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(72) **Inventeurs/Inventors:**
ACLAND, GREGORY M., US;
GOLDSTEIN, ORLY, US;
KUKEKOVA, ANNA V., US;
JOHNSON, JENNIFER LYNN, US

(73) **Propriétaire/Owner:**
CORNELL RESEARCH FOUNDATION, INC., US

(74) **Agent:** RIDOUT & MAYBEE LLP

(54) **Titre : METHODE PERMETTANT D'IDENTIFIER UNE DYSPLASIE OCULO-SQUELETTIQUE CHEZ LES CHIENS**
(54) **Title: METHOD FOR IDENTIFYING OCULOSKELETAL DYSPLASIA IN DOGS**

(57) **Abrégé/Abstract:**
Provided are methods for identifying dogs as likely to be genetically normal, carriers of, or affected with Oculo--skeletal dysplasia (OSD) by determining the presence or absence of a drd2 COL9A2 mutation and/or a drdl COL9A3 mutation. Also provided is a method for selective breeding of dogs and kits useful for carrying out the methods of the invention.

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(71) Applicant *(for all designated States except US)*: **CORNELL RESEARCH FOUNDATION, INC.** [US/US]; Cornell Center For Technology Enterprise And, Commercialization, 395 Pine Tree Road, Suite 310, Ithaca, NY 14850 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **ACLAND, Gregory, M.** [US/US]; 110 Marlbrooke Way, Kennett Square, PA 19348 (US). **GOLDSTEIN, Orly** [IL/US]; 11 Rosina Way, Ithaca, NY 14850 (US). **KUKEKOVA, Anna, V.** [RU/US]; 1143 Eastshore Drive, Ithaca, NY 14850 (US). **JOHNSON, Jennifer, Lynn** [US/US]; 240 Fenner Road, Lansing, NY 14882 (US).

(74) Agents: **LOPINSKI, John, D.** et al.; Hodgson Russ LLP, The Guaranty Building, 140 Pearl Street, Suite 100, Buffalo, NY 14202-4040 (US).

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(54) Title: METHOD FOR IDENTIFYING OCULOSKELETAL DYSPLASIA IN DOGS

Figure 5A.

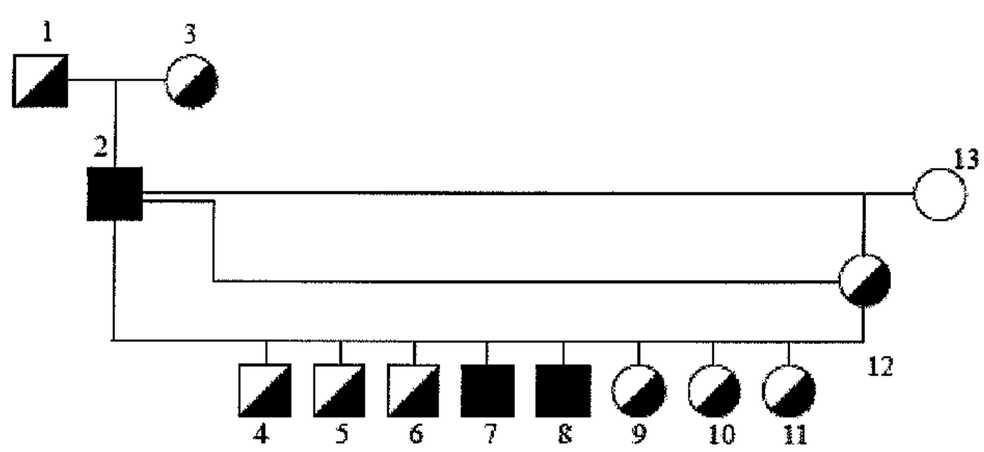

Figure 5B.

Figure 5C.

(57) Abstract: Provided are methods for identifying dogs as likely to be genetically normal, carriers of, or affected with Oculoskeletal dysplasia (OSD) by determining the presence or absence of a *drd2* COL9A2 mutation and/or a *drdl* COL9A3 mutation. Also provided is a method for selective breeding of dogs and kits useful for carrying out the methods of the invention.

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METHOD FOR IDENTIFYING OCULOSKELETAL DYSPLASIA IN DOGS

FIELD OF THE INVENTION

The present invention relates generally to a canine disease termed Oculo-skeletal dysplasia. More particularly, the invention relates to compositions and methods for use in testing dogs for Oculo-skeletal dysplasia.

DESCRIPTION OF RELATED ART

Oculo-skeletal dysplasia (OSD) is an autosomal recessive disorder that has been observed in 2 dog breeds, Labrador retriever (Carrig et al. JAVMA (1977) 170:49-57) and Samoyed (Meyers et al., JAVMA (1983) 183:975-979), but mutations associated with the disease may be present in other breeds.

Dogs that are affected with OSD have skeletal abnormalities characterized by short-limbed dwarfism and ocular defects including vitreous dysplasia, retinal detachment and cataracts. Dogs affected with OSD can usually be recognized upon physical examination by an experienced veterinarian, particularly an experienced veterinary ophthalmologist, or an experienced veterinary orthopedist, and sometimes by experienced dog breeders. However, recognition of affected dogs is not sufficient to allow adequate selection pressure to be applied to significantly reduce the frequency of a mutation that may be associated with OSD in the population. Furthermore, while it is widely held that OSD carriers can usually be recognized upon physical examination by an experienced veterinary ophthalmologist via detecting retinal folds or retinal dysplasia, this is highly unreliable as both false positive and false negative diagnoses are common. Thus, ophthalmoscopic examination to detect carriers is too inaccurate to allow adequate selection pressure to be applied to significantly reduce frequency of mutation(s) in the population that may be associated with OSD. Thus, improved methods for determining the likelihood of a dog to be a carrier, affected with, or normal for OSD are needed.

SUMMARY OF THE INVENTION

The present invention is based on our discovery of mutations associated with oculo-skeletal dysplasia (OSD). The OSD mutations analyzed in the method of the invention are referred to as the *drd1* COL9A3 mutation and the *drd2* COL9A2 mutation. These mutations are also referred to herein as the *drd1* mutation and the *drd2* mutation, respectively. *drd1* and *drd2* refer to dwarfism-retinal-dysplasia 1 and 2, respectively. The method comprises obtaining a biological sample from a dog and determining from the sample the presence or absence of the *drd1* mutation, the presence or absence of the *drd2* mutation, or the presence or absence of both of these mutations. Dogs that do not have either of these mutations (homozygous for the wild type sequence) are considered normal for OSD. Dogs that are heterozygous for the *drd1* mutation are considered *drd1* mutation carriers. *drd1* mutation carriers are considered to be carriers of a *drd1* form of OSD. Dogs that are heterozygous for the *drd2* mutation are considered *drd2* mutation carriers. *drd2* mutation carriers are considered to be carriers of a *drd2* form of OSD. Dogs that are homozygous for either the *drd1* mutation or the *drd2* mutation are considered to be affected with OSD.

The present invention also provides a method for selective breeding of dogs, whereby dogs that are identified as carriers of either OSD mutation, or as affected with OSD can be removed from the breeding stock. Also provided are kits useful for carrying out the methods of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides the genomic sequence of the 5' end of the canine COL9A2 gene.

Figure 2 provides the nucleotide sequence of the region of the COL9A2 gene which encompasses the COL9A2 *drd2* mutation.

