Identification of genetic variants in *PDC*, *RHO*, *PDE6A* and *PDE6B* in dogs with progressive retinal atrophy

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The progressive retinal atrophy (PRA) is an inherited eye disease and characterized by progressive retinal degeneration which leads to impaired vision in dogs. Using targeted next generation sequencing of nine PRA cases and six controls, we have identified SNPs in *PDC*, *PDE6A* and *PDE6B*, which were not previously associated with PRA. The gene in which the highest mutations found was *PDE6A* (113 and 104 SNPs), followed by *PDE6B*, *PDC* and *RHO* in all dog breeds and Spitz-only respectively. Five SNPs identified in *PDC* gene of Spitz-only breed showed significant association with PRA. However, no pathogenetically relevant mutations were found in *RHO* gene for PRA. The SNP in *PDE6B* chr3: 91763017 (G/A) in Spitz-only breed, and *PDE6A* chr4: 5912574 (T/C) and *PDC* chr7: 19511750 (T/A) were associated with PRA in the breeds of dog studied. Our results show that PRA is genetically heterogeneous and is caused by multiple, distinct mutations.

Keywords: Genome-wide association, next generation sequencing, progressive retinal atrophy, single nucleotide polymorphisms.

THE canine retinal dystrophy is the group of disorders collectively referred to as the progressive retinal atrophies (PRAs)[1] and is characterized by loss of vision due to deterioration of the photoreceptor cells (rods and cones) in the retina[2]. In typical PRA condition, functions of rod photoreceptor cells are lost before cone photoreceptor cells[3], eventually leading to complete blindness. The initial clinical signs include nyctalopia followed by hemerlopia with tapetal hyper-reflectivity, pigmented changes, retinal vascular attenuation and atrophy of the optic nerve[4].

PRA is found to be inherited and involves various genes and mutations[5]. In majority cases of PRA, a single gene is associated with one form of the disease condition in a breed[6]. Currently, various different types of PRA conditions have been documented in greater than 100 dog breeds. However, the aetiology, age of onset and rate of progression vary between and within breeds irrespective of the similar clinical signs[2].

Over the past two decades, a number of approaches have been used to search for candidate genes and mutations underlying various traits in dogs. Different mutations have been identified underlying retinal diseases in 58 dog breeds using genome-wide association studies (GWAS), linkage study and targeted gene approach[7]. Although several mutations have been identified in PRAs, the genetic cause of PRA in many breeds is unknown[8]. PRA in canine is equivalent to Retinitis Pigmentosa (RP) in human which leads to progressive loss of vision in ~1 in 4000 (refs 9–11). Canine disease models have been proved valuable for the study of various human disease conditions such as cardiac conotruncal malformations[12], myotubular myopathy[13] and hereditary retinopathies such as Leber congenital amaurosis (LCA) and achromatopsia[14,15]. Canine eye disorder models can be used for human eye diseases and it has been proved invaluable in gene-therapy studies[16–19]. We have the opportunity to identify genetic variants associated with PRA in dogs as the canine genome sequences are readily accessible[20].

There are a number of retina-specific genes involved in the visual transduction pathway which are the candidate genes for PRA. PRA was associated with the mutation in β-subunit of the cGMP-specific phosphodiesterase (*PDE6B*) gene in Irish setters and Sloughis[21,22] and in the α-subunit of the cGMP-specific phosphodiesterase (*PDE6A*) gene in Cardigan Welsh Corgis[23]. A missense mutation in Phosducin (*PDC*) was detected in the Miniature Schnauzer[24]. An autosomal dominant mutation in Rhodopsin (*RHO*) was found to be associated with PRA in the Bull Mastiff and English Mastiff breeds[25,26].

