Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Preclinical Sciences and Pathology

Philosophiae Doctor (PhD)
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Genetic studies of health challenges and behaviour in the Havanese dog breed

Genetiske studier av helseutfordringer og atferd hos hunderasen bichon havanais

Kim K. L. Bellamy

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Poor old Black Dog. I miss him. In the early morning when I work, he's not there on the kudu skin beside the typewriter; and in the afternoon when I swim, he's not hunting lizards beside the pool; and in the evenings when I sit in my chair to read, his chin isn't resting on my foot. I miss Black Dog as much as I miss any friend I ever lost.

-Ernest Hemingway, writer and Havanese owner

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In loving memory of Mercedes
27.11.2005-27.03.2023

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1 Abbreviations and definitions

AAE: Age at examination, here defined as “Year at examination” – “Year of birth”
AAO: Age at onset
AI-REML: Average information restricted maximum likelihood
CFA: Canine chromosome
CI: Confidence interval
DRD2, *DRD2*: Dopamine receptor D2, dopamine receptor D2-gene
ECVO: European College of Veterinary Ophthalmologists
FGF4, 18-FGF4RG: Fibroblast growth factor 4-retrogene, FGF4 retrogene on chromosome 18
GCTA: Genome-wide Complex Trait Analysis
GRCh38: Genome Research Consortium human build 38
GRM: Genomic relationship matrix
GWAS: Genome wide association study
HPD: Highest posterior density
ISWT: Irish soft coated wheaten terrier
KA: Thousand years ago
LD: Linkage disequilibrium
MCMC: Markov chain Monte Carlo
NKK: The Norwegian Kennel Club
NSDR: Nova Scotia duck tolling retriever
OS: Owner score
OQ: Owner questionnaire
PBE: Provocative behavioural evaluation
PPV: Positive predictive value
NPV: Negative predictive value
REML: Restricted maximum likelihood
Sensitivity: the proportion of true positives that test positive (at a diagnostic test)
SNP: Single nucleotide polymorphism

2 List of papers

Paper I
Bellamy KKL, Storengen LM, Handegård K, Arnet E, Prestrud KW, Overall K, Lingaas F. **DRD2 is associated with fear in some dog breeds**. Journal of Veterinary Behavior, 27, 67-73 (2018). DOI: 10.1016/j.jveb.2018.07.008

Paper II
Bellamy KKL, Lingaas F. **Short and sweet: foreleg abnormalities in Havanese and the role of the FGF4 retrogene**. Canine Medicine and Genetics, 7, 19 (2020). DOI: 10.1186/s40575-020-00097-5

Paper III
Bellamy KKL, Lingaas F, Madsen P. **Heritability of distichiasis in Havanese dogs in Norway**. Canine Medicine and Genetics, 8, 11 (2021). DOI: 10.1186/s40575-021-00110-5

Paper IV
Bellamy KKL, Lingaas F. **Cataracts in Havanese: genome wide association study reveals two loci associated with posterior polar cataract**. Under review in Canine Medicine and Genetics*

*This version of the article has been accepted for publication, after peer review but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: https://doi.org/10.1186/s40575-023-00127-y

3 Abstract

Havanese is the most registered companion dog breed in Norway, and is generally a healthy and long-lived breed. However, published studies, as well as health surveys conducted by the breed club, indicate a predisposition to some issues, including cataracts, foreleg abnormalities, social fear and distichiasis. This thesis addresses these challenges and provides new information that will be useful in planning an overall breeding strategy.

In paper I, we use a candidate gene-approach to investigate the genetic background of increased levels of social fear, which is observed in almost one in five Havanese. We identify significant associations between two SNPs in a dopamine receptor gene, *DRD2*, and an increased tendency to react fearful in social situations, classified both through owner questionnaires and a simple behavioural test. Heterozygote individuals display an intermediate phenotype, compared to homozygotes for the protective- or risk allele.

In paper II, we hypothesise that chondrodystrophy, caused by an expressed fibroblast growth factor 4 (FGF4) retrogene on CFA18, may be the cause of the bowed forelegs seen in some Havanese. We genotype 355 Havanese for a linked marker and show that both the wild type- and mutant allele segregate in the breed. The frequency of the risk allele has gradually increased during the past two decades, and today, most Havanese are chondrodystrophic. The results show that heterozygote individuals are significantly taller at the shoulder than chondrodystrophy homozygotes, which indicate association with foreleg shortening, in an incomplete dominant manner. The findings support the hypothesis that angular limb deformities in Havanese are caused by chondrodystrophy.

In paper III, we estimate the prevalence and heritability of distichiasis in Havanese in Norway. The estimated prevalence is moderate (0.145), but most affected individuals are graded mildly affected. The heritability estimates are moderate to high: between 0.276 calculated by a linear model and 0.720 calculated by a Bayesian threshold model.

In paper IV, a genome wide association study is conducted for three forms of cataracts in Havanese. Two SNPs are identified, on CFA20 and CFA21, respectively, that show significant association with posterior polar cataract. Two additional peaks, on CFA4 and CFA30, show putative association with cortical cataract. All the top SNPs are positioned close to cataract candidate genes. The associated regions will be subject to resequencing and fine mapping, with an aim of identifying causative variants. The study also shows that sensitivity of cataract screening is highly influenced by age at examination.

In this thesis, genetic variants associated with increased social fear and posterior polar cataract are identified, but further studies are needed before the findings can be fully utilised in the Havanese breeding program. Until causative variants are identified and can be tested for, increasing the average age of breeding animals is likely the most effective way to improve accuracy of selection and reduce the prevalence of cataracts. Our findings support that chondrodystrophy is contributing to angular limb deformities in the Havanese breed and indicate that the population risk of foreleg pathology could be reduced by increasing the frequency of the healthier, wild type allele. The estimated heritability of distichiasis in Havanese is relatively high, which means it should be possible to control the prevalence of the disorder through phenotypic selection.

Through the use of appropriate genetic methods, the studies provide new knowledge on the genetic background of four important traits in Havanese, which can be implemented in the breeding program to further improve dog health and welfare. The thesis provides an example of how health challenges in dog breed can be systematically addressed through targeted research.

4 Norsk sammendrag

Bichon havanais er den mest registrerte selskapshundrasen in Norge, og er generelt en frisk rase med høy levealder. Publiserte studier, samt helseundersøkelser foretatt av raseklubben, viser imidlertid at rasen er disponert for enkelte tilstander, som katarakt, bøyde forben, sosial frykt og distichiasis (feilstilte øyehår). Denne avhandlingen adresserer disse utfordringene og bidrar med ny informasjon som vil være nyttig ved utarbeidelse av en overordnet avlsstrategi.

I artikkel I undersøkes det om et kandidatgen kan ha sammenheng med den genetiske bakgrunnen for forøkede nivåer av sosial frykt, som er observert hos nesten en av fem havanais. Vi identifiserer signifikante assosiasjoner mellom to SNP'er i et dopamin reseptor-gen, *DRD2*, og en økt tendens til å reagere med frykt i sosiale situasjoner, klassifisert både ved hjelp av spørreundersøkelser blant eiere, og gjennom en enkel atferdstest. Heterozygote individer viser en intermediær fenotype sammenlignet med individer som er homozygote for enten det gunstige eller det ugunstige allelet.

I artikkel II tester vi en hypotese om at kondrodystrofi forårsaket av et uttrykt FGF4 retrogen på kromosom 18, kan være årsak til de bøyde forbena som sees hos enkelte bichon havanais. Vi genotyper 355 bichon havanais for en koblet markør, og finner at både villtype-allelet og allelet assosiert med kondrodystrofi, segregerer i rasen. Frekvensen av risikoallelet har økt gradvis de siste tyve årene, og i dag er de fleste havanais kondrodystrofe. Resultatene viser at heterozygote individer har en signifikant høyere skulderhøyde enn individer som er homozygot kondrodystrofe, hvilket indikerer assosiasjon med forkortning av forbena, med en ufullstendig dominant nedarving. Funnene underbygger hypotesen om at avvikende forbenstilling hos havanais er forårsaket av kondrodystrofi.

I artikkel III estimerer vi prevalens og arvegrad for distichiasis hos bichon havanais i Norge. Den estimerte prevalensen er moderat (0.145), men de fleste av de affiserte individene er gradert som mildt affisert. Arvegradsestimatene er moderate til høye:

mellom 0.276 beregnet ved hjelp av en lineær modell og 0.720 beregnet ved hjelp av en Bayesiansk terskelmodell.

I artikkel IV gjennomføres en genomvid assosiasjonsanalyse for tre former for katarakt hos bichon havanais. To SNP'er identifiseres, på henholdsvis kromosom 20 og 21, som viser signifikant assosiasjon med bakre pol-katarakt. Ytterligere to regioner, på kromosom 4 og 30, viser mulig assosiasjon med kortikal katarakt. Den sterkest assosierte SNP'en i hver av de fire kromosomregionene ligger i nærheten av katarakt kandidatgener. De assosierte regionene vil bli nøye kartlagt, med et mål om å identifisere kausale varianter. Studiet viser også at sensitiviteten av kataraktundersøkelser i stor grad avhenger av alder ved øyelysning.

I avhandlingen identifiseres genvarianter som er assosiert med økt sosial frykt og bakre pol-katarakt, men videre studier er nødvendig før disse resultatene kan implementeres fullt ut i avlsprogrammet for bichon havanais. Det å øke gjennomsnittlig alder på avlsdyr trolig være det raskeste og mest effektive tiltaket for å redusere forekomsten av katarakt, og etter hvert kan en ha stor nytte av å bruke kunnskap om genetiske varianter/kausale gener i avlsarbeidet. Våre funn underbygger at kondrodystrofi er medvirkende årsak til avvikende benstilling hos havanais, og indikerer at populasjonsrisikoen for forbenspatologi kan reduseres ved å øke frekvensen av det sunnere villtypeallelet. Den estimerte arvegraden for distichiasis hos havanais er ganske høy, hvilket innebærer at det skal være mulig å kontrollere prevalensen av sykdommen gjennom seleksjon basert på fenotype.

Gjennom bruk av egnede genetiske metoder gir studiene økt kunnskap om den genetiske bakgrunnen for fire viktige egenskaper hos bichon havanais, som kan benyttes i avlsprogrammet for å ytterligere forbedre hundehelse og -velferd. Avhandlingen gir et eksempel på hvordan helseutfordringer i en hunderase kan adresseres systematisk gjennom målrettet forskning.

Ah! you should keep dogs — fine animals — sagacious creatures

-Charles Dickens, writer and Havanese owner

5 Synopsis

5.1 Introduction

With its unique genome and population structure, profound variation and extreme degree of domestication, the dog is undoubtedly an intriguing species. The selection response obtained through domestication and subsequent artificial selection has been remarkable and has gained the dog its title of "Man's best friend". The unique evolution of the dog has secured it a high standard of living and status in society, but has not been without cost. Extreme selection and assortative mating have put our dogs at increased risk of genetic disease, and the urbanised environment we keep them in place heavy demands on their behavioural adaptivity. Increased understanding of the genetics underlying canine disease and behaviour will enable us to breed healthier dogs, that are even better adapted to their role as a modern pet, to secure the welfare of our best friend.

5.1.1 The creation of man's best friend

The time of domestication from the grey wolf is controversial. Many genetic studies indicate that domestication of dogs took place somewhere between 11 and 16 KA (1-3), while others suggest that domestication happened much earlier, over 30 KA (4, 5). Archaeological findings indicate that dogs were domesticated 12-14 KA, but some argue that dogs may have been domesticated earlier than this and simply remained morphologically unaltered for a long time after domestication, which would make them impossible to distinguish from wolves (6). However, results from the well-known genetic study on selection for tameness in foxes (7), indicate that selection for tameness through the domestication process would lead to morphological changes after few generations.

In addition to difficulties determining the exact time of domestication, placing the domestication of the first dogs geographically has also proved challenging (8). Because mitochondrial DNA does not show systematic differences between grey wolves in different parts of the world (9), it is hard to substantiate that wolves in some regions are more closely related to the modern dog than others, which could

have been a plausible way to estimate the place of domestication. This method would, however, also be sensitive to disruption from more recent events of gene flow and population dynamics (8). Some researchers have suggested that domestication took place in East Asia, because genetic diversity is larger there than in populations further west, which would be a typical pattern after a founding event (2, 3, 5, 10), but this conclusion has been challenged (11), and some argue in favour of a European origin (4). Based on several genetic studies it is well established that domestication of the dog happened somewhere in Eurasia, but the exact location remains unknown (8, 12).

Darwin believed that the large degree of phenotypic variation observed in dogs could only be explained by dogs having evolved from different types of wild canids (13). Today, genomic studies have shown that all modern dogs have evolved from the domestication of a single, or several very closely related, now-extinct populations of wolves (2, 3, 8, 14). The genetic diversity in the founding population of dogs was much larger than in most wolf populations today, due to narrow bottlenecks in wolf populations after divergence from dogs (1). Interestingly, a recent study showed that a genetic variant causing reduced body size in dogs, was present in Pleistocene wolves and introgressed to dogs during domestication, but is now almost completely eradicated in wolves (15). Some have suggested that there has been substantial bidirectional gene flow between dogs and wolves after domestication (1), but recent research indicates that the gene flow has been largely unidirectional, from dogs to wolves (8).

Studies show that the domesticated population of dogs had diverged into at least five different lineages by the onset of the Holocene (approximately 11-12,000 years ago) (8). It appears that early European dogs had large diversity, caused by gene flow from different lineages, but that most of these were later erased by expansion of a single ancestry. Today, European dogs have also contributed significantly to dogs worldwide, although precolonial ancestry is still present in some African and central American breeds (16), and dogs in parts of Asia show indigenous admixture (10). The extreme phenotypic diversity in today's dog breeds has been created recently from a shared genetic pool, which is exemplified by the fact that mutations that cause different forms of morphological traits, like hairlessness (17), short stature (18), body size (15) and coat variations (19), are shared across breeds, and exist in breeds from very different parts of the world that are not closely related (12).

The creation of modern dog breeds has happened gradually, with a stepwise increase in assortative mating and divergence between dog types and breeds (16, 20, 21). The Kennel Club was founded in 1873, as the first national kennel club in the world (22), with the original purpose of regulating dog shows and trials. In 1880 the Kennel Club started monthly publications of dog names, which can be considered the first registry of dogs as we know it, but the divergence of dogs into types and breeds had already taken place for many years (16, 23).

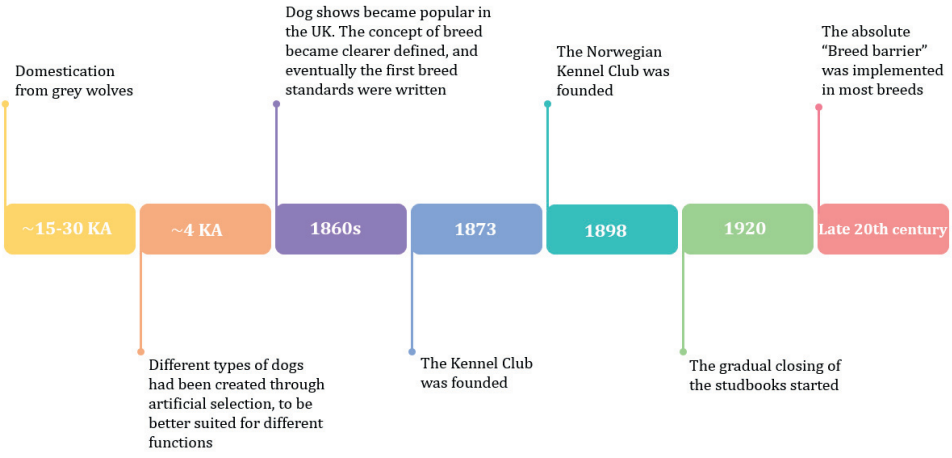

Figure 1: Timeline of important events in the evolution of dogs.

The history of the Havanese is not fully understood, but several theories on the evolution of the breed have been suggested (24, 25). Havanese is the national dog of Cuba, and the country's only native breed. It is likely that a proportion of the Havanese ancestors were brought to Cuba from Spain during the colonisation - some suggest from the island of Tenerife. The Cuban breed that developed was called Blanquito de la Habana and differed somewhat from the modern Havanese. In the 19th century, different types of lap dogs, including poodles, were brought from Europe to Cuba with European settlers, and these were mated with the Blanquitos to create the Havanese breed as we know it. During the Cuban revolution, some Cubans brought their Havanese with them to the United States, where much of the breeding of Havanese was continued. The Cuban Havanese Club was founded in

1991, leading to re-establishment of formal breeding programs in the breed's country of origin.

Domestication involves strong selection for tameness, and can be considered the first phase of selection for dogs that suit humans' needs and ways of living. In the beginning, this may have been a form of natural selection, without direct involvement of humans, that favoured individuals that had enough courage to approach humans for food (26, 27). It is estimated that the first division of dogs into distinct types, which can be considered the precursor of modern breeds, happened around 4,000 years ago (28-30). The different types of dogs were created through artificial selection, to make them more suitable for different tasks, like hunting, guarding, herding or companionship. Today, kennel clubs still classify dog breeds in groups based largely on their original purpose (31-33), and it is generally accepted that many of the working traits originally selected for, still contribute to their behaviour today (20, 34-36).

5.1.2 Every dog has its day

Many behavioural traits show significant heritability (37-41), including traits directly related to dogs' relationship with humans (42, 43), which means genetically distinct subpopulations like dog breeds, would be expected to show behavioural divergence. A large number of studies confirm this, and show differences in temperament between breeds (39, 44-47), and between groups of dogs based on ancestral function (20, 48-50), or common use today (44). Serpell et al. (51) showed significant breed differences in a broad spectrum of dog personality traits, including aggression, social- and non-social fears, barking, energy level and trainability.

However, there are also behavioural differences within each breed (51), leading to some controversy on to what degree breed is a reliable predictor of a dog's temperament. A recent study compared owner questionnaires and DNA-sequences from a large number of purebred and mixed breed dogs and concluded that breed might not be as good of a predictor as previously assumed (50). However, a subsequent study questioned this conclusion, and showed significant behavioural differences between lineages of dogs selected for specific purposes, predating breed creation (20).

Assuming breed is predictive of behaviour, it's clearly important that owners choose a breed that has good prerequisites to thrive in the life they are intended to live. Although most breeds were created to perform jobs like hunting, herding, or guarding, most of today's dogs are kept solely for companionship (44, 52-54). In Norway, there is long tradition for owning different types of gundogs (FCI-groups 5-8), like the Norwegian hounds used for elk- or hare hunting, pointing dogs, and also the worldwide popular retrievers and spaniels (55), and most of these make excellent pets in an active home. After all, traits that have been selected for in many working breeds, like trainability (48) and biddability (50), are also desired by most pet owners (56).

However, with increased urbanization worldwide, busy modern life, and dog ownership becoming steadily more popular (55), the gene-environment conflict that may arise if dogs bred to work are assigned a life as a couch potato, represents a potential threat to dog welfare (57).

One study showed correlation between herding lineage and non-social fears, which may be a result of selection for high reactiveness to subtle environmental changes (20). It's also been reported that terriers and herding dogs are more likely to have behavioural problems than other breed types (58), which could be a reflection of the high energy and work drive that have been selected for in these breeds. A UK-study showed that although gundogs, herding dogs and other working dog breeds received more exercise than for example toy dogs or terriers, they were less likely to receive the recommended amount for their breed (59).

A possible solution to the paradox of having many dog breeds that were created to perform a job, combined with a low demand for dogs with working abilities, is to soften the working traits of the working dog breeds through selection, to make them less active and more "companion dog-like" behaviourally (60, 61), which has been done in several breeds.

Another solution is to match less active owners with the handful of breeds that have actually been bred purely for companionship: the so-called companion- and toy breeds (FCI-group 9) (34). Most of the companion- and toy breeds are small and well adapted to small space living, and they don't require more exercise than what many modern pet owners can realistically provide (34). Havanese is an example of a breed that has been created purely for companionship, and fit the criteria of many

modern dog owners (56), as they are typically playful, affectionate, good with children, suited for urban living and require only moderate amounts of exercise (25, 62). They have become steadily more popular in Norway, experiencing a tenfold increase in registration numbers during the last two decades, and are now exceeding 600 registrations yearly (55).

The 21st century could certainly be the era of the companion dog. However, there are concerns that as dogs are kept purely for companionship, the focus of selection appears to have shifted from working ability to conformation (44, 60, 63, 64). This observation is supported by a genetic analysis that shows excessive differentiation in genomic sites associated with physical traits, but not in sites associated with behaviour, indicating substantial selection for looks (50). Because dog breeding can be a competitive hobby, it's not unlikely that show results have filled the void of working dog performance, as it may be the only trait that is formally merited (60). Today, even in most working dog breeds, significantly more dogs have merits from dog shows than from working trials (44).

Owners have high demands for what they consider ideal behaviour (56), and if a dog's behaviour deviates strongly from what the owner expects, it may affect the owner's attachment negatively (65). Given that behavioural problems significantly increase the risk of relinquishment or euthanasia (66), selection for dogs that are well adapted to life as a modern pet is important (44, 60). Some of today's owners, especially in urban environments, might more realistically be able to provide a good life for dogs with lower drive and energy (34, 59), and given that most dog owners say appearance is less important than behaviour when describing their ideal pet (56, 67, 68), it's unfortunate if selection in the increasingly relevant companion dog breeds is too focused on conformation, at the cost of behaviour, health or function (60). Increased use of tools to evaluate and objectively register companion dog behavioural traits, like the Swedish "Behaviour and Personality description Dog" (BPH) (69), could make selection for well-bred companion dogs more effective (44, 60).

5.1.3 Challenges and possibilities

Selection for good looks has been a popular recreational activity since the Victorian era (21), and can be positive if selection favours healthy conformation, e.g. parallel forelegs (70), as one of many breeding goals. Fortunately, most puppies in the

Nordic countries are produced by pet owners who breed dogs as a hobby (71), which undoubtedly contributes to improved welfare for breeding animals compared to conditions in commercial breeding establishments (72), and in this respect, the hobby-aspect of dog breeding has had positive effects on dog welfare, in addition to any beneficial effects the hobby might have for the owner (73).

Unfortunately, selection for an extreme appearance has led to health challenges in some dog breeds (74, 75). One example of a potentially harmful conformational trait that has been intentionally selected for, is brachycephaly, characterised by shortening of the scull. Brachycephaly is associated with increased risk of brachycephalic obstructive airway syndrome (BOAS), which compromises the dog's ability to breathe (76, 77), as well as increased risk of several eye diseases (78, 79), reduced thermoregulation (80), dental abnormalities (81) and skin issues (82). The degree of brachycephaly is often measured by the cranial-facial ratio (CFR) (76), ranging from ratios just below 0.5 which some consider mildly brachycephalic, to ratios closer to 0.1 which indicate extreme brachycephaly. Many of the breeds with the lowest cranial-facial ratios belong to the companion- and toy dog breeds (76, 83).

In the toy- and companion dog breeds, other conformational traits that have the potential to negatively affect health, are mainly related to their small size (proportional or disproportional dwarfism), which if exaggerated, can produce dogs that are either too fragile or too low on the legs to function normally (84).