Figure 3 provides the canine COL9A2 cDNA sequence.

Figure 4A provides the wild type canine COL9A3 *drd 1* cDNA coding sequence.

Figure 4B provides the mutant canine COL9A3 *drd 1* cDNA coding sequence.

Figure 4C provides the provides the genomic sequence of the 5' end of COL9A3 gene in a normal CFA24 chromosome.

Figure 4D provides the genomic sequence of the 5' end of COL9A3 in a CFA24 chromosome that comprises the *drd1* mutation.

Figure 5A provides a pedigree of a colony of dogs descended from a purebred Samoyed affected with OSD.

Figures 5B and 5C provide photographic representation of electrophoretic separation of PCR amplification products from *drd2* COL9A2 tests performed on biological samples obtained from the dogs depicted in the pedigree.

Figure 6 provides a photographical representation of electrophoretic separation of PCR amplification products from *drd1* COL9A3 tests.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for determining whether a dog is normal for OSD, or is a carrier of an OSD mutation, or is affected with OSD. The invention is based on our discovery of genetic mutations associated with OSD. In particular, we have discovered a mutation that is a 1,267 base pair deletion in the canine COL9A2 gene on canine chromosome 15 (CFA15) (the *drd2* COL9A2 mutation) and a mutation that is a one nucleotide (a guanine; "G") insertion in the canine COL9A3 gene on canine chromosome 24 (CFA24) (the *drd1* COL9A3 mutation).

The term "*drd1* COL9A3 mutation" is used interchangeably herein with "*drd1* mutation." Likewise, the term "*drd2* COL9A2 mutation" is used interchangeably with "*drd2* mutation." The *drd1* mutation and the *drd2* mutation can each be referred to individually as an "OSD mutation" and collectively as "OSD mutations."

As used herein, a dog is termed "normal" or "normal for OSD" if the dog does not have either OSD mutation (i.e., the dog is homozygous wild type for both OSD mutation sites). A dog is termed "affected" or "affected with OSD" if the dog is homozygous for either or both OSD mutations. A dog is a carrier of OSD if it is heterozygous for either the *drd1* mutation or the *drd2* mutation, or for both mutations. A "*drd1* mutation carrier" as used herein means a dog that is heterozygous for the *drd1* mutation. A *drd1* mutation carrier is considered to be a carrier of *drd1* form of OSD. A "*drd2* mutation carrier" as used herein means a dog that is heterozygous for the *drd2* mutation. A *drd2* mutation carrier is considered to be a carrier of *drd2* form of OSD. Determining that a dog is a *drd1* mutation carrier does not indicate whether or not the dog is a *drd2* mutation carrier, or whether or not the dog is affected via homozygosity for the *drd2* mutation. Likewise, determining that a

dog is a *drd2* mutation carrier does not indicate whether or not the dog is a *drd1* mutation carrier, or whether or not the dog is affected via homozygosity for the *drd1* mutation.

It will be recognized by those skilled in the art that carriers of either OSD mutation that are not affected with OSD may nevertheless exhibit varying OSD traits, such as an ocular phenotype that ranges from localized retinal dysplasia characterized by focal or multifocal retinal folds to large plaques of dysplastic retinal tissue, but have a normal appendicular skeleton. The present invention addresses this and other difficulties in ascertaining the OSD status of dogs by providing a method for determining whether a dog is normal for OSD, or is a carrier of the *drd1* mutation, or is a carrier of the *drd2* mutation, or is affected with OSD. The method comprises the steps of obtaining a biological sample from a dog and determining from the biological sample the presence or absence of the *drd1* mutation, the *drd2* mutation, or both of these mutations. A determination that the dog is homozygous for either OSD mutation is indicative that the dog is affected with OSD. A determination that neither OSD mutation is present is indicative that the dog is normal for OSD. A determination that the dog is heterozygous for the *drd1* mutation indicates that the dog is a *drd1* mutation carrier, a carrier of *drd1* form of OSD. A determination that the dog is heterozygous for the *drd2* mutation indicates that the dog is a *drd2* mutation carrier, and is therefore a carrier of *drd2* form of OSD.

Any dog, regardless of breed, could be heterozygous or homozygous for either or both OSD mutations. To date, we have found the *drd1* mutation in Labrador retrievers and the *drd2* mutation in Samoyeds. Nevertheless, upon identification of heterozygosity for one OSD mutation, it is preferable to determine the status of the other OSD mutation. For example, upon a determination that a dog is a *drd1* mutation carrier, it would be preferable to determine the *drd2* status of the dog to ascertain whether or not the dog is also a *drd2* mutation carrier, or whether the dog is homozygous for the *drd2* mutation, and is therefore affected with OSD, in addition to being a *drd1* mutation carrier.

In one embodiment, the method further comprises communicating the result of determining whether a dog is normal for OSD, a *drd1* mutation carrier or *drd2* mutation carrier, or is affected with OSD to an individual. The test result can be communicated to an individual by any method. Non-limiting examples of the individual to whom the test results may be communicated include the dog owner, a canine pedigree accreditization agency, a veterinarian, or a provider of genetic test results.

We have determined that the *drd2* COL9A2 mutation is a 1,267 base pair deletion. The deletion and its genomic context is illustrated by the DNA sequences depicted in Figures 1-3.

Figure 1 provides the genomic sequence of the 5' end of the canine COL9A2 gene (SEQ ID NO:1). This sequence is a corrected version of the 5' region of the canine COL9A2 genomic sequence, and extends from a location 1,310 nucleotides (nts) of SEQ ID NO:1 before the start codon (boxed), to the beginning of intron 2. In particular, we have determined that both the 2004 July CanFam1 and 2005 May CanFam2 assemblies (provided to the public through GenBank) have incorrect and incomplete sequences for this interval of CFA15. In particular, we have determined the 2005 assembly is missing exon 1 and surrounding sequence. We determined this using primers designed from sequence shared by both CanFam assemblies to amplify genomic DNA to retrieve the sequence presented as SEQ ID NO:1. In the corrected sequence presented in SEQ ID NO:1, nucleotide 1 corresponds to nucleotide (nt) 5,649,378 of canine chromosome 15 in the CanFam2 2005 assembly, and nt 5,641,234 of canine chromosome 15 in the CanFam1 2004 assembly. The start codon is at positions 1311-1313 (boxed), exon 1 ends at nt 1373 (and thus contains a 63 base coding sequence), intron 1 extends from nt 1374 through 2262 and exon 2 from nts 2263-2337. Capital letters signify coding regions.

Figure 2 provides a DNA sequence (SEQ ID NO:2) that illustrates the sequence deleted in the *drd2* COL9A2 mutation in the genomic CFA15 DNA context. The region of the canine genomic COL9A2 sequence presented in Figure 2 encompasses the *drd2* mutation (the deletion), and extends from the *drd2* 5' region, through exon 1, and into the start of intron 1. The ATG of codon 1 is boxed; the coding sequence is shown in uppercase. The *drd2* mutation is a deletion of the 1,267 nucleotides between nucleotide number 230 and nucleotide number 1,498 in the nucleotide sequence depicted in Figure 2. Thus, determining a deletion of 1,267 nucleotides between nucleotide number 230 and nucleotide number 1,498 in the nucleotide sequence depicted in Figure 2 (SEQ ID NO:2) identifies the presence of the *drd2* mutation. The *drd2* mutation removes the complete exon 1 of the transcript and its 5' untranslated region (UTR), as well as part of intron 1.

