ANIMAL GENETICS Immunogenetics, Molecular Genetics and Functional Genomics

doi: 10.1111/age.12659

Identification of the mutation causing progressive retinal atrophy in Old Danish Pointing Dog

P. Karlskov-Mortensen*, H. F. Proschowsky†, F. Gao‡ and M. Fredholm*

*Animal Genetics, Bioinformatics & Breeding, Department of Veterinary & Animal Sciences, Faculty of Health & Medical Sciences, University of Copenhagen, Grønnegårdsvej 3, DK-1870 Frederiksberg C, Denmark. †The Danish Kennel Club, Parkvej 1, Solrød Strand, Denmark. ‡Agricultural Genomes Institute at Shenzhen, Chinese Agricultural Academy of Sciences, Shenzhen, China.

Summary

Progressive retinal atrophy (PRA) is a common cause of blindness in many dog breeds. It is most often inherited as a simple Mendelian trait, but great genetic heterogeneity has been demonstrated both within and between breeds. In many breeds the genetic cause of the disease is not known, and until now, the Old Danish Pointing Dog (ODP) has been one of those breeds. ODP is one of the oldest dog breeds in Europe. Seventy years ago the breed almost vanished, but today a population still exists, primarily in Denmark but with some dogs in Germany and Sweden. PRA has been diagnosed in ODP since the late 1990s. It resembles late onset PRA in other dog breeds, and it is inherited as an autosomal recessive trait. In the present study, we performed whole-genome sequencing and identified a single base insertion (c.3149_3150insC) in exon 1 of *C17H2orf71*. This is the same mutation previously found to cause PRA in Gordon Setters and Irish Setters, and it was later found in Tibetan Terrier, Standard Poodle and the Polski Owczarek Nizinny. The presence of the mutation in such a diverse range of breeds indicates an origin preceding creation of modern dog breeds. Hence, we screened 262 dogs from 44 different breeds plus four crossbred dogs, and can subsequently add Miniature Poodle and another polish sheepdog, the Polski Owczarek Podhalanski, to the list of affected breeds.

Keywords blindness, *C17H2orf71*, *C2orf71*, PRA, retinitis pigmentosa

Introduction

Progressive retinal atrophy (PRA) comprises a complex group of hereditary retinal dystrophies featuring degeneration of rod and cone photoreceptors resulting in loss of vision. These diseases have been known in dogs since the beginning of the 20th century, when Magnusson (1909) described a spontaneous degeneration of the retina due to a generalized progressive atrophy of the retinal neuroepithelium in Gordon Setters in Sweden; later, Parry (1951) described a similar disease in Red Irish Setters in England. Today, PRA is reported in over 100 dog breeds and considered a significant health concern in purebred dogs (Miyadera *et al.* 2012). Most forms of PRA are inherited as autosomal recessive traits, but significant genetic heterogeneity is observed within as well as between breeds (Downs *et al.* 2014). So far, 31 mutations in 24 different genes have been identified as the cause of retinal diseases (Miyadera 2014), yet, the genetic basis underlying most PRA cases remains unresolved.

The Old Danish Pointing Dog (ODP) is one of five national Danish dog breeds recognized by the World Canine Organization (Fédération Cynologique Internationale, FCI). According to the FCI standard, the origin of the breed can be traced back to around AD 1710 when a Dane named Morten Bak crossed local farm dogs with dogs that arrived with itinerant Roma people in the 17th century. Through crossings in eight generations, he created the Bak-hound, now known as the Old Danish Pointing Dog. Hence, ODP is one of Europe's oldest dog breeds. Two hundred years after creation, the breed was on the brink of extinction with only seven male and 13 female dogs remaining. However, an organized breeding effort saved the breed. In 1961, the breed was acknowledged by the Danish Kennel Club. The breed became very popular in the 1980s, and the population underwent a rapid expansion with more than 500 new puppies registered in 1983 alone, compared to an average of 28.5 puppies per year in the period from 1970 to 1979.

