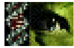
Molecular Vision **2002; 8:436-41** <http://www.molvis.org/molvis/v8/a52>
Received 20 June 2002 | Accepted 13 November 2002 | Published 15 November 2002

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The canine Recoverin (*RCV*1) gene: a candidate gene for generalized progressive retinal atrophy

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Purpose: We describe the cloning, sequence, and mutation analysis of the canine Recoverin (*RCV*1) gene, a candidate gene for generalized progressive retinal atrophy (PRA).
Methods: The gene was isolated from a genomic λ Fix II library using an exon 1 probe of the human *RCV*1 cDNA. Canine *RCV*1 sequences were identified by subcloning, polymerase chain reaction (PCR), and sequence analysis. Furthermore, selected DNA samples of 22 dog breeds (including all PRA-affected and several representative unaffected dogs from the pedigrees) were screened for mutations and polymorphisms using PCR-SSCP (single strand conformation polymorphism) and sequence analysis.
Results: The canine *RCV*1 gene revealed 3 exons and an open reading frame of 606 bp, potentially coding for a protein of 202 amino acids. The deduced amino acid sequence of the canine *RCV*1 gene shares 89% identity with the homologous human, 94% with bovine, and 91% identity with the mouse genes. The protein sequence reveals two typical Ca^{2+}-binding EF-hand motifs. In the ORF (open reading frame) of the *RCV*1 gene a C272A (exon 1) and a C4275A transversion (exon 3) were discovered. These exchanges result in amino acid substitutions (N3K and P202H), but they do not segregate with PRA in the breeds investigated. Additionally, two sequence variations were identified in the 5'-UTR, one in intron 1 and thirteen variations in intron 2 as well as one in the 3'-UTR.
Conclusions: Using intragenic polymorphisms, we excluded the *RCV*1 gene as a candidate gene for autosomal recessively transmitted (ar) PRA in 16 dog breeds. In addition the *RCV*1 gene was excluded for presumedly autosomal dominant (ad) PRA in 8 out of the 22 dog breeds investigated.

In most cases, progressive retinal atrophy (PRA) represents a genetically heterogeneous disorder [1] in dogs which in most forms is inherited as an autosomal recessive (ar) trait. Like the homologous disease retinitis pigmentosa (RP) in man, PRA is characterised by degeneration of the peripheral retina leading to night blindness and loss of visual fields. The age of onset and the progression rate varies in different dog breeds, but typically the disease progresses to complete blindness [1,2]. RP is characterised by different inheritance modes, autosomal dominant (ad), ar, X-linked, and digenic, as well as maternal [3-7]. Causal mutations for ar PRA have been identified in three genes affecting five different dog breeds: the β-subunit of the *cGMP-specific phosphodiesterase* (*PDE6B*) gene in Irish Setters [8] and Sloughis [9], the α-subunit of the *cGMP-specific phosphodiesterase* (*PDE6A*) gene in Cardigan Welsh Corgis [10]. In the X-linked form (XLPRA), the *RPGR* gene is mutated in Samoyed and Siberian huskies [11]. An ad transmitted retinal dysfunction is known from Mastiffs caused from an *opsin* mutation [12]. In cats, an as-yet unknown ad-transmitted mutation leads to early-onset retinal dystrophy [13].

Recoverin (RCV) is a retinal calcium-binding protein. Together with Rhodopsin Kinase (RHOK), RCV forms a complex in the presence of Ca^{2+}. The specific and Ca^{2+} dependent RCV/RHOK interaction is necessary for the inhibitory effect of RCV on rhodopsin (RHO) phosphorylation. This effect may be important for photoreceptor light adaption [14]. The Ca^{2+}-bound form of recoverin prolongs the photoresponse, presumably by blocking phosphorylation of photoexcited RHO. RCV contains a covalently attached myristoyl or related fatty acyl group at its N-terminus and at least two Ca^{2+}-binding sites as well as an EF-hand motif (a name is derived from the E and F helices and the intervening loop [15]). In man, the retinal *RCV*1 gene is localized on chromosome 17p13.1, a region where ad and ar forms of RP have been mapped (RetNet; [16,17]). As a possible member of the light transduction pathway [14], recoverin was analysed as a candidate for ar RP in 42 Spanish families and excluded as cause of ar RP in 38 pedigrees [18]. In addition, the human *RCV*1 gene was investigated in 596 patients with RP or related diseases and was excluded as a cause for these disorders [19]. In the phototransduction cascade RCV is involved in the inactivation of activated RHO. Therefore, loss of function of RCV possibly disturbs the function of RHO. Mutations in RHO lead to ad and ar transmitted RP [20]. Consequently, inactivated RCV may abolish or hamper the function of RHO thus causing ar or ad PRA in dog breeds. In order to investigate its role in PRA, we cloned the *RCV*1 gene and investigated the complete sequence with PCR-SSCP analysis in 22 dog breeds (Table 1).