The presence or absence of the *drd2* mutation can be detected by any appropriate method, including but not limited to by analysis of canine genomic DNA, mRNA, cDNA, or protein.

In one embodiment, the presence or absence of the *drd2* mutation can be determined by PCR analysis. Exemplary PCR primer binding sites useful for PCR based amplification across the deletion are underlined in Figure 2 (primer pair 1) or double underlined (primer pair 2).

Figure 3 provides the canine COL9A2 cDNA sequence (SEQ ID NO:3). The first methionine (ATG) and the normal stop (TGA) codons are boxed. Non-coding sequence is shown in lowercase, coding sequence in uppercase. A single nucleotide polymorphism (SNP) identified in exon 3 has 2 alleles (C or T) and is shown as a bold enlarged Y (indicating a pyrimidine). In CFA15 chromosomes that comprise the *drd2* mutation, the C allele of this SNP is present, but this allele is also observed in normal dogs of various breeds.

Turning to the *drd1* mutation, we have determined that the *drd1* mutation is a one nucleotide (guanine) insertion in the canine COL9A3 gene on canine chromosome 24 (CFA24). This insertion is illustrated in Figure 4B (SEQ ID NO:4), which depicts a cDNA sequence comprising the Canine *drd1* COL9A3 mutation. Figure 4A (SEQ ID NO:5) provides the wild type *drd1* COL9A3 cDNA sequence. In Figures 4A and 4B, the first methionine (ATG) and the normal stop (TAA) codons are boxed. In a chromosome that contains the *drd1* mutation, the extra guanine (G) is inserted into the string of 4 Gs shown at nucleotide positions 7-10 of the normal coding sequence (Figure 4A), resulting in a string of 5 Gs in the mutant (Figure 4B). In the mutant sequence shown in Figure 4B, the inserted G is arbitrarily indicated (bolded and enlarged) as the 5th G in the string at nucleotide position 11 of SEQ ID NO:4, although the insertion could be any one of the 5 Gs. Thus, it is considered that determining a G in position 11 of SEQ ID NO:4 as depicted in Figure 4B identifies the presence of the *drd1* mutation. It is also considered that determining a G in position 11 of SEQ ID NO:4 as depicted in Figure 4B identifies a nucleotide sequence consisting of GGGGG in positions 7-11 in the nucleotide sequence depicted in Figure 4B. However, it will be recognized that the invention includes any other method of identifying the presence or absence of the *drd1* mutation, including but not limited to by analysis of canine genomic DNA, mRNA, cDNA, or protein. In respect of genomic DNA, Figure 4C provides the genomic sequence of the 5' end of COL9A3 gene in a normal CFA24 chromosome (SEQ ID NO:6). Figure 4D provides the genomic sequence of the 5' end of COL9A3 in a CFA24 chromosome that comprises the *drd1* mutation (SEQ ID NO:7),

wherein the fifth G that signifies the presence of the GGGGG sequence that may be used to identify the *drd1* mutation is shown in bold and enlarged. The atg start codon is boxed in Figures 4C and 4D.

It will be recognized that identifying the presence or absence of the *drd1* mutation by analysis of genomic DNA sequence is well within the purview of one skilled in the art and is included in the present invention. It is accordingly considered that identification of the presence or absence of the *drd1* mutation by analysis of genomic DNA sequence also identifies a G in position 11 of SEQ ID NO:4.

Representative primers useful for use in one embodiment of the invention for determining the presence or absence of the *drd1* mutation from genomic DNA are underlined in Figures 4C and 4D (these primers are also shown as primer pair 3 in Table 2). In Figures 4C and 4D, SNPs are shown as bold where r=A or G, y=C or T. The first methionine in the COL9A3 gene is boxed.

The insertion of G in the *drd1* mutation alters the *drd 1* open reading frame by +1 nucleotide, which introduces a stop codon after 48 codons (TGA; shown in bold and enlarged in Fig. 4B). Thus, the G in position 11 of SEQ ID NO:4 is in the +1 open reading frame relative to the ATG start codon presented in nucleotide positions 1-3 of Figure 4B. The invention therefore also includes identifying the *drd1* COL9A3 mutation using any method by which a +1 translational frameshift introduced into the *drd1* COL9A3 gene by the *drd1* mutation can be detected.

In addition to the inserted G, we have also identified four SNPs that are shown as bolded and enlarged R or Y in the sequence depicted in Figure 4A for the wild type *drd1* COL9A3 cDNA sequence, where the R indicates a purine (alleles are A or G for the first, second and fourth SNPS shown as R) and Y indicates a pyrimidine (alleles are C or T for the third SNP shown as Y). In CFA24 chromosomes that have the *drd1* mutation, we have determined the alleles present for the 4 SNPs are: G, G, T, A (in haplotype order). These are shown in bold and enlarged in the sequence depicted in Figure 4B. However, this haplotype is also observed in normal dogs.

As noted above, the *drd1* mutation creates a +1 translational frameshift and a premature stop codon (TGA) relative to the normal coding sequence. This results in a predicted protein that is altered relative to the normal protein. The predicted altered protein is shorter and has a predicted amino acid sequence that is different from that of the

predicted normal drd1 protein. Therefore, the presence of the *drd1* mutation could be determined by detecting this altered protein. Similarly, the *drd2* mutation also results in a sequence encoding a predicted protein that is altered via a predicted different and shorter amino acid sequence relative to the predicted normal protein. Therefore, the presence of the *drd2* mutation could also be determined by detecting this predicted altered protein. Detecting either predicted altered protein would be indicative that the dog is not normal. Such altered proteins could be detected using any conventional technique, such as by immunodetection methods, including but not limited to immunohistochemistry, Western blotting, ELISA, and fluorescent in situ hybridization (FISH).

The biological sample tested in the method of the invention can be any biological sample that contains nucleic acids or protein. For example, a sample of blood, hair, spleen, mucosal scrapings, semen, tissue biopsy, saliva or the like can be used. In one embodiment, the biological sample is blood. Suitable collection techniques for obtaining biological samples from dogs are known in the art.

Techniques for isolating and preparing nucleic acids in a form that is suitable for testing for OSD mutations are well known. Nucleic acids for use in testing for OSD mutations may be tested directly using any suitable method, or may be amplified before testing using a variety of techniques that are well known. For example, genomic DNA or mRNA may be amplified through use of PCR or RT-PCR, respectively (Saiki et al. Science 239:487-491 (1988)). Other suitable in vitro amplification methods include the ligase chain reaction (LCR) (Wu and Wallace Genomics 4:560-569 (1989)), strand displacement amplification (SDA) (Walker et al. PNAS USA 89:392-396 (1992)), and the self-sustained sequence replication (3SR) (Fahy et al. PCR Methods Appl. 1:25-33 (1992)).