Address for correspondence

P. Karlskov-Mortensen, Animal Genetics, Bioinformatics & Breeding, Department of Veterinary & Animal Sciences, Faculty of Health & Medical Sciences, University of Copenhagen, Grønnegårdsvej 3, DK-1870 Frederiksberg C, Denmark.
E-mail: pkm@sund.ku.dk

Accepted for publication 20 February 2018

More recently, the number of new puppies has settled at a level of approximately 100 puppies registered per year by the Danish Kennel Club.

The first confirmed incidents of PRA in ODP were observed on the eve of the new millennium, when two male dogs from the same litter born in 1992 developed blindness. The next cases of PRA were observed in two dogs born in 1999, and among dogs born between 2001 and 2009, one to four cases evenly distributed between males and females were identified every year.

Here, we report the identification of a single base insertion in the chromosome 17 *C2orf71* homologue (*C17H2orf71*) causing PRA in ODP. The mutation is identical to the mutation previously identified by Downs *et al.* (2013) to cause PRA in Gordon and Irish Setter breeds.

Materials and methods

Animal material

Thirteen ODP were identified as PRA affected in the present study. Age at diagnosis was 6–9 years with an average of 7 years 8 months. Among the cases, five dogs presented with clear attenuation of blood vessels, increased reflection from the tapetum lucidum and pale optical nerve papilla; all five dogs had different degrees of immature cataract. Another three dogs were diagnosed with PRA by veterinary ophthalmologists but with no further details on the results of the clinical examination. One dog presented with an anamnesis typical for PRA together with a mature cataract, which made an unambiguous clinical diagnosis difficult. Four dogs were reported blind by the owner with an anamnesis typical for PRA but without documentation from a veterinarian. All these dogs were included as PRA cases. Pedigrees for all affected dogs were analysed to reveal the pattern of segregation and to identify common ancestors to all cases. Fourteen dogs were selected as unaffected controls; they were all more than 9 years old with owner reports of no signs of PRA or reduced vision in general. Additionally, from our in-house DNA bank, samples were available from a cohort of 324 ODP with unknown PRA status. All dogs in this cohort that could be identified as being unrelated at the grandparental level ($n = 24$) were used for estimation of allelic frequencies in the ODP population. All samples were obtained and used in the present study with owners' consent.

Test for known PRA-causing mutations

Two affected dogs were tested for the known mutation in the *PRCD* gene, which causes PRA in a wide range of breeds (Zangerl *et al.* 2006; Downs *et al.* 2014), and for the mutation in the *CNGB3* gene causing cone degeneration in the German Wirehaired Pointer (Sidjanin *et al.* 2002), a breed that could share a common ancestry with ODP. The standard commercial test was performed by OptiGen®.

Whole genome shotgun sequencing

Five affected and five unaffected dogs were selected for 30× coverage whole-genome sequencing. DNA was isolated from EDTA-stabilized blood using a salting out procedure (Miller *et al.* 1988) to obtain high quality genomic DNA. DNA degradation and contamination was monitored on 1% agarose gels. DNA purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN). DNA concentration was measured using a Qubit® DNA Assay Kit in a Qubit® 2.0 Flurometer (Life Technologies).

A total of 700 ng of DNA per sample was used for the DNA sample preparations. Sequencing libraries were generated using the NEB Next® Ultra DNA Library Prep Kit for Illumina®, following the manufacturer's recommendations. Briefly, the DNA was purified using the AMPure XP system (Beckman Coulter). After adenylation of 3′-ends of DNA fragments, the NEB Next Adaptor with hairpin loop structures were ligated to prepare for hybridization. Then, the adaptor-ligated DNA was loaded onto a 2% agarose gel at a voltage of 125 for 40 min. The DNA fragments, ranging from 400 to 500 bp in size, were selected and purified by a QIAquick gel extraction kit. A total of 3 μl of USER Enzyme was used with size-selected, adaptor-ligated DNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and library quality was assessed on an Agilent Bioanalyzer 2100 system.

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using a HiSeq 4000 PE Cluster Kit (Illumina), according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 4000 platform and 150-bp paired-end reads were generated.

