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Screening of the *arrestin* gene in dogs afflicted with generalized progressive retinal atrophy

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Abstract

Background: Intronic DNA sequences of the canine *arrestin* (*SAG*) gene was screened to identify potential disease causing mutations in dogs with generalized progressive retinal atrophy (gPRA). The intronic sequences flanking each of the 16 exons were obtained from clones of a canine genomic library.

Results: Using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequence analyses we screened affected and unaffected dogs of 23 breeds with presumed autosomal recessively (ar) transmitted gPRA. In the coding region of the *SAG* gene 12 nucleotide exchanges were identified, 5 of which lead to amino acid substitutions (H14C; A111V; A113T; D259T; A379E). 7 other exonic substitutions represent silent polymorphisms (C132C; Q199Q; H225H; V247V; P264P; T288T and L293L). 16 additional sequence variations were observed in intronic regions of different dog breeds.

Conclusions: In several breeds, these polymorphisms were found in homozygous state in unaffected and in heterozygous state in affected animals. Consequently these informative substitutions provide evidence to exclude mutations in the *SAG* gene as causing retinal degeneration in 14 of the 23 dog breeds with presumed ar transmitted gPRA.

Background

gPRA is usually inherited as an ar blinding disorder with different ages of onset and variable rate of progression observed in more than 100 dog breeds. Typically, gPRA commences with degeneration of the rod photoreceptors. Initial signs include night blindness whereas progression involves the cones and the central vision [1,2]. The human equivalent of canine gPRA is termed retinitis pigmentosa (RP). RP comprises a large and genetically heterogeneous group of blinding disorders. RP may be inherited in an ar, dominant, X-linked, digenic or maternal mode [3–7]. Similarly in dogs, at least 4 genes were identified so far as causing gPRA in 6 breeds. All of these genes encode photoreceptor specific proteins involved in the visual transduction cascade including the β-subunit of the cGMP-specific phosphodiesterase (PDE6B) in Irish Setters and Sloughis [8,9] as well as the α-subunit of the cGMP-specific phosphodiesterase (PDE6A) in Cardigan Welsh Corgis [10]. A missense mutation was detected in the *PDC* gene that may be associated with photoreceptor dysplasia, a form of gPRA in the Miniature Schnauzer [11]. The X-linked form of PRA in Samoyed and Siberian Husky is caused by mutations in the *RPGR* gene [12]. Recently in English Mastiff dogs an autosomal dominantly transmit-

ted form of gPRA was identified, mimicking human RP. The disease causing mutation is a T4R exchange in the *rhodopsin* (*RHO*) gene [13]. A number of other retinal genes have been excluded as harbouring mutations for gPRA in several dog breeds: RHO; [14], *RDS/peripherin* and *ROM-1* [15] as well as the α – and γ-subunits of *transducin*[16] and *SAG*[17]. Yet, the *SAG* gene had been analyzed on the exonic level exclusively, *i.e.* by sequencing of cDNA. The human *SAG* gene comprises 16 exons ranging in size between 243 and 10 bp. SAG protein (403 amino acids) has been identified only in retinal photoreceptor rods and pinealocytes [18].

Table 1: Characteristics of dog breeds examined

Breed (abbreviation)	Number of dogs	Diagnosis	Onset forms of gPRA	Age distribution (year)[f]
Afghan Hound (AW)	1	gPRA-affected	late[b]	6
	4	normal		2–12
Australian Cattle dogs (AC)	2	gPRA-affected	late[a]	10
	19	normal		2–4
Berger des Pyrénées (BDP)	1	gPRA-affected	mid-onset[b]	5
	42	normal		1–10
Berner Sennenhund (BS)	1	gPRA-affected	late[b]	10
Bolognese (Bo)	1	gPRA-affected	late[b]	9
Collie (Co)	3	gPRA-affected	early[cd]	4–8
	1	normal		8
Curly Coated Retriever (CCR)	1	gPRA-affected	late[b]	6
Dachshund (wire; D)	20	gPRA-affected	variable[b]	1–13
	49	normal		6–13
Engl. Cocker Spaniel (ECS)	6	gPRA-affected	late[cd]	3–11
	6	normal		6–14
English Springer Spaniel (SP)	1	gPRA-affected		7
Entlebucher Mountain Dog (EM)	17	gPRA-affected	late[e]	5–13
	10	normal		1–7
Giant Schnauzer (GS)	1	gPRA-affected	late[b]	10
Golden Retriever (GR)	2	gPRA-affected	late[b]	5–10
	8	normal		3–6
Irish Setter (IRS)	2	gPRA-affected	early[cd]/late[b]	0.6–7
	1	normal		3–13
Labrador Retriever (LR)	5	gPRA-affected	late[cd]	8–12
	140	normal		3–13
Miniature poodle (MP)	28	gPRA-affected	late[cd]	5–12
	15	normal		1–12
Newfoundland Dog (NF)	1	gPRA-affected	mid-onset[b]	3
Polski Owczarek Nizinny (PON)	1	gPRA-affected	late[b]	9
Rottweiler (Ro)	1	gPRA-affected	late[b]	3
Saarloos/Wolfhond (Sa)	7	gPRA-affected	late[b]	7–9
	118	normal		2–10
Schapendoes (SD)	3	gPRA-affected	early[d]	2–6
	7	normal		3–6
Sloughi (Sl)	5	gPRA-affected	mid-onset[b]	2
	183	normal		0.1–12
Tibetan Terrier (TT)	3	gPRA-affected	mid-onset[cd]	7–8
	93	normal		2–10