Detecting the presence or absence of an OSD mutation in nucleic acids can be accomplished by a variety of methods. Such methods include but are not limited polymerase chain reaction (PCR), hybridization with allele-specific oligonucleotide probes (Wallace et al. Nucl Acids Res 6:3543-3557 (1978)), including immobilized oligonucleotides (Saiki et al. PNAS USA 86:6230-6234 (1989)) or oligonucleotide arrays (Maskos and Southern Nucl Acids Res 21:2269-2270 (1993)), allele-specific PCR (Newton et al. Nucl Acids Res 17:2503-25 16 (1989)), mismatch-repair detection (MRD) (Faham and Cox Genome Res 5:474-482 (1995)), denaturing-gradient gel electrophoresis (DGGE) (Fisher and Lerman et al. PNAS USA 80:1579-1583 (1983)), single-strand-conformation-

polymorphism detection (Orita et al. Genomics 5:874-879 (1983)), chemical (Cotton et al. PNAS USA 85:4397-4401 (1988)) or enzymatic (Youil et al. PNAS USA 92:87-91 (1995)) cleavage of heteroduplex DNA, methods based on allele specific primer extension (Syvanen et al. Genomics 8:684-692 (1990)), genetic bit analysis (GBA) (Nikiforov et al. Nuci Acids Res 22:4167-4175 (1994)), the oligonucleotide-ligation assay (OLA) (Landegren et al. Science 241:1077 (1988)), the allele-specific ligation chain reaction (LCR) (Barrany PNAS USA 88:189-193 (1991)), dynamic allele-specific hybridization (DASH), microplate array diagonal gel electrophoresis (MADGE), Pyrosequencing™, any of various DNA "chip" technologies, such as those offered by AFFYMETRIX (Santa Clara, California), Polymorphism chipsgap-LCR (Abravaya et al. Nucl Acids Res 23:675-682 (1995)), and radioactive and/or fluorescent DNA sequencing using standard procedures well known in the art.

In one embodiment, amplification of genomic DNA for use in testing for an OSD mutation is performed by PCR. For this embodiment, PCR primers and a method of using the primers in amplification reactions are provided such that different amplification products are observed when DNA is amplified from affected, *drd1* mutation carrier or *drd2* mutation carrier, or normal dogs. It will be recognized by those skilled in the art that while particular sequences of PCR primers are provided herein, other PCR primer sequences can be designed to detect the presence or absence of an OSD mutation.

When PCR primers are used such that the amplification products are of distinct sizes, the amplification products can be analyzed by standard methods such as electrophoretic separation and detection using ethidium bromide and ultraviolet light, or any other suitable detection method. Alternatively, amplification products can be isolated and sequenced using any of a variety of techniques.

The method of the present invention can be carried out for any breed of dog. In general, dogs known to be affected with OSD include Labrador retrievers and Samoyeds. However, any other breed of dog, including mixed breeds, may be tested according to the method of the invention. Some non-limiting examples of dog breeds that could be tested in the method of the invention include Akita, American cocker spaniel, American eskimos, Australian cattle dog, Australian stumpy tailed cattle dog, basenji, Bernese mountain dog, border collie, Chesapeake bay retriever, Chinese crested, English cocker spaniel, English mastiff, English springer spaniel, Entlebucher mountain dog, Finnish lapphund, German

shorthaired pointer, giant schnauzer, Havanese, lowchen, miniature poodle, miniature schnauzer, Nova scotia duck tolling retriever, Portuguese water dogs, silky terrier, spitz, standard poodle, standard wirehaired dachshund, Tibetan terriers and toy poodle.

Also provided in the present invention are kits for detecting the presence or absence of an OSD mutation in a biological sample from a dog or a nucleic acid sample extracted from the biological sample. The kits of the present invention comprise reagents for nucleic acid based detection of the presence of an OSD mutation. In one embodiment, the kits comprise reagents for extraction/preparation of nucleic acid samples and pair(s) of specific primers for identification of OSD mutations.

The presently provided OSD mutation tests will allow diagnosis of dogs of any age, such as a fetal dog (in utero), neonatal dogs, adult dogs, etc., and will eliminate the false positives and false negatives that have complicated previous identification of the OSD status of dogs. Accordingly, by using the tools and method described herein, dogs which are genetically OSD normal, *drd1* mutation carriers or *drd2* mutation carriers, or dogs affected with OSD, can be identified and selected for breeding. It is preferable to select dogs that are normal for OSD for mating. However, the method also permits removal of carrier or affected dogs from a breeding stock. Alternatively, dogs which are heterozygous for an OSD mutation can be mated with genetically normal dogs to ensure the absence of dogs affected with OSD in the litter.

The invention will be further understood by the following examples, which are intended to be illustrative and not restrictive in any way.

EXAMPLE 1

This Example illustrates discovery of the *drd2* mutation and presents particular embodiments of the invention that are useful for detecting the presence or absence of the *drd2* mutation.

The CanFam2 May 2005 dog whole genome shotgun assembly v2.0 (available at genome.ucsc.edu/cgi-bin/hgGateway) identifies 30 predicted exons of the canine homolog of the human COL9A2 gene, but fails to predict homologs of human exons 1 and 26. The earlier (July 2004) CanFam1 assembly does identify a predicted canine exon 1 homolog, but comparisons between the 2004 (CanFam1) and 2005 (CanFam2) assemblies identified

sequence inconsistencies between them in the interval corresponding to the 5' end, exon 1 and part of intron 1 of the predicted canine COL9A2 gene.

We designed primers based on the 2004 assembly to amplify overlapping cDNA fragments to cover the complete coding sequence of the canine COL9A2 by RT-PCR (Table 1).

Shown in Table 1 are primer sequences used to amplify the complete coding region of COL9A2 (primer pairs 1 to 10), as well as to amplify COL9A3 (primer pairs 11 to 21). Also shown are the sequences of primer pairs used to retrieve the correct 5' end sequence of the Col9A2 gene. The primers were used to amplify cDNA from retina samples obtained from normal dogs, and dogs homozygous for the *drd2* mutation which were therefore affected with OSD. These normal and affected dogs were members of a controlled breeding colony we established. Briefly, the colony was established from a purebred Samoyed affected with OSD (a dog that was homozygous for the *drd2* mutation). This dog was bred to homozygous normal Irish-setter dogs and the heterozygous F1 progeny were then backcrossed to dogs homozygous for the *drd2* mutation (affecteds) or intercrossed to *drd2* mutation carriers to yield litters segregating the *drd2* phenotype. Five related three-generation families from this colony (two intercrosses and three backcrosses), which included 63 progeny, were studied.

Table 1.

Pair	Forward primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence
A. Col9A2 primers used to amplify the coding sequence.				
1	COL9A2_5UTR_F4	ccgccccgtccgagagcagc (SEQ ID NO:8)	COL9A2exon4R	gcttcccatcaggcccatctgg (SEQ ID NO:9)
2	COL9A2exon1F	gagcctccgccgcccgcatg (SEQ ID NO:10)	COL9A2exon4_5R	gctccagttagaccatcaatcc (SEQ ID NO:11)
3	COL9A2rg1F(exon1_2)	ctggcgcagatcagaggt (SEQ ID NO:12)	COL9A2rg1R(exon10_11)	cagttggtcggacacaagaa (SEQ ID NO:13)
4	COL9A2exon2F	cctggatccgacggcatcgac (SEQ ID NO:14)	COL9A2exon7_8R	gtggtccaggaggtccagcaaag (SEQ ID NO:15)
5	COL9A2exon4_5F	tgggattgatggtctaactgg (SEQ ID NO:16)	COL9A2exon22R	gggtccgatttctccttgag (SEQ ID NO:17)