We have used a study cohort of PRA affected and normal dogs including Spitz (n = 11), Spitz-Labrador cross (n = 1), Lhasapso (n = 1) and Cocker spaniel (n = 2)

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Table 1. Clinical data of PRA affected and normal dogs subjected to genetic analysis

Sample ID	Breed	Age (years)	Sex	Ophthalmic findings	Electroretinographic changes	Fundic imaging[#]
PRA affected dogs						
PR 65	Spitz	11.5	F	Immature cataract, Signs typical to PRA*	Positive for PRA	Not done
PR 66	Spitz	9	F	Reduced vision for last few months	Not done	Not done
PR 67	Spitz	13	M	Signs typical to PRA	Not done	Not done
PR 69	Spitz	10	F	Signs typical to PRA	Positive for PRA	Done
PR 70	Lhasa Aphso	6	M	Signs typical to PRA	Positive for PRA	Done
PR 72	Spitz	10	F	Signs typical to PRA	Positive for PRA	Done
PR 74	Spitz (cross with labrador)	12	F	Signs typical to PRA	Positive for PRA	Done
PR 82	Spitz	16	F	Bilateral mature cataract, dense vitreal bodies, signs typical to PRA	Not done	Not done
PR 84	Cocker spaniel	8.5	M	Signs typical to PRA	Not done	Not done
Normal samples						
PR 73	Spitz	7	F	No vision abnormality	Not done	Not done
PR 75	Spitz	9	M	No vision abnormality	Normal	Done
PR 76	Spitz	10	F	No vision abnormality	Not done	Not done
PR 79	Spitz	10	F	No vision abnormality	Not done	Not done
PR 81	Spitz	12	M	No vision abnormality	Normal	Done
PR 86	Cocker spaniel	10	M	No vision abnormality	Normal	Done

[#]Fundus examination is done by indirect ophthalmoscope for all studied animals and documented by fundus imaging device. However, the documentation was done for few of them.
*Nyctalopia, tapetalhyperreflectivity, attenuation of blood vessels, altered pigmentation and atrophied optic disc.
(Characteristics included dog breeds examined, age of PRA affected and normal dogs during the time of DNA isolation, sex and clinical data.)

breeds to identify a possible association of *RHO*, *PDC*, *PDE6A* and *PDE6B* genes with PRA by sequencing these whole genes using amplicon sequencing approach, variants detection and its association with PRA using case-control analysis.

Materials and methods

Clinical investigation and sample processing

Each dog in this study was diagnosed by veterinary ophthalmologists at the Department of Veterinary Surgery, College of Veterinary Science and A. H., Anand Agricultural University, Anand, India. The dog was diagnosed as PRA (cases, $n = 9$) affected when displaying ophthalmoscopic signs of PRA including tapetal hyper-reflectivity, vascular attenuation and atrophy of optic disc with typical history of nctylopia followed by hemerlopia, which are typical PRA signs and controls ($n = 6$) were the animals without any clinical sign of eye disease and with normal vision, and at least of 6–7 years during clinical examination (Table 1). For fundus examination, the dogs were subjected to indirect ophthalmoscopy by dilating the pupil with mydriatics. The animal was considered clinically positive for PRA when fundus examination revealed typical PRA changes (Figure 1). Blood samples were collected in EDTA vacutainer and genomic DNA was extracted using NucleoMag®Blood 200 µl kit (Macherey-Nagel, Germany) and treated with RNase A to remove RNA contamination. The quality of genomic DNA was checked by agarose gel electrophoresis and ND-1000 spectrophotometer, and quantity was measured by Qubit fluorometer (Life Technologies, USA).

High-throughput amplicon sequencing of RHO, PDC, PDE6A and PDE6B

To identify genetic variants of *RHO*[25,26], *PDC*[24], *PDE6A*[23] and *PDE6B*[22] in all clinically PRA positive and normal samples, amplicon sequencing was performed for these candidate genes known to cause PRA in dogs as reported previously, using two primer sets (Tables S1 and S2, see Supplementary Material online). Amplicons were generated and pooled, using 50 ng of genomic DNA as template from clinically PRA positive and normal samples. PCR primers were designed for all RefSeq exons of four genes (primer set 1) with amplicons expected to be 380–420 bp in size and primers were also designed to cover a whole gene in approximately, 2.5 kb amplicon size of intronic plus exonic regions (primer set 2). First, amplicons of primer set 1 and primer set 2 from each gene were pooled separately per sample and then the library was prepared with the Ion Xpress™ Plus fragment library kit (Life Technologies, USA), following the manufacturer's protocol. Briefly, for each sample, ~200 ng of pooled amplicons of primer set 2 from each gene was fragmented by enzymatic digestion, and mixed with amplicons of primer set 1 from all genes and used for library