[a][34]. [b] owners report/certificate of eye examination. [c] classifications of the different onset forms of gPRA in the reviews [1] and [2]. [d] online information among PRA Today [http://www.sheepdog.com/diseases/pra/clinical]. [e][35]. [f] at the time when blood was taken for DNA analysis.

SAG belongs to a family of inhibitory proteins that bind to tyrosine-phosphorylated receptors, thereby blocking their interaction with G-proteins and effectively terminating the signalling chain. In the phototransduction cascade, SAG and rhodopsin kinase (RHOK) act together in the recovery phase of RHO. After photoactivation, RHOK phosphorylates photoexited RHO which is then blocked by SAG binding thus inhibiting its ability to interact with transducin [19,20]. The existence of stable complexes between RHO and its regulatory protein SAG were demonstrated to be responsible for retinal degeneration in several mutations in Drosophila [21]. Accumulation of these complexes triggers apoptotic cell death showing that retinal degeneration requires the endocytic machinery (*op. cit.*). Interestingly, loss of function in the *SAG* gene causes ar inherited Oguchi disease in Japanese, a variant of congenital stationary night blindness [22,23]. Apparently the mutation causing Oguchi disease can also lead to arRP in Japanese families [24]. Here we report on the identification of intronic sequences and mutation screening of the canine photoreceptor-specific *SAG* gene in 19 different dog breeds.

Table 2: Primers for the characterization of the SAG gene

Exon	Forward primer, reverse primer (5'–3')	Exon (#) and size (bp)	Intron size (bp)	splice donor site (*gt*), splice acceptor site (*ag*)
1–2	GGGCAACCCTGTCCAGG ACACCCTGGGGTCTGTGTC	(1) 156	836	 CACAAG**gt**acatg
2–3	AGTGAAAGAAGCTACCAGGGA ACCCACAGGCTCTACTTGT	(2) 132	~ 5500	ttccc**ag**CTTGCT AAGTCG**gt**aagtgg
3–4	AGGTGACCATCTACTTGGG AAAGTTCTTTCCTAGCACTAAG[a]	(3) 61	~ 2600	cctat**ag**GTGACC CTGTGG**gt**aagttg
4–5	AGATGGTATCGTGTTGGTGG ACCGTGAGCAGGAAGGG	(4) 45	~ 1800	ggttt**ag**ATGGTA AGAAAG**gt**aagaca
5–6	AGTGTATGTCTCTCTGACCTG ACCTTCCCCATGTCTTGCG	(5) 194	~ 2000	cctcc**ag**TGTATGT CTCACG**gt**gggtg
6–7	CAAGTTTTACACACTGAGTGC[a] TCGCAGGCCACAGAGGAGAAG[a]	(6) 60	~ 1300	cccac**ag**TTCCCTG GGGAAG**gt**tagtg
7–8	GTGCTGTGGGGTCGACTTT CAGCACCCACAGCAGATTG[a]	(7) 77	~ 1500	actgc**ag**TGCTGTG CAAGAA**gt**aagagt
8–9	AGGAGGTCCGTGCGTTTTAC CTAATGCCTTTAATCTTCTTC	(8) 136	905	tctgc**ag**GAGCTCC AAAGAG**gt**gagcca
9–10	CCTAGAAAGCCATGAGATTAAA[a] AAGCCAAGCATCCTACTTCC	(9) 85	~ 1500	ttttc**ag**ATCTATT CATTAG**gt**aggaac
10–11	TGGAACAAGTGGCCAACGTT[a] ACATGGTGCTGCAAGG	(10) 73	~ 2500	tctgc**ag**TGGAACA AACACA**gt**gagtag
11–12	GGACTGATGGTGGCTTTATG[a] ACCCTGACACCGTGAGCTTC	(11) 138	~ 3200	cctac**ag**AGAAAAA CACCAT**gt**gagtcc
12–13	TGAGGGATGTGTTCATCTAG[a] TTCATAACACCTCTGAGCTAC[a]	(12) 78	1390	tgagc**ag**AATAAAG GTCAGG**gt**gagtgt
13–14	CTGGAGGTGAGCTCTCCCA GTGCTCAGGGACCACTAG	(13) 24	~ 1400	ttcct**ag**CTTTCTG CTCCAG**gt**aagcct
14–15	AGTGAAGTGGCAACTGAGGT AAGCCGAAGACTGGGAGCa	(14) 56	~ 3000	tttcc**ag**TGAAGTG ACCCAG**gt**cagtcg
15–16	CCTGCTCACGATTCTCTTTC GTCCAGGTCGACCGACCT	(15) 16	426	cttac**ag**CTACGGC GGAAAG**gt**gagccc
		(16) 237		tctttt**ag**TTTTCA