The breed standards describe the ideal for each breed, both morphologically and behaviourally (85). They have been used as guidelines in breeding, and both dog shows and working trials seek to evaluate how well each animal fits the criteria of the standard. In most standards, the desired conformation is in line with what is considered functional, e.g. straight forelegs and a level topline (86, 87), and breeding pedigree dog according to a standard improves predictability. However, the breed standards often consist of rather loose descriptions (85, 88, 89), and it appears that in some breeds, descriptions like "short muzzle" have been misinterpreted in a way that has favoured the dogs with the *shortest* muzzles (or most profound coats, longest ears, shortest legs etc), which has moved the breed average gradually towards the extreme (89).

There has also been, and still are, a few examples of breed standards that describe directly harmful traits, like the specifications for muzzle length in French bulldogs (76, 90) and foreleg conformation in Irish Glen of Imaal terriers (70, 91), although major efforts have been made in recent years to improve standards, and promote a more moderate appearance (92). Unfortunately, there are also examples of harmful conformational traits that have been awarded and selected for, despite being contradictory to the description in the breed standard, like the domed head shape of some cavalier King Charles spaniels, which is associated with increased risk of syringomyelia (93, 94). The same phenomenon was observed in the 1890s, when screw tails were controversially introduced to the bulldog breed as a result of fashion, even though the standard required a different type of tail (21).

The breed standard of the Havanese describes a moderate conformation (62), e.g. length of muzzle similar to length of skull, leg length proportional to length of body, relatively small to medium body size, almond-shaped and normally sized eyes, and no excess skin, which several studies indicate is associated with reduced risk of disease (76, 78, 93, 95-98).

The first kennel clubs were founded with the primary purpose of regulating dog shows and field trials, which were becoming increasingly popular in the middle of the 19th century (21, 22). There has been controversy regarding selection for unhealthy conformational traits since the beginning (21), and dog shows have undoubtedly contributed to the negative development in some breeds (99). However, the organised dog world has also played a crucial role in creating rules and regulations for dog breeding, facilitating screening programs and registering health results, keeping pedigree information, funding research in canine health, and providing education to breeders (61, 92, 100, 101).

To combat misinterpretation of standards and promote healthy conformation in pedigree dogs, the Nordic Kennel Union has published a written guideline for show judges, that explain which conformational exaggerations they must be aware of and avoid rewarding (84), and in Norway, evidence based screening programs (102, 103) have been implemented to test all pugs, English- and French bulldogs for BOAS prior to breeding (104). However, it is evident that health issues related to extreme conformation are still much too prevalent is some breeds (82, 96, 102, 103, 105), and that increased efforts to combat the issue are long overdue (61, 106).

In addition to the health issues related to breed specific conformation seen in some breeds, purebred dogs are also at risk of heritable disease caused by reduced genetic diversity within each breed (107). Today, more than 350 dog breeds are recognised by the FCI (108), each representing a finite population which underwent founder effects at breed creation and may also have experienced subsequent bottlenecks caused by disease, world wars and fluctuations in popularity (109, 110). Combined with overuse of popular sires (111), and in some cases additional subdivision within breeds (112, 113), the effective population size of each breed is often relatively low (114), which increases the risk of genetic drift and ineffective selection against slightly deleterious alleles (115).

Additionally, there is evidence of strong selection (110), and because of the "breed barrier"-rule, i.e. that a dog can only be registered as a purebred if both its parents are also registered, there is little gene flow between populations (116, 117). The combination of small, finite populations; strong selection, and minimal gene flow, means the level of genetic variation in many breeds is low (118-122) and decreasing (118). Studies indicate that founder effects at breed creation and popular sire effects have been the main drivers of reduced diversity; much more so than recent inbreeding (110, 111).

Inbreeding depression is defined as a reduction in mean fitness due to inbreeding, and is the result of increased homozygosity and expression of recessive alleles, as well as loss of heterozygous advantage (123, 124). There are indications of accumulation of potentially deleterious alleles in some dog populations (110, 125, 126), and in some highly inbred dog breeds, the effect of inbreeding depression is evident (127). In addition to reduced fitness, low levels of genetic diversity negatively affect the potential for genetic change (128), which is a great concern in breeds where the need for phenotypic improvement is large (120). However, it is important to note that there are also dog breeds with sufficient levels of genetic diversity, especially where there has been recent admixture, or the studbooks are open (121, 122). Several dog breeds also have reduced risk of disease and increased longevity compared to mixed breed dogs (129), possibly as a result of purging (123), or because many registered purebreds are bound by health screening programs and breeding regulations that mixed breed dogs are not (101, 104).

Several studies report high levels of genetic diversity in Havanese compared to other purebreds, with above average levels of heterozygosity (121, 130), and an

estimated F-value of 0.13, close to half the purebred average (122). Genetic analyses indicate that the Havanese has received genetic contributions from a number of different breeds (131), which could explain the observed diversity.

"the Bichon Havanese is, above all (and outside any hypotheses concerning its origin), the sum of a great variety of antecedents – as are Cuban people themselves."
- Zoila Portioned Guerra, founder of the Havanese Club of Cuba

Suggested remedies for issues related to reduced genetic diversity in purebred dogs, include limiting the use of popular sires, importing genetic material from abroad, opening studbooks between similar breeds, allowing unregistered dogs to be entered into the studbook, as well as carefully monitored cross breeding projects (61, 132, 133), which are, contrary to popular belief and controversy, not new concepts. The definition of "breed" has gradually evolved (16, 134), and although a large degree of biological divergence between breeds started in the 19th century, and soared in the early 20th century, it wasn't until the end of the 20th century that studbooks in most breeds were fully closed (21).

Genetic analyses show that the flat coated- and golden retrievers were one breed until turn of the last century (16), and some breeds, like Norwich and Norfolk terriers, cluster together in microsatellite analyses, indicating a very recent split (134). There is also genetic evidence of significant contributions from breeds which have little in common with the recipient breed, like the inclusion of collie in the nova scotia toller retriever, and German shepherd in the Peruvian- and Mexican hairless dogs (16). To improve genetic diversity and health, crossbreeding projects have taken place in several breeds in the Nordic countries since the early 1990s, including four of seven Norwegian breeds (127, 135-137).

5.1.4 The canine genome and research

The breeding practices that have put some breeds at increased risk of inherited disease and inbreeding depression, have also shaped the canine genome in a way that can be highly useful in genetic research (14, 138, 139). Several studies have shown long stretches of linkage disequilibrium (LD) in purebred dogs (14, 50, 140), and that LD-stretches are on average 10-100 times longer in purebreds than in mutts, across breeds, or in humans (14, 140). Genome-wide association studies (GWAS) is a genetic method that is based on utilizing LD, where thousands of single

nucleotide polymorphisms across the genome are tested for association with a trait of interest (141). The idea is that although the SNPs are not predicted to have direct effect on the trait, they are expected to be in LD with variants that do.

Because of the long LD stretches found in purebred dogs, far fewer SNPs are needed to successfully identify associated regions in genome-wide association studies within breeds, than what would be required in a human GWAS (14). Additionally, because dogs within a breed are closely related, locus heterogeneity is lower, which means fewer samples are required to identify disease mutations (139, 142). Lindblad-Toh et al. showed that for a simple mendelian trait with dominant inheritance and high penetrance, 100 cases and 100 controls was enough to identify the locus, using only ~15,000 SNPs (14). In a subsequent study by Karlsson et al., two mendelian loci were successfully mapped through a GWAS of ~27,000 SNPs, using only ten cases and ten controls (143).

The excessive LD in purebred dogs can also represent a challenge in GWAS, because the associated regions will be widespan, making subsequent fine mapping more difficult. If the same mutation is predicted to cause disease in additional breeds, secondary across-breed GWAS may be helpful to narrow down the associated region and detect causative mutations more efficiently (143, 144).

Genome wide association studies are often used to study traits with presumed simple inheritance, but because many other methods are unsuited to detect multiple loci with small effects, GWAS can also be highly useful in studies of complex traits (138, 141). Results from GWAS are most commonly used as a baseline for fine-mapping and targeted resequencing, but can also be utilised directly for estimation of heritability, genetic correlation, or polygenic risk scores (a prediction of an individual's liability to disease, based on their genotype at multiple loci).

The expression of recessive alleles caused by mating of related individuals, offers natural models for inherited disorders (145). Increased availability and reduced cost of whole genome sequencing, combined with improved computer technology for analysis of large datasets, as well as increased international collaboration to share whole genome data (146, 147), has led to the identification of several disease-causing mutations in dogs during the last decade (148-151). WGS-data is typically analysed through variant calling, to identify variants that differ between carriers, affected and unaffected dogs in the expected pattern of a recessive- or dominant

disease, but may also be used for imputation in a high-density GWAS. Especially for mendelian traits, the advancements achieved using whole genome sequencing, makes eradication of several diseases feasible through DNA-testing.

The identification of new mutations in dogs through GWAS or whole genome sequencing is important to improve dog health, but may also present novel candidate genes for human disease (149-152), and provide increased insight into pathogenesis and pharmacological treatment (141). With their unique combination of having a lifestyle and environment that is shared with humans, and a genetic makeup that resembles that of an inbred laboratory mouse, the dog is in many ways an ideal natural model for research on human genetic disease (134, 138).

In addition to studies that aim at identifying variants that cause heritable disease, many studies on heritability of various traits have been conducted in dogs (38, 153, 154). Selection studies (155) and studies that map differences between breeds (156), provides information on the degree of genetic influence on a trait. In addition, several studies calculate estimates of heritability though the use of pedigree information (38, 153, 154, 157) and/or genetic markers (153, 157).

Genomic prediction, i.e. estimating an animal's breeding value based on their genotype, has gained increased interest since Meuwissen et al. published a well-known paper on the concept two decades ago (158). In genomic prediction, the observed phenotype of a number of genotyped individuals in a "training set", is used to estimate effect size of the genetic variants (usually SNPs) through for example BLUP-analysis or Bayesian methods (159). Once the SNP-effects have been estimated, they can be used to predict genomic estimated breeding values (GEBVs) of animals with unknown phenotype, based on their SNP-genotypes (160).

Genomic prediction can be a useful tool in selection for polygenetic traits, especially in situations where phenotyping each animal directly is challenging due to practical considerations or a late onset of disease. It has improved the rate of genetic change in livestock species (161), which resembles dog breed populations in many aspects, including small effective population sizes and high levels of relatedness between individuals. The long stretches of LD gives good genome coverage, and having animals in the training set that are closely related to the selection candidates, may improve accuracy (162, 163). Some studies on genomic prediction in dogs have

already been conducted, including studies on hip dysplasia (164) and anterior cruciate ligament rupture (165).

To the author's knowledge, only two studies besides the articles that constitute this thesis, have been published on hereditary disease specifically in Havanese. Starr et al. investigated different abnormalities that had been reported by Havanese owners through an anonymous questionnaire and suggested that they were part of a breed specific syndrome with a shared genetic background (166). By evaluating eleven disease traits in 122 phenotyped dogs, the authors classified the dogs as affected or unaffected by the suggested syndrome using different classification systems, and found moderate heritability estimates. They also found that cataracts, liver abnormalities, angular limb deformities and heart murmurs co-segregated, and suggested potential candidate genes based on genes that were differently expressed in the liver of three Havanese classified as affected by the syndrome, compared to three healthy controls from other breeds.

Frazer et al. did a retrospective study that included 34 Havanese, and found evidence of a predisposition to the suspected immune mediated skin disease, sebaceous adenitis (167). The authors found that the ears were often affected and described the typical histopathological findings.

Several retrospective across-breed studies based on records from different teaching hospitals in North America, include Havanese. Inga et al. reported that Havanese were significantly overrepresented in a study on sterile granulomatous dermatitis and lymphadenitis, however, only four cases were identified in the study (168). Tobias and Rohrback showed that Havanese were the most commonly affected breed in a study on congenital portosystemic shunts and suggested a genetic predisposition (169). Lastly, Gellat and Mackay showed that Havanese are predisposed to the eye disease cataract (170), which is also considered a challenge by the breed community, and prioritised in the breeding strategy and screening program in Norway (171, 172).

Compared to other breeds, Havanese experience fewer non-routine veterinary care events (122) and a longer life span (121). In a study from the United Kingdom on the proportion of purebred litters born by caesarean section, the proportion reported for Havanese was 9.1%, which was less than half of the study average (173).

5.1.5 Aims and objectives

For better or worse, the selection response obtained in dog breeding has been remarkable, reaching a level of domestication and degree of diversity and specialization that is unparalleled. With "companion dogs" becoming steadily more important, due to increased urbanization and busy modern life, it's regrettable that some of the breeds that have been bred primarily for companionship, struggle with health issues as a result of selection for extreme conformation, or heritable diseases that have become close to fixation.

Havanese is a pure companion dog breed, with generally good health, sufficient levels of genetic diversity and a high life expectancy. Particularly in the Nordic countries, they have had a burst in popularity in recent years and are now the most registered breed in the companion- and toy group in Norway. The Havanese has large potential as a moderately built companion dog, but the increased popularity is also a potential threat to their good health. Not much research has been conducted specifically in Havanese, which makes it challenging to create evidence-based breeding strategies.

Recognizing the need for improved knowledge on the health status of the breed, to enable informed breeding decisions and take necessary precautions as breed popularity increase, the Havanese club of Norway has conducted several health surveys. Unfortunately, the raw data from the surveys have not been published, but a summary report from a survey conducted among both owners and veterinarians in 2012, has been made available.

In the 2012-survey, the responses form veterinarians in small animal practices indicated that, based on perceived prevalence and clinical relevance, nervousness, epiphora and distichiasis, as well as skin issues and allergies, were considered the most relevant challenges in the breed (it should be noted that the number of responders in the veterinary survey was reportedly low). The results from the owner survey, which received 226 replies, indicated that the health status of the breed was generally sound, with 96% of owners saying their dog's health was good or very good, and no owners describing their dog's health as poor.

Owners of 60% of the dogs responded that their dog's knees had been tested for patella luxation, and 11,6% of the tested individuals were affected. Among the

affected individuals, half displayed clinical signs. Cataracts were kept out of the survey because good information on this condition was available through the kennel club database (174), but owners were asked whether their dog showed clinical signs of eye disease, which 95% did not. However, among the few that did, prolapse of the gland of the third eyelid, distichiasis, and epiphora, were the most common complaints. Owners of 7.4% of the dogs reported itchy skin without a specific diagnosis, owners of 3% responded that their dog was allergic, and 2% reported frequent otitis. Regarding behaviour, 93.8% of owners described the temperament of their dog as good or very good, but 18.6% of owners also stated that their dog was a little or very nervous.

In Havanese, some heritable diseases have proven hard to eradicate, despite breeders' long-term commitment to health screening and breeding regulations – a phenomenon that is not unique to this breed. Increasingly advanced and available genetic methods enable us to map the genetic background of different traits more efficiently, and through improved knowledge of the genetic mechanisms that underlie health traits in dogs, we can better address issues and improve animal welfare.

This thesis demonstrate how health and welfare challenges within a dog breed can be systematically addressed through genetic research. The Havanese was chosen as a "model breed", because it is the most registered breed in the companion- and toy group in Norway (55): a group that is becoming increasingly relevant in the modern world.

We use a large dataset of Havanese, with comprehensive phenotype-, genotype- and pedigree information, to address four of the most important challenges in the breed: social fears, foreleg abnormalities, distichiasis, and cataracts, using different genetic methods. The study elucidates the complexity of breeding strategies in dogs, where multiple traits and challenges must be addressed simultaneously, and show how targeted research projects are essential to optimise breeding strategies.

The aim of the thesis was twofold:

- To use Havanese as a model breed, to demonstrate how genetic methods can be better utilised in dog breeding, to improve the health and welfare of dogs.
- To obtain increased knowledge on the genetic background of four important traits in Havanese, which has great potential as a healthy, genetically diverse, and moderately built companion breed.

To reach these aims, the following objectives were determined:

- Identify genetic variants that are associated with level of social fear in Havanese.
- Test the hypothesis that the bowed and shortened forelegs seen in some Havanese could be a result of chondrodystrophy caused by the FGF4 retrogene on CFA18, and that neither the risk- nor wildtype allele are fixed in the breed.
- Estimate the prevalence and heritability of distichiasis in Havanese.
- Identify genetic markers that are associated with the three forms of cataract in Havanese, as a first step towards uncovering causative variants
- Investigate why strategies to reduce the prevalence of cataracts in Havanese have so far been unsuccessful and test a hypothesis of low sensitivity of cataract screening due to a high onset of disease.

5.2 Overview of materials and methods

The main materials included in the thesis were:

- A biobank consisting of ~450 Havanese DNA samples.
- The Norwegian Kennel Club database, including pedigree and offspring information of all registered Havanese in Norway, as well as individual health screening results.

5.2.1 DNA-sample collection (paper I, II and IV)

The DNA-samples were collected in three batches. The first two batches (paper I and II) were randomly collected through advertisements on the breed club website and in Norwegian Havanese Facebook-groups, with the only inclusion criteria being Havanese > 1 year old and that the owner was willing to participate. The third batch (paper IV) was collected through targeted sampling of dogs that met inclusion criteria for the cataract project.

All DNA-samples were collected with owner's consent for use in research, in the form of EDTA-whole blood (paper I) or Performagene™ PG-100 buccal swabs (DNA Genotek Inc) (papers I-IV). Blood samples were collected by certified veterinarians, whilst most buccal swabs were collected by the owner.

For paper I, DNA-samples were collected at dog shows, gatherings arranged by breeders, Havanese "playdates" and home visits. Some samples were also sent by mail in the form of cheek swabs. 104 Havanese underwent a behavioural evaluation, and owners of 150 Havanese responded to the questionnaire. Some dogs displayed an intermediate phenotype and were therefore excluded from the association analyses.

DNA-samples from 235 Havanese that had been recruited for paper I were brought forward and included in the allele frequency calculation in paper II, together with a batch of 120 additional samples that were collected specifically for the second study. Only the second batch was included in the association analyses in paper II, because information on shoulder height was not available for the first batch.

The fourth study (paper IV) included 65 samples that had been recruited for the two previous studies (paper I and II), but also met inclusion criteria as cataract cases or controls, as well as 45 additional samples were collected specifically for the cataract project. The samples were recruited through both online advertisements and direct inquiries to owners, calling for Havanese that were either cataract-affected or free of cataracts at 7 years of age or older.

In paper I, 200 additional samples of collies (94), Irish soft-coated wheaten terriers (44), Nova Scotia duck tolling retrievers (33) and standard poodles (29) were included in the association analysis of noise reactivity.

5.2.2 Pedigree and database-analyses (paper III and IV)

ECVO eye screening results (paper III and IV) and pedigree information (paper III) was pulled from the Norwegian Kennel Club database (1).

5.2.3 Selection of research topics

Through a thorough evaluation of published literature, as well as health surveys conducted by the breed club; social fears, angular limb deformities, distichiasis, and cataracts; were selected as the research topics of each of the articles that constitute this thesis, based on an assessment of presumed prevalence, clinical relevance, and mode of inheritance. Please see the Discussion for a more elaborate consideration of each study topic.

5.2.4 Methods

In paper I, a candidate gene approach was used to investigate social fears in Havanese. Two different classification methods were used in the social fear association analyses: one based on a behavioural test conducted by an external evaluator, and one based on an owner questionnaire. An additional questionnaire was used to evaluate noise reactivity in five breeds, including Havanese.

In paper II, the allele frequency of the FGF4 retrogene on CFA18 was estimated through genotyping a closely linked marker (2). Complete linkage disequilibrium between the marker and the retrogene insertion site was verified in a smaller sample, in which the presence or absence of the retrogene was investigated directly using allele-specific PCR (3). Shoulder height measured by the owner was used as an indirect measure of degree of foreleg shortening.

In paper III, heritability of distichiasis was estimated by three different models, using both linear- and Bayesian threshold methods. The linear models were analysed using the average information restricted maximum likelihood (AI-REML)-module in DMU (4), whilst Bayesian threshold models were analysed by Gibbs sampling, and post Gibbs analysis using BOA (5). The Dogs were classified as either unaffected or affected, regardless of grade (most cases were mild or ungraded).

Dogs with conflicting results were classified as affected, in accordance with ECVO guidelines (6).

In paper IV, a genome wide association study (GWAS) was conducted for three different forms of cataracts in Havanese, using a mixed linear model which included a genomic relationship matrix (GRM) to correct for relatedness and population stratification. The dogs were genotyped using the Illumina CanineHD 230K BeadChip, and the analysis was conducted using the MLMA-LOCO-function in GCTA (7). Inclusion criteria for cases was a positive diagnosis of either posterior polar-, cortical or anterior suture line cataracts, and inclusion criteria for controls was an ECVO-certificate stating that the dog was free of cataracts at seven years of age or older. The effect of age at examination, on observed prevalence and sensitivity of cataract screening, was also investigated.

For further details on sample collection and methods, please see papers I-IV.

5.3 Summary of papers

Paper I

DRD2 is associated with fear in some dog breeds

Almost one in five Havanese show an increased tendency to react fearfully in social interactions with unfamiliar people or dogs. In paper one, we used a candidate gene approach to investigate the potential association between this undesirable phenotype and a dopamine receptor gene, *DRD2*. Significant associations were detected between 2 SNPs in exon 2 of the DRD2 gene and increased social fear in Havanese dogs (n = 158), classified both through observation by an external evaluator (respective allelic odds ratio: 4.35, 4.07) and owner questionnaires (respective allelic odds ratio: 1.96, 2.2). The correlations between the two means of classification ($\rho = 0.738$, P-value < 0.001) and the intra-rater reliability for the owner questionnaires ($\alpha = 0.82$), were good. The average owner score of the heterozygote individuals was intermediate compared to the average owner score of the homozygotes of the risk- and protective alleles, respectively. Because different types of fear-related behavioural disorders sometimes co-occur, the two SNPs in exon 2 were also investigated for association to noise reactivity in 5 breeds, including Havanese. Significant associations were detected between SNPs in exon 2

of *DRD2* and noise reactivity in the Irish soft-coated wheaten terrier (respective allelic odds ratio: 2.64, 2.88) and collie (allelic odds ratio: 3.03, (one SNP fixed)). The same alleles were associated with the beneficial phenotypes in the 3 breeds. No association was detected between the two SNPs and noise reactivity in Havanese, which could reflect the low prevalence of noise reactivity in the breed.

Paper II

Short and sweet: foreleg abnormalities in Havanese and the role of the FGF4 retrogene

Cases of foreleg deformities, characterised by varying degrees of shortened and bowed forelegs, have been reported in the Havanese breed. Even though most of the affected individuals appear asymptomatic, a fraction develops pain and discomfort that requires invasive surgical correction. Because the health and welfare implications are severe in some of the affected dogs, developing a better understanding of the genetic background of the trait was crucial. A previous study indicated that the problem could be part of a breed specific syndrome, but was inconclusive. A FGF4-retrogene on CFA18 is known to cause chondrodystrophy in dogs, and in most breeds, either the wild type- or mutant allele is fixed. Because other studies, as well as our own investigations, had shown relatively high levels of genetic diversity in Havanese, we hypothesised that both alleles could segregate in the breed, and that the shortened and bowed forelegs seen in some Havanese could be a consequence of FGF4RG-associated chondrodystrophy.

The study confirmed variation in the presence/absence of the retrogene, but the prevalence of the non-chondrodystrophic wild type was low, with allele frequencies of 0.025 and 0.975 for the wild type and mutant allele, respectively (linked marker). No dog was homozygote wild type. To verify association with phenotype, shoulder height was used as an indirect measure of the degree of foreleg shortening. The study showed that heterozygotes were significantly taller at the shoulder than mutant allele homozygotes, with average heights of 31.3 cm and 26.4 cm, respectively. We hypothesised that there had been a gradual decline in the population frequency of the lower-risk, wild type allele during the past two decades, which findings confirmed. Heterozygote individuals were born on average 4.7 years earlier than mutant allele homozygotes.