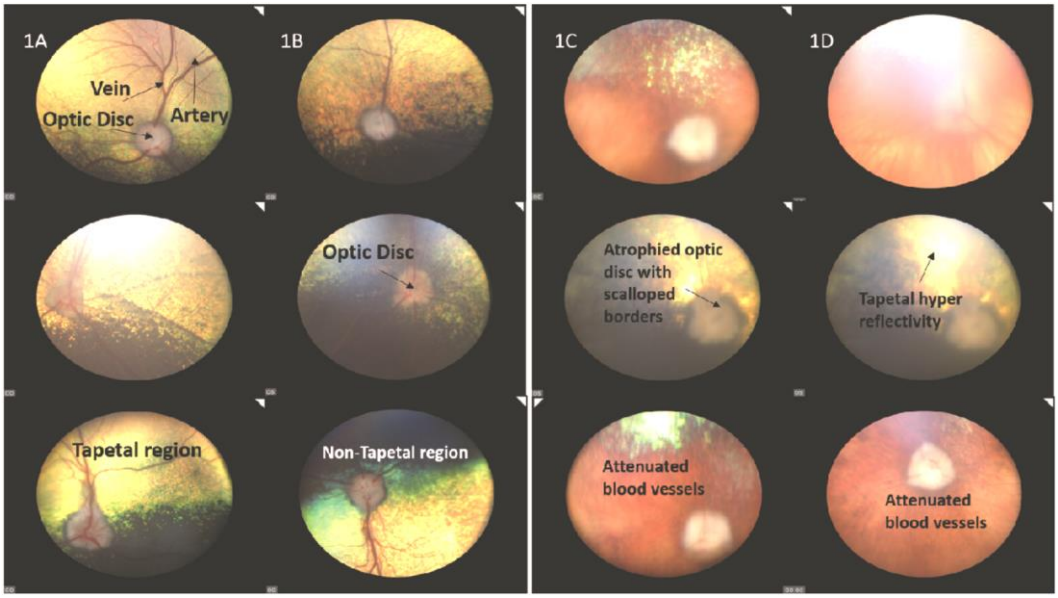

Figure 1. Fundus photograph of normal (1A, 1B) and PRA affected (1C, 1D) eye. 1A and 1B shows healthy fundus of normal dog with well separated veins and arteries. Hyper reflectivity of tapetal fundus (arrow) and pigmented changes are seen in image 1C which is of PRA affected dog eye. Attenuated arteries and vascular attenuation (arrow) along with greyish optic disc can be viewed in image 1D.