PCR amplification of the canine *SAG* gene: primers for the identification of exon/intron boundaries and for the determination of intronic lengths of the canine genomic *SAG* clones (exon sequences are shown in upper case) [a]the intronic sequences of the *SAG* gene received the EMBL accessions numbers AJ426068-AJ426078

Results and Discussion

Genomic organization of the SAG gene

The screening of the canine genomic library with probes for exons 2, 5 and 16 led to the isolation of seven DNA clones, each containing different parts of the *SAG* gene. This gene contains 16 exons with the 5'*UTR* split into exons 1 (156 bp) and in 2 (57 bp). The 3'*UTR* is comprised in exon 16 (137 bp). The coding region is 1215 bp long. Most introns were longer than 1.5 kb (Table 2). Compared to the human *SAG* gene the position of intron 1, which is in the 5'UTR, is 23 bp further upstream in the dog. This means the canine exon 1 is 23 bp shorter and exon 2 23 bp longer than the equivalent human exons. In dogs exon 15 is 6 bp longer and exon 16 6 bp shorter than the equivalent exons in human. Therefore, the predicted protein in both species are 405 aa long and have a similarity of 89.8%. The intron sizes in man and dog differ, leading to gene sizes of ~ 35 kb in dog and ~ 40 kb in man. The splice donor and acceptor sites follow the GT/AG rule (Table 2). Canoidea-specific, tRNA-derived short interspersed nucleotide elements (SINE; [25]) were identified in introns 1, 3 and 14 and additional repetitive elements in introns 2, 3 and 14. The human *SAG* gene maps to chromosome 2q37.1. On the basis of reciprocal chromosome painting [26], the canine gene is, therefore, predicted to map to CFA 28 or 33, the homologous chromosomal regions in dogs.

Mutation screening by PCR-SSCP analysis

The *SAG* gene was screened for mutations in 23 breeds, including all gPRA-affected, selected healthy dogs as well as obligatory carriers. The 16 exons were analysed by PCR-SSCP including all intronic splice signal sequences as well as the *UTR*s (*i.e.* complete cDNA plus >3070 bp of the introns). In the coding region of the *SAG* gene, 5 polymorphisms were identified that result in altered amino acid coding (H14C, A111V, A113T, D259T and A379E), and 7 silent polymorphisms were identified (C132C, Q199Q, H225H, V247V, P264P, T288T and L293L; Table 4). In addition, 13 sequence variations were identified in 9 introns of gPRA affected and unaffected animals (Table 4). Several gPRA-affected dogs in 14 of the 23 breeds were heterozygous for one of the aforementioned polymorphisms (Table 4). In 6 of these 14 dog breeds the major cause of gPRA has meanwhile been determined. Direct DNA tests are possible for Irish Setters and Sloughis [8,9]. Indirect tests for progressive rod cone degeneration (*prcd*) were recently offered for Australian cattle dogs, English Cocker Spaniels, Labrador Retrievers and Miniature poodles (patented by OptiGen, USA). These dog breeds were included as controls to characterise the identified polymorphisms to exclude linkage for causal gPRA mutations. A second gPRA form may be exist in Irish Setter because one affected Setter shows a late form of gPRA without the typical *PDE6B* mutation. Because of the clinical signs, also in Miniature poodles two types of gPRA are possible (OptiGen).