METHODS

Blood of 805 dogs from 22 different breeds including 112 PRA-affected animals (Table 1) was received from the own-

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ers in cooperation with the breed clubs [Verband für das Deutsche Hundewesen (VDH); Nederlandse Vereiniging van Saarlooswolfhonden; Schweizer Kynologischen Gesellschaft (SKG)]. The blood of most dogs was sent from different regions of Germany. In addition, several Saarloos/Wolfhounds (Sa), Schapendoes (SD), Sloughis (Sl) and Tibetan Terriers (TT) originated from the Netherlands (Sa, SD), Switzerland (Sl, TT), Sweden (Sl) and USA (Sl). By observing the cases of PRA in the pedigrees, the breeders have assumed ar inheritance in the following breeds: Australian Cattle dogs, Scottish Collies, Wire-haired Dachshunds, Engl. Cocker Spaniels, Entlebucher Mountain Dogs, Irish Setters, Labrador Retrievers, Miniature Poodles, Saarloos/Wolfshounds, Salukis, Schapendoes, Sloughis and Tibetan Terriers. Experienced veterinarians confirmed the PRA status of affected and unaffected dogs by ophthalmoscopy which is documented in certificates of the eye examinations.

Genomic DNA was extracted from peripheral blood according to standard protocols [21]. Sequences of the *RCV*1 gene were amplified (Table 2) by PCR in a thermocycler (Biometra, Goettingen, Germany) from the inserts of the λ phages and genomic DNA for each dog. 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 each dNTP, 0.4 mM of each primer and varying concentrations of $MgCl_2$ (Table 2). For SSCP analysis, 0.06 μl of [α^{32}P] dCTP (10 mCi/ml) was included in the PCR. Parts of the inserts of the λ phage were amplified with a one step PCR; for annealing temperatures see Table 2. A "touchdown" PCR procedure was applied: initial denaturation step (5 min at 95 °C), two initial cycles 6 °C and 3 °C above the annealing temperature (Table 2), 25 cycles of 95 °C (30 s), annealing temperature (30 s), elongation at 72 °C (30 s) and a final elongation step at 72 °C (3 min).

The *RCV*1 gene was cloned 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 Stratagene standard protocol. Recombinant λ DNA was fixed of Hybond™-N Nylon membranes (Amersham, Buckinghamshire, UK) and UV-crosslinked (1' 70 mJ/cm^2). The library was screened with PCR product of a probe from exon 1 corresponding to positions 22-381 of the human mRNA of the *RCV*1 gene (EMBL accession number AB001838). This probe was labelled using [α^{32}P] dATP (10 mCi/ml) and the Megaprime Labelling System (Amersham, Buckinghamshire, UK). Hybridizations were performed at 65°C in 0.5 M sodium phosphate buffer pH 7.2/7% sodium dodecyl sulfate [22]. After hybridization the filters were washed twice for 30 min each in 2X SSC/1% SDS, once for 15 min with 0.2X SSC/1%SDS at 65 °C and for 30 min with 6X SSC at room temperature. The filters were exposed to phosphoimager screens (STORM 860) and evaluated with the programs STORM Scanner Control and Image Quant (Molecular Dynamics Sunnyvale, CA). Hybridising clones were isolated and plaque purified as described [23].