6	COL9A2exon21F	ctccctggattctctggtcc (SEQ ID NO:18)	COL9A2exon26R	gcccttctcgcctttctctc (SEQ ID NO:19)
7	COL9A2exon24F	cttgccaggcatcaagggagac (SEQ ID NO:20)	COL9A2exon31R	ggtcacccttctctccacgtttt (SEQ ID NO:21)
8	COL9A2exon24_25F	atcaagggagacaagggcttcc (SEQ ID NO:22)	COL9A2exon30R2	ctcacagcgacctctgccagt (SEQ ID NO:23)
9	COL9A2exon29F	cgaccagcacatcgtgaccgt (SEQ ID NO:24)	COL9A2exon32R	tccttgccgttgattgcctg (SEQ ID NO:25)
10	COL9A2exon30_31F2	ccaagggaaaacgtggagaga ag (SEQ ID NO:26)	ColA2_3UTR_R2	gaaagctggcttcctggtctgag (SEQ ID NO:27)
B. Col9A3 primers used to amplify the coding sequence.				
11	COL9A3_5UTR_F	gcgcgcagagccgctgagag (SEQ ID NO:28)	COL9A3exon8R	attccaccggggacaccact (SEQ ID NO:29)
12			COL9A3rg1R(exon10)	ggaggacccctgggtgac (SEQ ID NO:30)
13			COL9A3exon12R2	cgacctctccttgatctcctt (SEQ ID NO:31)
14	COL9A3rg1F(exon1_2)	gcagaaagtgggacctcaag (SEQ ID NO:32)	COL9A3exon8R	attccaccggggacaccact (SEQ ID NO:29)
15			COL9A3rg1R(exon10)	ggaggacccctgggtga (SEQ ID NO:30)c
16	COL9A3exon4F	aagccagggaagccaggaga (SEQ ID NO:33)	COL9A3exon8R	attccaccggggacaccact (SEQ ID NO:29)
17			COL9A3rg1R(exon10)	ggaggacccctgggtgac (SEQ ID NO:30)
18	COL9A3exon8F	agaggcaggagagagtggtgt (SEQ ID NO:34)	COL9A3exon12R	cttgccgacctctccttgat (SEQ ID NO:35)
19			COL9A3exon25_26R	aacgccctggtctcccttag (SEQ ID NO:36)
20	COL9A3exon20_21F	gaagggagaactgctggagag (SEQ ID NO:37)	COL9A3exon29_30R	gatttgttcgctgagcatcc (SEQ ID NO:38)
21	COL9A3exon26F	ctggggacaaaggagagctg (SEQ ID NO:39)	COL9A3_3UTR_R2	cccgaggtacgatgttagagc (SEQ ID NO:40)
C. Col9A2 primers used to amplify genomic DNA.				
22	Col9A2_5UTR_F2	tgtggtaggctttcctgaaga (SEQ ID NO:41)	A2i2R2	cccgacccaggttagagact (SEQ ID NO:42)
23	Col9A2_5UTR_F3	aaaagcctgacctcctagctg (SEQ ID NO:43)		

24	Col9A2_5UTR_F7	ccggttctgtgctgctaagccag (SEQ ID NO:44)		
25	A2_exon1_F	gagcctccgccgccccgcatg (SEQ ID NO:45)	A2int1R11	cgcctaacccgaaaggagcac (SEQ ID NO:46)
26	A2_exon1_F2	ctgctgctgctccaggggctc (SEQ ID NO:47)		
27	A2_F11	gcccggttctgtgctgctaag (SEQ ID NO:48)		
28	A2_exon1_F2	ctgctgctgctccaggggctc (SEQ ID NO:47)	A2int1R	gtgaatgggcaccattgtct (SEQ ID NO:49)
29	A2_F10	ccggttctgtgctgctaag (SEQ ID NO:50)	A2int1R10	cctaacccgaaaggagcac (SEQ ID NO:51)

Amplification of affected retina cDNA resulted in a lack of amplification product from PCR analysis using three primer pairs (primer pairs 1, 2 and 3 in table 1). This indicated that the genomic sequence at the 5' end of the gene in affected dogs is mutated, which results in a lack of amplification products. After retrieving the correct 5' end of the gene from a dog normal for OSD using primer pairs 22 to 29 in Table 1, a comparison of this region of COL9A2 to the *drd2*-affected (homozygous for the *drd2* mutation) dog revealed a 1,267 bp deletion in the affected dog-(illustrated in Figures 1 - 3).

To identify dogs that were normal for OSD, that were *drd2* mutation carriers, and that were affected with OSD via homozygosity for the *drd2* mutation, as well as to analyze co-segregation of the COL9A2 mutation with the disease in the Samoyed derived colony dogs, genomic DNA of 70 Samoyed-colony-derived dogs were amplified using two primer pairs (primer pairs 1 and 2 in table 2). Analysis of the resulting PCR amplification products showed complete co-segregation with the disease: all affected dogs were homozygous for the *drd2* mutation, all obligated carriers were heterozygous for the *drd2* mutation, and all known normal dogs were homozygous for the wild type allele.

Thus, this Example demonstrates the discovery of the *drd2* mutation and its association with OSD, and illustrates a method for detecting the presence or absence of this mutation in to ascertain the OSD status of dogs.

Example 2

Based on our discovery of the *drd2* mutation as set forth in Example 1, we designed another PCR based test. This test was performed on samples obtained from dogs obtained from the control colony described in Example 1. A pedigree for dogs analyzed in this Example is presented in Figure 5A.

The primers used in this Example are presented in Table 2 (primer pairs 1 and 2). The two primer pairs were used in separate PCR reactions (Table 2). Representative PCR amplification products obtained using primer pair 1 are shown in Figure 5B. Representative PCR amplification products obtained using primer pair 2 are shown in Figure 5C.

To obtain the PCR results presented in Figures 5B and 5C, 50-100 ng of genomic DNA were mixed with 12.5 ul of GoTaq green master mix (Promega, Catalog number M7123), 1.25 ul of DMSO (5% final concentration) and 10uM of forward and reverse primers in a final volume of 25 ul. The DNA was then denatured at 95^0C for 2 minutes and 35 cycles of 95^0C for 30 seconds, 58^0C (for primer pair number 1) or 60^0C (for primer pair number 2) for 30 seconds, 72^0C for 30 seconds were performed in a thermal cycler (MJ Research, Watertown, MA, USA). An additional final extension of 5 minutes at 72^0C was performed to ensure full-length products.

Primer pair number 1 in Table 2 produces a 1,445 bp product from amplification of wild type (normal) chromosomes while a smaller molecular weight product (178 bp) is observed from amplification of affected chromosomes (Figure 5B). Since in carrier dogs both alleles are present, this PCR reaction will usually produce only the smaller molecular weight product in carriers due to competition between the targets and the differences in size between the two amplicons. Therefore, a second PCR reaction can be performed using primer pair number 2 in Table 2. This primer pair yields a 504 bp product from amplification of wild type (normal) chromosomes and will not result in any product from affected chromosomes because the binding site for the reverse primer is located within the deletion (Figure 5C). PCR products obtained using this strategy to amplify DNA obtained from normal dogs, dogs that are *drd2* mutation carriers, and dogs that were affected with OSD via homozygosity for *drd2*. The PCR products were visualized on a 1.8% agarose gel and stained with Ethidium Bromide using standard protocols. A normal dog yielded either a faint 1,445 bp band in the reaction using primer pair number 1 or no visible band and a 504 bp band from PCR amplification using primer pair 2 (Figure 5A dog 13). A carrier dog

yielded a 178 bp band from PCR amplification using primer pair 1 and a 504 bp band using primer pair 2 (Figure 5A dogs 1,3,4,5,6,9,10,11,12). Amplification from an OSD affected dog (homozygous for the *drd2* mutation) yields only a 178 bp band from PCR amplification using primer pair 1 and no amplification when using primer pair 2 (Figure 5A, dogs 2,7,8).