Conclusions

None of the amino acid changes identified here in dogs correspond to residues that are mutated in known RP, nor are they known to be important for binding activated dephosphorylated RHO [22,23,27]. As detailed above, Oguchi disease and some forms of arRP is caused by the deletion in codon 309 in Japanese. None of the aa exchanges in the dog breeds investigated here correspond with this region. Nevertheless, these novel sequence variations can be used as intragenic markers for segregation analyses with ar gPRA. The breeding history, small population sizes and gPRA abundance in the investigated breeds point together to few meiotic events in which intragenic recombinations could have occured between an unidentified mutation in the *SAG* locus in gPRA dogs and the polymorphisms investigated here. Given ar transmission our typing results suggest that the sequence variations in the *SAG* gene are not causative for gPRA in the following 14 dog breeds: AC, BDP, Bo, BS, ECS, D, IRS, GR, MP, NF, PON, Sa, SD and Sl. In 6 of these dog breeds only one gPRA affected animal was available for mutation analysis (Table 1). For these breeds the exclusion of the*SAG* gene is not definitive, since the possibility of false diagnosis is not ruled out completely. Nevertheless, gPRA-affected AW, CCR, SP, LR, Ro and TT show homozygous sequence variation patterns and 3 dog breeds (Co, EM, GS) did not harbour any sequence variations. Therefore, the *SAG* gene cannot be excluded as a cause for gPRA in these breeds, especially because of mutations in the elusive regulatory regions for gene expression.

Materials and Methods

Animals

Blood from 810 dogs of 23 different breeds, including 113 gPRA-affected animals (Table 1) was collected with the permission of the owners and in cooperation with breeding organisations. Experienced veterinarians confirmed the gPRA status of healthy and affected dogs by ophthalmoscopy.

Isolation of canine DNA and PCR

DNA was extracted from peripheral blood according to standard protocols [28]. Portions of the SAG gene were amplified by PCR in a thermocycler (Biometra, Goettingen, Germany) from the inserts of the λ phages in order to obtain intronic sequences. Genomic DNAs from all gPRA-affected, obligate carrier and gPRA-unaffected dogs were screened for sequence variations. PCRs were performed in 96-well microtiter plates (Thermowell Costar Corning, NY). Each well contained 50 ng DNA in 10 μl reaction volume (100 mM Tris [pH 8.3], 500 mM KCl, 1 U Taq Polymerase [Genecraft, Münster, Germany], 0.2 mmol of

Table 3: Primer sequences used for mutation analysis of individual exons/introns of the canine *SAG* gene