The findings of paper II corroborate that FGF4RG-associated chondrodystrophy contribute to angular limb deformities in Havanese, and that both the wild type and mutant allele segregate in the breed. The population frequency of the wild type allele is low and appear to be decreasing. We argue that efforts should be made to reverse the process and preserve the healthier wild type in the population. Gradually reducing the frequency of this unnecessary, underlying risk factor could reduce the breeds overall risk of foreleg pathology.

Paper III

Heritability of distichiasis in Havanese dogs in Norway

In paper III, the prevalence and heritability of distichiasis were estimated in the Norwegian population of Havanese dogs. Distichiasis is an eye condition, characterised by misplaced eyelashes, that is frequently diagnosed during routine eye screenings of Havanese, and the clinical relevance is varied. Inclusion criteria were Havanese that were registered in the Norwegian Kennel Club and had been eye screened through the ECVO eye scheme between 2005 and 2020. 1156 Havanese were included in the study, and out of these, 168 were affected with distichiasis, making the prevalence in our sample 14.5%. Most cases (86.9%) were graded "mild". There was no significant difference in prevalence between males (13.1%) and females (15.3%). The heritability was estimated using both linear and Bayesian threshold models and ranged from 0.276 (linear model) to 0.720 (Bayesian threshold model), depending on how age and year of diagnosis was treated. The linear estimates corresponded well to the results of the Bayesian models after conversion to the underlying scale (h^2_l = 0.664-0.674), as described by Dempster and Lerner (1950). The high heritability estimates indicate that a significant selection response could be obtained through simple phenotypic selection. To secure good animal welfare, the number of affected individuals, and especially the severely affected, should be controlled.

Paper IV

Cataracts in Havanese: a genome wide association study reveals two loci associated with posterior polar cataract

Havanese are predisposed to cataracts, and it's considered an important health and welfare challenge in the breed. The clinical relevance is variable, ranging from no apparent clinical signs or only mildly reduced eyesight, to total cataracts with

secondary changes. Severe forms require surgical removal of the lens, which is associated with risk of complications. Despite two decades of eye screening breeding animals and using only unaffected animals for breeding, the prevalence of cataracts had reportedly not gone down, which the findings of paper IV confirmed. The aim of the study was to elucidate the genetic background of cataracts in Havanese, to improve the efficacy of breeding strategies and move towards identification of causal variants.

Through a genome wide association study using a mixed linear model, we identified two SNPs on CFA20 (BICF2S23632983, p = 7.19e-09) and CFA21 (BICF2G630640490, p = 3.33e-09), that were significantly associated with posterior polar cataract. Closer inspection of genotypes for BICF2S23632983 showed that the allele associated with posterior polar cataract was present in a heterozygote state in 5/7 cases and only 1/57 controls. For BICF2G630640490, the allele associated with disease was present in a heterozygote state in 6/7 cases and only 2/57 controls. BICF2S23632983 is located in proximity to two relevant candidate genes, FOXP1 and RYBP, both of which are highly expressed in the lens. In mice where either of these genes have been inactivated, various eye abnormalities are observed, including lens opacities. BICF2G630640490 is positioned ~800 kb upstream of the LGR4-gene, which is a known cataract candidate gene.

Two peaks on CFA4 and CFA30, showed putative association with cortical cataract. The associations were not significant after Bonferroni correction, but the finding pinpoint regions that might still be worthy of further investigations. Both SNPs are linked to relevant candidate genes: the top SNP on CFA4 is located ~1500 base pairs away from *ANK3*, and the top SNP on CFA30 is positioned within the PCLAF-gene.

The study also showed that average age at onset for cataracts in Havanese is 4.8 years, and that sensitivity (the likelihood that a true positive will test positive) is highly influenced by age at examination. Most Havanese are eye examined at an age where they are unlikely to be diagnosed with cataract, even if they are genetically at risk. The findings suggest that a Havanese cannot with certainty be declared cataract negative earlier than at 7 years of age (sensitivity at 7 years of age = 0.95), which means genetic studies on cataracts should only include controls that are least this age. Increasing the average age of breeding animals, whilst continuing to eye screen breeding animals yearly, could increase the accuracy of selection and genetic improvement.

5.4 Discussion

An overall objective of the thesis was to address important health- and welfare challenges in Havanese, to further improve the health status of the breed. Havanese are generally healthy dogs, but published studies, as well as health surveys conducted by the breed club, indicate a predisposition to cataracts (1, 2), foreleg abnormalities (2), increased levels of social fear, distichiasis and epiphora, allergies, patellar luxation, liver shunts (2, 3), sebaceous adenitis (4) and sterile granulomatous dermatitis (5). No causative or associated mutations have previously been identified, and for some of the traits, even the mode of inheritance is unknown.

5.4.1 Selection of research topics

In pedigree dogs, breeding strategies are often complex, and typically include a large number of loosely defined breeding goals and selection criteria. The various breeding goals must be balanced based on effect on health, welfare, and function, and preferably a good understanding of the underlying genetics. The complexity of breeding goals, and often limited amounts of published research, makes it hard to create effective, evidence-based breeding strategies in many breeds. As an example of how important challenges in a breed can be systematically addressed, unravelling the genetic background of four important challenges in the Havanese breed; social fears, foreleg abnormalities, distichiasis, and cataracts, using appropriate genetic methods, was selected as the main objective of the thesis.

Social fears were mentioned as a potential issue by both veterinarians and owners in the 2012 health survey, with almost 1 in 5 owners characterizing their dog as either nervous or very nervous. Anxiety and fear are negative affective states which contribute to reduced dog welfare, and if there is large discrepancy between an owner's idea of ideal behaviour and the actual behaviour of the pet, it may also pose a threat to the human-dog-relationship (65).

Cataracts and angular limb deformities were selected because they were highlighted as important issues in both research articles and by the breed community (166, 170). Both have the potential to have significantly negative effects on health (70), and in severely affected individuals, surgical correction is the only definite therapy, which is associated with risk of complication and a potentially long convalescence

(175, 176). The observed prevalence of cataracts is not very high, but due to the combination of highly standardised phenotyping through the ECVO screening program and the potentially simple mode of inheritance, we considered it feasible to obtain significant results even with a limited sample size.

Distichiasis is moderately prevalent in the breed (174) and although most reported cases are mild, the surveys conducted in 2012 indicated that both owners and small animal practitioners considered the condition relatively important. In addition to the direct, negative effects distichiasis may have on health, the fact that breeders consider the condition important is relevant, as it will influence the direction of breeding.

Despite evidence that Havanese are predisposed to the diseases (167-169), portosystemic shunts, sebaceous adenitis and sterile granulomatous dermatitis were not prioritised in this study, because there were few indications that these diseases are prevalent in the Norwegian population. The presumed low prevalence in Norway might be due to population differences, the limited sample size included in the studies, underreporting in Norway, or that the diseases are generally rare.

Allergies and related skin issues were also listed as somewhat important by owners and veterinarians in the 2012 survey, but the reported prevalence was low. Among the allergic dogs, some were allergic to food and others reacted to allergens in the environment, which may be genetically distinct traits, leading to a further reduction in potential sample size. Havanese are not mentioned in studies on breed predisposition to canine atopic dermatitis (177, 178).

Luxating patellas is an issue that is recognised and prioritised by Havanese breeders in Norway (171), and kennel club data indicate a prevalence of ~ 0.1 (174). A Swedish study showed that age and environmental factors influence patella status to some degree, leading to a small risk of misclassification, but also report moderate heritability estimates (179). The findings indicate that the screening program (which is similar to the Norwegian screening program), is able to detect genetic differences between dogs, and could be used to classify dogs in for example a GWAS.

Despite clinical relevance, neither patellar luxation nor skin allergies were prioritised as research topics in the thesis, because the presumed highly polygenic

nature of both traits means a large sample size would be required to detects associated variant, which could be challenging to obtain at the observed prevalence.

5.4.2 General discussion and methodological considerations

DRD2 was chosen as a candidate gene for paper I, because the phenotype we were interested in was a general form of social "anxiousness". DRD2 had previously been found to be associated with anxious (180) and antisocial (181) personality traits, and has since been shown to affect level of sociability in the general population (182), which resembles the highly undesirable, but not pathological, phenotype we observed in some of the Havanese.

We found a strong association between SNPs in exon 2 of the dopamine receptor D2-gene (*DRD2*) and social anxiety in Havanese (odds ratio of 4.07-4.35), when classification was based on a behavioural test. This is a large effect compared to most markers that are associated with behavioural traits, but comparable to results from several other studies on *DRD2* (181, 183). In humans, the *DRD2* Taq1A polymorphism (rs1800497) has been extensively studied, and found to be associated with various phenotypes, including addiction, several psychiatric disorders, Parkinson's disease, migraines and some personality traits (184).

We observed a weaker association when classification was based on owner questionnaires, which is not surprising, given the lack of standardization when classification is performed by multiple testers who have no prior training in evaluating dogs. In retrospect, using a better validated questionnaire, like C-BARQ (185), could possibly have improved the accuracy of classification. The downside to using C-BARQ, would be that it is much more extensive, which could negatively affect the number of responders. Especially breeders who responded for more than one dog, would likely be less inclined to respond if the survey was too comprehensive.

Sample collection was typically done at gatherings arranged by breeders so that many samples could be collected simultaneously, which naturally resulted in a potential overrepresentation of breeder-owned dogs. A few dogs that were classified as controls based on the owner score, were classified as cases in the behavioural test, and owner scores were generally high, indicating that some owners might have been reluctant to classify their dog as anxious. In some cases, the

same breeders responded for several related dogs which could lead to a risk of spurious association. However, the average number of dogs per owner in the study was only 2.08, indicating that this limitation was minimal. A more extensive use of cheek swabs would have increased both the total number of samples included in the survey-based analysis, and increased the proportion of samples from single dog households.

Our study did not identify polymorphisms that were likely to have a functional effect, which means further investigations of biological differences between the cases and controls should be performed. In humans, the functional effect of the *DRD2* Taq1A polymorphism is not fully understood, but it is likely that it is linked to disturbances in dopamine regulation (180). Interestingly, rs1800497 is not actually located within *DRD2*, but rather in the neighbouring gene *ANKK1*, positioned ~10 kb away, which means the effect may be caused by linked variants. *DRD2* is a highly relevant candidate gene, but given the long stretches of linkage disequilibrium found in purebred dogs (14, 140), the possibility that distantly positioned variants are causing the effect we observe in Havanese cannot be excluded.

Even though the underlying biological explanation is unknown, the association between *DRD2* genotype and social fears in Havanese is strong. Unfortunately, the finding has limited useability without further studies, as DNA-tests for single associated markers for traits that are highly polygenetic, are challenging to implement in breeding programs due to a risk of overestimating the effect. Also, because it is not unlikely that the effect is due to linked variants, decay in LD could negatively affect accuracy.

Regardless of the genetic method used, the main challenge in identifying, validating, and utilizing associated markers to improve behaviour, is correct classification of a sufficient number of dogs. Given the highly polygenetic nature of behaviour, relatively large samples sizes are required, which may only be obtainable by basing classification on owner questionnaires. However, the results of paper I, indicate that the inaccuracy in classification using questionnaires is not necessarily compensated by the increased sample size. Increased use of behavioural tests like the Swedish BPH-test (69), could provide valuable phenotype-data for future genetic studies.

In paper I, we also found that the two SNPs in exon two of the *DRD2* gene was associated with noise reactivity in the Irish soft-coated wheaten terrier (ISWT) and

collie (one of the two SNPs were fixed in the latter). There was no association between *DRD2* and fear of loud noises in Havanese, probably as a reflection of the low prevalence of noise reactivity in this breed. It's not surprising that an increased tendency to react fearful is expressed differently in separate breeds, despite a common genetic background, as the phenotype in each breed will also be influenced by many other genes. Studies show that ISWT and collie are more frequently affected by noise reactivity (186), and less frequently affected with social fears (187), whilst social fears are more common in small breeds. Due to the lack of association, and no indication that noise reactivity is a relevant issue in Havanese, noise reactivity was not given further priority in this study.

In paper II, we hypothesised that foreleg abnormalities in Havanese could be a result of chondrodystrophy caused by the FGF4 retrogene on CFA18 (18), rather than a breed specific "syndrome" as previously suggested (166). A marker in complete linkage disequilibrium with the insertion site of the retrogene (verified in a sample of 22 heterozygotes and 22 chondrodystrophy homozygotes), was genotyped in 355 Havanese. One of the main limitations of the study was that we used shoulder height as an indirect measure of the degree of foreleg shortening, rather than directly measuring bowing of the legs or clinical signs. We discussed different options for obtaining a more direct measure, but did not find a method we believed would be sufficiently standardised, yet feasible to conduct in a large number of dogs. In retrospect, the low allele frequency we observed for the wild type allele, means that we would not have had enough power to detect an association if the sample size had been much smaller, which probably would have been the case if we had used a more advanced classification method.

Since we wrote paper II, Pulkkinen et al. has shown that external rotation and carpal valgus can be reliably measured using a goniometer, which provides a simple, non-invasive and inexpensive method for measuring angular limb deformities in chondrodystrophic- and non-chondrodystrophic dogs (188). Using this method to compare the degree of foreleg bowing in Havanese that are homozygote for the chondrodystrophic genotype with Havanese that are heterozygote, could be an option to substantiate the findings from paper II. However, the measurements performed in the study by Pulkkinen et al. also corroborate that the chondrodystrophic dogs display on average more carpus valgus and external rotation compared to the non-chondrodystrophic dogs, which is also supported by Bannasch et al. (189). Additionally, strong association between degree of foreleg

bowing and clinical signs like lameness, pain, reduced range og motion and elbow joint osteoarthritis, was recently reaffirmed (70). Thus, the conclusion we made in paper II about how having a large proportion of chondrodystrophic dogs in the breed population is associated with an increased population risk of angular limb deformities, is now better validated. In line with the findings of paper II, the Bannasch-study also concluded that chondrodystrophy is inherited in an incomplete dominant manner.

An additional FGF4 retrogene on CFA12, cause a less severe degree of foreleg shortening than the one on CFA18, but is also associated with increased risk of intervertebral disc calcification and disease (190-192). Because the primary aim of the study was to investigate the genetic background of foreleg abnormalities, in which *FGF4*-18 is considered most important, combined with the presumed low frequency of *FGF4*-12 in Havanese (193), we did not give priority to study this variant. However, as most commercial distributers of the *FGF4*-18-test simultaneously test for *FGF4*-12, increased knowledge on allele frequencies of both mutations will be obtained if *FGF4*-18 testing becomes more common among Havanese breeders, which is positive.

In paper III, high heritability estimates for distichiasis were detected, using both linear- and Bayesian threshold models. Distichiasis is a trait that is well suited for heritability studies, given the presumed polygenetic inheritance, the relatively high prevalence, and the fact that both affected and unaffected individuals are bred. Havanese is the second most registered dog breed in Norway where ECVO eye screening is mandatory prior to breeding (104). Additionally, the age of onset is low (194, 195), which means that all dogs that have been examined at least once can be considered either a case or a control, providing a reasonably sized dataset available for study.

When using linear models to estimate heritability of threshold traits, the estimate will always be underestimated (196), and the deviation from the true heritability will be more severe the more the threshold, reflected in the disease prevalence, deviates from 0.5 (196, 197). To combat this issue, a method was suggested by Dempster and Lerner to convert estimates on the observed scale to the underlying liability scale, taking population prevalence into account (197). In paper III, we used the method described by Dempster and Lerner to convert the estimates from the

linear models, and found values that were comparable to the estimates from the Bayesian threshold models.

We observe that even though distichiasis in Havanese is a trait in which a relatively large number of phenotype registrations were available for study, the standard errors and highest posterior density regions were still quite large. This illustrates an important limitation in estimation of heritability, genetic correlation, and breeding values in dogs, of small breed populations and low test-percentages resulting in insufficient numbers of registrations. There are strict quality control requirements that must be met for new phenotype registrations to be entered into the Norwegian Kennel Clubs database, including standardisation and validation of health screening, safe identification of animals, as well as protocols to ensure all test results are submitted regardless of result. Thus, increasing the number of phenotypes registered, may be challenging without compromising on quality control, which could negatively affect accuracy of heritability estimates or breeding values. Increased data sharing between neighbouring countries where there is significant exchange of genetic material, could improve sample size and give more accurate estimates (198).

In retrospect, it's unfortunate that we did not investigate association between distichiasis and epiphora in paper III, as these complaints tended to co-occur in replies in the breed club health survey. Distichiasis can cause excessive lacrimation (199), but it's also possible that owners of dogs that exhibit both conditions have assumed that they are related, and might therefore have been more inclined to report distichiasis as an issue, even though the two conditions are both relatively prevalent in the breed and could occur simultaneously, but independent of each other.

In paper IV, we used a genome wide association study (GWAS) to identify regions associated with different forms of cataracts in Havanese and found two single SNP that were significantly associated with posterior polar cataracts. The long stretches of linkage disequilibrium and low genetic heterogeneity makes GWAS more efficient in dogs than in most other species (14), but there are also aspects of the canine population structure that pose challenges in GWAS. The high relatedness between individuals within a breed will improve the ability to identify true associations due to low genetic heterogeneity, but may also lead to spurious associations from population stratification. In human GWAS, closely related individuals are normally

removed prior to analysis, to reduce the risk of false association, but applying the threshold values for relatedness that are commonly used in human GWAS in dogs, would have removed a large proportion of sequenced individuals, leading to severely reduced power (159).

Instead of filtering on relatedness, we used a mixed linear model which included a genomic relatedness matrix (GRM) as a random effect, taking the pairwise relatedness between individuals into account to correct for sample structure and avoid spurious association. Research on different species, including species with high levels of relatedness between individuals, has shown that mixed linear models control false positives from population stratification effectively, but may also increase type II errors (200).

One concern with using mixed linear models to control for population stratification, which is especially important to consider in dogs, is that including the candidate SNP or SNPs in strong LD with the candidate SNP in the GRM, will lead to double fitting the SNP as both a fixed effect tested for association and a random effect in the GRM (201). As a result of long stretches of LD in dogs (14, 140), many SNPs are expected to be in LD with each other, which means loss of power may be a real concern. To address this issue, we used the "leaving one chromosome out" (MLMA-LOCO)-option in GCTA, which computes a separate GRM for each chromosome and excludes all SNPs on the same chromosome as the candidate SNP from the computerization of the GRM (202) . However, the results of the association analyses in paper IV, showed very similar results regardless of whether the --MLMA-LOCO or --MLMA function was used.

The results from paper IV underlines the importance of strict inclusion criteria for controls used in canine GWAS-studies, especially when working with traits that have a high age of onset. In humans, the allele frequencies of specific disease mutations are often so low that even individuals with unknown phenotype can be included as controls (141). In dogs, the frequency of disease mutations can become high within the finite breed populations as a result of genetic drift, which means strict inclusion criteria for controls is essential, to avoid controls that carry disease variants. Unusually high frequencies of risk alleles can also pose a challenge in canine GWAS if the variants associated with disease become fixed in the breed, which would effectively disguise them in a GWAS-analysis, as was observed in a study on canine osteosarcoma (203).

We attempted to use SNP-data from the GWAS-analyses to calculate SNP-heritability and genetic correlation, for posterior polar-, cortical- and anterior suture line cataracts. Unfortunately, the sample size was too small to give informative results. Even though calculation of SNP-heritability for the three forms of cataracts gave moderate to high estimates, the standard error was too high for us to include the results in the paper. We were unable to estimate genetic correlation in GCTA, as the analysis would not converge, but observe that the top SNPs for each form of cataract are positioned on different chromosomes, and that risk variants associated with one form of the disease, are not overrepresented in dogs affected with other forms. Thus, we find no indication of major correlation, which means breeding dogs with the clinically less relevant form of cataract, anterior suture line cataract, is unlikely to lead to increased prevalence of posterior polar- or cortical cataract, which has been a concern.

5.4.3 Breeding implications

Several of the main health challenges in Havanese, and cataracts in particular, have proven hard to eradicate despite breeders' long-term commitment to screening programs and breeding recommendations. In paper IV, we showed that the high age of onset negatively affects sensitivity of cataract screening and accuracy of selection. Even though cataract is an extreme example, in that dogs are typically at the end of their breeding career at the time they are diagnosed, breeding progress for other diseases would also significantly benefit from genetic testing, to identify at-risk individuals at a younger age, and even prior to adoption at 8 weeks.

The simple behavioural test described in paper I, which consisted of greeting the dog and carefully handling it, identified clear phenotypical differences. This indicate that variation in social fears in Havanese can be reliably classified in the adult dog, simply through visual inspection. Given the moderate to high heritability estimates of many behavioural traits (37, 38, 40), and clear selection response observed in studies on selection for tameness (7), genetic improvement should be feasible through simple phenotypic selection. Genetic tools to identify at-risk individuals would nevertheless be highly valuable, to select breeding prospects at an early age, and provide a more objective evaluation of potential breeding partners.

Angular limb deformities, i.e. foreleg bowing, can lead to lameness, pain and osteoarthritis (70), and treatment of severely affected individuals includes relatively invasive surgery at a young age. Even though the prevalence of surgical cases is presumably low, the high clinical relevance in the few dogs that are severely affected, means the issue should still be highly prioritised in breeding strategies.

Angular limb deformities are visible when the dog is fully grown, which means phenotype manifests prior to breeding, but after adoption. Degree of foreleg bowing can be classified using standardised methods such as radiographs (95) or goniometric measurements (188), but may presumably also be evaluated with some degree of reliability through visual inspection of the dog when the coat is soaped up or cut. Preliminary evaluations of Havanese show critiques reveals low repeatability in classification of foreleg bowing (174), which indicate that show results are not reliably predictive of a dog's liability to angular limb deformities.

The results in paper IV showed that there is variation in the presence/absence of the *FGF4*-retrogene on CFA18 in Havanese, and that the mutation is associated with degree of foreleg shortening. Paper II also show that there has been a gradual decline in the frequency of the healthier, wild type allele during the last two decades. A possible explanation is that "flashy" dogs have been favoured in the show ring, and that the coat appears longer and flashier when the legs are shorter, even though the breed standard describes a dog with proportions similar to the longer-legged heterozygote individuals.

Because it is well established that chondrodystrophy significantly increases the risk of angular limb deformities (95, 188, 204), we conclude that gradually increasing the frequency of the healthier, wild type allele would reduce the population risk of foreleg pathology, by addressing the causative underlying risk factor. Although this process will be time consuming and challenging due to the now very low allele frequency, it will more directly address the issue than simply reducing the degree of foreleg bowing within the chondrodystrophic population. The two approaches should therefore be implemented simultaneously to maximise improvement. A few carefully monitored crosses with for example non-chondrodystrophic bichon frises, could be considered to speed up the process of increasing the frequency of the wild type allele.

The findings in paper II exemplify how traits may be unintentionally introduced or increase in frequency due to correlation between traits and hitchhiking effects. Breeders are now trying to reverse the process, and since paper II was published in December 2020, several litters in Norway and Finland have been bred with the intention of conserving the wild type allele. In the prosses it will be important to avoid overuse of individual breeding animals. Continued efforts to identify more carriers of the wild type allele are crucial.