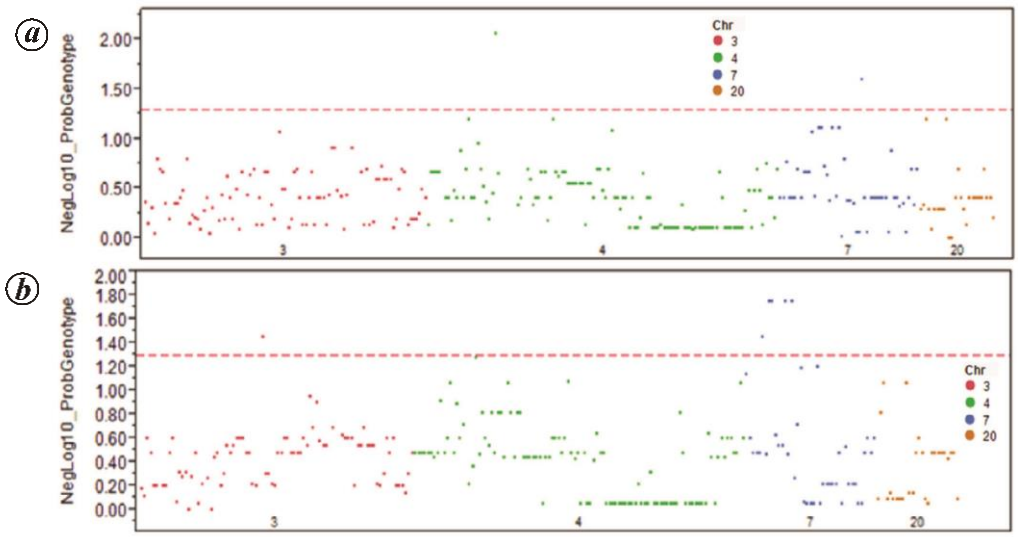

Figure 2. A Manhattan plot of genome-wide case-control association analysis performed (***a***) using nine cases and six controls (of all breeds samples) indicated two statistically significant SNPs located on chromosome 4 and 7 respectively, and (***b***) six cases and five controls (of Spitz breed samples) indicate the most highly associated region in *PDC* gene located on chromosome 7.

preparation. Each sample's pool was individually barcoded and amplified (5 cycles). Samples were run on an ion PGM™ next generation sequencing platform with ion PGM™ sequencing 400 kit.

Variant calling and annotation

The criteria used to filter raw reads using PRINSEQ were: remove reads with sequence adaptors; remove low quality reads, which have mean quality score <20; remove reads with an exact duplicate; remove reads, which have read length <60. Further downstream analysis was carried out on these quality filtered reads. Filter reads in FASTQ format from each exome sequencing sample were aligned to the dog reference genome (canFam3.1) downloaded from Ensembl genome browser with BWA-MEM module of BWA using default parameters. PCR duplicates were marked with Picard MarkDuplicates

(http://broadinstitute.github.io/picard/). Thereafter, the Genome Analysis Toolkit (GATK, version 2.8)[27] was used to call SNP by the UnifiedGenotyper module. SnpEff was used to annotate the SNPs with gene annotations downloaded from the Ensemble Genome Browser.

Statistical analysis

We carried out a case-control analysis of 15 samples: nine cases versus six controls and in Spitz dog breed samples, six cases versus five controls. We used a logistic regression model with case status regressed on each allele, SNPs genotype, and linear trend. The corresponding Manhattan plot is shown in Figure 2. Analysis was carried out in the SAS statistical analysis software package using JMP Genomics 6.1 (SAS, Cary, NC).

Result

We analysed nine PRA affected and six normal dogs to determine the genetic cause of progressive retinal atrophy. Clinical signs of the affected dogs were typical for PRA, including attenuated vessels, pigmentary changes and tapetal hyper-reflectivity (Figure 1). The age at PRA diagnosis of PRA-affected samples was between 6 and 16 years (average 10.6 years). Four retina-specific genes named *RHO*, *PDC*, *PDE6A* and *PDE6B* were targeted in the present study.

A total of 284 high quality SNPs were subjected to case-control association analysis and we found two SNPs with suggestive evidence of association ($P < 0.05$) (Table 2 and Figure 2). The most significant SNP was found at chr4: 5912574 (T/C), $P = 8.75 \times 10^{-3}$ and the second most significant SNP was found at chr7: 19511750 (T/A), $P = 2.53 \times 10^{-2}$. The frequencies of the alternate alleles of both the intronic SNPs were higher in controls (0.30, 0.50) as compared to PRA cases (0.0, 0.22) (Table 4).