Sequence data were analysed using the GENOME ANALYSIS TOOLKIT (GATK) (McKenna *et al.* 2010) adhering to GATK best practices (Van der Auwera *et al.* 2013) but adapted to the canine dataset. Shortly, for each individual dog, high quality Illumina reads were aligned to the canine reference genome (CanFam3.1) using the BURROWS-WHEELER ALIGNER (BWA-MEM; Li & Durbin 2009). Subsequently, PICARD tools (http://broadinstitute.github.io/picard/) were used to mark duplicates and build bam indexes. GATK was used for indel realignment, base quality recalibration and variant discovery. Subsequently, variants in all cases and controls were compared to identify variants for which all cases and no controls were homozygous for a non-reference allele.

Mutation screening

A primer set designed by Downs *et al.* (2013) was used to genotype a 1-bp insertion identified in the *C17H2orf17* gene: 5′-FAM-CCGAGTGCTCCCTCTGTG, 5′-GGCTGCAGGC CTCGTC. PCR was performed on genomic DNA using Qiagen Hot Star polymerase, Qiagen Q-solution 1× final concentration and a final concentration of 2.5 mM $MgCl_2$. PCR products were run on an ABIprism 3130xl (Applied Biosystems) to determine fragment lengths. The test was used to ascertain *C17H2orf17* genotypes in confirmed cases and controls and to determine allele frequencies of the *C17H2orf17* insertion in the Danish ODP population by genotyping the 24 ODP that were identified as not related at the grandparental level. Additionally, the test was used to screen 262 dogs representing 44 different breeds and four crossbred dogs (Table S1) for the mutation.

Results

All identified PRA cases in the ODP were diagnosed at the age of approximately 7 years. At this age, the dog presents with an anamnesis of nyctalopia and gradually developing loss of vision. A clinical and ophthalmoscopic examination typically reveals decreased pupillary reflex, increased reflection from the tapetum lucidum, decreased vascularization of the retina, general atrophy of the retina and sometimes a developing cataract. A cataract was a common sequel late in disease development. Pedigree analyses suggested that PRA segregated as an autosomal recessive trait in ODP (Fig. 1), and through various paths in the pedigree, seven different dogs qualified as a most recent common ancestor to all obligate carriers of the disease (Table 1).

When the first PRA cases in ODP came to our attention around 2009, two affected dogs were tested for known mutations in the *PRCD* and *CNGB3* genes. Both dogs were homozygous for the wild type alleles. However, because it is well documented that the candidate gene strategy in the context of PRA has a very low success rate (Aguirre-Hernandez & Sargan 2005; Chew *et al.* 2017), this strategy was not followed further.

Table 1 Most recent common ancestors to obligate carriers of the PRA causing mutation.

Common ancestor	Year of birth	Gen.[1]
CA1	1969	4.9
CA2	1973	5.5
CA3	1970	5.8
CA4	1968	6.0
CA5	1968	6.8
CA6	1971	5.5
CA7	1968	6.1

[1]Average number of generations between the common ancestor and parents to PRA cases.

Instead, to discover the disease-causing variant, five PRA-affected dogs and five controls were subjected to whole-genome sequencing, 30× coverage for each dog. Sequencing resulted in 77.7–98.1 billion aligned reads from each dog resulting in an average coverage between 32.5 and 41.0× for each individual dog and an overall average coverage per dog of 35.5×. On average, only 95.4 Mb per dog were covered less than 10×.

Following sequence analysis using the GATK tool package, comparison of identified variants across individuals resulted in a list of 2050 variants for which all cases but no controls were homozygous for the non-reference allele. Of these, 41 variants were located within known canine protein coding genes (refGene table from the canFam3 database, UCSC genome browser, www.genome.ucsc.edu) and 1001 variants were located within regions homologous to known