Figure 2: A 18-FGF4RG heterozygote, 5 months old Havanese puppy, bred with the intention of preserving the wild type allele. Photo: Anne Greus.

In paper III, we found high heritability estimates for distichiasis, which was not very surprising given the low age of onset and highly standardised phenotyping through the ECVO screening program. The findings in paper III indicate that it would be possible to significantly reduce the prevalence of the disorder through simple phenotypic selection, if selection intensity was high enough. However, we argue in favour of a less stringent selection strategy, as most affected individuals are only mildly affected and display few clinical signs. A gradual reduction in prevalence, based on combinations of animals rather than strict exclusion, in accordance with recommendations of certified eye scrutinisers (205), is likely sufficient to secure good animal welfare, without negatively affecting genetic diversity.

In paper IV, we show that the prevalence of cataracts has not decreased during the last two decades, despite mandatory eye screening prior to breeding, and consistent exclusion of all cataract affected individuals. We show that sensitivity of cataract screening is low at the age the dogs are typically bred, which leads to inaccurate and ineffective selection - a phenomenon not limited to this breed or trait. Increasing the

average age of breeding animals could improve efficacy of selection against cataracts, as well as other diseases.

In addition to inaccurate selection among adult dogs, a challenge in dog breeding is that a significant proportion of the selection process happens before the dogs are adopted at 8-10 weeks of age, as they are often not even considered as breeding prospects later in life, unless they are owned by a breeder. Thus, although dogs owned by breeders are excluded from breeding if they develop disqualifying flaws, e.g. significant health issues, selection among the dogs that fulfil the minimum criteria, might be somewhat inconsistent. Increased use of older, unmerited pets as studs, based on a thorough evaluation of health, temperament, and conformation, could counteract this tendency, and simultaneously contribute to an increased effective population size.

Maximizing the rate of genetic change isn't a goal in itself, but should reflect the status of the breed. In some breeds the need for change is pressing, whilst in other breeds, where the health status is good and most individuals fit within the frames of some sort of breed ideal, it might be sufficient to address the issues that exists using appropriate genetic tools, to limit the risk of health problems in the future and simultaneously preserve sufficient diversity.

The results of paper IV indicate that, although increasing the age of breeding animals could lead to improved selection response, genetic testing would likely reduce the prevalence of cataracts much more effectively. For posterior polar cataract we find two, single SNPs that show strong association, indicating that one or two polymorphisms might have major effects on the trait. Resequencing the associated regions could result in identification of functional variants, which could enable the development of a DNA-test. For cortical cataract, our findings indicate that the mode of inheritance could be polygenetic, which would make development of DNA-based breeding tools more challenging. If major functional polymorphisms are not detected for one or both forms of cataract, calculation of polygenetic risk scores for breeding animals would be an attractive alternative to traditional DNA-testing.

Genomic prediction of cataract risk has potential as a useful future breeding tool, to estimate genomic breeding values of Havanese that are too young to be phenotyped accurately. Before genomic prediction of cataract risk could be implemented in

breeding programs, the results would have to be carefully validated in independent samples of Havanese. It would also be necessary to calibrate SNP effects and validate the accuracy of prediction regularly after implementation, by continuously eye screening a sufficient number of dogs that are 7 years or older.

In addition to multiple SNP-prediction for all three forms of cataract, prediction based on only the top SNPs could be attempted for posterior polar cataract, but it's expected that this would be less accurate than prediction based on a larger number of SNPs, as the effect of genome wide significant SNPs are always overestimated due to "winner's curse" (159).

If genomic prediction were eventually incorporated in the breeding program of Havanese, with a primary aim of reducing the prevalence of cataracts, it could have synergistic effects for other traits. Validation of each additional trait would be crucial, and an important limitation for several traits would likely be inability of obtaining secure phenotypes. Thus, although there is potential for increased use of both breeding indexes and estimation of polygenic risk scores in dog breeding, there are also challenges and pitfalls. Accurate phenotyping is challenging for many traits in dogs, due to both practical considerations (the breeding animals are privately owned pets) and the nature of breeding goals (e.g. "friendly towards children").

To summarise, mandatory eye screening prior to breeding should be continued, to monitor and control the prevalence of distichiasis, cataracts and other eye diseases, and to provide additional samples for genetic research with an aim of creating better breeding tools for the future. Increasing the average age of breeding animals could improve accuracy of selection against cataracts until DNA-based breeding tools become available. Efforts should be made to preserve the non-chondrodystrophic type in the Havanese population, to gradually reduce the population risk of angular limb deformities. Sociability is a highly desirable trait in Havanese, that should be prioritised in breeding through selection for low levels of social fear.

Lastly, it's important to note that the breeding strategy must also consider other traits, and balance these based on effect on health and welfare. It's advisable that Havanese are screened for patellar luxation, however, several traits that cannot reliably be screened for, like skin issues, may be equally important and must be addressed by excluding dogs that have clinical disease.

5.5 Future perspectives

5.5.1 Challenges discussed in the papers

Our main priority for future studies will be sequencing and variant calling of the associated regions identified in the cataract study (paper IV), with an aim of detecting functional variants that influence disease risk and development. The top SNPs for both posterior polar- and cortical cataracts are located in close proximity to several relevant candidate genes, which will naturally be areas of special interest. Especially for posterior polar cataracts, our findings indicate that few variants may have major effects. The results of the cortical cataract analysis indicate a more polygenetic inheritance, but another mode of inheritance, e.g. a simple inheritance with incomplete penetrance, cannot be excluded.

Whole genome sequencing multiple family groups of affected and unaffected individuals would be ideal, but unrelated cases and controls also provide valuable information. Variant calling, focusing on the associated regions, and searching for variants that are present in affected individuals and absent in controls and unrelated breeds, in the expected manner of a recessive or dominant model respectively, could be an effective method. However, such strategies are challenging in situations with incomplete penetrance. Another fine-mapping strategy could be to use whole genome sequences for genotype imputation to improve marker density in GWAS, to identify imputed SNPs that show high differences in allele-frequencies between cases and controls. Our primary goal would be to identify causative mutations in functional or regulatory regions, enabling the development of a traditional DNA-test, but a genomic prediction approach is a good alternative.

Going forward, the material that has already been collected for paper IV could function as a training set in preliminary attempts at genomic prediction of cataract risk in Havanese. A feasible first step could be to do a BLUP-analysis in GCTA to estimate genomic breeding values and SNP-effects, although Bayesian methods perform better in some analyses (206), and should also be explored. Validation can be done using cross-validation methods within the same dataset, however, validation in an independent sample would be advisable prior to implementation in the breeding program (163). Continued sample collection and eye screening of Havanese aged 7 years or older is important to improve the likelihood of obtaining useful results and increase accuracy of prediction.

A genomic prediction study could also include prediction of social fear, as our preliminary investigations indicate that the trait has moderate to high SNP-heritability, which is crucial for accurate prediction (206). Relative to cataract studies, where samples must be collected from the small number of dogs that are eye screened at a high age, there are nearly unlimited numbers of dogs that could potentially be phenotyped for level of social fear, using owner questionnaires and cheek swabs. Basing classification on a behavioural test could improve accuracy of classification, but would be more practically challenging.

In paper I, we identified two SNPs in exon 2 of the dopamine receptor 2-gene, that were significantly associated with social fear, with an allelic odds ratio between 2.0 and 4.4, depending on the classification method. Regardless of potential studies on genomic prediction, attempt should also be made to identify additional associated variants with major effect on social fear, to increase our understanding of the genetics underlying the distinct phenotypic differences we observed in the Havanese. Using a genome wide association study is a potential approach, which would have the benefit of allowing for identification of novel candidate genes.

A GWAS approach has successfully identified regions associated with similar behavioural traits in other studies (50, 153, 207, 208), but the associations detected for social fear or human sociability is often relatively weak compared to other behavioural traits, even in studies that include large numbers of dogs (50, 153). However, at least one canine GWAS-study report clear associations with social fear, even with a moderate sample size (208). An important challenge in GWAS for behavioural traits, is that due to the presumed highly polygenetic inheritance, each variant is only expected to have a minor effect on the trait, which might not be detectable at a significant level after correction for multiple testing. A broader candidate gene approach is a potential alternative.

5.5.2 Other potential health challenges in Havanese

New information on the health status of Havanese is continuously obtained and shared. When the health survey was conducted in 2012, the breed's popularity had just begun increasing, which means the number of adult Havanese in Norway was much lower than at the present time. A larger population size combined with more information sharing, provides a better understanding of various challenges.

Although the main challenges, including the ones investigated in this thesis, remains highly relevant, others may have arisen or been resolved.

There have been a few reports of intervertebral disc disease (IVDD) in the breed. Although there are no indications that the condition is widespread, investigating potential association between intervertebral disc disease and the FGF4 retrogene on CFA12 could be worthwhile, given the limited resources required and potential benefit. If an association was affirmed, marker-assisted selection could be used to screen breeding animals with increased risk. Given IVDDs high age at onset and significantly negative effect on health in affected individuals, a better tool to reduce the prevalence of the disease would be valuable. An efficient approach could be to initially genotype a selection of Havanese that have been diagnosed with intervertebral disc disease, as it would be purposeless to genotype a large number of controls, if even the cases turned out to be wild type homozygotes.

In the 2012 health survey, there were some reports of allergies (3%), itchy skin of unknown cause (7.4%) and frequent otitis (2%). In the breed community, these and related issues have since been frequently discussed, with a strikingly high number of owners reporting negative reactions to chicken. Although purely anecdotal, the observation inspires further investigations.

The reported prevalence of adverse food reactions in dogs is low (209), but likely underreported due to difficulties in correct diagnosis (210). Secondary to beef and milk, chicken is the most commonly offending food allergen (211). There is little evidence that some breeds are predisposed (212), and to the authors knowledge, the genetic background of adverse food reactions in dogs has not been extensively studied.

The apparently distinct phenotypical differences observed within the population, sparks an interest for a future GWAS-study on the subject.

5.6 Concluding remarks

The study shows how systematic sample collection, thorough phenotype registration, and use of appropriate genetic methods, can provide improved insight

into the genetic makeup of relevant traits and health challenges in a dog breed. The findings from some of the studies have already been successfully implemented in the Havanese breeding program in Norway, and we are confident that more will follow, after further studies and publication.

The studies in paper II and IV provides examples of how health challenges that breeders had been aware of and were willing to prioritise, still had increased in prevalence because the genetic background of the traits was unknown, leading to ineffective selection. In paper II, we suggest that selection for positively correlated traits have unintentionally increased the frequency of the risk allele, and show that even though the *FGF4* retrogene and its effect was already known (18), identifying the knowledge gap and conducting a simple study to estimate allele frequencies and confirm association with the trait of interest, was crucial to improve selection. In paper IV, we suggest that selection has been inaccurate due to the high age of onset and low sensitivity of screening, and recommend increasing the average age of breeding animals until DNA-based breeding tools are made available.

We have successfully identified genetic variants that are significantly associated with both social fear (paper I) and posterior polar cataract (paper IV) in Havanese, but further studies are needed before the findings can be fully utilised in the breeding program. In paper III, we show moderate heritability estimates for distichiasis, which means it should be possible to control the prevalence of the disease through phenotypic selection.

Most breeders want to breed healthy dogs that are well suited to the life they are intended to live, and the diversity and adaptability of dogs illustrates that selection response has been significant. Systematic health screening and careful selection has successfully reduced the prevalence of several diseases (213), but there are also examples of issues that have proven hard to eradicate despite long term commitment from breeders, demonstrating a need for increased knowledge of heritability, mode of inheritance and identification of associated, or preferably, causative genetic variants. The signals of selection observed in regions of the genome associated with physical, but not behavioural, traits (50), are not necessarily purely a result of breeders' priorities, but may also reflect the efficacy of selection for conformation, relative to selection for other traits. Addressing the most important challenges in a breed using appropriate genetic methods, should provide

better breeding tools, and improve accuracy in selection for health and behavioural adaptivity.

Evidence-based breeding is dependent on several factors, with sufficient resources in canine genetic research being an obvious one. There is potential to better the health and welfare of large numbers of dogs simultaneously through genetic improvement, and dogs are particularly well-suited for genetic research because breeding practises has formed the canine genome in a way that makes it feasible to obtain significant results with limited resources. Increased research in canine genetics will lead to improved welfare in dogs, but may also benefit humans, as dogs are valuable natural models.

Evidence-based breeding also depends on improved communication between dog owners, breeders, kennel clubs, breed clubs and researchers. The breed community is an excellent source of a priori information, which can improve the efficacy, quality, and useability of genetic research. Researchers should be better at communicating their results back to the breed community, and always keep the end goal of contributing to improved canine welfare, in mind. In this respect, the trend of open access publishing and adding "plain English summaries" to papers, is a positive contribution.

Lastly, evidence-based breeding is naturally dependent on a willingness among breeders to implement novel knowledge in breeding programs. Prioritizing unhealthy phenotypes or breed purity over welfare, has no place in ethical breeding. Based on experience from the research projects that constitute this thesis, and an observation of many breed communities initiating and funding research, it appears that most breed clubs in Norway are willing to adapt breeding practises according to new evidence.

Havanese is an example of how, given sufficient levels of genetic diversity, a moderate conformation, relevant health testing and systematic use of appropriate genetic tools to combat challenges, we can breed dogs that are both healthy and long-lived, and excel at their job as "Man's best friend".

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7 Papers I-IV

Paper I

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Canine Research

2018 Journal of Veterinary Behavior Early Career Scientist Award in Behavioral Genetics

DRD2 is associated with fear in some dog breeds

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ABSTRACT

Behavioral problems occur frequently in dogs and represent a significant threat to dog welfare. Anxiety, phobias, and fears comprise most of the canine behavioral conditions. The identification of an association between specific behavioral phenotypes and genetic variants of candidate genes would be a valuable tool in selection for dogs less susceptible to anxiety and fear, which may improve animal welfare. The DRD2 gene encodes the dopamine receptor 2. In this study, we found 8 SNPs in the DRD2 gene of the Havanese, a breed that shows large variation in a behavioral phenotype that manifests itself as a tendency to react fearfully by withdrawing in social situations. Significant associations were detected between 2 SNPs in exon 2 of the DRD2 gene and increased social fear in Havanese dogs (n = 158), as evaluated through observation by an external evaluator (respective allelic odds ratio: 4.35, 4.07) and through owner questionnaires (respective allelic odds ratio: 1.96, 2.2). Because different types of fear-related behavioral disorders commonly co-occur, the SNPs in exon 2 were also investigated for possible association to noise reactivity in 5 breeds: Havanese (n = 121), collie (n = 94), Irish soft-coated wheaten terrier (n = 44), Nova Scotia duck tolling retriever (n = 33), and standard poodle (n = 29). Significant associations were detected between SNPs in exon 2 of the DRD2 gene and noise reactivity in the Irish soft-coated wheaten terrier (respective allelic odds ratio: 2.64, 2.88) and collie (allelic odds ratio: 3.03). The same SNP alleles were associated with the beneficial phenotypes in the 3 breeds.

Introduction

Behavioral problems occur frequently in dogs (Bamberger and Houpt, 2006) and represent a significant threat to dog welfare. Behavioral problems are an important cause of both dog abandonment (Scarlett et al., 1999) and euthanasia (Houpt et al., 1996). In 1 study from the United States, at least 1 behavioral reason was recorded for 40% of relinquished dogs and behavioral reasons accounted for 27% of single-reason canine relinquishments (Scarlett et al., 1999). In 1 set of UK shelters, problematic behavior was responsible for 34.2% of relinquishments (Diesel et al., 2010), which is similar to the 35% calculated for purely behavioral relinquishments in the United States (Dolan et al., 2015). The most recent data indicate that 20% of 3.3 million shelter dogs in the United States are euthanized (ASPCA, 2018), with dogs with behavioral concerns especially at risk. Marston et al., 2004 reported that 54% of 4,846 relinquished dogs in 3 shelters in Australia were euthanized for temperament, aggression, or other behavioral problems. Behavioral problems pose the single largest health and longevity threat to modern pet dogs (Dreschel, 2010).

Various types of anxiety constitute a large portion of these behavioral issues, including anxiety in general, various phobias, separation anxiety, noise reactivity, and various social and environmental fears. Bamberger and Houpt, 2006 found that anxiety, phobias, and fears comprise well over 20% of cases presented at a large university behavioral clinic. Fear aggression toward owners (5.2%) and strangers (16.8%) were also common complaints. Being fearful is a welfare issue in itself, but anxious dogs might also be

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subject to secondary welfare issues such as isolation or unethical training methods. Anxiety-related issues are also of relevance to society, as aggression resulting from anxiety can create unpleasant or dangerous situations for people or other dogs. Studies show that different types of anxiety commonly co-occur in both clinical studies (Overall et al., 2001) and survey studies of the general population (Tiira et al., 2016).

Some behavioral traits have high heritability in laboratory animals, humans, and other species studied. A large genetic influence has been detected for personality traits of shyness, inhibition, and fear in people (Eley et al., 2003), and heritability estimates for anxiety disorders, in general, are often high in humans (Davies et al., 2015). One study (Saetre et al., 2006) on behavioral traits in dogs estimated the heritability of the shyness/boldness aspect of a dog's personality to be 0.25. The heritability of fearfulness has been estimated to be 0.5 in one study of guide dogs (Goddard and Beilharz, 1982), whereas fearlessness has been reported to have a heritability estimate of 0.20 in a study of rough collies (Arvelius et al., 2014). One study reported a heritability estimate of 0.56 for gun shyness in Labrador retrievers (van der Waaij et al., 2008).

In a well-known selection study in foxes (Trut et al., 2009), systematic selection was performed to improve tameability. The clear selection response can be considered as evidence that tameness has a high realized heritability, indicating that it is possible to reduce aggressive-avoidance responses through breeding. In an open-field study (DeFries et al., 1978), mice were categorized as fearful or not fearful based on their activity level, allowing the establishment of 3 selection lines (fearful, not fearful, and controls). A strong selection response was shown in both the fearful and the not-fearful lines. A similar strong selection response has also been shown for dogs when breeding for either anxious or outgoing temperament in English pointers (Murphree et al., 1974).

Neurotransmitters are chemical compounds that transfer signals from neurons, by binding to the neurotransmitter receptors on surrounding neurons. A number of neurotransmitters, including dopamine, adrenaline, noradrenaline, serotonin, acetylcholine, and glutamate, are known to influence behavior and mood through regional brain and neurochemical effects.

Regulation of the amount, release, and reuptake/termination of these neurotransmitters is crucial for optimal neurological and mental function. Each neurotransmitter is regulated by various mechanisms, including high numbers of different receptors, transporters, and reuptake systems that work together in complex interactions. Each of the receptors is encoded by specific genes, and different genetic variants in these genes may influence the function of the receptor and "success" of neurotransmission.

Dopamine levels in the amygdala can influence individual differences in anxious temperament in humans (Kienast et al., 2008). Low dopamine reuptake by neurons is associated with increased anxiety and irritability (Laakso et al., 2003). Genes related to dopamine regulation may also have an association with anxiety and behavioral issues in dogs (Lit et al., 2013a, b, c). DRD2 is one of the several dopamine presynaptic receptors, which functions as an autoreceptor to ensure negative feedback when dopamine levels are elevated (Stahl, 2008). A polymorphism in the 3′UTR-region of the human gene has been associated with dopamine receptor density and anxiety, in close interaction with the dopamine transporter gene DAT (Kulikova et al., 2008).

There is large interest in animal models in human psychiatry, to understand molecular contributions to psychiatric disorders, including those related to anxiety. The identification of associations of specific behavioral phenotypes with genetic variants of candidate genes would be an important step in an increased understanding of etiology and could improve psychopharmacological application and aid new drug development. For dogs, identification of genetic variants could also improve animal welfare by improving selection for less fearful and anxious individuals.

Breeds represent pools of canalized genetic variation. Dog breeds can be important sources of information about behavioral phenotypes and genetic variants. In 2013, the Norwegian Havanese Club conducted a survey on health and behavior in their breed, in which 18.6% of owners reported that their dog was either "nervous" or "very nervous" (unpublished results). The survey indicated that there are interesting phenotypic variations within the breed, motivating further investigations. In the preliminary research for this study, owner interviews were conducted to obtain more detailed information on the phenotype of these "nervous" dogs. We found that the dogs functioned relatively well in everyday life and at home but had an exaggerated tendency to react fearfully in certain social situations with unfamiliar dogs and people. This behavior represents a major deviation from the typical and desired behavior of the Havanese, as these dogs are considered to generally have a very sociable and outgoing personality.

The main goal of this study was to investigate potential associations between the DRD2 gene and an increased tendency in several dog breeds to react fearfully to social or environmental stimuli, in the absence of truly threatening circumstances. Fear can be normal and adaptive in context. For the purposes of this study, pathological fear was defined as responses to stimuli (social or physical) that are characterized by active (backing or turning away, escape, hiding, flight) or passive (lowered/hunched body posture, tail tucked/down, ears back) avoidance/withdrawal behaviors associated with sympathetic physiological signs (increased heart rate/respiration, shaking, trembling, salivation, mydriasis) (Overall, 2013).

One form of environmental fear is noise phobia/reactivity. Noise-phobic dogs are characterized by a profound, nongraded, extreme response to noise, that manifest as intense avoidance, escape, or anxiety, associated with the sympathetic branch of the autonomic nervous system. Dogs that are characteristically distressed when exposed to specified noises but that do not meet the criteria for a "phobia" may be classified as 'reactive' in the absence of more specific provocative information (Overall et al., 2001; Scheifele et al., 2016).

We investigated dopamine gene variants with respect to fear in social situations and noise phobia and reactivity.

Materials and methods

Dogs

Data on social fears in Havanese and noise reactivity in 5 breeds (including Havanese) were collected from privately owned dogs in collaboration with breed clubs and owners (Table 1). First, a candidate gene study on DRD2 and social fear was conducted in the

Table 1
Breeds and number of dogs included in the study

Breed (abbreviation)	Number of dogs (females, males)
Havanese	158 (92, 66)[a]
Collie (smooth and rough)	94 (62, 32)
Irish soft-coated wheaten terrier	44 (27, 17)
Nova Scotia duck tolling retriever	33 (17, 16)
Standard poodle	29 (19, 10)
Total	358 (217, 141)

[a] Number of individual Havanese where information on at least one phenotype (provocative behavioral evaluation [PBE], owner score on social fear and/or classification for noise reactivity) was available. For most of the dogs, there was information on all 3 phenotypes, but a portion of dogs were classified as intermediate and therefore excluded—see detailed information in the text.

Table 2
Breed and number of cases and controls for noise reactivity

Breed (abbreviation)	Number of dogs (cases, controls)
Havanese	121 (25, 96)
Collie	94 (49, 45)
Irish soft-coated wheaten terrier	44 (20, 24)
Nova Scotia duck tolling retriever	33 (16, 17)
Standard poodle	29 (15, 14)

Havanese. Cases and controls from 5 breeds (Table 2) was then tested to look for associations of noise reactivity to the identified SNPs. Owners were contacted through the respective breed clubs and the Norwegian Kennel Club (including adverts in the Norwegian Kennel Clubs journal and in dog shows), and samples were collected from all dogs whose owner responded and allowed DNA sampling.

EDTA-blood samples were collected from all dogs by certified veterinarians, with owner's consent, in agreement with the provisions enforced by the Norwegian Animal Research Authority. Genomic DNA was extracted using E.Z.N.A blood DNA kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's recommendations and subsequently stored at −20°C. The samples were collected according to rules for ethical approval for collecting blood samples (FOR-2010-07-08-1085, FOR-1996-01-15-23, Regulation on Animal Experimentation). Performagene buccal swabs (DNA Genotek Inc) were used when blood sampling was impossible due to geographic distance. DNA was extracted following the manufacturer's recommendations.

