Haplotype-defined linkage region for gPRA in Schapendoes dogs

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Purpose: In order to determine the molecular basis of canine generalized progressive retinal atrophy (gPRA), we initiated whole-genome scanning for linkage in gPRA-informative pedigrees of the Schapendoes breed.

Methods: Detailed pedigree and ophthalmological data were assembled in selected Schapendoes pedigrees. A whole-genome scan was initiated by two-point linkage analysis using microsatellite markers in combination with haplotype analyses. Mutation screening was carried out in respective candidate genes by DNA sequencing of amplified products and quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Results: Genotyping data of the microsatellite genome scan evidenced a peak two-point lod score of 4.78 for marker REN93E07 on CFA20. Haplotype analyses inferred the gPRA locus in a 5.6 megabase (Mb) region between markers FH3358 and TL336MS. Mutation screening in the genes CACNA2D3, HT017, and WNT5A revealed no causative sequence deviations. In addition, CACNA2D3 mRNA levels were equivalent in retinas of affected and healthy dogs.

Conclusions: By genome-wide linkage analysis a region for gPRA was identified and fine-localized in Schapendoes dogs. Although the mutation causing gPRA in Schapendoes dogs has not yet been identified, we established indirect DNA testing for gPRA in this breed based on linkage analysis data.

Generalized progressive retinal atrophies (gPRAs) in domestic dogs (Canis familiaris) are a group of inherited retinal dystrophies that share a similar phenotype. gPRA causes progressive loss of vision, usually leading to blindness. Initially, rod photoreceptor vision is affected, causing night blindness followed by progressive loss of cone photoreceptors with deteriorations in daytime vision. gPRAs can be classified by age of onset and rate of progression [1]. Certain breeds show early onset forms, e.g. Collies, Irish Setters, Norwegian Elkhounds and Miniature Schnauzers. In these breeds, the disease results from abnormal or arrested development of the photoreceptor cells in the retina, and gPRA affects pups very early in life. In other breeds (including Miniature Poodles, English and American Cocker Spaniels, Labrador Retrievers) gPRA onset occurs much later. Affected dogs in these latter breeds appear normal when young, but develop gPRA as adults.

Two X-linked [2] and an autosomal dominantly inherited trait [3,4] have been described, yet most gPRA forms are transmitted in an autosomal recessive (AR) mode. Up to now, causative mutations have been identified only in few breeds of dogs with AR transmitted gPRA [5-11].

In addition to the respective pedigree material highly informative polymorphic DNA markers [12,13] are the necessary tools for mapping chromosomal locations of disease gene loci by linkage analysis. In order to determine the molecular basis of canine gPRA, we initiated whole-genome scanning (WGS) using markers spread evenly across the canine genome for linkage in gPRA-informative pedigrees of the Schapendoes breed. Here we demonstrate linkage of the gPRA trait to markers on canine chromosome 20 (CFA20) in Schapendoes. In addition, the critical region was fine mapped, and the novel candidate genes CACNA2D3, HT017, and WNT5A were investigated.

METHODS

Animals: All dogs were collected from the general breeding population of pure-bred Schapendoes. Five pedigrees, comprising 57 Schapendoes dogs including 13 gPRA-affected animals, were available in which gPRA is transmitted in an AR manner. Ophthalmologically experienced veterinarians confirmed the gPRA status of affected and unaffected dogs by ophthalmoscopy as documented in certificates of eye examinations. gPRA in Schapendoes is characterized by late onset and slow progression as documented by veterinarians of the Dortmunder Ophthalmologenkreis (DOK). Affected Schapendoes dogs appear normal when young, but develop gPRA at an age of onset between 2-5 years. Early in the disease, affected dogs are night-blind, lacking the ability to adjust their vision to dim light; later, their daytime vision also fails. This process of complete photoreceptor degeneration takes up to 2 years.

Genomic DNA was extracted from peripheral blood according to standard protocols [14]. For isolation of RNA and retina sections we obtained an eye of a gPRA-affected, five-year-old Schapendoes with complete loss of night vision yet anecdotally remaining, very limited day-time vision. Retinas of gPRA-free Saarloos/Wolfshounds were used as controls.
**Histology:** The enucleated eyes from a five year old, gPRA-affected Schapendoes dog and control eyes from a gPRA-free Sarloos/Wolfhound were sagittally cut at the level of the optic nerve, immersion-fixed in 100% ethanol and paraffin-embedded. Serial sections, 15 µm thick, were cut over the whole extension of the retina, stained with hematoxylin and eosin and photo-documented.