Table 2.

	Primer Pairs				Expected Size		
#	Forward primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence	Expected size in wt	Expected size in carrier	Expected size in affected
1	COL9A2_ Part11F	gctgaccttgtggattttc tcc SEQ ID NO:52	COL9A2intron1R	gtgaatgggcacca ttgtct SEQ ID NO:53	1,445 bp	178 bp	178 bp
2	COL9A2_ Part10F	catctctccctcactccct cct SEQ ID NO:54	COL9A2_Part10R	tcacccctctcccag tctcttag SEQ ID NO:55	504 bp	504 bp	No band
3	COL9A3t est1F	gctgccactgggctcctt tcttcg SEQ ID NO:56	COL9A3test1R	agcaggagcaggg ccagcgtg SEQ ID NO:57	248 bp	248 bp and 249 bp	249 bp
4	COL9A3t est1F	gctgccactgggctcctt tcttcg SEQ ID NO:56	COL9A3test1R3	cgctcacatgcgcc ccggtc SEQ ID NO:58	298 bp	298 bp and 299 bp	299 bp
5	COL9A3_ AS_WF	ggcgcagccatggccg ggac SEQ ID NO:59	COL9A3_AS_R	ggtcagggtggcg gccaggagc SEQ ID NO:60	72 bp	72 bp	No band
6	COL9A3_ AS_AF	ggcgcagccatggccg ggcg SEQ ID NO:61	COL9A3_AS_R	ggtcagggtggcg gccaggagc SEQ ID NO:60	No band	73 bp	73 bp

Thus, this Example demonstrates an embodiment of the invention that is useful for determining OSD status by testing for the presence or absence of the COL9A2 *drd2* mutation.

Example 3

This Example demonstrates an embodiment of the invention using the PCR based method described in Example 2 to test unrelated Samoyeds (meaning the dogs had no parents or grandparents in common).

Fifty-five unrelated Samoyed dogs were screened for the presence or absence COL9A2 *drd2* mutation using this test. One Samoyed was found to be a *drd2* mutation carrier and is therefore considered to be a carrier of the *drd2* form of OSD. Another 126 dogs, considered to be normal for OSD based on phenotypic analysis, from 26 additional

breeds were screened for the deletion and all the dogs had the wild type (normal) genotype (Table 3).

Table 3.

Breed	Number of dogs	*drd2* mutation Carriers
American Cocker Spaniel	5	0
American Eskimos	10	0
American Pitbull Terrier	5	0
American Staffordshire Terrier	5	0
Australian Cattle Dogs	5	0
Basenji	1	0
Border Collies	5	0
Boxer	1	0
Chesapeake Bay Retriever	5	0
Chinese Crested	5	0
English Cocker Spaniel	5	0
English Springer Spaniel	7	0
Entelbucher Mountain Dogs	7	0
Glen Of Immal Terrier	6	0
Golden Retriever	5	0
Golden Retriever/Labrador Retriever Crosses	5	0
Labrador Retriever	5	0
Nova Scotia Duck Tolling Retriever	4	0
Samoyed	55	1
Papillon	7	0
Poodle	7	0
Portuguese Water Dogs	5	0
Tibetan Terrier	8	0
American Bulldog	2	0
Pomeranian	1	0
Corgi	4	0
Dachshund	1	0
Total	181	1

Thus, this Example demonstrates another embodiment of the invention that is useful for determining OSD status in dogs by testing for the presence or absence of the *drd2* mutation.

Example 4

This Example describes discovery of the *drd1* mutation and particular embodiments of methods for testing for the presence or absence of this mutation.

Our comparison of the 2005 CanFam2 dog sequence assembly to the human COL9A3 genomic sequence identified 29 predicted canine exons and failed to identify predicted exons homologous to human exons 16, 17 and 32. Based on this analysis, 12 primers (Table 1 primer pairs 11 to 21) were designed to amplify overlapping cDNAs obtained from one normal, and one affected dogs derived from Labrador retriever colony dogs to cover the complete coding region of the canine Col9A3. Briefly, the Labrador retriever colony dogs are descended from two unlreated purebred Labrador retrievers affected with OSD. These dogs were bred to homozygous normal unrelated Beagles, Beagle-crossbred dogs, Irish-Setter and Irish-setter-crossbreds dogs and poodle-crossbred dogs, and the heterozygous F1 progeny were then backcrossed to affected dogs or intercrossed to carriers of the *drd1* mutation to yield litters segregating the OSD phenotype. Eight related three-generation families from this colony (five intercrosses and three backcrosses), which included 68 progeny, were analyzed using 12 primers were in 11 different combinations of primer pairs to produce redundant overlapping fragments.

Our comparison of retinal cDNA amplification products from normal dogs and dogs homozygous for the *drd1* mutation revealed a one-base insertion (guanine) in the coding sequence (exon 1) that changes a string of 4 guanines (nucleotides 49,699,847-49,699,850 of canine chromosome 24 in the May 2005 CanFam2 assembly v2.0; <genome.ucsc.edu/cgi-bin/hgGateway>) to a string of 5 Gs. This mutation is indicated by the presence of the G at nucleotide position 11 in the nucleotide sequence depicted in Figure 4B (SEQ ID NO:4). The string of five Gs is as depicted in Figure 4B for nucleotides 7-11.

To identify *drd1*-affected (homozygous for the *drd1* mutation), *drd1* mutation carrier and normal dogs in the Labrador retriever derived colony, and to analyze co-segregation of the *drd1* mutation with the disease, genomic DNA of 80 colony dogs was amplified using the primer pairs COL9A3test1F and COL9A3test1R (Table 2, primer pair 3). Sequencing of the 248 bp amplicon with the forward primer identified normal animals (4 Gs) or affected animals (5 G's, 249 bp amplicon) while an overlapping chromatogram was observed in carrier dogs at the insertion point. All outbred normal dogs were determined to be homozygous wild type. All affected dogs were determined to be homozygous for the *drd1* mutation (5 Gs). All the obligated carriers were determined to be heterozygous for the *drd1* mutation (overlapping chromatograms; not shown). 30 unaffected dogs from intercrosses were genotyped as follows: 5 were homozygous normal (4 Gs) and 25 were

heterozygoug for the *drd1* mutation. These results accordingly showed a complete co-segregation of the *drd1* mutation with the disease in the colony.

In another test, we employed the primer pair COL9A3test1F and COL9A3test1R3 (Table 2, primer pair 4). Sequencing of the 298 bp product with the forward and the reverse primers identified normal sequence or affected sequence (299 bp) while an overlapping chromatogram was observed in *drd1* mutation carrier dogs after the insertion point (sequencing data not shown).

Thus, this Example demonstrates the discovery of the *drd1* mutation and its association with OSD, and illustrates a method for detecting the presence or absence of this mutation to determine the OSD status of dogs.

