).

During license hunting in Sweden there is no selection of which wolves that are to be shot. This can potentially lead to genetically valuable animals being lost. Because there is a difference in how much genetic variation the wolves carry, there should be a better way of choosing what animals to kill, to avoid further inbreeding.

Another, and probably more efficient, way of measuring the genetic variation instead of measuring MHC class II variation, of the Scandinavian wolf population would be to sample the wolves and run them on a 170.000 dog SNP chip (CanineHD Genotyping BeadChip, Illumina). This would pick up the total variation of the wolves in a more manageable, labor and cost effective way.

6 Conclusion

The wolves from the Scandinavian wolf population are not affected by any of the monogenic eye diseases; Gr_PRA1, Prcd-PRA, Cord1, Type-A PRA or PLL. This does not mean that they do not carry any other mutation causing hereditary blindness such as in captive wolves. The population has about half of the variation exhibited by a common dog breed at the MHC class II loci and is in dire need of all the immigrating variation it can get. Because of this, license hunting should be abandoned in favor of careful testing of genetic variation, and depending on the result, cautious selection of what animals to euthanize.

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7 Appendix

Table of the dog controls used for the various genetic tests.

Gene	Breed	ID	Genotype
RPGGRIP1	English Springer Spaniel	ESS-003	Normal
		ESS-004	Carrier
		ESS-005	Normal
		ESS-006	Carrier
		ESS-007	Normal
		ESS-008	Affected
		ESS-009	Normal
		ESS-010	Normal
		ESS-011	Carrier
		ESS-012	Normal
		ESS-013	Carrier
		ESS-014	Affected
		ESS-015	Normal
		ESS-016	Carrier
		ESS-017	Normal
		ESS-018	Normal
		ESS-019	Normal
		ESS-020	Carrier
		ESS-021	Normal
SLC4A3	Golden Retriever	GR109	Affected
		GR203	Affected
		GR305	Carrier
		GR326	Normal
	Field Spaniel	FS-01	Normal
		FS-02	Normal
ADAMTS17	Miniature Bull terrier	4574	Carrier
		4778	Carrier
		8321	Carrier
		5675	Affected
		6855	Normal
	Lancshire heeler	7018	Normal
		7020	Normal
		5313	Carrier
		7021	Normal
		5429	Affected
		5476	Affected
		5479	Affected

PDC	**Miniature Schnauzer**	dvs01 **Affected** dvs02 **Affected** dvs03 **Affected** dvs04 **Affected** dvs05 **Affected** dvs06 **Affected** dvs07 **Carrier** dvs08 **Affected** dvs09 **Carrier** dvs10 **Affected** dvs11 **Affected** dvs12 **Affected** dvs13 **Affected** dvs14 **Affected** dvs15 **Affected** dvs16 **Affected** dvs17 **Affected** dvs18 **Affected** dvs19 **Affected** dvs20 **Affected** dvs21 **Carrier**
Prcd	**English Springer Spaniel**	ESS-001 **Normal** ESS-002 **Normal** ESS-003 **Normal** ESS-004 **Normal** ESS-005 **Normal** ESS-006 **Normal** ESS-007 **Normal** ESS-008 **Normal** ESS-009 **Normal** ESS-010 **Normal** ESS-011 **Normal** ESS-012 **Normal** ESS-013 **Normal** ESS-014 **Normal** ESS-015 **Normal** ESS-016 **Normal** ESS-017 **Normal** ESS-018 **Normal** ESS-019 **Normal** ESS-020 **Normal** ESS-021 **Normal**
	Labrador Retriever	L4 **Normal** L5 **Normal** L1 **Affected** L3 **Normal** L2 **Normal**

Prcd	**Chinese Crested**	CCR-001 **Normal** CCR-002 **Normal** CCR-003 **Normal** CCR-004 **Normal** CCR-005 **Normal** CCR-006 **Normal** CCR-007 **Normal** CCR-008 **Normal** CCR-009 **Normal** CCR-010 **Normal** CCR-011 **Normal** CCR-012 **Normal** CCR-013 **Normal** CCR-014 **Normal** CCR-015 **Normal** CCR-016 **Normal** CCR-017 **Normal** CCR-018 **Normal** CCR-019 **Normal** CCR-020 **Normal** CCR-021 **Normal** CCR-022 **Normal** CCR-024 **Normal** CCR-025 **Normal** CCR-026 **Normal** CCR-027 **Normal** CCR-028 **Normal** CCR-029 **Normal** CCR-030 **Normal** CCR-031 **Normal** CCR-032 **Normal** CCR-033 **Normal** CCR-034 **Normal** CCR-035 **Normal** CCR-036 **Normal** CCR-037 **Normal** CCR-038 **Normal** CCR-039 **Normal** CCR-040 **Normal** CCR-041 **Normal** CCR-042 **Normal** CCR-043 **Normal** CCR-044 **Normal** CCR-045 **Normal** CCR-046 **Normal** CCR-047 **Normal** CCR-048 **Normal** CCR-049 **Normal** CCR-050 **Normal** CCR-051 **Normal** CCR-052 **Normal** CCR-053 **Normal** CCR-054 **Normal**

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