As Spitz dog breed samples dominated our study, we analysed Spitz samples (5 normal and 6 PRA affected) separately. A total of 258 SNPs detected in Spitz-only were subjected to case-control association analysis. Six SNPs were identified as suggestive evidence of association ($P < 0.05$) (Table 3 and Figure 2): chr7: 19509452 (T/A), chr7: 19509598 (A/G), chr7: 19510446 (C/T) and chr7: 19510503 (G/A) ($P = 1.77 \times 10^{-2}$), chr7: 19509281 (G/T) at *PDC* ($P = 3.57 \times 10^{-2}$) and chr3: 91763017 (G/A) at *PDE6B* ($P = 3.57 \times 10^{-2}$) (Table 4). The alternate allele frequency of all five intronic SNPs at *PDC* was quite high in cases (0.50) as compared to controls (0.13). We observed 83.3% and 20% heterozygosity in cases and controls respectively, across five loci of *PDC* gene. However, we found G allele of chr3: 91763017 (G/A) at *PDE6B* in five out of six PRA cases, whereas only single control sample had G allele present in heterozygous condition.

SNPs were also classified according to the type of effect they produce, i.e. intronic, synonymous, non-synonymous, 5′ UTR, 3′ UTR and downstream regions. Out of 284 SNPs identified in all dog breeds in the present study, 9 (3.17%) and 256 (90.14%) are located in exon and intron regions respectively (Figure 3). Whereas a total of 258 SNPs were identified in Spitz-only; out of which 8 (3.1%) and 235 (91.08%) are located in exon and intron regions respectively (Figure 3). In all dog breeds studied and only in Spitz breed, a total of 17 (5.98%) and 13 (5.03%) SNPs respectively, are located downstream from candidate genes. The remaining SNPs (0.70% and 0.77%) are located in 3′ UTR of candidate genes in all dog breeds and only in Spitz breed respectively. In *PDC*, we found all SNPs intronic in all dog breeds studied (48 SNPs) and Spitz-only (41 SNPs). Interestingly, most of the SNPs (one out of two in all dog breeds of this study and five out of six in Spitz-only) which showed significant association were present in *PDC* gene. The gene in which the highest mutations found was *PDE6A* (113 and 104 SNPs), followed by *PDE6B* (97 and 87 SNPs), *PDC* (48 and 41 SNPs) and *RHO* (26 SNPs in both group) in all dog breeds and Spitz-only respectively (Tables 2 and 3). Further, we calculated unique SNPs which are present only in normal (35 and 46) and cases (70 and 51) in all dog breeds studied and Spitz-only respectively (Table 5). In all samples and in Spitz-only, we found 66 SNPs that are reported in dbSNPs.

Discussion

The progressive retinal atrophy (PRA) is inherited and equivalent to retinitis pigmentosa (RP) which leads to

Table 2. Distribution of SNPs by effect and gene obtained from all dog breed samples

	RHO	*PDC*	*PDE6A*	*PDE6B*	Total
Intron	23	48	108	77	256
Synonymous_Coding	3	–	1	2	6
Non_Synonymous_Coding	–	–	2	1	3
UTR_3_prime	–	–	2	–	2
Downstream	–	–	–	17	17
ALL	26	48	113	97	284
Affected	23	43	100	83	249
Normal	17	38	85	74	214

Table 3. Distribution of SNPs by effect and gene obtained from Spitz-only samples

	RHO	*PDC*	*PDE6A*	*PDE6B*	Total
Intron	23	41	100	71	235
Synonymous_Coding	3	–	–	2	5
Non_Synonymous_Coding	–	–	2	1	3
UTR_3_prime	–	–	2	–	2
Downstream	–	–	–	13	13
ALL	26	41	104	87	258
Affected	23	36	88	65	212
Normal	17	38	82	70	207

Table 4. Association results and functional alteration of SNPs identified in this study for Spitz-only samples