Example 5

This Example describes another embodiment by which the presence or absence of the *drd1* mutation can be determined. In particular, we designed an allele-specific PCR based test using primer pairs 5 and 6 presented in Table 2, namely: Forward (wild type primer): 5'-GGCGCAGCCATGGCCGGGAC-3' (COL9A3_AS_WF; SEQ ID NO:59); Forward (mutant primer): 5'-GGCGCAGCCATGGCCGGGCG-3' (COL9A3_AS_AF; SEQ ID NO:61); and Reverse primer: 5'-GGTCAGGGTGGCGGCCAGGAGC-3' (COL9A3_AS_R; SEQ ID NO:60).

DNA samples were obtained from normal, *drd1* mutation carrier and affected dogs from our control colony of Labrador retrievers. Each DNA sample was analyzed in separate PCR reactions, with primer pairs 5 (wild type forward and reverse primers) and 6 (mutant forward primer and wild type reverse primer). The reactions were performed in a total volume of 15 µl, containing 1xPCR buffer (Qiagen); 10-100 ng/µl total DNA; 0.3 µM forward (wild or mutant type) and reverse primers; 0.2 mM each of four dNTPs; 1.5 mM MgCl2; 17% DMSO and 0.375 units Taq-polymerase. The reaction mixture was overlaid with mineral oil. The enzyme Taq-pol (5 u/µl) is diluted 1:10 with water and 0.75 µl was added to the reaction in a "hot-start" manner: the enzyme was added after the denaturing step at 94C). The PCR cycles were: 1 cycle of 94C for 3 minutes; 32 cycles of 94C for 15 seconds, 58C for 30 seconds and 72C for 15 seconds. From each PCR amplification, 6-8 µl was analyzed by electrophoresis on a 6% PAGE in TBE buffer with ~1 mg/ml of ethidium bromide for staining. Representative photographs of electrophoretic separation of

amplification products obtained from using this protocol are presented in Figure 6, where M=mutant PCR (primer pair 6), W= Wild type PCR (primer pair 5). Samples 1, 2 and 6 are *drd1* mutation carriers and are therefore considered carriers of the *drd1* form of OSD, samples 3, 4 and 5 are affected with OSD, and samples 7, 8, 9, 10, and 11 are normal for OSD. The sizes of the amplification products are provided in Table 2.

Thus, this Example demonstrates another embodiment of the invention that is useful for determining OSD status in dogs by testing for the presence or absence of the *drd1* mutation.

Example 6

This Example provides an illustration of determining the presence or absence of the *drd1* mutation in unrelated dogs to determine OSD status.

In particular, 59 phenotypically normal unrelated Labrador retrievers (no parents or grandparents in common), and 19 unrelated Labrador retrievers with retinal folds were tested for the presence or absence of the *drd1* mutation. One dog with retinal folds was found to be homozygous for the mutation (affected). 78 dogs considered normal for OSD via phenotypic examination from 23 additional breeds were also screened and all 78 dogs were normal for the mutation (Table 4), meaning they were homozygous wild type for the *drd1* gene.

Table 4.

Breed	**Number of dogs**	*drd1* mutation carriers	**Affected** (homozygous for the *drd1* mutation)
American cocker spaniel	2	0	0
American Eskimos	3	0	0
Australian cattle dogs	6	0	0
Basenji	2	0	0
Border Collies	10	0	0
Boxer	1	0	0
Chesapeake bay retriever	4	0	0
Chinese crested	4	0	0
Collie	5	0	0
English cocker spaniel	8	0	0
Entelbucher mountain dogs	1	0	0
Glen of Immal terrier	2	0	0
Golden Retriever	4	0	0

Irish Setter	1	0	0
Labrador retrievers	78	0	1
mix	6	0	0
Nova scotia duck tolling retriever	4	0	0
Poodle	3	0	0
Portuguese water dogs	4	0	0
American Bulldog	2	0	0
Pomeranian	1	0	0
Corgi	4	0	0
Dachshund	1	0	0
Total	156	0	1

Thus, this Example demonstrates yet another embodiment of the invention that is useful for determining OSD status by testing for the presence or absence of the *drd1* mutation.

The invention has been described through specific embodiments. However, routine modifications to the compositions, methods and devices will be apparent to those skilled in the art and such modifications are intended to be covered within the scope of the invention.

We claim:

1. A method for determining whether a dog is normal for Oculo-skeletal dysplasia (OSD), a carrier for *drd1* form or *drd2* form of OSD, or affected with OSD, the method comprising the steps of:

determining, from a biological sample obtained from a dog, the presence or absence of:

i) mutation 1 identified as a G in position 11 as depicted in nucleotide sequence of SEQ ID NO:4; and/or

ii) mutation 2 identified as a deletion of 1,267 nucleotides between nucleotide number 230 and nucleotide number 1,498 in nucleotide sequence of SEQ ID NO:2,

wherein homozygosity for mutation 1 or mutation 2 is indicative that the dog is affected with OSD, heterozygosity for mutation 1 is indicative that the dog is a carrier for *drd1* form of OSD, heterozygosity for mutation 2 is indicative that the dog is a carrier for *drd2* form of OSD, and an absence of mutation 1 and mutation 2 is indicative that the dog is normal for OSD.

2. The method of claim 1, wherein identification of G in position 11 is carried out by determining the presence of 5 consecutive Gs in SEQ ID NO:4 starting at position 7.

3. The method of claim 1, wherein mutation 1 or mutation 2 is detected by analysis of polymerase chain reaction amplification product.

4. The method of claim 1, wherein the dog is a breed of dog selected from the group of dog breeds consisting of Akita, American cocker spaniel, American eskimos, Australian cattle dog, Australian stumpy tailed cattle dog, basenji, Bernese mountain dog, border collie, Chesapeake bay retriever, Chinese crested, English cocker spaniel, Samoyed, English mastiff, English springer spaniel, Entlebucher mountain dog, Finnish lapphund, Labrador retriever, German shorthaired pointer, giant schnauzer, Havanese, lowchen, miniature poodle, miniature schnauzer, Nova scotia duck tolling retriever, Portuguese water dogs, silky terrier, spitz, standard poodle, standard wirehaired dachshund, Tibetan terriers and toy poodle.

5. The method of claim 1, wherein the biological sample is selected from the group consisting of blood, hair, mucosal scrapings, semen, tissue biopsy and saliva.

6. The method of claim 5, wherein the biological sample is collected from a fetal dog, a neonatal dog, or an adult dog.

7. The method of claim 1, further comprising selecting a dog for breeding, wherein a dog selected for breeding exhibits an absence of mutation 1 and mutation 2, or is heterozygous for mutation 1 or mutation 2 and is selected for breeding with a dog that exhibits an absence of mutation 1 and mutation 2.

8. The method of claim 1, further comprising communicating to an individual a result obtained from determining the presence or absence of mutation 1 and/or mutation 2.

9. A method for determining whether a dog is a carrier for *drd1* form of Oculo-skeletal dysplasia (OSD) or is affected with OSD, the method comprising the steps of determining, from a biological sample obtained from a dog, the presence of a G in position 11 of the nucleotide sequence depicted in SEQ ID NO:4,

wherein heterozygosity for the presence of the G in position 11 of the nucleotide sequence depicted in SEQ ID NO:4 indicates that the dog is a carrier of *drd1* form of OSD, and homozygosity for the presence of the G in position 11 of the nucleotide sequence of SEQ ID NO:4 indicates that the dog is affected with OSD.