Behavioral classification for social fear in Havanese

In Havanese, the dogs' tendencies to react fearfully in social situations were classified both through a PBE and through a questionnaire (owner score).

Inclusion criteria for each classification system (behavioral parameters and survey questions, respectively) were based on the phenotypic characteristics described in owner interviews during the initial planning of the study. An increased tendency to act fearfully towards unfamiliar dogs and people, displayed as active (backing or turning away, escape, hiding, flight) or passive (lowered/ hunched body posture, tail tucked/down, ears back) avoidance behaviors, were the main complaint and therefore the main focus of the Havanese study.

Lower body and tail posture has been associated with fearful, withdrawn, or uncertain behaviors (Beerda et al., 1999). Owner interviews revealed that "dropping the tail" was an important indicator of fear in Havanese. For this reason, tail position was also registered in the PBE.

Provocative behavioral evaluation

A standardized evaluation of the dogs' behavior was performed for each dog. The evaluator first presented herself to the owner, ignoring the dog. The evaluator then approached the dog directly by bending down, holding one hand forward, and calling the dog. Finally, the dogs' reaction to gentle restraint at an examination table before DNA sampling was registered. Tail position was noted at the time of initial greeting.

The dogs were observed and classified for 3 criteria (contact seeking, tail position, and reaction to gentle restraint that physically supported and stabilized the dog). Dogs that displayed fearful behavior in all criteria were classified as cases and dogs that displayed no fearful and only affiliative behavior in all criteria were classified as controls. The same person (K.K.L.B.) evaluated all the dogs that were included in the study. DNA samples were obtained in the home of the owner after finishing the behavior evaluation.

Questionnaire (owner score)

A questionnaire was sent to all owners of dogs that participated in the provocative behavioral evaluation. It was also sent to Havanese owners who were not able to participate in the provocative behavioral evaluation due to geographic distance, which explains the difference in sample size.

The questionnaire consisted of 9 questions concerning the dogs' tendency to react fearfully in social situations (Supplemental Table 1). The owners were asked to what degree they could agree with various statements on the dogs' behavior. Answers were given on a 5-point scale, representing high to low levels of fear. The average of all answers was then calculated to indicate the individuals' general tendency to react fearfully in social situations (owner score [OS]).

Behavioral classification for noise reactivity determined by short questionnaire across breeds

Owners of collies (smooth and rough), Irish soft-coated wheaten terriers (ISWTs), Nova Scotia duck tolling retrievers (NSDRs), standard poodles, and Havanese answered 4 questions concerning reactions to loud noises including gunshots, fireworks, thunderstorms, and heavy traffic (Supplemental Table 2). Answers were given on a 5-point scale, indicating high or low levels of noise reactivity. Cases were defined as dogs with a score of ≤ 2 in at least 1 of the 4 categories and controls had a score of ≥ 4 in all categories. This methodology mirrors that of published studies (Overall et al., 2006, 2014; Scheifele et al., 2016).

Selection of candidate genes

DRD2 is an interesting gene that has been associated with a large variety of behavioral traits in humans (Munafo et al., 2007; Markett et al., 2011; Takeuchi et al., 2015). Several studies have found associations between the DRD2 gene and personality traits of apprehension and neuroticism (Kulikova et al., 2008; Kazantseva et al., 2011; Montag et al., 2012). Because the phenotype of interest in the Havanese was a general "nervousness" and increased tendency to react fearfully, we chose DRD2 as the candidate gene for this study.

Primers

Primers embracing all exons and UTRs were designed based on the reference dog genome (CanFam3.1), using Primer3plus (https://primer3plus.com/). Amplification was successful for all parts except for exon 1 and parts of the 3′UTR. Optimal temperatures were detected using a temperature gradient PCR program with temperatures ranging from 54°C to 64°C.

Sequencing of the PCR products was performed following a standard Sanger method on an ABI 3500 XL DNA analyzer (Applied Biosystems, Life Technologies of Thermo Fisher Scientific), followed by manual inspection using the Sequencher software from Gene Codes Corporations, at the Norwegian University of Life Sciences. Primers and optimal temperatures are listed in the Supplemental Table 3.

Statistical analyses

For the association testing, odds ratios were calculated according to Altman (Altman, 1991) and the *P*-values were calculated according to Sheskin (Sheskin, 2004). The correlation between the 2

means of classification for social fear in Havanese was calculated using Pearson correlation coefficient, and the intrarater reliability of the questionnaire was calculated using Cronbach's alpha, both in JMP Pro v. 14. The positive and negative predictive value (PPV/NPV) of the owner questionnaire (OQ) compared to the PBE was calculated using the formulas (true case/classified as case using questionnaire) and (true control/classified as control using questionnaire), respectively.

Results

Provocative behavioral evaluation

A total of 104 Havanese underwent a provocative observational classification (Table 3). Of these, 28 dogs were classified as cases and 33 were classified as controls. Forty-three dogs did not meet the criteria for either cases or controls (indicating an intermediate phenotype) and were therefore excluded.

Questionnaire (owner score)

Owners of 150 dogs responded to the questionnaire. Dogs with more than 2 missing answers were excluded ($n = 3$). The lowest recorded individual owner score (most fearful dog score) was 1.22 and the highest (least fearful dog score) was 5.0. The average score was 4.12 (Figure). Cutoff for cases was set as 0.5 σ below average OS and the cutoff for controls was set as 0.5 σ above average OS. Forty-three dogs were classified as cases and 60 dogs were classified as controls.

Correlation and predictive value between questionnaire and provocative behavior test for social fear

The correlation between the 2 means of classification for social fear in Havanese was calculated using Pearson correlation coefficient and was good ($\rho = 0.738$, P-value < 0.001). All Havanese dogs that were classified as cases using the questionnaire were also classified as cases in the PBE. Four dogs that were classified as controls using the questionnaire were classified as cases in the PBE. Based on the dogs that had results from both evaluations, the PPV/NPV of the OQ compared to the PBE were estimated to 1.0 (case in OQ also case in PBE) and 0.88 (control in PBE also control in OQ), respectively.

Questionnaire reliability

Most of the questions included in our questionnaire were identically worded as questions included in a health survey conducted in the Havanese breed a few months later. Because some owners ($n = 35$) had answered both questionnaires, we were able to calculate an estimate of intrarater reliability using Cronbach's alpha. We found good reliability for survey scores on both social fear and noise reactivity ($\alpha = 0.82$ and $\alpha = 0.80$, respectively). Other studies on questionnaires designed in a similar way also indicate that OQs on behavior have acceptable reliability (Hsu and Serpell, 2003).

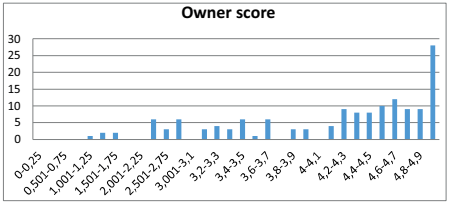

Figure. Distribution of average owner scores; x axis is score; y axis is frequency.

Detection of SNPs

All exons were initially sequenced for a small group of 8 unrelated Havanese to identify regions with variation. Eight SNPs were identified in the DRD2 gene—3 located in introns, 3 located in exons, and 2 located in the 3′UTR (Table 4). The 3 exonic SNPs were synonymous.

Exon 2

The 2 synonymous SNPs in exon 2 were evaluated for association to phenotype. First, we evaluated these with respect to social fear in Havanese and then to noise reactivity in the breeds collie, ISWT, NSDR, standard poodle, and Havanese. The allele frequencies varied between the breeds and can be found in Table 5.

Social fear in Havanese

Genetic assessment using the PBE

Significant association was detected between 2 SNPs in exon 2 of the DRD2 gene and social fear in Havanese dogs, classified through a PBE. In the first SNP (5:19782667), the allelic odds ratio was 4.35 (P-value 0.0008) (T = beneficial allele). In the second SNP (5:19782829), the allelic odds ratio was 4.07 (P-value 0.0010) (C = beneficial allele).

Genetic assessment using OQ

Significant association was detected between the 2 SNPs in exon 2 of the DRD2 gene and social fear in Havanese dogs, classified through an OQ (owner score). In the first SNP (5:19782667), the allelic odds ratio was 1.96 (P-value 0.0283) (T = beneficial allele). In the second SNP (5:19782829), the

Table 3
Criteria for observed phenotype classification in the provocative behavioral evaluation

Observation	Case (N = 28)	Control (N = 33)
Reaction to visitor	Avoiding contact with visitor, hiding behind owner	Actively contact seeking, does not pull away when petted
Tail position	Down	Up
Reaction to gentle restraint on examination table before DNA sampling	Strong avoidance, climbing on to owner, frantic escape behavior, or vocalization	No or only mild avoidance calmly accepting gentle restraint or positive reaction

Table 4
SNPs identified in the DRD2 gene (Havanese)

Index	Intron/exon	Location (CanFam 3.1)	Alleles (CanFam3.1 in bold)	Amino acid change
1	Intron 1	5:19782497	**G**/A	-
2	Exon 2	5:19782666	**C**/T	Synonymous
3	Exon 2	5:19782828	**T**/C	Synonymous
4	Intron 4	5:19787766	**T**/C	-
5	Intron 4	5:19787788	**C**/T	-
6	Exon 7	5:19791794	**C**/T	Synonymous
7	Exon 8, 3′UTR	5:19794262	**A**/G	-
8	Exon 8, 3′UTR	5:19794287	**T**/C	-

Table 5
Allele frequencies (%) of the SNPs in exon 2 in the DRD2 gene

SNP chromosome and position[a]	Alleles	Havanese	Collie	ISWT	NSDR	Standard poodle
5:19782666	C/T	64/36	0/100	61/39	55/45	36/64
5:19782828	T/C	63/37	13/87	60/40	50/50	48/52

[a] Canfam 3.1.

allelic odds ratio was 2.22 (*P*-value 0.0095) (C = beneficial allele). The average behavioral score of each genotype can be found in Table 6.

Noise reactivity across breeds

Significant association between noise reactivity and the DRD2 gene was detected for the ISWT and collie. Significant association was found between the first SNP (5:19782667) and noise reactivity in the ISWT (this SNP showed no variation in the collie), with allelic odds ratio of 2.64 (*P*-value 0.0371) (T = beneficial allele). Association between noise reactivity and the second SNP (5:19782829) was significant in the ISWT with allelic odds ratio of 2.88 (*P*-value 0.0227) and in the collie with allelic odds ratio of 3.03 (*P*-value 0.0319) (C = beneficial allele).

Discussion

Significant associations were detected between SNPs in exon 2 of the DRD2 gene and social fears in Havanese and noise reactivity in ISWT and collie. Because the SNPs are synonymous, the functional effect associated with the SNPs is most likely due to the effect of variation in linked sequences/modifications. We found no association to noise reactivity in the Havanese, but the observed portion of dogs with noise reactivity in this breed was very low compared to the other breeds (Table 2), which may indicate that noise reactivity is not an issue of large importance in this specific breed.

The level of social fear in the Havanese dogs was classified through both an OQ and a PBE by an external evaluator. Observations made by the owner and observations made by an external evaluator have different strengths and weaknesses (Spady and Ostrander, 2008).

A challenge when working on behavioral traits is consistent recording of traits and describing them correctly. To obtain a consistent characterization and diminish misclassification, we used strict inclusion criteria for a dog to be classified as a case or control in the PBE. Because of the stated definitional and inclusion criteria, we believe that our records are consistent, which is important for a reliable analysis of the described and studied phenotype. This test will identify fearful dogs, although they will not all be equally fearful, and some may be less affected than others.

Previous studies have shown that complex behavioral patterns in dogs can be reliably evaluated by an experienced person and that a few, well selected characteristics may be sufficient to describe the differences between dogs (Wilsson and Sundgren, 1997).

Table 6
The average behavioral score of each genotype

5:19782667 (beneficial allele in bold)	Average OS	5:19782829 (beneficial allele in bold)	Average OS
CC	4.00	TT	3.94
CT	4.15	T**C**	4.18
TT	4.39	**CC**	4.42

OS, owner score.

The major weakness of owner evaluation may be that owners may evaluate dogs differently based on their skills and frame of reference. Owners may also not be objective because they are reluctant to classify their dog as fearful. One study showed that owners are less likely to report unfavorable behavioral traits in a nonconfidential survey, compared to a confidential survey (Segurson et al., 2005). Owners also may recognize or understand only the easiest to detect signs of any fear- or anxiety-related condition and so underestimate the presence of the condition if their dogs show different signs (Mariti et al., 2012). Finally, some behavioral signs are simply less apparent than others if constant monitoring of the dog is not occurring, suggesting that false negatives may be a risk (Overall et al., 2016). This issue is further discussed, below.

Subjective bias in owner evaluation (e.g., systematic underreporting of fear) could be a challenge if one owner/breeder was reporting several dogs from a certain line/genotype, which could lead to a false association. The number of Havanese per owner in this study was 2.08, and therefore, we do not believe that the owner classification represents a systematic problem. Most owners did, however, rate their dogs quite high, indicating low levels of fear.

Another challenge using owner ratings by questionnaires is that dogs may change by age and that the level of challenges/exposure (e.g., time of exposure, and loudness/type of fireworks) the dog have met at the time the owner replies to a questionnaire may influence results. If dogs are not exposed or not witnessed to react, owners would report a potential false negative (Overall et al., 2016). We note that in the survey-based classification for social fear in Havanese, there was no significant age difference between the case and control groups.

To reduce the risk of misclassification, the survey questions were based on wording frequently used by owners, to ensure a mutual understanding of the terminology. In addition, the distinct phenotypic variations in the Havanese breed combined with relatively strict inclusion criteria for cases and controls should help reduce the risk of misclassification. Studies show that questionnaires designed in a similar matter have acceptable validity (Duffy et al., 2014; Hsu and Serpell, 2003).

In the owner-based classification, we observed that the criteria/threshold for inclusion of dogs as cases/controls could have a marked influence on the results. This demonstrates the challenge of a biologically correct behavioral phenotyping and may, together with genetic heterogeneity, explain the variable reproducibility of many studies on genetics of behavior. To reduce the frequency of misclassification and obtain a clear difference between cases and controls, we were conservative and cutoffs were set as 0.5 σ above and below average owner score.

We found good correlation between the survey-based and observational classification of the Havanese ($\rho = 0.738$, *P*-value < 0.001). All Havanese dogs that were classified as cases using the questionnaire were also classified as cases in the PBE. Four of the dogs that were classified as controls using the questionnaire were classified as cases in the PBE, suggesting that the concern with false negatives on owner-based assessments is real (PPV = 1.0; NPV = 0.88), but minor. This result confirms that there is generally very good concordance between the 2 classification systems but also underpins our hypothesis of a slight underreporting/underobserving of fear in the OQ producing the occasional false negative as has been noted in other studies (Overall et al., 2016). That owners appear to produce false negatives suggests that more studies should validate questionnaire results with behavioral test results within the same population under study, something especially important for genetic studies.

In this initial study, we did not identify functional mutations in any of the successfully sequenced exons of DRD2, as the identified

SNPs associated with fear were both synonymous. However, some studies have shown the likely effect of synonymous mutations in DRD2 on RNA stability (Duan et al., 2003). If DRD2 is functionally involved, the functional effects may also be due to closely linked variants in gene regulatory regions (3′ or 5′ to the gene, including promoter regions) or to epigenetic effects.

It is also possible that the functional effect is caused by variants in closely linked genes. The region 3′ to the gene involves genes including NCAM1-TTC12-ANKK1 and is frequently discussed in behavioral issues (Savitz et al., 2013). Functional interactions with other relevant genes are also reported (Montag et al., 2010). On the 5′-side of DRD2 are HTR3A and HTR3B, 2 serotonin receptors and potential candidate genes (Jajodia et al., 2015; Kondo et al., 2015), within 300K distance. This is a region with many candidate genes reported to be associated with anxiety, which supports that the present results may indicate important functional variants in the region.

Exon 2 was prioritized in the association testing and sequenced for all individuals. Exon 7 was only sequenced for a small group of dogs to look for variation. Because the SNPs in exon 2 are located only 8966 base pairs away from the SNP in exon 7, the probability of recombinations between them is negligible. We expect a large degree of LD between all markers within the gene, i.e., variation in exon 7 would be covered by the variation in exon 2. The SNPs in exon 2 were prioritized over the SNP in exon 7, partly because of a higher estimated MAF.

Conclusion

SNPs in exon 2 of the DRD2 gene are significantly associated with an increased tendency to react fearfully in social situations in Havanese and noise reactivity in Irish soft-coated wheaten terrier and collie. The same alleles were associated with the beneficial phenotypes in the 3 breeds. There was no significant association between noise reactivity and the SNPs in the Havanese, NSDR, or standard poodle. Because the SNPs are synonymous, the functional effect associated with the SNPs is most likely due to linked mutations and/or epigenetic effects.

Acknowledgment

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Ethical considerations

EDTA-blood samples were collected by certified veterinarians, with owner's consent, in agreement with the provisions enforced by the Norwegian Animal Research Authority. The samples were collected according to rules for ethical approval for collecting blood samples (FOR-2010-07-08-1085, FOR-1996-01-15-23, Regulation on Animal Experimentation).

Conflict of interest

Karen L. Overall is the Editor-in-Chief of Journal of Veterinary Behavior: Clinical Applications and Research. Another editor, Christel Moons, was appointed to manage all steps of this submission to ensure that the highest ethical standards were upheld by the Journal.

Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jveb.2018.07.008.

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Paper II

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Canine Medicine and Genetics

RESEARCH Open Access

Short and sweet: foreleg abnormalities in Havanese and the role of the FGF4 retrogene

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Abstract

Background: Cases of foreleg deformities, characterized by varying degrees of shortened and bowed forelegs, have been reported in the Havanese breed. Because the health and welfare implications are severe in some of the affected dogs, further efforts should be made to investigate the genetic background of the trait.
A FGF4-retrogene on CFA18 is known to cause chondrodystrophy in dogs. In most breeds, either the wild type allele or the mutant allele is fixed. However, the large degree of genetic diversity reported in Havanese, could entail that both the wild type and the mutant allele segregate in this breed. We hypothesize that the shortened and bowed forelegs seen in some Havanese could be a consequence of FGF4RG-associated chondrodystrophy.
Here we study the population prevalence of the wild type and mutant allele, as well as effect on phenotype. We also investigate how the prevalence of the allele associated with chondrodystrophy have changed over time. We hypothesize that recent selection, may have led to a gradual decline in the population frequency of the lower-risk, wild type allele.

Results: We studied the FGF4-retrogene on CFA18 in 355 Havanese and found variation in the presence/absence of the retrogene. The prevalence of the non-chondrodystrophic wild type is low, with allele frequencies of 0.025 and 0.975 for the wild type and mutant allele, respectively (linked marker).
We found that carriers of the beneficial wild type allele were significantly taller at the shoulder than mutant allele homozygotes, with average heights of 31.3 cm and 26.4 cm, respectively.
We further found that wild type carriers were born on average 4.7 years earlier than mutant allele homozygotes and that there has been a gradual decline in the population frequency of the wild type allele during the past two decades.

Conclusions: Our results indicate that FGF4RG-associated chondrodystrophy may contribute to the shortened forelegs found in some Havanese and that both the wild type and mutant allele segregate in the breed. The population frequency of the wild type allele is low and appear to be decreasing. Efforts should be made to preserve the healthier wild type in the population, increase the prevalence of a more moderate phenotype and possibly reduce the risk of foreleg pathology.

Keywords: FGF4, Chondrodystrophy, Chondrodysplasia, Havanese, Short ulna, Genetic diversity, Exaggerations in conformation

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Plain English summary

Previous research and statements from owners, breeders and breed clubs, show that some Havanese have short and bowed forelegs. Most of these dogs show no signs of pain or discomfort, but a few of them do.

Some dog breeds are so-called chondrodystrophic, meaning that their legs are "too short" compared to the size of their body. Examples of chondrodystrophic breeds are dachshunds, bassets and corgis. The gene that causes chondrodystrophy is known and can be tested for (FGF4-retrogene on chromosome 18).

There is a lot of variation in the Havanese breed as regards color, size, head shape etc. We hypothesize that because there is so much variation in Havanese, it is possible that some of them are chondrodystrophic and some are not.

Could it be that Havanese with short and bowed forelegs do not have a "breed specific syndrome" as we have thought, but are simply chondrodystrophic?

We DNA-tested 355 Havanese to check this and to investigate whether things have changed over time. Is it possible that selection for certain conformational traits have unintentionally turned a primarily non-chondrodystrophic breed, chondrodystrophic, and subsequently made them more prone to foreleg bowing?

We found that some of the Havanese we DNA-tested are chondrodystrophic and some are not. In our sample, only about 5% of the dogs carry the non-chondrodystrophic gene variant.

We also found that carriers of the non-chondrodystrophic gene variant are taller at the shoulder than other Havanese, with average heights of 31.3 cm and 26.4 cm, respectively.

Carriers of the non-chondrodystrophic gene variant are born on average 4.7 years earlier than the other dogs in our sample. More Havanese are chondrodystrophic now, compared to two decades ago.

We recommend that Havanese are DNA-tested, to identify carriers of the non-chondrodystrophic gene variant. By breeding these dogs, we can prevent the variant being lost from the breed forever.

Carefully monitored outcrossings to non-chondrodystrophic individuals in closely related breeds may also be considered.

If we gradually increase the number of Havanese that are not chondrodystrophic, the breeds' overall risk of foreleg problems will reduce, which would benefit the health and welfare of the breed.

Background

Previous research has shown that foreleg deformities occur frequently in the Havanese breed [1]. In Norway, bowed forelegs is a common remark in dog show critiques and sporadic cases of short ulna syndrome have been reported [2]. In a survey conducted in the United States, 44% of Havanese owners replied that their dog had bowed, shortened or asymmetric forelegs [1].

Starr et al. [1] propose the idea of a breed specific syndrome in Havanese, including symptoms like bowed forelegs, cataracts, liver abnormalities and heart disease. Moderate heritability estimates were found and a few candidate genes were suggested [1].

Bowed forelegs in dogs is often a result of some form of leg shortening. When the growth of the long bones is stunted, it is often asynchronous as well. Disparity in length between the radius and ulna cause the shorter bone to act as a bowstring, which lead to the subsequent bowing of the longer bone. Stunted growth of the long bones, may be caused either by trauma to the growth plate before the dog is fully grown, or by genetic predisposition [3].

Several forms of hereditary disproportional dwarfism have been described in dogs [4–10]. A recessive mode of inheritance is reported in many breeds [4–10] and associated genes or possible causative mutations are known in some of them. A nonsense-mutation in the ITGA10-gene cause chondrodysplasia in Norwegian elkhounds and Karelian bear dogs [4]. In Labrador retrievers, a mild form of chondrodysplasia is associated with a mutation in the COL11A2-gene [6]. A deletion in the SLC13A1-gene has been associated with chondrodysplasia in miniature poodles [5].

In addition to breed specific forms of chondrodysplasia, disproportionally short legs also occurs as a desired and fixed trait in several dog breeds. Chondrodystrophy is caused by an expressed fibroblast growth factor 4 (FGF4) retrogene on chromosome 18, across dog breeds [11]. The FGF4-retrogene is responsible for the typical "short-legged" appearance of chondrodystrophic breeds like dachshunds, bassets and corgis.