Chr.	Ref.	Alt.	Gene symbol	ID	Position	SNP location	Genotype of affected samples*			Genotype of normal samples			*P*-value	Allele frequency (normal)		Allele frequency (affected)	
							0/0	0/1	1/1	0/0	0/1	1/1		Ref.	Alt.	Ref.	Alt.
7	T	A	*PDC*	snp225	19509452	INTRON	–	5	–	3	1 (PR81)	–	0.0177	0.88	0.13	0.50	0.50
7	A	G	*PDC*	snp226	19509598	INTRON	–	5	–	3	1 (PR81)	–	0.0177	0.88	0.13	0.50	0.50
7	C	T	*PDC*	snp230	19510446	INTRON	–	5	–	3	1 (PR81)	–	0.0177	0.88	0.13	0.50	0.50
7	G	A	*PDC*	snp232	19510503	INTRON	–	5	–	3	1 (PR81)	–	0.0177	0.88	0.13	0.50	0.50
7	G	T	*PDC*	snp223	19509281	INTRON	1	5	–	4	1 (PR81)	–	0.0357	0.90	0.10	0.58	0.42
3	G	A	*PDE6B*	snp66	91763017	INTRON	5	1	–	1	4	–	0.0357	0.60	0.40	0.92	0.08

*Homozygous for reference allele – 0/0; Homozygous for alternate allele – 1/1; Heterozygous – 0/1. Ref., Reference allele frequency; Alt, Alternate allele frequency.