**Markers and genotyping:** For the WGS highly informative autosomal microsatellite markers were analyzed from the minimal screening set 2 (MSS-2) [12]. Microsatellites for fine mapping (Table 1, Figure 1) were identified using published dog markers [15], the dog genome sequence (May 2005) and the Tandem Repeats Finder included in the UCSC Genome Browser. Only microsatellites with a repeat length exceeding 15 units were selected. PCR primers were designed using Primer Express software (PE Biosystems). For PCR we used the “tailed primer PCR” as described before [16]. This method requires three oligonucleotides for amplification: 1. tailed forward primer (tailed F), 2. reverse primer and 3. labeled primer.

| Marker type | Primer sequence (5'-3') | Location on CFA20 (bp) | PCR products (bp) |
|-------------|-------------------------|------------------------|-------------------|
| REN100J131  | TGATTGACTCTACTTTACACA   | 25,817,177              | 164               |
| FH33582     | CTATCACCCTAACTGAAAGCA   | 33,096,985              | 268               |
| REN149D232  | CCAATGAGAAGGCTTAAAAAGTT| 33,097,252              | 168               |
| REN316E232  | AAAAAGGATGATGCCATGAG    | 38,133,160              | 154               |
| TL335MS     | CACCTTTCCCTCCCTCCCTCG   | 38,313,607              | 126               |
| REN93D071   | CATCACCCAAATTCAAAGCA    | 38,525,708              | 170               |
| FH33582     | CATAGTCACACCCCAATG      | 38,727,171              | 133               |
| TL335MS     | CCATCAAGGCCCTAATATTTAAAGATT | 38,956,429          | 335               |
| TL327MS     | TGCCCTTTGATGAGATGCTGG   | 39,812,660              | 167               |
| TL195II     | AACTGAGGTTCCCTTGTTCC   | 47,049,704              | 232               |
| REN114M191  | GAGCCCCAGTTTGTTGAGAAG   | 56,553,756              | 192               |

Location ascertained from UCSC Genome Browser (assembly: dog May 2005).

Figure 1. Haplotypes of the gPRA Schapendoes families SD1-5 as established by microsatellite markers for chromosome 20. Affected dogs are represented by black, unaffected by white and those with known carrier status are represented by half-filled symbols. Circles represent females and squares represent males. Genotype that could not be ascertained are scored as “0”. Black bars indicate the affected haplotype. In the box, genotyped markers and the disease haplotype are indicated. The observed recombination events evidence the disease causing locus in the region between markers FH3358 and TL195IIMS.
The intronic primer sequence for amplification of corresponding exons and 50 intronic bases on either end of the exons and the product sizes are shown.

### Table 2. Primers for amplification of exonic regions of CACNA2D3, HT017, and WNT5A including exon/intron boundaries

| Gene      | Exon | Sequence (5’-3’) | Size (bp) |
|-----------|------|------------------|-----------|
| CACNA2D3  | 1F   | ACAGTC ATGTTGGCCTTACCTC | 636       |
|           | 2F   | AAAGGACGGGGTCTTGGGACG | 599       |
|           | 3F   | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 4F   | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 5F   | ATACAT GGTCTTGGGACG | 503       |
|           | 6F   | CAAAGGACGAGGAAGGACG | 503       |
|           | 7F   | ACAGAGGACGACGAGGAAGAAC | 589       |
|           | 8F   | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 9F   | AAAGGACGGGGTCTTGGGACG | 599       |
|           | 10F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 11F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 12F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 13F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 14F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 15F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 16F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 17F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 18F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 19F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 20F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 21F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 22F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 23F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 24F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 25F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 26F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 27F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 28F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 29F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 30F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 31F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 32F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 33F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 34F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 35F  | AAACAGGACGACGAGGAAGAAC | 589       |
|           | 36F  | GACGCGTGGAAGGAGCAGGGAG | 503       |
|           | 37F  | AAACAGGACGACGAGGAAGAAC | 589       |

The exonic primer sequence for amplification of corresponding exons and 50 intronic bases on the exons and the product sizes are shown.