Unlike chondrodysplasia, chondrodystrophy is often considered an accepted phenotypic variation, rather than a pathological condition. The trait is, however, still associated with increased risk of some health issues. Chondrodystrophic dogs are more likely to have bowed forelegs, and 3.5 times more likely to be affected with elbow disease, than non-chondrodystrophic dogs [12]. Angular limb deformity and elbow incongruity may cause abnormal strain on the joints and secondary degenerative joint disease [3]. In the chondrodystrophic dog breed Skye terrier, clear association was found between lameness and the degree of elbow incongruity [13].

Additionally, chondrodystrophic dog breeds are at increased risk of developing intervertebral disc disease [14], although recent research has shown that a FGF4-retrogene on CFA12 is of greater importance in intervertebral disc disease in dogs than the one on CFA18 [15, 16].

In the research that led to the discovery of the FGF4-retrogene on CFA18, four breeds (jack russel terrier, west highland white terrier, Havanese and Sussex spaniel) were excluded from the initial association analyses because leg length in these breeds was uncertain or variable. Later, sequencing of the insert revealed that out of seven Havanese included in the original study, six were homozygote for chondrodystrophy and one was heterozygote. The authors air the idea that the previously reported "Havanese syndrome" may disguise the absence of the retrogene and that this could be the reason that the trait is not fixed [11].

The Havanese breed was created from various small dogs and anecdotally there was significant conformational variation in the founder dogs that is still evident [17]. Several reports also show a relatively high degree of heterozygosity in the breed [18, 19]. It is plausible, that contrary to the situation in most other breeds, both alleles of the FGF4-retrogene segregate in this breed. We hypothesize that the short and bowed forelegs seen in some Havanese could potentially be a result of chondrodystrophy, rather than a breed specific syndrome as previously suggested.

The prevalence of bowed and shortened forelegs in the Havanese breed is high [1, 2]. Although most cases show little signs of discomfort or pain, the negative effect on health and welfare is severe in some cases. If the shortened and bowed forelegs seen in Havanese are directly associated with chondrodystrophy, increasing the population frequency of the non-chondrodystrophic allele, could reduce the breeds overall risk of foreleg pathology.

The aim of this study was to investigate the presence/absence of the chondrodystrophic genotype in the Norwegian population of Havanese dogs, as well as its effect on phenotype. We also studied how the population frequency of the wild type and mutant allele has changed over time.

Results

Prevalence

We genotyped an A/G SNP on chromosome 18 (CFA18), base position 23,432,408 (CanFam2), that has previously been reported as part of a "chondrodystrophy-haplotype" [11], for a random sample of 355 Havanese. We found that although most individuals were homozygote for the allele associated with chondrodystrophy (A), 5% of the population carried one copy of the wild type allele (G). The allele frequencies were 0.975 and 0.025 for the chondrodystrophy-associated allele and the wild type allele, respectively. No dogs were homozygote wild type.

To verify the linkage disequilibrium between the marker and the insert, 22 A/A-dogs and 22 A/G-dogs were assayed for the FGF4 insertion on CFA18. The LD between the SNP and the causative insert was complete in our sample ($n = 44$).

Association

We found significant association between genotype and shoulder height in Havanese ($n = 103$). Havanese with one copy of the beneficial allele (A/G) were on average 4.9 cm taller than risk allele homozygotes (A/A) ($p < 0.0001$), with an average heights of 31.3 cm and 26.4 cm, respectively (Fig. 1).

Change in allele frequency over time

To investigate potential changes over time, we genotyped a random sample of 285 Havanese with available information on birth year. Havanese that carried the wild type allele were born on average 4.7 years earlier than A/A-homozygotes (p-value < 0.0001). Analysis of allele frequencies in different birth year groups, show that there has been a gradual decline in the allele frequency of the wild type allele during the past two decades (Fig. 2).

Discussion

We show that both the wild type and mutant allele of the FGF4-retrogene segregate in the Norwegian population of Havanese dogs and that it is associated with shoulder height. Our results support that the short and bowed forelegs seen in some Havanese could potentially be a result of chondrodystrophy, rather than a breed specific syndrome as previously suggested.

It should be noted, that the prevalence of the risk allele is high, but the number of severely affected individuals (e.g. those requiring surgery) is low, which means that modifying genes probably affect the degree of foreleg bowing and elbow incongruity in the chondrodystrophic dogs. Our result does not uncover other associated genes, but highlight the increasing population frequency of an unnecessary, underlying risk factor.

For most dogs in the study, we genotyped a very closely linked variant rather than the causative insert itself. The studied SNP is, however, located only 1272 base pairs away from the insert site (~ 0.001 cM), which means the likelihood of a recombination is very low. We have verified a complete LD between the variant and the retrogene in a selection of 44 Havanese with genotypes A/G ($n = 22$) and A/A ($n = 22$). The strong association between the marker and phenotype also point towards true variation in the presence/absence of the retrogene.

Shoulder height was selected as a phenotypic marker for foreleg shortening, because it could be easily and reliably measured by the owner. A more standardized measure, e.g. using radiographs to evaluate the degree of foreleg bowing or having one person measure all the dogs, could have improved precision of the

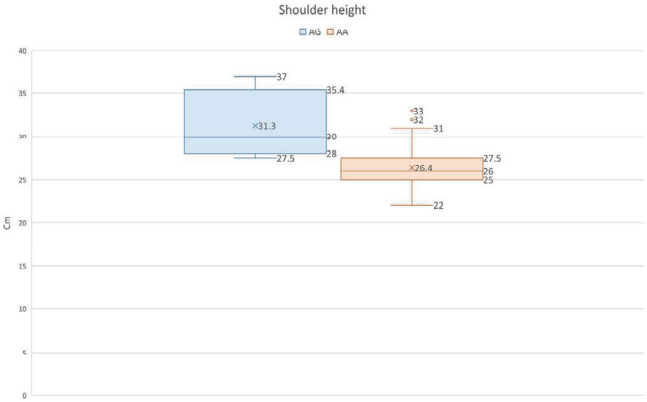

Fig. 1 Average and median shoulder height in genotype groups AG and AA

measurements, but would significantly reduce the number of dogs we were able to include in the study. We believe the degree of error in owner measurements is similar in the two genotype-groups and should therefore not affect the result of our association analysis.

The primary aim of the study was to investigate the frequency of the FGF4 retrogene and discuss potential effects on the population risk of foreleg pathology. A thorough clinical evaluation of the dogs, which would be necessary to accurately classify the degree of foreleg bowing and give a conclusive description of prevalence, was beyond the scope of this study. All owner-reported cases of severe foreleg bowing have been from dogs that are risk allele homozygotes.

We did not identify any wild type homozygotes. This is not surprising, given the low population frequency of the wild type allele. The absence of G/G-individuals prevent us from investigating possible phenotypic differences between G/G-dogs and heterozygotes. Traditionally, chondrodystrophy has been considered a dominant trait in dog, but the significant height difference we found between A/G- and A/A-individuals show that at least in this breed, the dominance is incomplete. Some forms of chondrodysplasia in human, also show incomplete dominance [20].

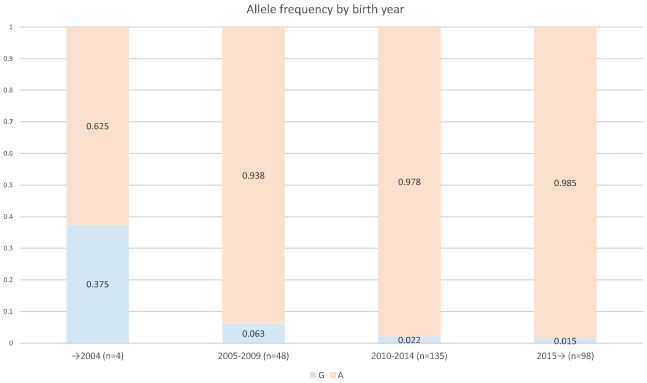

Fig. 2 Allele frequencies for the wild type (G) - and mutant (A) allele, by birth year

The Fédération Cynologique Internationale (FCI) breed standard for Havanese [21], states that the height at the withers should be between 23 cm and 27 cm (tolerance 21 cm to 29 cm), which means the average height of the A/A-dogs is correct. Increasing the number of A/A- x A/G-matings, would reduce the prevalence of dogs with disproportionally short legs, with a risk that some offspring might be too tall according to standard. We believe that preserving the wild type allele before it is lost should be of high priority. We therefore suggest allowing a limited increase in height for the first generations that may be corrected in succeeding generations through traditional selection.

A slight increase in the height acceptance in the breed standard could also be considered. This would allow a faster change in allele frequency and still leave room to focus on other traits, because the need to select for height would decrease. Increasing the height acceptance to 30 cm, which equals the median height of the A/G-dogs, would be enough to ensure most A/G-dogs are still within standard. This is also in accordance with what some consider to be the original, Cuban standard [17].

Lastly, it should be noted that the standard lists a "French front" (pasterns to close and feet turned outwards) as an important fault [21].

We show a decline in the population frequency of the wild type allele during the past two decades, with A/G-dogs being on average 4.7 older than A/A-dogs. This finding is supported by statements from breeders, who indicate that there has been a "trend" of selection for longer backs and shorter legs in recent years. It is possible that a selection for certain conformational traits have unintentionally turned a primarily non-chondrodystrophic breed, chondrodystrophic.

Chondrodystrophy is associated with increased risk of angular limb deformity and elbow disease [12]. If the shortened and bowed forelegs seen in Havanese are directly associated with chondrodystrophy, increasing the prevalence of the non-chondrodystrophic wild type in the population could reduce the number of dogs with increased risk of foreleg pathology, subsequently reducing the number of clinically affected individuals. This would benefit the health and welfare of the breed.

Marker-assisted selection should be implemented to gradually increase the population frequency of the beneficial allele and ensure that the non-chondrodystrophic type is not lost. We believe any increase in the frequency of the wild type allele has the potential to reduce risk of foreleg pathology and that ideally, the wild type should eventually become be the predominant variant. However, it is challenging to obtain a fast change in allele frequency without negatively influencing genetic variation and/or other traits. The initial goal should therefore be to recover a sustainable population of non-chondrodystrophic individuals and avoid that the risk allele becomes fixed.

DNA-testing as many Havanese as possible for the FGF4-retrogene on CFA18, would be valuable to identify the rare, wild type carriers for breeding purposes. Litters from wild type carriers should be tested prior to adoption, to ensure continuation of the breeding program.

To avoid loss of genetic variation through selection for the low frequency wild type, it may also be worth considering a limited outcross to wild type carriers in closely related breeds like the bichon frisé. If done right, such an outcross could increase the prevalence of the wild type allele and speed up the reversal process, without much negative effect on other traits because the breeds are so similar.

Parallel to breeding for a gradual increase in the population frequency of the non-chondrodystrophic genotype, efforts should be made to reduce the degree of foreleg deformities and elbow incongruity among the chondrodystrophic Havanese. Selection response in other chondrodystrophic breeds have shown that it is possible to reduce the degree of foreleg bowing by selection based simply on visual inspection. A suggested protocol for classification of elbow incongruity in chondrodystrophic breeds [13], could potentially be used to screen chondrodystrophic Havanese prior to breeding.

Conclusions

Our findings show that leg length in Havanese is strongly associated with FGF4-retrogene variants, in an incomplete dominant manner. The allele frequency of the wild type allele is low and appear to be decreasing. Efforts should be made to preserve the healthier wild type allele in the population, increase the prevalence of a more moderate phenotype and reduce the risk of foreleg pathology.

Methods

Dogs

Two batches of samples, all collected with owners' consent, were included in the study. The first batch of samples was recruited specifically for this project, for the association analysis. Owners were asked to measure the shoulder height of their dog and send in a cheek swab for DNA-studies ($n = 120$). The samples were collected using Performagene™ buccal swabs (DNA Genotek Inc), administered by the owner. DNA was extracted following the manufacturer's recommendations. The second batch of samples was originally recruited for a research project on behaviour [22] and was readily available through our DNA biobank ($n = 235$). The second batch of dogs was only included in the allele frequency calculation and birth year analyses. The only inclusion criteria

in both batches were age > 1 year old and that the owner was willing to participate. DNA was stored at − 20 degrees Celsius.

Genotyping

An A/G SNP at base position CFA18:23432408 (CanFam2), that has previously been reported as part of a "chondrodystrophy-haplotype", was genotyped for 355 Havanese. The SNP is positioned 1272 base pairs downstream of the insert [11]. Primers used were forward: 'TTACCCACAAGGAAGATACAGC' [11] and reverse: 'TGCAGTGACCCCATCAGTTC'. Primer3plus was used to create the reverse primer. Sequencing of the PCR products were performed following a standard Sanger method on an ABI 3500 XL DNA analyzer (Applied Biosystems, Life Technologies of Thermo Fisher Scientific), followed by manual inspection using the Sequencher software from Gene Codes Corporations.

Linkage disequilibrium between the SNP and the causative insert was checked and verified in a material of 44 dogs. We amplified the insert site on CFA18 in Havanese with genotypes A/A ($n = 22$) and A/G (n = 22) (G/G not available), using allele-specific PCR. Primers used were: forward: F_flank: 'TTGGGAATGTCAAACCACTG', F_insert 'GTCCGTGCGGTGAAATAAAA' and reverse: R_flank: 'GTTCCCTCCATTTCGGTTT' [23]. When no insert was present, the primers F_flank/R_flank gave a PCR-product~ 388 bp. When an insert was present, the primers F_insert/R_flank gave a PCR-product~ 168 bp. Following the PCR reaction, results were visualized by gel electrophoresis and manual inspection.

The allele frequencies were calculated using the formula: $p = f\,(\mathrm{AA}) + 0.5\,f\,(\mathrm{AG})$, $\mathrm{q} = f\,(\mathrm{GG}) + 0.5\,f\,(\mathrm{AG})$.

Association analyses and statistics

Shoulder height measured by the owner, was selected as a phenotypic marker for the degree of foreleg shortening. Shoulder height was defined as the distance from the ground to the "withers", i.e. the ridge between the shoulder blades at the tallest part of the dogs back, near the base of the neck.

For the association analyses on shoulder height and birth year, the mean and standard deviation for each genotype was calculated in Excel (AVERAGE, STDEV.S). The pooled standard deviation and standard error, as well as the significance level using the t-test, were calculated using MedCalc [24].

Acknowledgements

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Authors' contributions

KB designed the study, organized the collection of samples, obtained information on phenotype from owners, did the labwork, analyzed the results and wrote the manuscript. FL gave guidance and input throughout the project and substantially revised the manuscript. Both authors have read and approved the final manuscript.

Funding

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Availability of data and materials

The dataset analyzed during the current study is not publicly available due to difficulty in fully anonymizing the individual dogs, but is available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All dog owners have given their consent to the use of their dogs DNA-sample in research. DNA-samples were either newly collected using cheek swabs administered by the owner or from earlier studies (available as DNA from our biobank).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Paper III

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Canine Medicine and Genetics

RESEARCH Open Access

Heritability of distichiasis in Havanese dogs in Norway

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Abstract

Background: Distichiasis is a presumed inherited eyelid disease, characterized by misplaced eyelashes. The effect on eye health and animal welfare varies between individuals; most mild cases show no clinical signs, but some affected animals develop painful corneal disease.

In this study, we investigated the prevalence and heritability of distichiasis in the Norwegian population of Havanese dogs.

Results: A total of 1156 Havanese were included in the study. Out of these, 168 were affected with distichiasis, making the prevalence in our sample 14.5% (95% CI 12.5–16.6%). There was no sex predisposition. Most affected individuals were graded "mildly affected".

The estimates generally showed high heritabilities, which varied between 0.276 (linear model) and 0.720 (Bayesian threshold model). The linear estimates, after conversion to the underlying scale ($h^2_l = 0.664–0.674$), corresponds well to the results of the Bayesian models.

Conclusions: The estimated heritability of distichiasis in Havanese is high and the prevalence is moderate. The high heritability indicate that a significant selection response could be obtained by simple mass selection. To secure good animal welfare, it's important to control the number of affected individuals and especially the severely affected.

Plain English summary: prevalence and heritability of distichiasis in a population of Havanese Dogs

Distichiasis is an eye condition, characterized by misplaced eyelashes, that is frequently seen in dogs. Some dog breeds appear to be more at risk than others. The degree of clinical signs in affected dogs varies a lot. Many mild cases appear to be completely asymptomatic, while others suffer pain and damage to the eye, which necessitates removal of the hairs.

In this study, we investigate both how common distichiasis is in the Havanese dog breed and estimate the degree of genetic influence on the trait. We find that 14.5% of eye screened Havanese, registered in the Norwegian Kennel Club, are affected with distichiasis. Most cases are graded "mild". There is no significant difference in how many males and females are affected.

We find high heritability estimates of distichiasis in Havanese (≈0.28 calculated by linear models and 0.59-0.72 calculated by Bayesian threshold models), showing a high genetic influence on the trait. The high estimated heritability mean that it should be possible to reduce the prevalence of the condition, and contribute to improved animal welfare, though systematic breeding.

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We recommend that all Havanese are eye screened prior to breeding, to control the prevalence of distichiasis, as well as other eye conditions that are relevant in the breed, like cataracts. Dogs with severe distichiasis or ectopic cilia should not be bred. Dogs with mild or moderate distichiasis may be bred to an unaffected partner.

Keywords: Distichiasis, Havanese, Heritability, Prevalence

Background

Distichiasis is a condition characterized by misplaced eyelashes. The term distichiasis stems from the Greek words di and stichos, meaning two rows. In dogs the term in somewhat misleading, as there is no complete row of extra lashes, but rather one or several individual stray hairs [1, 2].

The hairs arise from ectopic hair follicles in the tarsus and emerge through the meibomian duct openings [2, 3]. The condition may be uni- or bilateral and the number of misplaced cilia vary considerably between eyes and between individuals [3, 4]. The clinical relevance is variable. Many affected individuals show no clinical signs, but some dogs experience corneal damage and pain that requires removal of the hairs [1, 2, 5]. The degree of pain and corneal damage vary and are not directly proportional to the number of cilia [4].

In some cases, one or a few single hairs grow through the palpebral conjunctiva a few millimeters from the eyelid margin, directly onto the cornea. These hairs are referred to as ectopic cilia [1–3, 5]. Distichiasis and ectopic cilia are two different forms of disease that are both caracterized by misplaced eyelashes and are grouped together in the ECVO (European College of Veterinary Ophthalmologists) certificates. Ectopic cilia generally cause significant corneal disease and pain [1, 3, 5].

Clinical signs of distichiasis and ectopic cilia may include epiphora, squinting of the eyes, photophobia, keratitis, and corneal damage. Because many dogs affected with distichiasis don't show clinical signs, it's important to rule out possible additional diagnoses when clinical signs are evident [3].

Distichiasis and ectopic cilia normally occur early in life and are often congenital, but may develop at any age [4, 5]. According to the European College of Veterinary Ophthalmologists (ECVO) scheme, a dog is considered to be "affected" by distichiasis if the diagnosis has been made by a panel member once, even if no stray cilia are detected on subsequent examinations [5].

Both distichiasis and ectopic cilia are classified as presumed hereditary eye diseases [5]. Studies show evidence of a genetic component [6] and that affected dogs are more likely than unaffected dogs, to parent affected offspring [7].

Some breeds, like Pekingese, poodles and both American and English cocker spaniels, are reported to be affected more frequently than others [3, 4, 8], which support that the trait is heritable. Heritability estimates for distichiasis are high in cocker spaniels [7, 9], but low in Tibetan terriers [10]. Distichiasis is reported in Havanese through the open databases of kennel clubs in several countries, which indicate that the problem is widespread in this breed.

In this study, we calculate heritability estimates for distichiasis in Havanese with ECVO eye results registered in The Norwegian Kennel Clubs (NKK) database. Increased knowledge of the genetic component of the trait would be important to select an optimal breeding strategy, to secure a low frequency of the trait and good animal welfare.

Results

Prevalence and grading

Of the 1156 dogs in the material, 168 were affected with distichiasis, making the prevalence of distichiasis in our sample 14.5% (95% CI 12.5–16.6%).

Of the affected dogs, 86.9% ($n=146$) were graded "mild", 10.1% ($n=17$) were not graded, 2.4% ($n=4$) were graded "moderate" and 0.6% (n = 1) was graded "severe". Of the graded dogs, 96.7% were graded "mild", 2.6% were "moderate" and 0.7% were graded "severe". Ectopic cilia were noted in a single dog.

Heritability estimates

The heritability was estimated using both linear and Bayesian threshold models.

Linear model

Alternative linear models, with slight differences in how year of diagnosis was treated, all gave heritability estimates around ~0.28 (Table 1).

Table 1 Estimated variance components and heritability, with standard deviation in brackets

Model	Genetic variance	Residual variance	Heritability
1	0.034 (0.007)	0.088 (0.006)	0.276 (0.050)
2	0.034 (0.007)	0.087 (0.006)	0.279 (0.051)
3	0.034 (0.007)	0.088 (0.006)	0.280 (0.051)

Table 2 Posterior means and highest posterior density (HPD) regions for dispersion parameters

Model	Genetic variance (σ_a^2)				Heritability			
	Posterior mean	HPD region		Effective sample size	Posterior mean	HPD region		Effective sample size
		Lower	Upper			Lower	Upper	
1	1.598	0.578	2.890	1455.0	0.594	0.415	0.762	1535.0
2	3.078	0.761	6.407	496.8	0.720	0.540	0.900	459.0
3	2.373	0.686	4.652	707.5	0.674	0.491	0.854	991.9

Bayesian threshold models

Heritability estimates using a Bayesian threshold method, were 0.594, 0.720 and 0.674 for the three models, respectively.

Posterior means, highest posterior density (HPD) regions and effective sample size for genetic variance and heritability from the three models are shown in Table 2. Trace plots for heritability and genetic variance for model 1 are shown in Fig. 1 (trace plots for model 2 and 3 are similar and not shown).

Comparison of the heritability estimates from the linear- and Bayesian threshold models

To compare the heritability estimates from the linear models with the estimates from the Bayesian threshold models, the results of the linear models were converted to the underlying normally distributed liability scale, as described by Dempster and Lerner [11] (Table 3).

The relatively small data set leads to large standard errors for the heritabilities estimated by the linear models, as well as large HPD regions for the estimates from the threshold models.