Exon/intron boundaries were analysed by comparison with the mRNA sequence of the human *RCV*1 gene ([24]; EMBL accession number AB001838) of the genomic clone of chromosome 17 (EMBL accession number AC005747) using the program BLAST Search (NCBI). Optimized positions of primers were identified via comparison of sequences from mouse ([25]; EMBL accession number X66196), bovine ([26]; EMBL accession number M95858) and man (EMBL accession number AB001838). Intronic sequences were characterised by overlapping PCRs including neighbouring exons (see conditions described above using 0.2 ng phage DNA). Amplified fragments were extracted from 0.8% agarose gels using the Easy Pure extraction kit (Biozym, Germany) and sequenced with intron-overlapping primers (Table 2). Then canine primer sequences for intron 1 and intron 2 were created to amplify the complete intron (Table 2). The isolated λ clones were digested with *Hind*III and subcloned in pBluescript II+ phagemid (Stratagene, La Jolla, CA) to identify the 5' untranslated regions (5'-UTR) of the gene [27]. The 3'-UTR of the *RCV*1 gene was characterised by nested PCR using the T3 primer sequence of the λ FIXII vector. Sequencing reactions from 2-3 clones were carried out by the dideoxy-chain termination method using the BDT (Perkin-Elmer, Norwalk, CT) accord-

Table 1. Characteristics of 22 dog breeds

Breed (abbreviation)	Number of dogs	Diagnosis	Onset forms of PRA	Age distribution (year)
Australian Cattle dog (AC)	2	PRA-affected	late	10
	19	normal		2-4
Berger des Pyrénées	1	PRA-affected	mid-onset	5
(Pyrenean Shepherd; BDP)	42	normal		1-10
Bichon Bolognese (Bo)	1	PRA-affected	late	9
Curly Coated Retriever (CCR)	1	PRA affected	mid-onset	6
Scottish Collie (Co)	3	PRA-affected	early	4-8
	1	normal		8
Wire-haired Dachshund (D)	20	PRA-affected	variable	1-13
	49	normal		6-13
Engl. Cocker Spaniel (ECS)	6	PRA-affected	late	3-11
	6	normal		6-14
Entlebucher Mountain Dog (EM)	17	PRA-affected	late	5-13
	10	normal		1-7
Giant Schnauzer (GS)	1	PRA affected	late	11
Golden Retriever (GR)	2	PRA-affected	late	5-10
	8	normal		3-6
Irish Setter (IRS)	2	PRA-affected	early/late	0.6-7
	1	normal		3-13
Labrador Retriever (LR)	5	PRA-affected	late	8-12
	140	normal		3-13
Miniature Poodle (MP)	28	PRA-affected	late	5-12
	15	normal		1-12
Newfoundland (NF)	1	PRA-affected	mid-onset	3
Polish Lowland Sheepdog (PON)	1	PRA-affected	late	9
Rottweiler (Ro)	1	PRA-affected	late	3
Saarloos/Wolfhound (Sa)	7	PRA-affected	late	7-9
	118	normal		2-10
Saluki (Persian Greyhound; Sal)	1	PRA-affected	late	8
	1	normal		8
Scottish Terrier (ScT)	1	PRA-affected	late	6
Schapendoes	3	PRA-affected	early	2-6
(Dutch Sheepdog; SD)	7	normal		3-6
Sloughi (Arabian Greyhound; Sl)	5	PRA-affected	mid-onset	2
	183	normal		0.1-12
Tibetan Terrier (TT)	3	PRA-affected	mid-onset	7-8
	93	normal		2-10

From the total number of 805 animals all 112 PRA-affected and many representative unaffected dogs from the pedigrees were investigated. Characteristics included age of onset forms and age distribution of normal and PRA affected dogs during the time of DNA isolation of the RCV1 gene in 22 breeds. The ages of onset were classified according to [33] for AC; according to the owners' report/certificate of eye examination for BDP, Bo, CCR, D, GR, GS, IRS (late form), NF, PON, Ro, Sa, ScT, SD and Sl; according to reviews [1,34] and online information from PRA Today for Co, ECS, IRS (early form) LR, MP and TT; and according to [35] for EM.

ing to the manufacturer's instructions to identify the intronic sequences. All sequencing reactions were run on an automated DNA sequencer (Applied Biosystems 373 XL, Foster City, USA) and analysed using the ABI Prism™ 373XL Collection and Sequencing Analysis 3.0 software. Additional programs used included BLAST Search, ProDom NCBI-BLASTP2, RetNet, and the Transcription Element Search System (TESS), as well as NNPP/Eukaryotic.