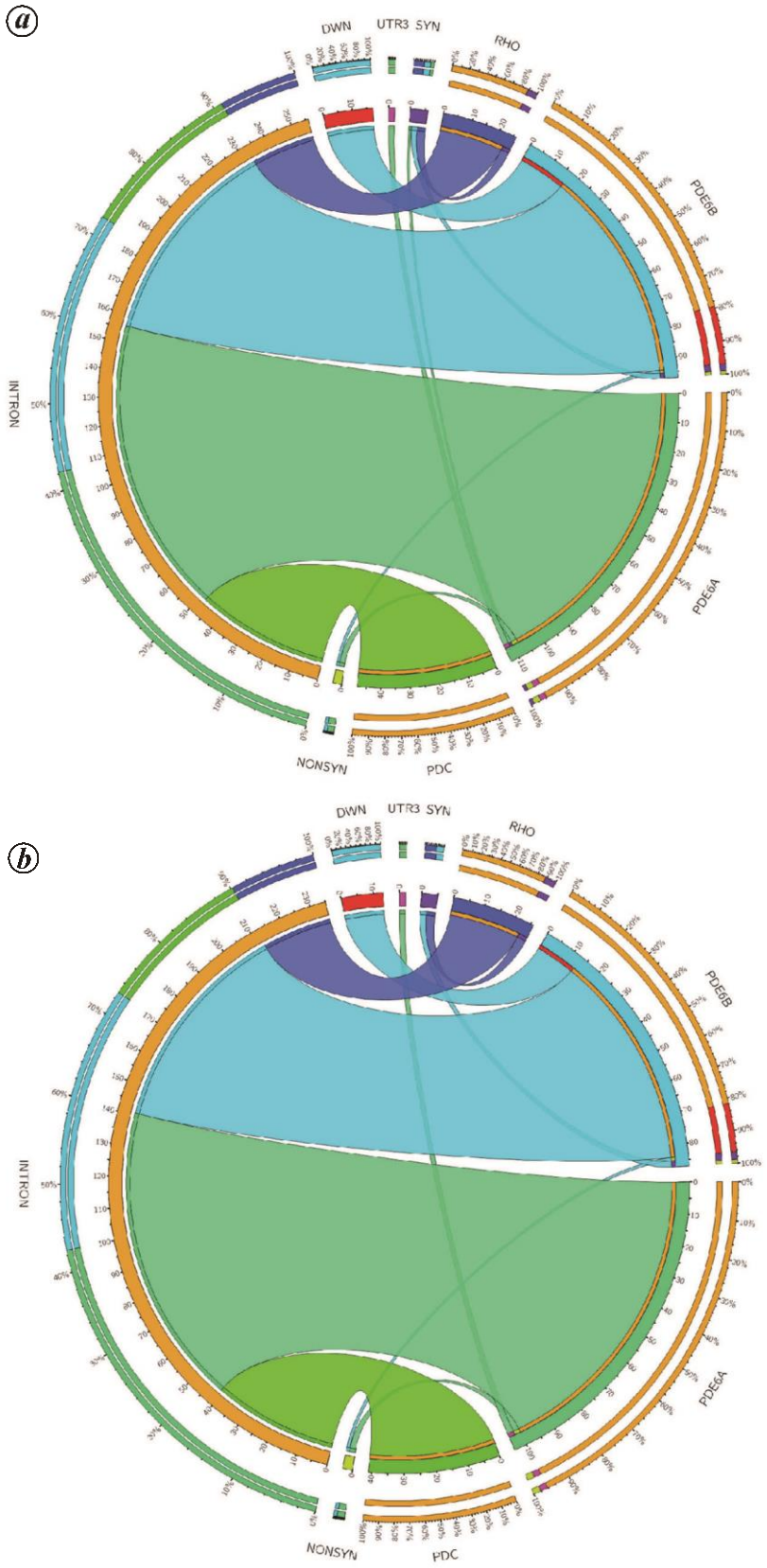

Figure 3. The circos diagram showing effect-wise distribution of SNPs of four genes for (***a***) all dog breed samples and (***b***) Spitz-only samples. Distribution of annotated SNPs in various regions (Intron; DWN, Downstream; SYN, Synonymous; NONSYN, Nonsynonymous, UTR 3′) versus four genes (*RHO*, *PDC*, *PDE6A* and *PDE6B*) is shown by various colour lines.

Table 5. Proportion of unique SNPs present in normal and PRA affected samples

Gene	Effect	All samples		Spitz samples	
		Unique in affected	Unique in normal	Unique in affected	Unique in normal
RHO	Intron	9	2	9	2
	Synonymous_Coding	–	1	–	1
	Non_Synonymous_Coding	–	–	–	–
	UTR_3_Prime	–	–	–	–
	Downstream	–	–	–	–
PDC	Intron	10	5	3	5
	Synonymous_Coding	–	–	–	–
	Non_Synonymous_Coding	–	–	–	–
	UTR_3_Prime	–	–	–	–
	Downstream	–	–	–	–
PDE6A	Intron	27	12	21	14
	Synonymous_Coding	–	–	–	–
	Non_Synonymous_Coding	1	–	1	–
	UTR_3_Prime	–	1	–	2
	Downstream	–	–	–	–
PDE6B	Intron	18	11	14	20
	Synonymous_Coding	–	–	–	–
	Non_Synonymous_Coding	1	–	1	–
	UTR_3_Prime	–	–	–	–
	Downstream	4	3	2	2
Total		70	35	51	46

impaired vision in human and dogs due to progressive bilateral retinal degeneration. Most mutations associated with canine PRAs are recessive and breed-specific, still the genetic cause of PRA is unknown for many dog breeds. The genetic variation underlying some forms of progressive retinal atrophy can be mapped to the chromosomal location of others using molecular techniques[1].

SNPs are present to a greater extent than microsatellite and are distributed across the genome to aid association studies[7]. A definite advantage of GWAS over Linkage Association (LA) study is that it does not essentially require large and multigenerational pedigrees. We have carried out amplicon sequencing of four candidate genes known to associate with PRA to identify genetic variants associated with PRA in three breeds of dog.

We analysed nine cases clinically positive for PRA and six controls. In order to identify variants in the *PDC*, *RHO*, *PDE6A* or *PDE6B* genes, we performed amplicon sequencing using Ion-Torrent platform. We have identified variants in both coding as well as non-coding regions, but the most of variants were present in non-coding regions.