Primer	Location	Forward primer, reverse primer (5'–3')[a]	PCR conditions [T-°C/MgCl$_2$-mM]	PCR amplicon length (bp)	Restriction enzymes for SSCP analyses
UTR1F	exon 1	GGGCAACCCTGTCCAGGT	54/1.0	699	*NlaIII/RsaI*
UTRI-1aR	intron 1	TCTATCATGACGGGACGCCT			
UTRI-1aF	intron 1	GAAAATGATATTTGCAAAGCAG	50/1.0[b]	283	*Tru11*
UTRI-1aR	intron 1	TCTATCATGACGGGACGCCT			
1-IF	intron 1	CTAATGGGCACACAGCATCTC	53/1.0	249	*PvuIII; NlaIII*[c]
2-I2R	intron 2	TTCTGTTAAGCCACTCACTTC			
3-IF	intron 2	TGTTTTTATCTAACACTGACTACTTC	48/1.0[b]	224	*NlaIII*
3-IR	intron 3	AAATAACAAAGTAGCAGCTGTC			
4-IF	intron 3	AACTGCAGATAAATATATGAAG	52/1.0	189	*AluI*
4-IR	intron 4	AAAGTTCTTTCCTAGCACTAAG			
5-IF	intron 4	GGTTACCCCATGTTCACTTG	56/1.0	343	*MnlI*
5-IR	intron 5	GCTCCTGGTCACACTGCAAG			
6-IF	intron 5	CAAGTTTTACACACTGAGTGC	55/1.0	213	*AluI*
6-IR	intron 6	ATTTTCCCCAGAGAAAAGGCTA			
7-IF	intron 6	CGGGAAGGGAGGTGCTGA	58/1.0	233	*RsaI*[c]
7-IR	intron 7	TCGCAGGCCACAGAGGAGAAG			
8-IF	intron 7	ATCACAGCGTGAGTACGGGGAG	55/1.0	288	RsaI; *PstI*[c]
8-IR	intron 8	CAGCACCCACAGCAGATTG			
9-IF	intron 8	CCTAGAAAGCCATGAGATTAA	55/1.5	214	*Tru11; StyI*[c]
9-IR	intron 9	GACCAGACTGAGAAATTCTAG			
10-IF	intron 9	GGCACCATGCACATGCGTG	58/1.0	235	*AluI*
10-IR	intron 10	AAGCCAAGCATCCTACTTCC			
11-IF	intron 10	GGACTGATGGTGGCTTTATG	58/1.0	266	*BsuRI*
11-IR	intron 11	AGCAGACCAGCACCTCCTC			
12-IF	intron 11	TGAGGGATGTGTTCATCTAG	55/1.0	183	-
12-IR	intron 12	GCCCATGGTGTTGGCTCTTG			
13-IF	intron 12	CATGCTTGGGACATGTCCAC	64/1.0	209	*MvaI*
13-IR	intron 13	TTCATAACACCTCTGAGCTAC			
14-IF	intron 13	CTCTGCAGCCACAGCCCTTC	50/2.0	229	*AvaII*
14-IR	intron 14	GTGCTCAGGGACCACTAG			
15-IF	intron 14	CCTGCTCACGATTCTCTTTC	58/1.0	244	*HphI*
15-IR	intron 15	AAGCCGAAGACTGGGAGC			
16-IF	intron 15	GATCGGTCCCTTGTTGCA	52/2.0[b]	347	*AluI*
16-ER	Exon 16	CACAGCTGAACAGACAAACTT			

[a]See EMBL accession numbers AJ426068-AJ426078 [b]with 5% formamide [c]with RLFP analysis

each NTP, 0.4 mM of each primer and varying concentrations of $MgCl_2$ [Table 3]). For SSCP analysis, 0.06 µl of [α^{32}P] dCTP (10 mCi/ml) was included in the PCR. Parts of the λ phage inserts were amplified in a one step PCR (annealing temperatures in Table 3). For genomic mutation analysis PCR conditions included initial denaturation (5 min at 95°C), the 10 initial cycles 1°C above the annealing temperature (Table 3), 22–25 cycles of 95°C (30 s), annealing temperature (30 s), elongation at 72°C (40 s) and a final elongation step at 72°C (3 min).

Cloning and identification of exon/intron junctions

Clones containing the *SAG* gene were isolated from a genomic canine λ-DNA library (λ FIX®II Library; host: *E. coli* XL1-Blu MRA (P2) Stratagene, La Jolla, Ca, USA) according the manufacturer's protocol. Recombinant λ DNA was fixed to Hybond™-N Nylon membranes (Amersham, Buckinghamshire, UK) and UV-crosslinked (1' 70 J/cm^2). The library was screened with probes prepared from PCR products corresponding to portions of exons 2, 5 and 16 (nucleotides 909–1157 of EMBL accession number CFA426068, nucleotides 395–588, and 1333–1579 of EMBL accession number X98460, respectively). These probes were labelled using [α^{32}P] dATP and the Megaprime Labelling System (Amersham, Buckinghamshire, UK). Hybridisations were performed at 65°C in 0.5 M sodium phosphate buffer (pH 7.2)/7% sodium dodecyl sulfate [29]. After hybridisation the filters were washed twice for 30 min each in 2× SSC/1% SDS, once for 15 min with 0.2× SSC/1%SDS at 65°C and for 30 min with 6×SSC at

Table 4: *SAG* sequence variations and amino acid exchanges in respective dog breeds