Positions of primers which were used for mutation screening were designed after DNA sequence analysis of the *RCV*1 clones (Table 2). SSCP samples were treated according to [28,29]. PCR products were digested dependent on the lengths of the fragments with different restriction enzymes (Table 2) to optimise the mutation screening by SSCP analysis [30]. 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 through two 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 1X TBE buffer at 50-55 W for 4-6 h at 4°C. All gels were dried and subjected to autoradiography over night. For haplotype determination selected DNA samples with band shifts evidenced by SSCP electrophoresis were purified and cycle sequenced as described above.

RESULTS & DISCUSSION

In the presence of Ca^{2+}, RCV operates together with RHOK by interacting with RHO in the phototransduction cascade. Mutations in the *RCV*1 gene could therefore cause the failure of RHO, possibly leading to retinal disease. In this context we investigated the *RCV*1 gene as a candidate gene for gPRA. A canine genomic library was screened with a human exon 1 DNA probe in order to identify the canine *RCV*1 gene. Three λ clones were isolated containing the gene with incomplete 5'- and 3'-UTRs. After alignment of mRNA sequences of human, mouse and bovine retinal *RCV*1 genes, we positioned primers according to conserved parts of the gene and amplified the canine gene by PCR using hybridising clones as templates. After sequencing of the overlapping PCR products, we designed intronic primers to identify the lengths of the two introns (EMBL accession number AJ414401; Table 2). Exon/intron boundaries were aligned with the mRNA of the human *RCV*1 gene against the genomic clone of chromosome 17 using NCBI BLAST Search. The canine intron/exon organization corresponds to that of the human gene Table 3. The canine gene is 4523 bp long and exon 1 comprises 644 bp (UTR 263 bp, ORF 381 bp), intron 1 951 bp, exon 2 112 bp, intron 2 2456 bp and exon 3 360 bp (ORF 116 bp, UTR 244 bp). The canine *RCV*1 gene is shorter than its human counterpart. This difference is caused primarily by smaller introns. Two EF-hand type motifs were identified by searching with the pro-

Table 2. Primers and conditions for PCR amplification

PCR-system	Primer sequence (5'->3')*	Location	PCR Fragment length (bp)	Restriction enzymes for SSCP analysis	Annealing T/$MgCl_2$ (°C/mM)
H 1F	CCCTGTCCAAGGAGATCCT	E 1; 13	359	-	47/2**
H 1R	CATGACGATCTCCAGCACTT	E 1; 381			
I 1-331EF	TCTCGCTCTACGACGTGGA	E 1; 580	837	-	54/2
I 1-IR	GACAGGGAACGGAGTGAAG	I 1; 1419			
H 1F	CCCTGTCCAAGGAGATCCT	E 1; 13	1422	-	46/2**
H 2R	CTTTCCAAAGTACTTCCAGATC	E 2; 483			
H 2F	TTCAAAATGATCACTCCCGAGG	E 2; 388	2684	-	48/2**
H 3R	TCATCTTTTCCTTCACTTTTTGAG	E 3; 592			
I 2F	ACCGCCGAGCTCCCTGG	I 2; 2006	1774	-	54/2**
I 2R	ATGCGGAGACACTCCCACG	I 2; 3777			
I/M 2 2F	TGAGAACTGGCCTTCGAGG	I 2; 2398	1020: 208, 98, 160,	DdeI	52/1
I/M 2 2R	CGTGACTGTGGCATTCACC	I 2; 3417	13, 188, 276, 77		
I/M 2Ht 2F	GGGAGGCATTCCAGAACAG	I 2; 2658	760: 30, 160, 13,	DdeI	54/1
I/M 2 2R	CGTGACTGTGGCATTCACC	I 2; 3417	188, 276, 77		
M 5'-UTR1F	CTCCCTGAAGGCCAAGATG	UTR/E 1; 1	372: 184, 132,	PstI	55/1
M/Ht Kl1ER	TTCGGAAGGAAACTGGTACC	E 1; 374	56		
H/M 1F	CCCTGTCCAAGGAGATCCT	E 1; 13	424: 183, 71,	NlaIII	54/1
M 1-IR	GAACCCCCTGGACCCAGGA	I 1; 709	145, 65		
M 2-IF	GGAATCTGTATTTTCACCTCTG	I 1; 1483	308: 136,	DdeI	55/1
M 2-IR	CTAGGAAGACCAATCTCACT	I 2; 1790	172		
M 3-IF	GTGATGGGTTGTACCCTCAG	I 2; 4019	340: 206,	MboI	55/1
M UTR 3'R	GCATGTGCACGTGCTCACG	3'-UTR; 4358	134		