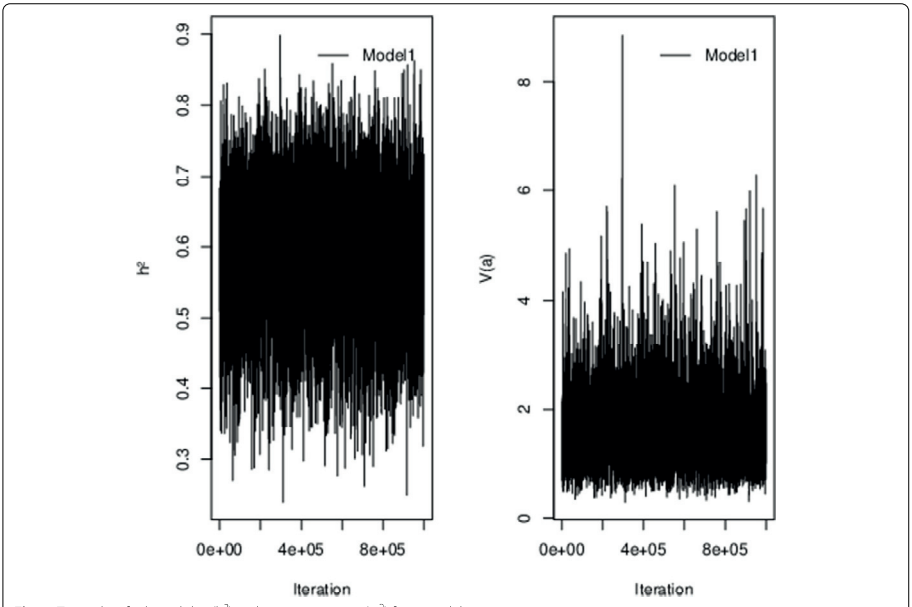

Fig. 1 Trace plots for heritability (h^2) and genetic variance (σ_a^2) from model 1

Table 3 Heritabilities from the linear models on the observed (h^2_o) and underlying liability scale (h^2_l) with standard error in brackets and heritabilities from the threshold models (h^2_t) with highest posterior density region in brackets

Model	h^2_o	h^2_l $p=0.145$ $=>z_{ord}=0.227$	h^2_t
1	0.276 (0.050)	0.664 (0.120)	0.594 (0.415–0.762)
2	0.279 (0.051)	0.671 (0.123)	0.720 (0.540–0.900)
3	0.280 (0.051)	0.674 (0.123)	0.674 (0.491–0.854)

Correlation of breeding values

The correlation between breeding values estimated by the linear models and breeding values estimated by the Bayesian threshold models was high (>0.97) (Table 4). Spearman rank correlation >0.98 indicates almost identical ranking based on breeding values predicted by linear and threshold models.

Sex effects

The 1156 dogs consisted of 426 males and 730 females. There was no significant difference in prevalence between males (13.1%) and females (15.3%) ($X^2(1, N=1156)=1.05$, $p=.307$).

The genetic correlation between the estimates analyzed within sex were 0.885 (0.144), 0.934 (0.139) and 0.930 (0.141) for model 1–3 respectively, which means there is no significant effect of sex on the trait.

Conflicting results and age

By comparing the distichiasis result of the first and last examination in dogs that have been eye screened at least twice, we found that out of the 1156 Havanese in our material, 49 had gone from unaffected to affected, and 11 had gone from affected to unaffected, in the first and last examination respectively. Out of the 2259 eye certificates registered in the kennel club database between 2005 and 2020, only 33 came from dogs that were younger than 1 year old at the time of examination.

Table 4 Correlation coefficients between breeding values calculated by a linear model and breeding values calculated by a threshold model

Model	Pearson's correlation	Spearman's rank correlation
1	0.984	0.993
2	0.976	0.984
3	0.980	0.988

Test percentage

By comparing registration numbers and the number of ECVO eye certificates in the kennel club database from 2005 to 2020, we find a test percentage of 21.3%.

Out of all litters registered between 2005 and 2020, at least one puppy was eye screened in 48.8% of them.

Discussion

Our results show a prevalence of distichiasis in Havanese of 14.5%. To the authors knowledge, few studies have been conducted on the prevalence of distichiasis in different dog breeds, which gives little reference for comparison. In a study of Tibetan terriers, 11.43% of the study population were affected with distichiasis [10], while a study of English cocker spaniels in Denmark showed a prevalence of distichiasis of 49.31% [7]. Havanese have a long and furnished double coat. Considering that most of the predisposed breeds, like poodles, Pekingese and cocker spaniels are also heavily coated, it's possible that selection for a profound coat could contribute to an increased risk of distichiasis.

Since April 2016, the Norwegian Kennel Club require a valid ECVO certificate for all Havanese used for breeding, for the offspring to be eligible for registration [12]. Due to breed club recommendations [13], which have been in place since the breed club was founded in 2009, most breeding animals were eye examined yearly prior to 2016 as well. The number of individual Havanese that have been eye screened at least once between 2005 and 2020, equals 21% of the number of Havanese registered in the same time period. The number of individual Havanese litters, in which at least one puppy has been eye screened, equals 49% of the number of litters registered in the same time period. Based on these numbers, we consider the test-percentage sufficient.

As there is no official dog registry in Norway, it is uncertain what percentage of purebred dogs are registered in the Norwegian Kennel Club. However, by comparing numbers from a microchip registry to the kennel club database, we find that ≈70% of microchipped dogs are registered in the NKK. Because the total number of microchipped dogs also include mixed breeds, it's reasonable to assume that more than 70% of purebred dogs in Norway are registered in the kennel club.

More females than males are eye screened, and we presume this is because more females than males are used for breeding. This indicates that dogs that are intended for breeding are eye screened more often than other dogs. Breeders often keep and test one or two puppies from each litter with the intention of continuing their breeding program. We therefore believe that a large portion of the active breeding population is tested, as opposed to certain lines or litters being overrepresented.

Based on these factors, we believe our material is representative of Havanese registered in the Norwegian Kennel Club and the Norwegian population of Havanese in general.

We have considered the possibility of owners removing misplaced hairs prior to examination, which could result in misclassification of affected dogs, but based on our knowledge of the breed community we believe this to be unlikely. Because a distichiasis diagnosis does not exclude a dog from breeding, the owner's motivation to falsify the result of the examination is limited. We also believe that potential removal of hairs in a limited number of dogs, would be relatively equal in different breeding lines/families, thus we believe the potential effect on heritability estimates is neglectable. However, the "once affected=always affected" policy, is established to limit this source of error.

In a total of 60 dogs, the distichiasis status changed from the first to the last eye examination. In 49 dogs the result went from unaffected to affected and in 11 dogs the results went from affected to unaffected, from the first to last examination respectively. The 49 dogs that went from unaffected to affected, could have developed distichiasis later than usual, or it's possible that a few very mild cases may have gone undetected in the first examination. However, according to the ECVO "once affected=always affected" policy, no dog should go from affected to unaffected with distichiasis. The 11 cases could be caused by human error in filling out the certificates and we consider the number low enough not to represent an important source of error.

Distichiasis normally occurs early in life and is often congenital. However, if a large portion of dogs were tested at a very young age, it could potentially result in an underestimation of prevalence. In our material, only 33 out of 2259 eye certificates came from dogs that were under 1 year old at the time of examination, which indicate that this source of error is most likely neglectable.

Our results show high heritability estimates for distichiasis in Havanese dogs, using both linear and Bayesian threshold models. This means that it should be possible to control the prevalence of the disease through traditional mass selection, without complex routines for index estimation.

Transformation of the heritabilities estimated by the linear models to the underlying liability scale, show results that are similar to the Bayesian estimates. Additionally, for all three models there are very high correlations (>0.97) between breeding values calculated by the linear models and breeding values calculated by the Bayesian threshold models. Our results indicate that with prevalence as in the present data, computationally heavy Bayesian threshold models could be successfully substituted by linear models.

Most of the affected individuals in our material were graded mild, with comments often indicating that only one or a few cilia were present. The hairs are often soft, which may partly explain why many Havanese affected with distichiasis don't show clinical signs [1, 14]. However, as we know distichiasis and ectopic cilia cause pain and corneal damage in some individuals, measures should be made to control the prevalence.

The ECVO breeding guidelines states that it is "optional" to breed affected animals, with the exception of severe cases [5]. Mild and moderate cases may only be bred to an unaffected partner [15]. Results from Petersen et al. [7] supports this recommendation, as the risk of producing affected offspring is higher when two affected dogs are bred, than when an affected animal is paired with an unaffected partner. It is further recommended to exclude all dogs affected with ectopic cilia [15]. Because the number of severely affected Havanese is very low, this policy will exclude very few dogs from breeding and at the same time prevent high risk combinations.

Our findings support that the mandatory ECVO eye screening prior to breeding should be continued. Results from routine eye screenings can be helpful in monitoring the prevalence of distichiasis, since they are easily available through the NKK open database. The ECVO eye screening scheme is highly standardized and good routines are implemented for secure identification of animals and publication of results. This makes it a valuable breeding tool, to help breeders reduce the prevalence of distichiasis as well as other eye diseases that are relevant in the breed, like cataracts [16, 17].

Conclusion

We show that 14.5% of Havanese that are registered in the Norwegian Kennel Club and have been eye screened between 2005 and 2020, are affected with distichiasis.

The heritability estimates for the disease are generally high: around 0.28 calculated by linear models, which is comparable to the values from the Bayesian threshold models of 0.59–0.72, after conversion to the underlying liability scale. The high heritability suggest that it should be possible to reduce the prevalence of distichiasis through routine eye screenings and traditional mass selection.

Dogs that have ectopic cilia or severe distichiasis should be excluded from breeding, while dogs with mild or moderate distichiasis may be bred to an unaffected partner if they have other valuable traits that may be beneficial for the breed.

Methods

Dogs

The study material was collected from The Norwegian Kennel Clubs (NKK) database. For an eye certificate to be

registered in the NKK database, thorough protocols must be followed to secure quality assurance of the diagnostic testing. Prior to examination, owners must consent to the result being made publicly available. Animals are identified by microchip numbers that are linked to the kennel club registry and controlled by the examiner prior to examination. Only veterinarians who are certified eye scheme examiners and have completed the Nordic Eye Examination Committees extensive educational program [18], can register results in the database.

The Havanese breed was selected for this study because it is the most registered breed in the Companion and Toy group in The Norwegian Kennel Club, and one out of two breeds in the top 16 most registered breeds were a yearly eye examination is mandatory prior to breeding. Between 2005 and 2020, a total of 5422 Havanese, from 1756 different litters, were registered in the Norwegian Kennel Club [14].

The high registration numbers, high frequency and quality of diagnostic testing, as well as availability through an open database, entailed good quality data was available for analysis.

Inclusion criteria were Havanese that are registered with a pedigree in the NKK and have at least one ECVO certificate registered between 2005 and 2020. The material was readily available from the NKK open database and all available certificates were included (prior to duplicate removal and removal of dogs with missing information).

The classification of dogs as either "affected" or "unaffected" with distichiasis is done by visual inspection by a certified eye scheme examiner, as described in the ECVO manual [5], and the presence or absence of distichiasis is mandatory to record in all examinations. The diagnosis is classified as either "affected" or "unaffected", but the examiner may also grade the diagnosis. In patients were signs of corneal irritation, ectopic cilia and/or hard and stiff distichia are present, the grade is always classified as "severe" [5]. Because there was little variance in the grading in our material, and grading is not mandatory, we classified the dogs as "affected" or "unaffected" in the analysis.

Because dogs are often examined more than once during their lifetime, duplicate observations were removed. From an original sample of 2259 observations, 1166 unique Havanese remained after duplicate removal. The ECVO scheme states that once a dog is determined to be "affected" with distichiasis by a panel member, the diagnosis is final, i.e. once affected = affected [5]. We classified our sample accordingly, by keeping the "worst" diagnosis in individuals with conflicting results.

Out of the 1166 dogs that remained after duplicate removal, 10 were removed because the date of examination ($n=7$) or date of birth ($n=3$) was missing. The remaining 1156 dogs came from 857 different litters.

Tracing of the 1156 Havanese in the Norwegian Kennel Clubs pedigree files, resulted in a pedigree file of 3327 dogs.

Heritability estimates

Linear models

Three different linear models were used:

$$y = Sex + Age + a + e \quad (1)$$

$$y = Sex + R\_Year + Age + a + e \quad (2)$$

$$y = Sex + R\_Year + b * C\_Age + a + e \quad (3)$$

where: y = vector of observed diagnoses, Sex = fixed effect of sex, Age = fixed effect of age in years, R_Year = year of diagnosis, b = regression on C_Age, where C_Age is age at diagnosis as a continuous variable, a = random additive genetic effect and e = the random residual.

Assumption for random effects are:

$a \sim N(0, \sigma_a^2 A)$, where σ_a^2 is the genetic variance and A is the additive relationship matrix

$e \sim N(0, \sigma_e^2 I)$, where σ_e^2 is the residual variance and I is an identity matrix.

The analysis was conducted with the average information restricted maximum likelihood (AI-REML) module in DMU [19].

Bayesian threshold models

The same three models were analyzed by a Bayesian threshold model, using the Gibbs Sampler.

module in DMU [19]. To ensure identifiability of dispersion parameters and threshold, the residual variance (σ_e^2) was restricted to unity. Because only one registration was included per individual, which is known to create problems in threshold animal models, the genetic variance was sampled based on individuals that have offspring, as described by Ødegård et al. 2010 [20].

For each of the 3 models, the Gibbs Sampler was run for 1,100,000 rounds with the first 100,000 discarded as burnin. Every 10'th of the remaining 1,000,000 samples was stored for the Post Gibbs analysis.

Post Gibbs analysis was conducted by BOA software [21] and own developed software for computation of effective sample size.

For each of the stored samples, heritability was computed as $\frac{\sigma_a^2}{\sigma_a^2+1}$.

Mixing properties of the Markov chain Monte Carlo (MCMC) chains were visually inspected by trace plots.

Comparison of the heritability estimates from the linear and Bayesian threshold models

The heritabilities from the threshold models are expressed on the underlying scale. For comparison with the results from the linear model, the estimated heritabilities from the linear models were converted to the underlying normally distributed scale by the formula by Dempster and Lerner [11]:

$$h^2_l = \left(h^2_o x\ p\ x\ (1-p)\right)/z^2_{ord}$$

where:

h^2_l = heritability on the underlying scale.

h^2_o = heritability on the observed scale.

p = frequency.

z_{ord} = height of the standard normal distribution at the threshold value corresponding to p.

Correlation of breeding values

The correlation between estimated breeding values from the linear and Bayesian threshold models were calculated as both Spearman and Pearson correlation coefficients.

Sex effects

To correct for potential sex effects and possible confounding with age, both age and sex were included in the models used for the heritability estimates (model 1–3).

Sex effect on diagnosis was investigated by running a bivariate restricted maximum likelihood (REML) analysis on the three models from the heritability estimates (containing age-effect), treating diagnosis in each sex as two separate traits.

The significance level for potential difference in prevalence between males and females, were calculated using the chi-squared test.

Abbreviations

AI-REML: Average information restricted maximum likelihood; CI: Confidence interval; ECVO: European College of Veterinary Ophthalmologists; HPD: Highest posterior density; MCMC: Markov chain Monte Carlo; NKK: The Norwegian Kennel Club; REML: Restricted maximum likelihood.

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Authors' contributions

All authors were involved in designing the study, as well as preparation, analysis, and interpretation of data. KB wrote the manuscript, with important contributions from FL and PM. PM did the computer analyses for the heritability estimates. All authors have read and approved the final manuscript.

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Availability of data and materials

The phenotype- and pedigree information that support the findings of this study is freely available through The Norwegian Kennel Clubs open database, Dogweb [14].

Declarations

Ethics approval and consent to participate

The study was conducted using only results from diagnostic testing that had already been completed and made publicly available at the owners' request.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Paper IV

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Cataracts in Havanese: genome wide association study reveals two loci associated with posterior polar cataract

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Abstract

Background:
Cataract is considered an important health issue in Havanese, and studies indicate a breed predisposition. Possible consequences of cataracts include lens induced uveitis, reduced eyesight, and blindness in severe cases. Reducing the prevalence of cataracts could therefore improve health and welfare significantly. The most frequently diagnosed forms of cataract in Havanese are cortical- and anterior suture line cataract, but cases of posterior polar cataract are also regularly reported. Out of the three, posterior polar- and cortical cataracts are considered the most clinically relevant.

Results:
We performed a genome wide association study that included 57 controls and 27 + 23 + 7 cases of cortical-, anterior suture line- and posterior polar cataract, respectively. An association analysis using a mixed linear model, revealed two SNPs on

CFA20 (BICF2S23632983, p = 7.19e-09) and CFA21 (BICF2G630640490, p = 3.33e-09), that were significantly associated with posterior polar cataract, both of which are linked to relevant candidate genes. The results suggest that the two variants are linked to alleles with large effects on posterior polar cataract formation, possibly in a dominant fashion, and identifies regions that should be subject to further sequencing.

Promising regions on CFA4 and CF30 were also identified in the association analysis of cortical cataract. The top SNPs on each chromosome, chr4_12164500 (p = 4.30e-06) and chr30_28836339 (p = 5.62e-06), are located within, or in immediate proximity to, potential cataract candidate genes.

The study shows that age at examination is strongly associated with sensitivity of cataract screening. Havanese in Norway are on average 3.4 years old when eye examinations are performed: an age where most dogs that are genetically at risk have not yet developed clinically observable changes. Increasing the average age of breeding animals could improve accuracy of selection and genetic progress.

Conclusions:
The study identified two loci, on CFA20 and CFA21, respectively, that were significantly associated with posterior polar cataract in Havanese. Two peaks, on CFA4 and CFA30, showed putative association with cortical cataracts. All the top SNPs are located in close proximity to cataract candidate genes. The study also show that sensitivity of cataract screening is highly dependent on age at examination.

Keywords: GWAS, cataract, Havanese, dogs, ECVO, *FOXP1, RYBP, LGR4, ANK3, PCLAF*

Background

Cataracts is considered an important health challenge in the Havanese dog breed (1), and is characterized by opacities of the lens (2). Approximately 5% of ECVO eye examinations of Havanese in Norway result in a cataract diagnosis (1, 3), and in a study from North America, Havanese were the second most frequently diagnosed breed, with an estimated prevalence of 11.57% (4). Closely related breeds, like the bichon frisé, Maltese and Bolognese, are also predisposed (4-6).

Cataracts are defined by lens opacities (2). The lens is normally a dehydrated structure, which consists primarily of proteins, and the structure and organisation of these are crucial to maintain lens transparency. Swelling of lens fibers or disruption of proteins, for example because of fluid absorption, will result in light scatter and an opaque lens. Cataracts must not be confused with the normal increase in nuclear density in older dogs, called nuclear sclerosis (7).

Cataracts in dogs are often classified based on age at onset, localization in the lens and degree of opacification (2, 7). The most commonly reported forms of cataracts in Havanese in Norway, are cortical-, anterior suture line-, and posterior polar cataracts (3), which is in accordance with literature (7). Cortical cataract is also the most frequently diagnosed type of cataract in the closely related breed bichon frise (6). The clinical significance is dependent on the localization, density and extent of the opacity, as well as the rate of progression, which means posterior polar- and cortical cataracts are generally considered more clinically relevant than anterior suture line cataract. (8).

Cataracts can cause reduced eyesight or blindness, depending on the extent of the opacities. In addition to its effect on eyesight, cataracts can also lead to lens induced uveitis caused by leakage of lens proteins (2, 7). In cases where it is necessary to treat the cataract, the only definite therapy in dogs is surgical removal of the entire lens. The surgery is invasive, requires substantial follow-up by the veterinarian and owner, and is associated with risk of postoperative complications. Postoperative complications can include postoperative uveitis, glaucoma, retinal detachment, or phthisis bulbus (2, 7).

Causative mutations for cataracts have been identified in some dog breeds. In the wirehaired pointing griffon, a mutation in the FYCO1 gene, which encodes a protein that is important for lens autophagy, was identified as the likely cause of a form of juvenile cataracts (9). *FYCO1* is also associated with many forms of cataracts in humans (10-12). Mutations in the HSF4 gene cause a recessive form of cataract in Staffordshire bull terriers and Boston terriers, a dominant form of cataract in Australian Shepherds (13, 14), as well as several forms of cataracts in humans (15, 16). In many breeds, studies have failed to identify causative variants (17-20), even though there is evidence of a strong genetic influence on the trait (6, 18, 21).

Since April 2016, eye examining both parents maximum one year prior to breeding, has been a requirement for registration of Havanese puppies in The Norwegian Kennel Club (22). However, yearly eye examinations in breeding animals have been custom for Havanese breeders prior to 2016 as well, due to breed club recommendations (23). We have previously shown that 21% of Havanese that are registered in the Norwegian Kennel Club database are eye examined at least once in their lifetime, and

that at least one puppy is examined in half of all registered Havanese litters (24).

Despite almost two decades of good compliance to the screening program and using only unaffected individuals for breeding, the prevalence of cataracts in Havanese in Norway has been stable (1). We hypothesized that the main reason for this may be the late onset of disease. Many affected individuals have already had several litters at the time of diagnosis, which means the true selection pressure is very low. In addition, some sources indicate that the mode of inheritance could be recessive (7), which would mean that most risk alleles in the population are hidden in heterozygotes and that excluding affected dogs from breeding would only remove a minor proportion of risk alleles (25). A better understanding of the genetic background of cataracts in Havanese, and ideally the identification of causative mutations, would be valuable to improve the efficiency of the breeding program.

The aim of this study was to explore the genetic background of cataracts in Havanese. To identify genomic regions that may be important in cataract development and could be subject to further analysis and fine mapping, we conducted a genome wide association study (GWAS). We also examined age of onset and effect of age on test sensitivity, to estimate a reasonable minimum age for controls, for use in both genetic studies and breeding strategies.

Results

No obvious changes in prevalence from 2003-2022

Preliminary investigations had indicated a lack in selection response. Our results confirm that there has been no reduction in the observed prevalence of cataracts during the last two decades (table 1a and 1b).

Table 1a: Eye screening results for Havanese, year 2003-2022, from the Norwegian Kennel Club database

Year of examination	*N exams*	*Affected*	*Unaffected*	*Suspected*	*Prevalence (95% CI)*
2003-2007	104	1	103	0	1.0% (0-2.8%)
2008-2012	545	25	509	11	4.6% (2.8-6.3%)
2013-2017	800	38	739	23	4.8% (3.3-6.2%)
2018-2022	1132	66	1033	33	5.8% (4.5-7.2%)
Total	**2581**	**130**	**2384**	**67**	**5.0% (4.2-5.8%)**

Table 1b: Eye screening results for Havanese, year 2003-2022, from the Norwegian Kennel Club database

Year of examination	*Cortical*	*Post polar*	*Ant.sut.l*
2003-2007	1	0	0
2008-2012	12	3	9
2013-2017	21	3	15
2018-2022	20	4	31
Total	**54**	**10**	**55**

Age at examination

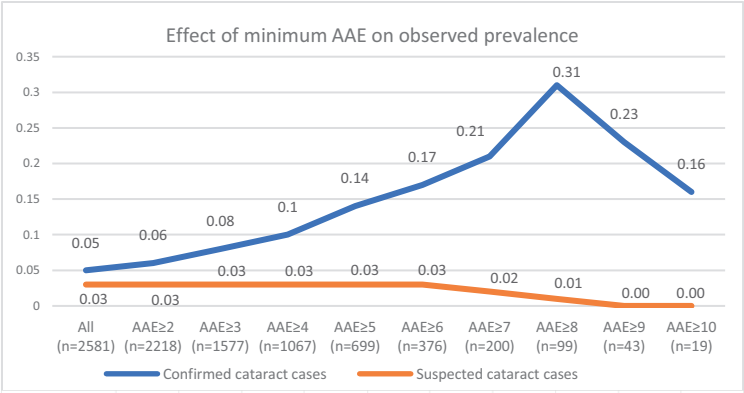
We found that the average age at examination (AAE) was 3.44 years, and that only 7.7% of examinations were performed in dogs that were 7 years or older (AAE≥7). The majority of Havanese that underwent an eye examination, were only examined once (764/1409).

Age at onset

Average age at onset (AAO) for all cataracts in Havanese was 4.8 years. Average AAO for the different forms of cataracts: cortical cataracts (4.5), anterior suture line cataracts (5.0), and posterior polar cataracts (3.0), were not significantly different.