Location	Sequence variation*	Amino acid exchange	Sequence variations in breed(s)[a] In affected and gPRA unaffected dogs	gPRA affected dogs in heterozygous state
Intron 1	IVS1+393T→C	-	BDP, BS, GR, IRS, Ro[b], TT	BDP, GR,
Exon 2	UTR-5A→G	-	Bo, BS, GR[c], BDP, IRS, SD[c], SI, TT[b]	Bo, GR, SD
	c255A→G	H14C	SI	SI
Exon 5	c526C→T	A111V	Sa[c]	Sa
	c531G→A	A113T	Sa[c]	Sa
Intron 5	IVS5-30C→T	-	ECS, MP, Ro[b], Sa[c]	ECS, MP, Sa
Exon 6	c610C→T	C132C	GR	GR
Intron 7	IVS7+10C→T	-	IRS[b], GR, SP[c]	D, IRS, GR
	IVS7+52Ins.G	-	GR[c]	GR
	IVS7-4Del.G	-	CCR[b] D, GR, IRS, MP, SD[c], SI[c], SP[b], TT[c]	D, GR, IRS, SD
Exon 8	c811G→A	Q199Q	GR	GR
Intron 8	IVS8+8A→G	-	D, ECS[c], GR[c], LR[c], Sa	D, ECS, GR, Sa
Exon 9	c874T→C	H225H	Bo, BDP, CCR[b], ECS, D, GR[c], IRS, MP, NF, Ro[b], Sa, SI[b], TT[bc]	Bo, ECS, D, GR, IRS, MP, NF, Sa
Exon 10	c949G→T	V247V	IRS, GR	GR
	c983G→T	D259T	AC[b]	AC
	c1000A→G	P264P	AC, LR, IRS[b], GR	GR
Intron 10	IVS10-18G→C	-	GR, IRS, BDP	GR
	IVS10-33C→T	-	GR, IRS	GR
Exon 11	c1063G→A	T288T	BS, BDP, GR, IRS[b], Sa	BS, GR, Sa
	c1076C→T	L293L	BS, BDP, GR[c], Sa, TT	BS, Sa
Intron 11	IVS11-51Ins.TT	-	Sa, IRS[b]	Sa
Intron 13	IVS13+66C→G	-	Sa, IRS[b]	Sa
Intron 14	IVS14-45Del.A	-	PON	PON
Intron 15	IVS15+14C→T	-	Sa	-
	IVS15+86T→A	-	Sa	-
	IVS15-45Ins.C	-	PON, AW[bc], SP[b]	PON
Exon 16	c1344C→A	A379E	IRS[b], ECS[b], GR[c], PON, Sa[bc], TT[b]	ECS, GR

[a] For abbreviations see Table 1 [b] Homozygous state in gPRA affected dogs [c] Homozygous state in unaffected dogs *(EMBL accession number for cDNA: X98460; EMBL accession numbers for genomic DNA: AJ426068-AJ426078)

room temperature. The filters were exposed to phosphoimager screens (STORM 860) and evaluated with the programs STORM Scanner Control and Image Quant (Molecular Dynamics). Hybridising clones were isolated and plaque purified as described [30]. The approximate insert sizes of the different clones were estimated with exon primers via PCR (see conditions described above using~0.2 ng phage DNA, 2 mM $MgCl_2$ and annealing temperature of 54°C in the PCR).

Exon/intron boundaries were analysed by comparison of canine mRNA ([17], EMBL accession number X98460) with 16 genomic sequences of the human *SAG* gene (5'-flanking region and exon 1 [18]; EMBL accession number X12453); exons 2–16 ([31]; EMBL accession numbers U70963-U70976) using the program Blast Search (NCBI [http://www.ncbi.nlm.nih.gov/blast]). Intronic sizes were estimated by overlapping PCR including parts of neighbouring exons. PCR products were extracted from 1.5% agarose gels using the Easy Pure extraction kit (Biozym, Germany) and sequenced with intron-overlapping primers (Table 2). Sequencing reactions from 2–3 clones were carried out by the dideoxy-chain termination method using the BDT (Perkin-Elmer, Norwalk, CT) according to the manufacturer's instructions. All sequencing reactions were run on an automated DNA sequencer (Applied Biosystems 373 XL, Foster City, USA) and analysed using ABI Prism™ 373XL.

PCR-SSCP and DNA sequence analysis

Positions of intronic primers which were used for mutation screening were selected after DNA sequence analysis of the genomic *SAG* clones (Table 3). SSCP samples were treated as previously described [16,32]. PCR products were digested dependent on the lengths of the fragments [33] with different restriction enzymes (Table 3). Using restriction length fragment polymorphism (RLFP) analysis the sequence variants in exon 2 (*Nla*III), intron 7 (*Rsa*I),

exon 8 (*Pst*I) and exon 9 (*Sty*I) were investigated. 3 μl of the PCRs were denatured with 7 μl of loading buffer (95% deionised formamide 10 mM NaOH, 20 mM EDTA, 0.06% (w/v) xylene cyanol, and 0.06% (w/v) bromophenol blue). The samples were heated to 95°C for 5 min and snap cooled on ice. 3 μl aliquots of the single-stranded fragments were separated in 2 sets of 6% polyacrylamide (acrylamide/bisacrylamide: 19/1) gels, one set containing 10% glycerol, another containing 5% glycerol and 1 M urea. Gels were run with 1× TBE buffer at 50–55 W for 4–6 h at 4°C. All gels were dried and subjected to autoradiography over night. Selected DNA samples with band shifts evidenced by SSCP electrophoresis were purified and cycle sequenced as described above.