PCR systems to characterize the canine *RCV*1 gene including mutation screening. Systems designated by H: PCR primer sequences selected from the human mRNA (EMBL accession number AB001838) for the identification of exon/intron boundaries and canine gene sequences. Systems designated by I: PCR primers (on the basis of the canine *RCV*1 gene EMBL accession number AJ414401) to amplify the complete intron. Systems designated by M: PCR primers (on the basis of the canine *RCV*1 gene) for mutation analysis. Ht: Additional PCR primers used for haplotype analysis. *Nucleotide position to which the 5' end of the primer (red type) hybridizes. ** Addition of 5% DMSO.

gram ProDom NCBI-BLASTP2 in the 202 aa deduced from the ORF of the canine *RCV*1 gene. Highest sequence homologies were observed with the bovine amino acid sequence: 95% in aa positions 26-95 and in aa 143-191. In intron 1 we identified a short sequence (position 1103 bp-1180 bp) corresponding to a canine tRNA-derived short interspersed nucleotide element (SINE; [31]). The human *RCV*1 gene maps to chromosome 17p13.1. On the basis of reciprocal chromosome painting [32], the canine *RCV*1 gene is therefore predicted to map to CFA 5, the homologous chromosomal region in dogs.

After isolation and characterisation of the canine *RCV*1 gene, we investigated the gene for disease causing mutations in PRA affected and unaffected dogs. For PCR-SSCP analysis primers were designed flanking the exons including the conserved splice sites and part of intron 2 (Table 2). DNAs of

Table 3. Exon/intron organization of the canine *RCV*1 gene

Exon	Exon length (bp)	Exon nucleotide position*	Splice sites**	Intron	Intron length (bp)
1	644	1	ATCGTCATGgtcagtctcg	1	951
2	112	1596	tgtgcctgcagGCTATTTTC		
		1707	AAAGATGATGgtgaattcc	2	2456
3	360	4163	ccatgcacagATAAACTT		

The exon/intron boundaries followed the gt-ag rule. Exon sequences are depicted in upper case, intron sequences in lower case.

Table 4. *RVC1* sequence variations

Location	Sequence variation	Amino acid exchange	Breeds*
5'-UTR	A127G	-	Bo, D, EM, MP, NF, GS, Sa, ScT, SD, TT
	C229T	-	Bo, D, IRS, NF, MP, ScT, Sl
Exon 1	C272A	N3K	D, ScT, Sl
Intron 1	C1552T	-	TT
Intron 2	C2735T	-	D, Co, GS, LR, NF, SD, Sl
	G2774A	-	D, Co, GS, LR, NF, SD, Sl
	C2814T	-	D, Co, GS, LR, NF, SD, Sl
	C2843T	-	D, Co, GS, LR, NF, SD, Sl
	G2940C	-	D, GS, NF, SD, Sl
	C2950T	-	CCR, D, IRS, LR, MP, NF, Sa, Sl, TT
	C2958A	-	MP
	G3010A	-	D, Sa
	A3102G	-	Bo, Co, GS, D, LR, MP, Sa, Sal, ScT, TT
	G3225A	-	Sa
	G3325A	-	NF
	T3370C	-	D, LR, Sa
	C3370G	-	Bo, IRS, MP, Sa, Sal, ScT
Exon 3	C4275A	P202H	Bo, D, GS, SD, Sl
3'-UTR	A4309G	-	Bo, D, ES, LR, Sa, Sal, SD, Sl

Identified polymorphisms in heterozygous patterns in PRA-affected dogs, used for exclusion of the *RVC*1 gene as cause for ar PRA in these dog breeds. *For abbreviations see Table 1.