PDC is a phosphoprotein expressed in retinal photoreceptor cells[28,29]. *PDC* modulates the phototransduction cascade by interacting with the $\beta\gamma$ subunit complexes of the retinal G-protein transducin ($Td\beta\gamma$)[30]. A missense mutation CGA $\rightarrow$ GGA in codon 82 changes amino acid residue Arg to Gly and this change is close to the residue (Glu 85) which directly interacts with the beta-gamma-subunits of transducin in photoreceptor dysplasia (pd) affected Miniature Schnauzer breed and it was not detected in the present study[24]. Polymorphisms detected in the 3′ UTR of two dog breeds: Miniature Poodles and Irish Wolfhound[31] were also not identified in any of our studied breeds. Only a single SNP showed significant association when all breeds were considered, but when Spitz-only data were analysed, we could identify five SNPs with significant case-control association. However, none of these five SNPs overlapped with the single SNP that was found to be associated when all breeds were considered. These five SNPs are located at intron 1 of phosducin gene in Spitz-only. One SNP found in all samples is also located at intron 1 of this gene. We did not identify the SNPs in *PDC* gene which were previously reported to associate with PRA. As a regulatory protein in phototransduction, it may be a candidate gene for retinal degenerative diseases.

RHO is the visual pigment of rod photoreceptors and member of G protein-coupled receptors genes family[32,33]. *RHO* is involved in photo-activated signal transduction process which is critical for vision[34]. We amplified *RHO* gene covering intronic and exonic regions and failed to get a statistically significant SNP associated with PRA in this gene. The autosomal dominant PRA mutation identified in the Bull Mastiff and English Mastiff dogs with a missense mutation (Thr4Arg) in this gene was not detected in our studied breeds.

Rod photoreceptor cyclic GMP-phosphodiesterase (*PDE6A* and *PDE6B*) is a key effector protein of the

phototransduction cascade in retina. Once initiated by the absorption of light by rhodopsin, activated cGMP-PDE rapidly hydrolyses cGMP, depletion of which shuts cGMP-gated cation channels. Aberrant functions of these proteins are associated with retinal degenerative diseases in human and animals. Mutations in the *PDE6A* and *PDE6B* cause retinitis pigmentosa (OMIM: 268000) in human[35–37]. Mutations in *PDE6B* also cause retinal degeneration in the *rd* mouse[38,39] and the *rcd1* dog[21,40,41]. A single base deletion at codon 616 in the *PDE6A* gene is co-segregated with PRA status with zero discordance in PRA affected Cardigan Welsh Corgis[23]. A different mutation in *PDE6B* for rcd1a was found to be associated with PRA in the Sloughi by using a candidate gene approach[22]. A stop mutation in exon 21 of canine *PDE6B* gene was also identified by sequencing in Iris Setters breed[21,40,41]. However, these mutations were not detected in our studied breeds. We found two SNPs in the intronic region which showed genome wide association. One SNP is present at Intron 7 of the *PDE6A* gene in all samples and another SNP is located at Intron 3 of the *PDE6B* gene in Spitz samples.

Conclusions

We screened four candidate genes (*PDC*, *RHO*, *PDE6A* and *PDE6B*) known to be associated with PRA through amplicon sequencing. Our data revealed that all associated mutations found in our study were present in non-coding regions of candidate genes. Five mutations [chr7: 19509452 (T/A), chr7: 19509598 (A/G), chr7: 19510446 (C/T), chr7: 19510503 (G/A) and chr7: 19509281 (G/T)] in *PDC* showed significant association with PRA only in Spitz breed, but further study is needed to confirm these findings. Validation of these mutations in other dog breeds would be needed for its diagnostic application for PRA. One SNP [chr3: 91763017 (G/A)] was present in the *PDE6B* gene in Spitz samples and another two SNPs [chr4: 5912574 (T/C), chr7: 19511750 (T/A)] were present in the *PDE6A* and *PDC* genes in all samples respectively. However, not a single mutation in *RHO* associated with PRA in our study could be detected. Our study confirms that PRA may be caused by polygenic mutations and it may be breed specific too. Our finding will encourage additional research to understand the molecular mechanisms behind PRA.

Conflicts of interest. The authors declare that they have no conflict of interests.

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ACKNOWLEDGEMENTS. The authors thank Dr Amrut Patel for his contribution to this work and to the owners of the dogs for blood samples. This work was supported by grants from the Department of Biotechnology (DBT).

Received 4 December 2015; revised accepted 4 May 2016

doi: 10.18520/cs/v111/i10/1640-1648