Authors' contributions

Author 1 carried out the molecular genetic analyses, sequence alignments and drafted the manuscript during her predoctoral studies supervised by the second senior author.

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References

1. Clements PJM, Sargan DR, Gould SM, Petersen-Jones SM: **Recent advances in understanding the spectrum of canine generalised progressive retinal atrophy.** *J Small Anim Pract* 1996, **37:**155-62
2. Petersen-Jones SM: **A Review of research to elucidate the cause of the generalized progressive retinal atrophies.** *Vet J* 1998, **155:**5-18
3. Farrar GJ, Jordan SA, Kumar-Singh R, Inglehearn CF, Gal A, Greggory C: **Extensive genetic heterogenety in autosomal dominant retinitis pigmentosa.** *In: Retinal degeneration (Edited by: Hollyfield JG, Anderson RE, LaVail MM) New York Plenum Press* 1993, 63-77
4. Kajiwara K, Berson EL, Dryja TP: **Digenic retinitis pigmentosa due to mutations at the unlinked peripherin/RDS and ROM1 loci.** *Science* 1994, **264:**1604-1607
5. Dryja TP, Li T: **Molecular genetics of retinitis pigmentosa.** *Hum Mol Genet* 1995, **4:**1739-1743
6. Shastry BS: **Signal transduction in the retina and inherited retinopathies.** *Cell Mol Lif Sci* 1997, **53:**419-429
7. Manserg FC, Millington-Ward S, Kennan A, Kiang AS, Humphries M, Farrar GJ, Humphries P, Kenna PF: **Retinitis pigmentosa and progressive sensorineural hearing loss caused by a C12258A mutation in the mitochondrial *MTTS2* gene.** *Am J Hum Genet* 1999, **64:**971-985
8. Suber ML, Pittler SJ, Qin N, Wleft GC, Holcombe V, Lee RH, Craft CM, Lolley RN, Baehr W: **Irish setter dogs affected with rod/cone dysplasia contain a nonsense mutation in the rod *cGMP* Phosphodiesterase beta-subunit gene.** *Proc Natl Acad Sci USA* 1993, **90:**3968-3972
9. Dekomien G, Runte M, Gödde R, Epplen JT: **Generalised progressive retinal atrophy of Sloughi dogs is due to an 8-bp insertion in exon 21 of the *PDE6B* gene.** *Cytogenet Cell Genet* 2000, **90:**261-267
10. Petersen-Jones SM, Entz DD, Sargan DR: **cGMP phosphodiesterase-α mutation causes progressive retinal atrophy in the cardigan welsh corgi dog.** *Invest Ophthalmol Vis Sci* 1999, **40:**1637-1644
11. Zhang Q, Acland GM, Parshall CJ, Haskell J, Ray K, Aguirre GD: **Characterization of canine photoreceptor *Phosducin* cDNA and identification of a sequence variant in dogs with photoreceptor dysplasia.** *Exp Eye Res* 1998, **67:**473-480
12. Aguirre G: **Genes and diseases in man and models** *Prog Brain Res* 2001, **131:**663-678
13. Kijas JW, Cideciyan AV, Aleman TS, Pianta MJ, Pearce-Kelling SE, Miller BJ, Jacobson SG, Aguirre GD, Acland GM: **Naturally occurring rhodopsin mutation in the dog causes retinal dysfunction and degeneration mimicking human dominant retinitis pigmentosa.** *Proc Natl Acad Sci* 2002, **99:**6328-33
14. Gould DJ, Petersen-Jones SM, Sohal A, Barnett KC, Sargan DR: **Investigation of the role of opsin gene polymorphism in generalized progressive retinal atrophies in dogs.** *Anim Genet* 1995, **26:**261-267
15. Runte M, Dekomien G, Epplen JT: **Evaluation of *RDS*/ Peripherin and *ROM1* as candidate genes in generalised progressive retinal atrophy and exclusion of digenic inheritance.** *Anim Genet* 2000, **31:**223-227
16. Dekomien G, Klein W, Epplen JT: **Polymorphisms in the canine rod *transducin* gene and exclusion as cause for generalised progressive retinal atrophy (gPRA).** *J. Exp. Anim. Sci.* 1998, **39:**86-90