Table 5. Haplotypes of the *RVC*1 gene in PRA-affected dogs in 11 dog breeds

	5'-UTR Exon 1			Intron 1	Intron 2											Exon 3 3' UTR	
Breed	A127G	C229T	C272A	C1552T	C2735T	G2774A	C2814T	C2843T	C2940G	C2950T	C2958A	G3010A	A3102G	G3225A	T3370C C3370G	C4275A	A4309G
Entlebucher Mountain Dog	G	C	C	C	C	G	C	C	C	C	C	G	A	G	T	C	G
	A	C	C	C	C	G	C	C	C	C	C	G	A	G	T	C	A
	A or G	C or T	C	C	C	G	C	C	C	C	C	G	A	G	T	C	A or G
Irish Setter	G	C	C	C	C or T	G or A	C or T	C or T	C	C or T	C	A	A	G	C or G	C	A
	G	T	C	C	C	G	C	C	C	C	C	G	G	G	C	C	G
Labrador Retriever	G	C	C	C	T	A	T	T	C	C	C	G	A	G	T	C	A
	G	C	C	C	T	A	T	T	C	T	C	G	A	G	T	C	A
	A	C	C	C	C	G	C	C	C	C	C	G	A	G	T	C	A
	A	C	C	C	C or T	G or A	C or T	C or T	C	C or T	C	G	A	G	T or C	C	A
Saarloos/Wolfhound	A or G	C	C	C	C	G	C	C	C	C or T	C	G	A or G	G or A	T or C	C	A or G
	A	C	C	C	C	G	C	C	C	C or T	C	G or A	A	G	T	C	A
	A	C	C	C	C	G	C	C	C	C	C	G	A	G	T or C	C	A or G
	A or G	C	C	C	C	G	C	C	C or G	C or T	C	G	A or G	G or A	C or G	C	A or G
Schapendoes (Dutch Sheepdog)	A	C	C	C	C	G	C	C	C	C	C	G	A	G	T	C	A
	A	C	C	C	C	G	C	C	C	C	C	G	G	G	T	C	A
	A or G	C	C	C	C or T	G or A	C or T	C or T	C or G	C	C	G	A or G	G	T	C or A	A or G
Sloughi (Arabian Greyhound)	A	C	C	C	C or T	G or A	C or T	C or T	C or G	C	C	G	A	G	T	C or A	G
	A	C	C	C	C or T	G or A	C or T	C or T	C or G	C or T	C	G	A or G	G	T	C or A	A or G
	G	C	C or A	C	C	G	C	C	C	C	C	G	A	G	T	C or A	A or G
	A or G	C or T	C or A	C	C or T	G or A	C or T	C or T	C or G	C or T	C	G	A or G	G	T	C or A	A or G
Wire-haired Dachshund	A or G	C	C	C	C or T	G or A	C or T	C or T	C or G	C	C	G	A or G	G	C	C	A
	A	C	C	C	C	G	C	C	C	C	C	G	G	G	T	C	A
	A or G	C	C	C	C	G	C	C	C	C	C	G	A or G	G	T or C	C or A	A or G
	A or G	C	C	C	C	G	C	C	G	C	C	G or A	A or G	G	T	C	A
	A or G	C	C	C	C	G	C	C	C	T	C	G	A	G	T	C	A or G
	G	C	C or A	C	C or T	G or A	C or T	C or T	C	C or T	C	G	A	G	T	C	A
Tibetan Terrier	A	C	C	C	C	G	C	C	C	C	C	G	A	G	T	C	A
	A	C	C	C	C	G	C	C	C	T	C	G	A	G	T	C	A
	G	C	C	T	C	G	C	C	C	C	C	G	A	G	T	C	A
	A	C	C	T	C	G	C	C	C	C	C	G	A	G	T	C	A
	A	C	C	C or T	C	G	C	C	C	C	C	G	A or G	G	T or C	C	A
Miniature Poodle	A	C	C	C	C	G	C	C	C	C	C	G	A	G	T	C	A
	A	C	C	C	C	G	C	C	C	C	A	G	A	G	T	C	G
	A	C	C	C	C	G	C	C	C	C	C	G	A or G	G	C or G	C	A or G
	A or G	C or T	C	C	C	G	C	C	C	C	A	G	A or G	G	C or G	C	A or G
	A or G	C or T	C	C	C	G	C	C	C	C	C	G	A	G	T	C	A or G
Polish Lowland Sh.	A	C	C	C	C	G	C	C	C	C	C	G	A	G	C	C	A
Berger des Pyrénées	A	C	C	C	C	G	C	C	C	C	C	G	A	G	C	A	A

Haplotype analyses are provided for dog breeds with two or more affected dogs. The identified alleles are marked and the two alleles in heterozygous state were characterized by an “or” in the appropriate dogs.