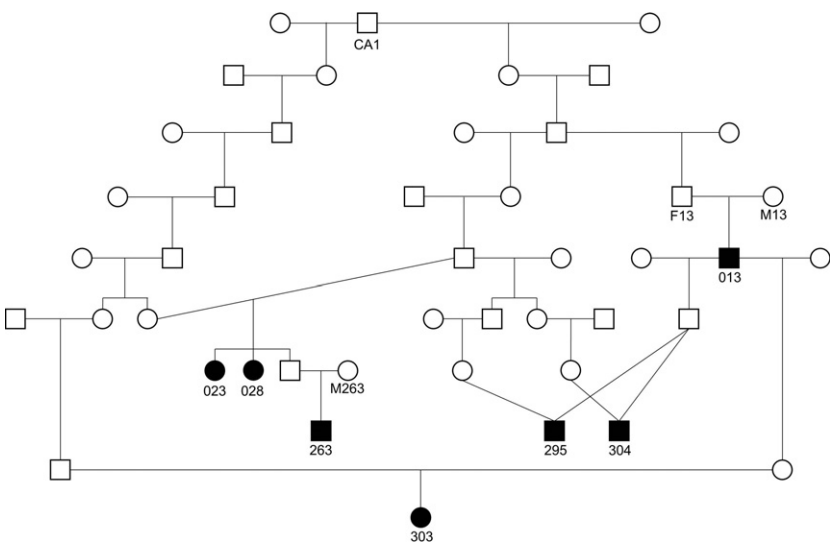

Figure 1 A simplified pedigree diagram to illustrate segregation patterns and relationships to one of seven potential common ancestors (CA1). M13 and M263 are daughters removed four and five generations respectively from CA1.

protein-coding genes from other organisms (XenoRefGene table from the canFam3 database, UCSC genome browser, www.genome.ucsc.edu). The total list of identified variants was submitted to the Ensembl Variant Effect Predictor (http://www.ensembl.org/Canis_familiaris/Tools/VEP), which identified one variant of high impact and 281 variants in 48 genes of moderate impact. The identified high impact variant was a 1-bp insertion on chromosome 17 at position 22907388 (assembly CanFam3.1). This insertion corresponds to the c.3149_3150insC variant in the *C17H2orf71* gene described by Downs *et al.* (2013) to cause the PRA variant *rcd4* in Gordon Setter and Irish Setter. The insertion results in a premature stop codon in exon 1 of *C17H2orf71*. Data on the identified variation have been deposited in the European Variation Archive, accession no. PRJEB24328.

The identified insertion was genotyped in all cases and controls. *C17H2orf71* genotypes were found to be concordant with PRA status in 11 out of 14 PRA-free dogs and 12 out of 13 PRA cases. That is, three dogs classified as healthy controls were found to be homozygous for the insertion, and one case was found to be heterozygous.

From an in-house DNA bank of ODP comprising over 300 dogs, all contemporary dogs unrelated at the grandparental level were genotyped ($n = 24$) to determine the allele frequency of the mutated allele in today's ODP population. The *rcd4* allele frequency in these dogs was found to be 0.396, and the estimated percentages of carrier and affected dogs were 47.8% and 15.7% respectively. Furthermore, over a 4-month period 91 dogs were tested for the *rcd4* mutation. Of these, eight tested homozygous for the mutation and 47 were identified as carriers, indicating an *rcd4* allele frequency of 0.346 in the ODP population.

Screening 262 dogs from 44 breeds plus four crossbred dogs, we identified the *rcd4* mutation in Gordon Setter (five carriers and one affected out of 10 tested dogs), Irish Setter (four carriers out of eight tested dogs), Miniature Poodle (two carriers out of seven tested dogs) and Polski Owczarek Podhalanski (one carrier out of three tested dogs).

Discussion

We here present the first report on PRA in ODP and an association with an indel in *C17H2orf71* that has previously been associated with PRA in Gordon and Irish Setters. Disease development and symptoms were similar to late-onset progressive rod-cone degeneration in other dog breeds (Petersen-Jones 1998), including PRA in Gordon and Irish Setters (Downs *et al.* 2013), and to retinitis pigmentosa, which is the most common hereditary cause of blindness in humans (Hartong *et al.* 2006).