17. Veske A, Narfstrom K, Finckh U, Sargan DR, Nilsson SE, Gal A: **Isolation of canine retinal arrestin cDNA and exclusion of three candidate genes for Swedish Briard retinal dystrophy.** *Curr Eye Res* 1997, **16:**270-274
18. Yamaki K, Tsuda M, Kikuchi T, Chen KH, Huang KP, Shinohara T: **Structural organization of the human S-antigen gene: cDNA, amino acid, intron, exon, promoter, in vitro transcription, retina, and pineal gland.** *J Biol Chem* 1990, **265:**20757-20762
19. Wilson CJ, Applebury ML: **Arresting G-protein coupled receptor activity.** *Curr. Biol* 1993, **3:**683-686
20. Hofmann KP, Jäger S, Ernst OP: **Structure and function of activated rhodopsin.** *Isr J Chem* 1995, **35:**339-355
21. Alloway PG, Howard L, Dolph PJ: **The formation of stable rhodopsin-arrestin complexes induces apoptosis and photoreceptor cell degeneration.** *Neuro* 2000, **28:**129-138
22. Fuchs S, Nakazawa M, Maw M, Tamai M, Oguchi Y, Gal A: **A homozygous 1-base pair deletion in the arrestin gene is a frequent cause of Oguchi disease in Japanese.** *Nat Genet* 1995, **10:**360-362
23. Sippel KC, DeStefano JD, Berson EL, Dryja TP: **Evaluation of the human arrestin gene in patients with retinitis pigmentosa and stationary night blindness.** *Invest Ophthalmol Vis Sci* 1998, **39:**665-670
24. Nakazawa M, Wada Y, Tamai M: **Arrestin gene mutations in autosomal recessive retinitis pigmentosa.** *Arch Ophthalmol* 1998, **116:**498-501
25. Bentolila S, Bach JM, Kessler JI, Bordelais C, Weissenbach J, Panthier J: **Analysis of major repetitive DNA sequences in the dog (*canis familiaris*) genome.** *Mamm Genome* 1999, **10:**699-705
26. Yang F, O'Brien PC, Milne BS, Graphodatsky AS, Solanky N, Trifonov V, Rens W, Sargan D, Ferguson-Smith MA: **A complete comparative chromosome map for the dog, red fox, and human and its integration with canine genetic maps.** *Genomics* 1999, **62:**189-202
27. Vishnivetskiy SA, Paz CL, Schubert C, Hirsch JA, Sigler PB, Gurevich VV: **How does arrestin respond to the phosphorylated state of rhodopsin?** *J Biol Chem* 1999, **274:**11451-11454
28. Miller SA, Dykes DD, Polesky HF: **A simple salting out procedure for extracting DNA from human nucleated cells.** *Nucleic Acids Res* 1988, **16:**1215
29. Church GM, Gilbert W: **Genomic sequencing.** *Proc Natl Acad Sci USA* 1984, **81:**1991-1995
30. Sambrook J, Fritsch EF, Maniatis : *Molecular cloning: A laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press* 1989
31. Yamamoto S, Sippel KC, Berson EL, Dryja TP: **Defects in the rhodopsin kinase gene in the Oguchi form of stationary night blindness.** *Nat Genet* 1997, **15:**175-178
32. Dekomien G, Epplen JT: **Exclusion of the *PDE6A* gene for generalised progressive retinal atrophy in 11 breeds of dogs.** *Anim Genet* 2000, **31:**135-139
33. Jäckel S, Epplen JT, Kauth M, Miterski B, Tschentscher F, Epplen C: **PCR-SSCP or how to detect reliably and efficiently each sequence variation in many samples and many genes.** *Electrophoresis* 1998, **19:**3055-3061

34. Laratta LJ, Sims MH, Brooks DE: **Progressive retinal degeneration in the Australian cattle dog.** *Proc Am Coll Vet Ophthal* 1988, **19:**9
35. Spiess BM: **Inherited ocular diseases in the Entlebucher Mountain Dog.** *Schweiz Arch Tierheilk* 1994, **136:**105-110

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