### Table 3. Primers for amplification of CACNA2D3 cDNA

| Primer | Sequence (5’-3’) | Size (bp) |
|--------|------------------|-----------|
| CACNA2D3 Ex2 F | ATGGAAAGGAGATGGTCTCAGAAAAGAAGC | 605       |
| CACNA2D3 Ex5 R | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |
| CACNA2D3 Ex7 F | ATGGAAAGGAGATGGTCTCAGAAAAGAAGC | 605       |
| CACNA2D3 Ex6 F | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |
| CACNA2D3 Ex9 R | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |
| CACNA2D3 Ex10 R | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |
| CACNA2D3 Ex12 F | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |
| CACNA2D3 Ex13 F | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |
| CACNA2D3 Ex14R | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |
| CACNA2D3 Ex15 F | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |
| CACNA2D3 Ex16F | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |
| CACNA2D3 Ex17 F | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |
| CACNA2D3 Ex18 F | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |
| CACNA2D3 Ex19 R | GCAATTGATGTTGGAAAGGACGATCCTAC | 672       |

The exonic primer sequences for amplification of overlapping polymerase chain reaction products of the cDNA and the product sizes are shown.
NP_065729; WNT5A, NM_003392) with the publicly available dog genome sequences in UCSC Genome Browser (assembly: dog May 2005). For sequencing of the coding regions of the three genes, intronic PCR primers flanking the exons were designed in order to amplify at least 50 intronic bases on either end of the exon in order to cover the splice junctions (Table 2). PCRs were performed under standard PCR conditions [18] with BioTherm DNA Polymerase (Genecraft) and 1.5 mM MgCl₂ at an annealing temperature of 57°C.

For sequencing of the CACNA2D3 cDNA total RNA of retinal tissue was isolated. For this purposepeqGOLD TriFast reagent (Peqlab, Erlangen, Germany) was added to the frozen tissue samples, the mixture was immediately homogenized and total RNA was then extracted using guanidinium isothiocyanate (RNeasy Mini Kit, Qiagen, Hilden, Germany). cDNA was synthesized by oligo-dT priming with the Sensiscript RT Kit (Qiagen, Hilden, Germany). Overlapping PCR products of the cDNA of the CACNA2D3 gene were generated using the primers in Table 3.

All sequencing reactions were carried out by the dideoxy chain termination method using the Dyenamic ET Terminator Kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer’s instructions. Reaction products were run on an automated capillary DNA sequencer (MegaBACE 1000, Amersham Biosciences, Freiburg, Germany).

Quantitative real-time RT-PCR of candidate gene CACNA2D3: Total RNAs from the retinas of a gPRA-affected Schapendoes and an unaffected Saarloos/Wolfshound were subjected to quantitative real-time RT-PCR analysis using the QuantiTect SYBR Green assay (Qiagen, Hilden, Germany) as described by the manufacturer and the iCycler iQ real-time PCR detection system (Bio-Rad, München, Germany). PCR primers were designed using Primer Express software (PE Biosystems). In order to avoid amplification of contaminating genomic DNA, the primers span an intron. CACNA2D3 mRNA/cDNA was amplified using primers CACNA2D3 Ex9-F (5’-CAC TTC AGG GAG CAT CTG GAC-3’) and CACNA2D3 Ex10-R (5’-GGC TGC AGA TGC TTC CTG CTG-3’). ATP-binding cassette, sub-family A, member 4 gene (ABCA4) and guanine nucleotide binding protein, α transducing activity gene (GNAT1) are retina specific. They were amplified using primers ABCA4-F (5’-TGG AGG AAA GCT CCC AAT CC-3’) and ABCA4-R (5’-GCC TCT CTG GTG ATA GGG CC-3’) and GNAT1-F (5’-GCT CGC GTG TCA AGA CCA C-3’) and GNAT1-R (5’-ATC CAC TTC TTG CGC TCT GAG-3’). Hypoxanthin phosphoribosyltransferase 1 (HPRT1) served as internal reference and was amplified using primers HPRT1-F (5’-AGC TTG CTG GTG AAA AGG AC-3’) and HPRT1-R (5’-TTA TAG TCA AGG GCA TAT CC-3’). One-step PCR cycling was carried out by reverse transcription at 50°C for 30 min, initial activation step at 95°C for 15 min x1 cycle, 4-step cycling at 94°C for 15 s, at 60°C for 30 s, at 72°C for 30 s x 40 cycles. As soon as the PCR was completed, baseline and threshold values were set automatically and threshold cycle (CT) values were calculated. CT values were exported to Microsoft Excel for calculating the real copy number. The CT value represents the PCR cycle at which an increase in fluorescence can first be detected above
a baseline signal level. Signals were quantified by normalizing with GAPDH expression.

RESULTS

Compared to a normal retina (Figure 2A), the gPRA-affected eyes of a five year old Schapendoes displayed typical degeneration signs in peripheral and central areas (Figure 2B). The outer retina with the photoreceptor layer and the outer nuclear layer was missing in all retinal parts investigated. The inner retina showed reduced inner nuclear and inner plexiform layers, whereas the ganglion cell layer appeared comparatively preserved.