The c.3149_3150insC variant in *C17H2orf71* was previously found and is associated with development of PRA in five dog breeds: Gordon and Irish Setters (Downs *et al.* 2013), Standard Poodle, Tibetan Terrier (Downs *et al.* 2014) and Polski Owczarek Nizinny dog (Svensson *et al.* 2016). In the present work, we identified the same association in ODP. Although the two Setter breeds are closely related phylogenetically with a recent common ancestor and although admixture with Setter breeds in the ODP may be a possibility, the genetic and phylogenetic relationship to Poodle and Tibetan Terrier is very low with no apparent admixture (Parker *et al.* 2017). Hence, unless the mutation has occurred independently in different dog clades, the origin of the mutation seems to trace back to before the creation of modern dog breeds and could thus be present in many other breeds. Downs *et al.* (2013) previously tested 90 dogs from 31 breeds without finding the mutation. In the present study, we screened 266 dogs representing 44 breeds plus four crossbred dogs, and added Miniature Poodle and Polski Owczarek Podhalanski dogs to the list of breeds carrying the *rcd4* mutation. Given the presence of the mutation in such a diverse range of breeds we suggest that PRA cases of unknown aetiology in other dog breeds should be tested for the *rcd4* mutation.

A lack of complete concordance between *C17H2orf71* genotype and case status in the ODP dogs included in the present work was observed. Revisiting metadata for three discordant animals, it was noted that blood samples from a PRA case heterozygous for the *C17H2orf71* mutation and one of the healthy controls homozygous for the mutation had arrived from the same veterinary clinic, sent in a single package and sampled the same day. Hence, a simple mix up of samples in the clinic cannot be excluded. Unfortunately, it was not possible to obtain new samples that could clarify this. For the remaining two discordant controls it is noted that Downs *et al.* (2013) reported that age of onset for PRA caused by the *C17H2orf71* mutation can vary from 5 to 12 years in Gordon Setters (Downs *et al.* 2013). The same is true for *rcd4* in Polski Owczarek Nizinny dog, for which Svensson *et al.* (2016) also documented a markedly slow progression of retinal changes based on repeated electroretinography. Hence, assuming the same rate and variation in age of onset and progression of the disease in ODP, the two remaining *C17H2orf71*$^{-/-}$ controls would not necessarily have been displaying clinical signs of PRA at the time of their examination. The same has been observed in Gordon and Irish Setters, for which only 78.1% of Gordon Setters and 66.7% of Irish Setters homozygous for the mutation had developed PRA by age 9.8 years (Downs *et al.* 2013). The observed age of diagnosis in the relatively small number of cases in the present study ranged from 6 to 9 years.

In the present study, high coverage whole-genome sequencing of a relative low number of cases and controls proved highly efficient for identification of a variant that is likely to be the PRA-causing mutation, considering the predicted effect on the protein and the observed association between the same variant and PRA in other dog breeds. Based on the breed history, we expected that the sequenced

dogs would show signs of a small effective population size, i.e. a relatively high degree of homozygosity. Additionally, based on the observed pattern of autosomal recessive segregation of PRA, we expected to find homozygosity for a non-reference variant among cases. This could be, for example, a high impact variant in a protein-coding gene. As expected, only a few protein-coding non-reference variants were found to be homozygous in all five cases and heterozygous or homozygous for the reference allele in the five sequenced controls. Aided by the Ensembl Variant Effect Predictor and manual curation, identification of a single top candidate variant was straightforward. It should be noted that identification of other types of mutations, such as structural variations and copy number variations, would require additional analyses (Antaki *et al.* 2017; Trost *et al.* 2018).

The *rcd4* allele frequency observed in ODP is comparable to the frequency observed in Gordon Setters (Downs *et al.* 2013). The high frequency reflects the late age of onset and hence no selection against the mutation in traditional breeding programs without genetic testing. The high *rcd4* allele frequency is a challenge in breeding, especially in a very small population like ODP. Therefore, at least for a period of time, breeders must accept the use of carriers and even affected animals for breeding as long as affected dogs are mated with homozygous normal animals.

Acknowledgements

The authors would like to thank owners of Old Danish Pointing dogs who donated blood and tissue samples from their dogs to this project. We also thank the Club for Old Danish Pointing Dog (Klubben for Gammel Dansk Hønsehund) for excellent collaboration and financial support making this project possible. Additionally, we thank Minna Jakobsen, Tina Mahler and Charlotte B. Larsen for excellent work in the laboratory.

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Supporting information

Additional supporting information may be found online in the supporting information tab for this article:
Table S1 Breeds screened for the *rcd4* mutation.