Effect of AAE on observed prevalence

Between AAE ≥ 1 and AAE ≥ 8, observed prevalence was positively correlated with AAE ($r(6) = .95$, $p = .0003$). The prevalence was highest in the AAE ≥ 8-group, in which almost one third of the dogs were diagnosed with cataracts. The frequency of suspected cataract cases was negatively correlated with AAE ($r(8) = -.87$, $p = .0011$) (Figure 1).

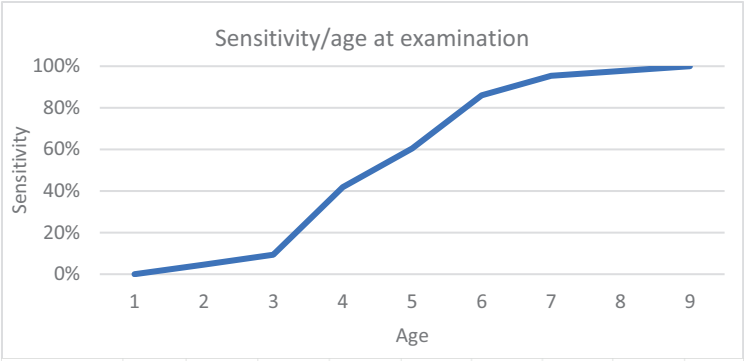


Age at examination	All	AAE≥2	AAE≥3	AAE≥4	AAE≥5	AAE≥6	AAE≥7	AAE≥8	AAE≥9	AAE≥10
Confirmed cataract cases	0.05	0.06	0.08	0.1	0.14	0.17	0.21	0.31	0.23	0.16
95% CI	[0.042, 0.059]	[0.048, 0.068]	[0.064, 0.090]	[0.086, 0.123]	[0.115, 0.168]	[0.131, 0.209]	[0.156, 0.273]	[0.224, 0.414]	[0.118, 0.386]	[0.034, 0.396]
Suspected cataract cases	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.01	0.00	0.00
95% CI	[0.020, 0.033]	[0.022, 0.036]	[0.025, 0.043]	[0.020, 0.041]	[0.015, 0.040]	[0.015, 0.052]	[0.006, 0.050]	[0.000, 0.055]	-	-

Figure 1: Proportion of examinations that result in a cataract diagnosis dependent on minimum age at examination.

Effect of AAE on sensitivity

Sensitivity (i.e. the proportion of eventually affected individuals that test positive), increased significantly with increasing age at examination (Figure 2). Eye examinations performed at 3 years of age detect only 9% of eventually affected dogs, but at 7 years of age, the likelihood of a correct diagnosis in at-risk individuals increases to 95%.



Age at examination	1	2	3	4	5	6	7	8	9
Sensitivity	-	0.05	0.09	0.42	0.60	0.86	0.95	0.98	1.00
95% CI		[0.00, 0.11]	[0.01, 0.18]	[0.27, 0.57]	[0.46, 0.75]	[0.76, 0.96]	[0.89, 1.00]	[0.93, 1.00]	

Figure 2: Sensitivity at different ages at examination.

Genome wide association analysis

Posterior polar cataract

The association analysis of posterior polar cataract revealed two associated SNPs on CFA20 (BICF2S23632983, p = 7.19e-09) and CFA21 (BICF2G630640490, p = 3.33e-09), that reached genome-wide significance (Figure 3). Closer inspection of genotypes for BICF2S23632983 showed that the allele associated with posterior polar cataract was present in a heterozygote state in 5/7 cases and only 1/57 controls. For BICF2G630640490, the allele associated with disease was present in a heterozygote state in 6/7 cases and only 2/57 controls.

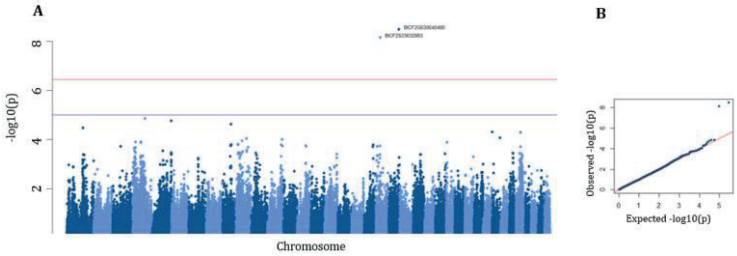


Figure 3: Genome wide association result for posterior polar cataract. A: Manhattan plot. B: Quantile-quantile plot. λ = 1.10.

BICF2S23632983 is positioned at CFA20:20379186 (CanFam3.1), ~ 60,000 base pairs downstream of the FOXP1-gene, which encodes the Forkhead Box Protein P1. Suzuki-Kerr et. al showed that *Foxp1* is highly expressed in the mural lens during development, and that *Foxp1* knockout mice develop several lens abnormalities, including lens opacities (26). Lenses from *Foxp1* knockout mice appear normal at birth, but closer inspection reveals increased proliferation and apoptosis of epithelial cells and malformed lens sutures. Two weeks after birth, the knockout mice develop small and opaque lenses with misaligned epithelial cells, abnormal fiber cells, and disorganization of fiber cells in the lens cortex (26). The essential role of *Foxp1* in normal lens development makes it a highly relevant candidate gene for posterior polar cataract in Havanese.

Another potential candidate gene for posterior polar cataract is the RYBP-gene, positioned ~700,000 base pairs downstream of BICF2S23632983. RYBP is a zinc finger protein that is essential for normal neurological development (27). It is highly expressed in the epithelial- and fiber cells of the lens during development, and *Rybp* knockout mice display several eye abnormalities, including malformed lenses (28). Overexpression of *Rybp* in the

lens cause abnormal fiber cell differentiation, altered levels of lens proteins and severe lens opacities (28), which makes it an interesting cataract candidate gene.

BICF2G630640490 is positioned at CFA21:47286098 (CanFam3.1), ~800 kb upstream of the LGR4 gene. *LGR4* encodes the Leucine Rich Repeat Containing G Protein-Coupled Receptor 4, which is important for normal development of many organs in the body and is a known cataract candidate gene. One study showed that one in four *Lgr4* knockout mice are affected by cataracts, with disorganized and enlarged lens fibres at the cortex of the lens (29). Insoluble lens proteins and abnormal protein deposits were also observed. The extent of the lens opacities varied between the affected individuals. Another study found that *Lgr4* knockout mice developed lens opacities earlier than wild type mice after exposure to oxidative stressors and had an increased incidence of cataracts, which indicate that *Lgr4* may play an important role in cataract formation (30).

Cortical cataract

The association analysis of cortical cataract did not reveal associations that were significant after Bonferroni correction (Figure 4), but we observed a suggestively associated peak on CFA4, in which the top SNP (chr4_12164500, p = 4.30e-06), is located ~ 1500 base pairs upstream of *ANK3*. The ANK3 gene encodes the protein ankyrin-G (also known as ankyrin-3), which functions as a scaffolding protein that links membrane proteins to the intracellular cytoskeleton (31). One study showed that ankyrin-G is expressed in the lens of mice, particularly in the lateral membranes of the lens epithelium (32). It plays an obligatory role in normal lens development, and Ank3 knockout mice develop bilateral microphthalmia and cataracts.

Suppression of ankyrin-B, which is another member of the ankyrin family, has also been shown to induce cataract formation, through disorganization of lens fibers (33).

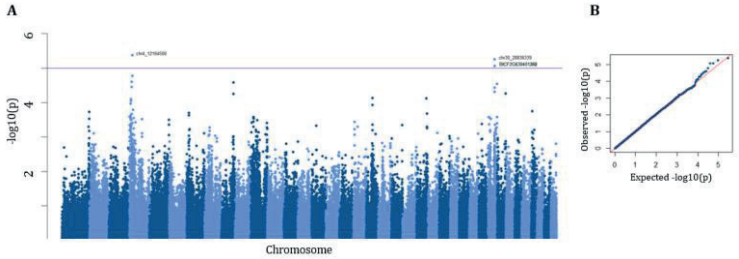


Figure 4: Genome wide association result for cortical cataract. A: Manhattan plot. B: Quantile-quantile plot. λ = 1.02.

A suggestively significant peak was also observed on CFA30, (chr30_28836339, p = 5.62e-06; BICF2G630401302, p = 8.81e-06; BICF2G630401280, p = 8.81e-06) (Figure 4), in which the top SNP is positioned at CFA30:28836339, within the 3'-UTR of *PCLAF*. The PCLAF-gene encodes a PCNA-binding protein that regulates DNA repair following DNA damage, including damage caused by ultraviolet (UV)-B radiation, which is a well-known cataract risk factor. Chen et. al examined differences in protein-expression and ubiquitination in normal lenses versus lenses subjected to UV-B irradiation, as a model for lens changes in age-related cataracts (34). Compared to the control lenses, lens epithelial cells that had been subjected to UV-B irradiation showed decreased expression and upregulated ubiquitination of PCNA-binding protein. This indicate that *PCLAF* could influence the development of age-related cataract following UV-B exposure.

Two additional potential candidate genes in proximity to the peak on CFA30, include *SNX22* and *TPM1*. *TPM1* is located ~1.2 Mb downstream of the top SNP and encodes the tropomyosin alpha-1 chain. Shibata et al. 2021 documented the essential role of *Tpm1* in lens fiber differentiation and homeostasis during aging (35). They showed that *Tpm1* lens-specific knockout mice develop lens opacities, and propose that disturbances in TPM1 regulation could induce cataract formation. *TPM2*, which is a closely related gene, has also been associated with cataract development. One study showed that *Tpm2* knockout mice had an earlier onset and faster progression of cataracts, and that the opacities first appeared in the centre of the anterior cortex (36). Another study found that the expression of *TPM2* is higher in lenses from humans with severe nuclear cataract, than in lenses from people with milder forms of the disease (37).

SNX22 is located ~200 kb upstream of chr30_28836339. One study report that *Snx22* may play a role in the development of congenital cataracts, due to altered expression in cataractous lenses compared to controls (38).

Anterior suture line cataract

The association analysis of anterior suture line cataract did not reveal any significantly associated regions. Interestingly, the SNP that showed the second strongest association (BICF2S23512744, p = 1.19e-05), was located on CFA6 within in the potential cataract candidate gene, *SDK1* (39), but given the lack of statistical support, the finding should be interpreted with caution.

Discussion

The study revealed two SNPs, on CFA20 (BICF2S23632983, p = 7.19e-09) and CFA21 (BICF2G630640490, p = 3.33e-09), that were significantly associated with posterior polar cataract in Havanese. Two candidate genes on CFA20, *FOXP1* and *RYBP*, are located approximately 60kb and 700kb away from the associated SNP respectively, well within a distance in which high linkage disequilibrium (LD) is expected (40). On CFA21, the associated SNP was positioned <1Mb upstream of the highly relevant candidate gene, LGR4.

The study identifies two regions of the genome that may harbour genetic variants that are important in the development of posterior polar cataract in Havanese. In humans, posterior polar cataract is considered a distinct type of cataract, which is most often inherited in a dominant fashion (41). Based on the allele distributions in cases versus controls, a dominant inheritance pattern could potentially fit our results as well, although not with a perfect association. This could be due to modifying genes, incomplete penetrance or incomplete LD between the SNPs and the functional polymorphisms.

We believe the reason we observe single SNPs in the association analysis of posterior polar cataract (rather than a peak consisting of multiple SNPs), is the small number of cases, which means strong LD between the marker and the functional variant is necessary to observe a significant association. Other closely linked markers will have different allele frequencies and may therefore have weaker LD with the functional variant, which could explain why they do not show significant association at this sample size.

Sequencing of a selection of cases and controls in the candidate regions is in progress, with the aim of identifying functional polymorphisms in the region. The clear association we find for posterior polar cataract, despite the limited sample size, indicate that there may be one or two mutations that have a major effect on the development of the disease, and that these variants, when identified, may provide a basis for future DNA-testing to support breeding and reduce the prevalence of disease.

The analysis of cortical cataract gave promising results, identifying two suggestively associated peaks on CFA30 and CFA4. The top SNP on CFA30 is positioned within the 3'-UTR of *PCLAF*, and within a 1.2 Mb distance of TPM1 and SNX22, and the top SNP on CFA4 is located ~ 1500 base pairs away from ANK3.

The relatively weak association we find in the cortical- and anterior suture line cataract analyses, might indicate a complex mode of inheritance. Also, mixed linear models which include relationship matrixes to control population stratification and relatedness between individuals, can sometimes be overly conservative after stringent adjustments for multiple testing, and may fail to control for false negatives (42). It's also possible that the lack of observed association in the analysis of anterior suture line cataract is partly contributable to challenges with correct classification, as the pathological changes observed in this form of cataract are subtle.

Continued studies of all three forms of cataracts, using fine mapping, targeted resequencing, or support from WGS-data, may help to identify functionally associated variants, and develop DNA-tests to help breeders avoid breeding Havanese that develop the disease. However, until the genetic background for the various forms of cataracts in Havanese is uncovered,

selection must be based on phenotype through ECVO eye screening. Our findings indicate that increasing the average age of breeding animals would improve accuracy of selection significantly.

One aim of this study was to recommend a reasonable minimum age for controls. Our findings support 7 years as reasonable cut-off, as 95% of true positives test positive at this age, compared to 86% one year earlier. Increasing the minimum age for controls to 8 years would improve accuracy of classification further (sensitivity at 8 years of age = 0.98) but could also negatively affect the number of controls available for study. In this study, half of the controls would be excluded using a cut-off age for controls of 8 years.

Even though observed prevalence of cataracts is strongly correlated with AAE between 1 and 8 years, we see a decrease in observed prevalence in dogs aged 9 years or older. A likely explanation is that dogs that are examined at this age is a selection of males that tested negative at age 7 or 8, and are therefore continuously examined because they are still used for breeding.

Most Havanese in Norway are eye examined at an age (average AAE = 3.44) when it is unlikely to observe clinical changes, even in dogs that are genetically at risk, which will result in a high number of false negatives. We recommend that all Havanese undergo an eye examination prior to breeding, even in situations where the animals are bred at a low age, but emphasize that results from young dogs must be interpreted with caution.

It's important that breeders are aware of the low sensitivity of cataract screening in young dogs. For bitches there are natural

limits to how much age at breeding can be increased without compromising welfare, but increasing the proportion of litters sired by older, unaffected males, would likely reduce the risk of producing affected offspring significantly.

Conclusions

The study identifies two loci on CFA20 and CFA21 that are significantly associated with posterior polar cataract in Havanese. The SNP on CFA20 is located close to two candidate genes, FOXP1 and RYBP, both of which are highly expressed in the lens and are suggested to influence development of lens opacities. The SNP on CFA21 is in proximity of the LGR4 gene, which is associated with cataract development in mice. The results suggests that the identified genomic regions may harbour genes with large effect on development of posterior polar cataract in Havanese, possibly in a dominant fashion.

Two peaks on CFA4 and CFA30 show putative association with cortical cataract and the top SNPs in each of them are within or close to cataract candidate genes. Polygenetic influence on cortical- and anterior suture line cataract in Havanese is not unlikely.

We also show that age at examination strongly influences sensitivity, and suggest that increasing the average age of breeding animals, and especially of stud dogs, would improve accuracy of selection and genetic improvement.

Methods

Population data analyses

Analyses of eye screening statistics, average age at examination (AAE), age at onset (AAO), effect of AAE on prevalence and effect of AAE on sensitivity, were conducted using data from The Norwegian Kennel Clubs open database, "Dogweb" (3). All available eye screening results for Havanese (n = 2581), were included, spanning from year 2003 to 2022. Out of these, 25% (n = 649) were performed between 2003 and 2012 and 75% (n = 1932) were performed between 2013 and 2022.

Age at examination and prevalence

A complete dataset, comprising all available Havanese eye screening records in the NKK database (n = 2581), were used to calculate eye screening statistics for years 2003-2022. The same dataset was used to estimate average age at examination, where "Age at examination" (AAE) was defined as "Year of examination"– "Year of birth". The full dataset was also used to calculate observed prevalence in groups of dogs based on minimum age at examination.

Age at onset and sensitivity

Average age at onset (AAO) was estimated based on a subset of Havanese that had been diagnosed with cataracts within two years after a negative diagnosis (n = 43), by calculating the median of "Age at first positive diagnosis" and "Age at last negative diagnosis". The same subset of dogs was also used to

calculate sensitivity, i.e., the proportion of true positives that test positive, and study how sensitivity is influenced by AAE.

Genome wide association analysis

Dogs

DNA-samples were recruited for the genome wide association study in three ways: 1) DNA-samples stored in our biobank that were originally recruited for other projects (24, 43), matched with Dogweb eye screening records, 2) through online announcements in breed club webpages and online groups for Havanese owners in Norway, Finland, Denmark and Sweden, and 3) through direct inquiries to owners of Havanese that were already registered in the NKK database with an ECVO certificate and met the inclusion criteria. All samples were collected with owners' consent for use in research.

All DNA-samples recruited specifically for this study were collected using Performagene™ buccal swabs (DNA Genotek Inc), administered by the owner. DNA was extracted following the manufacturer's recommendations and stored at -20°C.

Inclusion criteria for cases were purebred Havanese that had been diagnosed with cataracts (cortical, anterior suture line or posterior polar) at any age, either as a part of a routine ECVO screening, or because of clinical signs and subsequent surgery. Inclusion criteria for controls were purebred Havanese that were unaffected by any form of cataracts at 7 years of age or older, confirmed by an ECVO eye certificate. Controls were the same in the association analysis of the three different forms of cataracts.

Genotyping and association analysis

Dogs were genotyped using Illumina CanineHD 230K BeadChip. Quality control and filtering was performed using PLINK 1.9 beta 6 (44, 45). 211256 autosomal variants were present prior to filtering. 10389 variants were removed because they were missing in more than 5% of samples (--geno 0.05), 59545 variants were removed because the minor allele frequency was below 0.05 (--maf 0.05), and 847 variants were removed due to deviation from Hardy Weinberg equilibrium (--hwe 0.001). 140475 variants remained after quality control.

Dogs were excluded if more than 5% of genotype data was missing (--mind 0.05). 27 cortical cataract cases, 23 anterior suture line cataract cases, 7 posterior polar cataract cases and 57 controls were included in the association analysis.

High levels of relatedness between individuals and long stretches of linkage disequilibrium are expected in a closed population of purebred dogs (40, 46, 47). To control for population stratification, analyses were run using a mixed linear model, including a genetic relationship matrix (GRM) as a random effect. To avoid loss of power from including SNPs in high LD with the candidate SNP in the GRM, we used the "leaving one chromosome out" (MLMA-LOCO) option in GCTA (48, 49), which exclude the chromosome of the candidate SNP from calculation of the GRM. Sex chromosomes were excluded from the association analyses.

Even though high levels of linkage disequilibrium between SNPs are expected in purebred dogs (40, 46, 47), we calculated a conservative Bonferroni-corrected genome wide significance level of 3.6e-07, corrected for the number of all autosomal

variants that remained after quality control (n = 140475). The threshold for suggestive significance was set at the default p-value of 1.0e-05.

Manhattan plots and QQ-plots were created in R version 4.2.1 (50), using the R-package qqman version 0.1.8 (51).

Mapping of linked genes

Genes near the top SNPs (posterior polar and anterior suture line: 1Mb upstream and downstream, cortical: 0.5Mb upstream and downstream) were inspected and checked for any relevant involvement in cataract development using PubMed® (52). Additionally, a larger region (≈ +/- 2Mb) surrounding the top SNPs were controlled against the online chromosome map and reference database for inherited and age-related cataracts, Cat-Map (53), after conversion of coordinates from CanFam3.1 to GRCh38 using the LiftOver-function in the UCSC genome browser (54)

Abbreviations and definitions

AAE: Age at examination, here defined as “year of examination” – “year of birth”
AAO: Age at onset
CFA: Canine chromosome
ECVO: European College of Veterinary Ophthalmologists
GCTA: Genome-wide Complex Trait Analysis
GRCh38: Genome Research Consortium human build 38
GRM: Genomic relationship matrix
GWAS: Genome wide association study
LD: Linkage disequilibrium
SNP: Single nucleotide polymorphism

Declarations

Ethics approval and consent to participate

The study was conducted using results from diagnostic testing that had already been completed and made publicly available at the owners' request. The eye examination is performed by a certified veterinarian and is considered non-invasive, safe and pain free. DNA-samples collected specifically for this project were collected using buccal swaps administered by the owner. Owners' consent for use in research were obtained for all dogs included in the study.

Consent for publication

Not applicable.

Data availability

Analyses of eye screening statistics, average age at examination (AAE), age at onset (AAO), effect of AAE on prevalence and effect of AAE on sensitivity, were conducted using data from The Norwegian Kennel Clubs database, which is publicly available.

Competing interest

The authors declare no competing interests.

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Authors' contributions

KB designed the study, analysed the data, and wrote the manuscript. FL gave guidance throughout the project and made major contributions to the manuscript.

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Supplementary materials paper I

SUPPLEMENTARY MATERIALS

Supplemental table 1: Owner questionnaire, Havanese

1. My dog is scared of other dogs				
Strongly agree				Strongly disagree
1	2	3	4	5
2. My dog is scared of unfamiliar children				
Strongly agree				Strongly disagree
1	2	3	4	5
3. My dog is scared of unfamiliar women				
Strongly agree				Strongly disagree
1	2	3	4	5
4. My dog is scared of unfamiliar men				
Strongly agree				Strongly disagree
1	2	3	4	5
5. My dog is scared of people that display unusual behavior				
Strongly agree				Strongly disagree
1	2	3	4	5
6. My dog will pull away from other dogs on a walk				
Strongly agree				Strongly disagree
1	2	3	4	5
7. My dog will pull away from unfamiliar people who try to pet or touch him/her on a walk				
Strongly agree				Strongly disagree
1	2	3	4	5
8. I would describe my dog as jumpy				
Strongly agree				Strongly disagree
1	2	3	4	5
9. I would describe my dog as timid				
Strongly agree				Strongly disagree
1	2	3	4	5

Supplemental table 2: Owner questionnaire, noise reactivity (5 breeds)

1. My dog is scared of the sound of fireworks				
Strongly agree				Strongly disagree
1	2	3	4	5
2. My dog is scared when exposed to loud traffic				
Strongly agree				Strongly disagree
1	2	3	4	5
3. My dog is scared of the sound of gun shots				
Strongly agree				Strongly disagree
1	2	3	4	5
4. My dog is scared during thunderstorms				
Strongly agree				Strongly disagree
1	2	3	4	5

Supplemental table 3: primers

Exon	Forward	Reverse	Optimal temperature
1	CGGACGGCTGCCAGG	CGGACAAACTTGTGGTCCCA	No product
2	CCGGTGGTTGATTTCAGCTC	GCAACTTGTTGGCAGGAACC	57
3	GGAAGGAGAGCCCCGCTATA	ATGCACGCACAAACACATGG	62
4	AAGGCACAAGGTGTCTCTGG	CGGCCTCAGTCCCTATCTCT	59
5	GCGTACTCTGTCACATGGCT	CCACCCATCACAGGCCAG	63
6	CTTCACTCTTGCCTCCCCTG	GTGCCTGCTTGTGACTTGTG	58
7	ACCCGGTGAGGCTGAGTG	GAAGGGGATGGCAGGTAAGG	58
8	GCCCGTAGCACCCAATCTT	TAGCACTACCCCGGCAGAT	58
8	GGTGGGGATGGACAGTTCAC	AGTGGTTTTGTGGCAGGAGG	62

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