A detailed ophthalmological examination was performed on all 57 Schapendoes of five different families revealing 13 dogs with bilateral affection. Evaluation of gPRA in these five families suggested that the disease segregates as an AR trait (Figure 1). A case of inbreeding was identified in family SD3. Initially, WGS for linkage was performed in the five pedigrees using markers of the MSS-2 [12]. Having completed the typing of 165 microsatellites (CFA1-20 and 24-26) of 325 MSS-2 markers, the WGS was abbreviated after a two-point lod score of 4.78 at q=0.000 was obtained for marker REN93E07. The high lod score indicated that the gPRA phenotype was linked to a mutation on CFA20. For fine mapping of the gPRA region in the Schapendoes breed nine additional microsatellite markers from CFA20 were genotyped between marker REN100J13 and TL1951IMS (Table 1, Figure 1) in the five pedigrees. With one exception all microsatellites represent dinucleotide repeats. Heterozygosity values range around 0.5, and 4-10 different alleles were analyzed in the Schapendoes population. Two-point lod scores for linkage between the gPRA locus and microsatellite markers gave still a maximum lod score of 4.78 with marker REN93E07.

Figure 3. Schematic overview of the critical region on chromosome 20 in which the gPRA locus maps in Schapendoes dogs. On the left hand side of the chromosome the analyzed genes and their genomic location are shown, on the right hand side genotyped microsatellite markers are depicted. Location ascertained from UCSC Genome Browser (assembly: dog May 2005).

Genotyping of ten microsatellite markers for all 57 dogs of the five pedigrees revealed that the “2-2-3-2-2-2-2” haplotype (marker REN149D23 to TL327MS) segregates with the gPRA trait and has a frequency of 50% in the analyzed pedigrees (Figure 1). Analysis of this haplotype placed the gPRA locus in a region between marker FH3358 and TL1951IMS. A potential double recombination event in the dog SD90 of family SD5 may be interpreted to confine the size of the critical haplotype to 5.6 Mb flanked by markers FH3358 and TL336MS.

Figure 4. Analysis of gene expression by quantitative real-time reverse transcriptase polymerase chain reaction. Real-time RT-PCR was used to determine the expression of the genes CACNA2D3, GNAT1, and ABCA4 by calculating the real copy number. Expression levels were normalized to those of the HPRT1 gene. No differences were obvious between the unaffected Saarloos/Wolfshound (Sa; N=2) and the g-PRA affected Schapendoes (SD; N=1) for CACNA2D3 mRNA. GNAT1 and ABCA4 mRNA expression is substantially reduced or absent in retinal tissue of the affected Schapendoes.

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For the “recombined region” of dog SD90 no canine gene has been published in the different gene banks so far. Comparison of the canine DNA sequence of the critical region with the human genome in UCSC Genome Browser (assembly: human May 2004) shows homology with chromosome 3p. In man this region comprises candidate genes for retinitis pigmentosa (RP)-and thus also for gPRA in Schapendoes. The marker with the peak lod score REN93E07 is located in intron 7 of candidate gene CACNA2D3 (Figure 3) which spans a genomic region of about 830 kb (kb) and consists of 37 exons. The mRNA of this gene encodes the calcium channel α,δ, subunit, which is mainly expressed in brain [19] and also in the eye UniGene. In intron 26 of the human CACNA2D3 gene the HT017/LRTM1 gene is located (Figure 3). This gene encodes the leucine-rich repeat and transmembrane domain 1. Upstream of CACNA2D3 the WNT5A gene is situated (Figure 3) encoding member 5A of the wingless-type MMTV integration site family. Sequencing of the candidate genes HT017 and WNT5A did not reveal any pathogenic mutations, neither in the coding sequences nor in intron/exon junctions of affected individuals. Five single nucleotide polymorphisms (SNPs) were detected in the CACNA2D3 gene: IVS16-7>T, IVS23-51A>T, IVS29+18A>G, IVS29+57C>T, IVS30-57>T. Yet, these SNPs occur in homozygous state not only in gPRA-affected dogs, but also in healthy Schapendoes (data not shown). Thus these SNPs do not cause gPRA in Schapendoes. Additionally, a thym-ininsertion in intron 6 (IVS6-38-34insT) was identified in comparison to the UCSC dog genome sequence (assembly: dog May 2005). Further investigations revealed that this insertion was present in homozygous state in healthy dogs of other breeds, implying a non-pathogenic polymorphism. Furthermore, sequencing of the CACNA2D3 cDNA from a diseased Schapendoes eye revealed no sequence deviations, thus excluding “hidden” mutations affecting the splicing process. In addition, altogether 25 kb in introns 5, 7 and 8 of the CACNA2D3 gene comprising evolutionarily conserved sequences were analyzed without any hint on the gPRA mutation in question (data not shown).