altogether 805 dogs from 22 breeds including 112 PRA-affected animals were covered in the study. In 4 breeds (Australian Cattle dogs, Golden Retrievers, English Cocker Spaniels, Rottweilers), PCR-SSCP analysis of the *RCV*1 gene detected no mutations in the affected dogs. Two single nucleotide substitutions were identified within the ORF. The C272A transversion causes a N3K amino acid exchange in Dachshunds, Saarloos and Scottish Terriers. The C4275A variation, resulting in a P202H exchange in the carboxyterminal amino acid, was identified in homozygous state in Berger des Pyrénées and in heterozygous state in Bologneses, Dachshunds, Giant Schnauzers, Schapendoes and Sloughis. Further sequence variations were identified within the 5'-UTR, intron 1 and the 3'-UTR. Intron 2 harboured 13 breed specific sequence variations. The C2735T-C2843T variations were always in a linkage disequilibrium (Table 4 and Table 5). Sloughis and Irish Setters have been included in the investigation although the breed specific mutation for PRA has already been identified. One affected Irish Setter showed a late form of PRA without the typical mutation in the *PDE6B* gene. Therefore, a second PRA form may be present in this breed. Sloughis were included as controls.

The founder effect in connection with inbreeding, i.e. effective genetic separation, leads to breed-specific PRA mutations. Therefore, identical mutations are not likely to have happened independently in different breeds. The N3K exchange in exon 1 was identified in Scottish Terriers, Dachshunds and Saarloos and the P202H exchange in exon 3 in Bologneses, Berger des Pyrénées, Dachshunds, Schapendoes, Giant Schnauzers and Sloughis. Therefore, these latter exchanges probably pre-date the separation into these present-day breeds. In addition, these sequence variations were usually found in heterozygous state in PRA-affected dogs thus excluding them as the cause of ar transmitted disease. Heteroallelism is an unlikely cause of ar PRA within a single breed, as one would expect such disease to result from a single founder mutation. However, since SSCP analysis does not detect every sequence variation and the gene promotor has not been screened, heteroallelism invoking a second unidentified mutation cannot be entirely ruled out.

The identified intronic sequence variations were found in heterozygous state in PRA affected dogs of different breeds (see Table 4). Because of linkage of the polymorphic nucleotides to certain *RCV*1 alleles in a given breed, heterozygosity excludes an ar trait in affected dogs. The Irish Setter with the known breed-specific *PDE6B* mutation [8] is homozygous for all variable positions in the *RCV*1 gene. In contrast, the Setter with the late form of PRA (without the *PDE6B* mutation) is heterozygous for most positions. All in all, we excluded the *RCV*1 gene as a candidate gene for ar PRA via these intragenic polymorphisms in Bologneses, Curly Coated Retrievers, Giant Schnauzer, Entlebucher Mountain Dogs, Irish Setters, Labrador Retrievers, Miniature Poodles, Newfoundlands, Saarloos/Wolfhounds, Salukis, Scottish Collies, Scottish Terriers, Schapendoes, Sloughis, Tibetan Terriers and Wirehaired Dachshunds.

During polymorphism analyses of 11 different breeds altogether 12 different haplotypes were identified in affected dogs (Table 5). 3-5 haplotypes were verified in PRA-affected dogs of each single breed (see Table 5). Therefore, a potentially ad transmitted PRA can also be excluded in 8 breeds: Dachshund, Entlebucher Mountain Dogs, Miniature Poodles, Labrador Retrievers, Saarloos/Wolfshounds, Schapendoes, Sloughis, and Tibetan Terriers. The Polish Lowland Sheepdog and the Berger des Pyrénées show homozygous sequence variations without segregation with PRA. As Australian Cattle dogs, English Cocker Spaniels, Golden Retrievers and the Rottweiler presented no variations, no firm statements are possible concerning the cause for PRA in these latter 6 breeds.

ACKNOWLEDGEMENTS

We thank the owners of the dogs for blood samples, the veterinarians of the Dortmunder Ophthalmologenkreis (DOK) for the ophthalmologic investigations of the dogs and for the support of different breeding clubs. These studies were supported in part by the Gesellschaft für kynologische Forschung, Bonn (Germany).

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The print version of this article was created on 15 Nov 2002. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.