In order to exclude potential transcriptional impact of the elusive gPRA mutation, the expression of CACNA2D3 mRNA in retinal tissue was determined by real-time RT-PCR (normalized to the level of the housekeeping gene HPRT). mRNA expression of two unaffected Saarloos/Wolfshounds was compared to a gPRA-affected Schapendoes. GNAI1 and ABCA4 gene expression was substantially reduced in retinal tissue of the affected Schapendoes. In contrast, no reduction was obvious for the CACNA2D3 mRNA levels (Figure 4).

DISCUSSION

The responsible locus for gPRA in Schapendoes, a canine counterpart for RP in man, maps to the central region of CFA20. Haplotype analyses defined the critical interval between marker FH3358 and TL1951IMS. The haplotype potentially confining a smaller critical interval (between FH3358 and TL336MS) occurred exclusively in individual SD90 of family 5. A caveat remains to accept this confinement: Alleles 3 for markers TL336MS and TL337MS might not represent a recent or ancestral recombination event, but rather be due to non-mendelian inheritance. Yet non-mendelian inheritance appears less likely for two adjacent microsatellites in the absence of any additional slippage mutations of these microsatellites in all the other analyzed meiotic events. Consequently, we concentrated initially on the smaller critical interval between FH3358 and TL336MS, albeit the genomic region between TL337MS and TL1951IMS must not be excluded formally to comprise additional candidate genes.

An interesting candidate in the critical genomic interval represents the CACNA2D3 gene. The marker with the highest lod score REN93E07 was located in intron 7. Direct sequencing of all coding exons for homozygously affected and normal SD dogs exclude a gPRA-causing mutation in the coding sequence of the CACNA2D3 gene. This fact implies that the mutation causing gPRA in Schapendoes may be located intronically in the CACNA2D3 gene affecting splicing. Yet, extensive sequence analysis of retinal cDNA revealed no splice mutation in the CACNA2D3 gene. Furthermore, in order to exclude the CACNA2D3 gene as causative for gPRA in Schapendoes, we analyzed the expression of CACNA2D3 mRNA in retinal tissue: mRNA levels were nearly identical between a gPRA-affected Schapendoes and an unaffected Saarloos/Wolfshound. In contrast, the expression of the retina-specific genes GNAI1 and ABCA4 appeared reduced substantially or even absent. Photoreceptor cells in the retina of the affected Schapendoes have vanished so that an expression of retina-specific genes cannot be demonstrated. Since its mRNA expression is unaltered in a retina without photoreceptor cells, the CACNA2D3 gene appears expressed mainly in the cell types not affected by gPRA. Yet also photoreceptor cells may well produce small but crucial amounts of CACNA2D3 transcripts that do not significantly affect the mRNA levels from unseparated retinal extractions. Given the availability of respective tissue samples, we could nevertheless use the haplotype-defined linkage region data to examine obligatorily homozygotic mutation carriers for altered retinal mRNA expression already presymptomatically.

Since gPRA in Schapendoes is probably not caused by a CACNA2D3 mutation, mutation analysis of the HT017 and WNT3A genes were performed. Yet, gPRA-causing mutations were excluded in the coding sequence of these two genes. In comparison to the UCSC dog genome sequence (assembly: dog May 2005) the critical region between marker FH3358 and TL336MS comprises further candidate genes, which are be investigated. In case the causative mutation is not identified in this critical interval, additional candidate genes are to be investigated in the region between TL337MS and TL1951IMS. Although the mutation causing gPRA in Schapendoes has not yet been identified, the critical region for the location of the mutation was reduced to 5.6 Mb. Our findings for gPRA in the Schapendoes breed constitute an interesting naturally occurring model for RP, the human counterpart of gPRA.

Based on linkage analysis data, we established an indirect DNA test for gPRA in this breed. Nearly 600 dogs with a mean age of five years have been tested so far. Based on the
age of onset of 2-5 years in the Schapendoes breed, affected dogs among the tested individuals are likely to show initial gPRA symptoms. All 18 phenotypically affected dogs were tested as harbouring the linked haplotype in homozygous state, and all healthy obligatory carriers were typed as being heterozygous. The degree of certainty for a test result depends on the rate of recombination (so far no recombinations were observed) or new mutations. Theoretically small uncalculatable risks remain for false negative and positive results, respectively. Notwithstanding, the established indirect DNA test facilitates the eradication of gPRA among Schapendoes. Known mutation carriers can still produce offspring by selective crossing to dogs with mutation-free haplotypes.

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