10. The method of claim 9 wherein upon determination of heterozygosity for the presence of the G in position 11 of the nucleotide sequence depicted in SEQ ID NO:4, a further step of determination of status of *drd2* form of OSD is carried out by detecting the presence or absence of a deletion of 1,267 nucleotides between nucleotide number 230 and nucleotide number 1,498 of nucleotide sequence of SEQ ID NO:2.

11. The method of claim 9, wherein the dog is a Labrador retriever.

12. The method of claim 9, wherein determining the presence of the G in position 11 of the nucleotide sequence as depicted in SEQ ID NO:4 comprises determining 5 consecutive Gs starting at position 7 of SEQ ID NO:4.

13. The method of claim 9, wherein determining the presence of the G in position 11 of the nucleotide sequence as depicted in SEQ ID NO:4 is carried out by polymerase chain reaction.

14. A method for determining whether a dog is a carrier for *drd2* form of Oculo-skeletal dysplasia (OSD) or is affected with OSD, the method comprising the steps of determining, from a biological sample obtained from a dog, the presence of a deletion of 1,267 nucleotides between nucleotide number 230 and nucleotide number 1,498 of the nucleotide sequence as depicted in SEQ ID NO:2;

wherein heterozygosity for the presence of the deletion indicates that the dog is a carrier for *drd2* form of OSD and homozygosity for the presence of the deletion indicates that the dog is affected with OSD.

15. The method of claim 14, wherein the dog is a Samoyed.

16. The method of claim 15, wherein the presence or absence of the deletion is detected by analysis of polymerase chain reaction amplification product.

17. The method of claim 15 wherein upon identification of the deletion, a further step of determining a status of *drd1* form of OSD is carried out by detecting the presence or absence of a G in position 11 of the nucleotide sequence depicted in SEQ ID NO:4.

Figure 1. *(SEQ ID NO:1)*

```
   1 cctgtcagct tacttcctat aaactccaaa ttcctcctcg gtccattagg cagagcaatc
  61 aaattctccc aaattcagct ccaggaacac ctctcaaagc ccagggccct ttgctcacaa
 121 tgccacgcgg ggttggactt tcccaggctc cagaggggtt tctcctggac tattttagct
 181 gaccttgtgg attttctccc ccatgctggg cacaggactg gtaaacaaag tatattctcc
 241 ataaatcata atgaaagctg aaatttatga agcccctact acttcatatg aagtacttag
 301 catcatctca ttcaatcttc acaaaaattc tataagatag gattattatt atccccttt
 361 acagaggagg acactatatg gtttcaggtg cctgtggtca ccgttagcga cagattccaa
 421 agcacttcac caacattgct taagggaagg aaaagggagc taagagactg ggagaggggt
 481 gatgctggac aaacggggga gggggagtgc ttcatggagg aggtcacccc gagggatacc
 541 tagagactct ggtgcttcaa gaagctgcag tgaagaaaag gcaaggtgag gagaaggggg
 601 aggaggagga ggaggaagag gaggaggtgg aggaggagga ggaggacgct ggacggtggg
 661 ggcctgggca gggaggcttc aaggagagca tggccaaggg cggctccagg agcaacagga
 721 gcttcagtgc ttccgggggc actaagaggg cctggggctc gctggtggcc tccaaaagcc
 781 tgacctccta gctgcggcgg gagcagggag gtgagaggga gatgaggggg tctggaggtg
 841 cggcacgggg tgcatggggg ggctcccggg accgccgacc cgtaaggctt gtggtaggct
 901 ttcctgcaga agaggcctgc cggtctgtgc tgctaagcca ggtgggtggg ggcctctgcg
 961 aactggggtg caggacccgg gcggtggggg cctctgcgac ctggggtgca ggacccacgg
1021 ctcgctctcg gcctgcgccc tggagcagag gagcgacggg ccgaggctcg gcccggggtc
1081 aggctgcagg ggcccccccg acggccggcg cctcccctcg ggggcagagg gtgggcgctg
1141 gctgcggcgc cgggcggggc ggagctgcgc gggcctcggc ccctcggccg ccctctcggg
1201 tgacggcccc gcggcccccg ccccgccccg ccgcatattc aggcgcccgc ccgcccgccc
1261 cgtccgagag cagcccgcgc cgcccgcccg cccgagcctc cgccgcccgc ATGGCCGCCG
1321 CCCGCCGCCT CCTGCTGCTG CTCCAGGGGC TCGCGCTCGC CCTGGCGCAG ATCgtaagtg
1381 ccgcggccgc cgcgcggggt cggggtgcgg ggggcggggg cggggacggg tcggggtgcg
1441 ggcctcggcg cttcccctcg gcggggcgca cagcgggcgc gtccgggcgc cgcgagggag
1501 gcggaggggc ccggggagcc agggggggccc tgtccggagg cgcgcggccc ggcctgggac
1561 aggcggcgcg gccgggagcc ccgagagccc gggtggcggg cgagacaatg gtgcccattc
1621 acggggctcc ggtgcccsgg cgccgcctcc ccccgggggg ctccagccag acgccgctcg
1681 gccccgctgc cgggggccac cggggcgcgg ggacccaccg ggaccggccc gggcgccaag
1741 cctggcacct cgctgcgcgg agggcgcggg cccggggcgg gggggggggc tgccccgcgg
1801 agggcaagcc aacgggaaac ggaggggctc cggggagggg cgcccccccgc gtgctccttt
1861 cgggttaggc gcgccaggcg caggggggcag ggcacccgct caccgtcggt acctgtcgtg
1921 cgagcgagcg gtcccggagc ccgccgtcgc ctgcccgctg ggacccgcgg cgccccrgtc
1981 tcccgctccg tccaccgccc tccgccctcc gccctccgcc ctccgcgacc gcgcacgggc
2041 cccgcgggcc agcgcaccgg gacgcgctcc gcgctcgctc tccgggagcc gggtccccccc
2101 gcggccaacc cggggccgcg cctcgggtcc tgagcgccgg gaggggcacc cagggcgccg
2161 cacagtcggt tccccgtgag cgcgccgcgc cccgggggggg ctctccgcag ggacccccgc
2221 cccctgcacc gtctgagggc ctccccctgt gtgtgttctc agAGAGGTCC GCCCGGAGAA
2281 CCGGGGCCCC CGGGCCCCCC GGGGCCGCCG GGAGTGCCTG GATCCGACGG CATCGACgt
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Figure 2. *(SEQ ID NO:2)*

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aatgccacgcggggttggactttcccaggctccagaggggtttctcctggactattttagctgaccttgt
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Figure 3. *(SEQ ID NO:3)*

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CAAACCCGGGCCAGATGGGCCTGATGGGAAGCCTGGGATTGATGGTCTAACTGGAGCCAAGGGGGAGCCTGGCCCCA
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Figure 4A. *(SEQ ID NO:5)*

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GGGCCCACCGGCTACAAAGGAGATCAAGGAGAGGTCGGCAAGGACGGCGAGAAGGGCGATCCTG
GCCCCCCTGGGCCCGCTGGCGTCCCTGGCTCTGTGGGGCTGCAGGGGCCTCGGGGACTCCGAGG
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GCCGGTCGCAACGGTGCCCCAGGAGAGAAGGGTCCCAACGGGCTGCCGGGCCTCCCGGGTCGAG
CAGGGTCCAAGGGCGAGAAGGGAGAACTGGGCCGAGCTGGAGAGCTGGGGGAGGCTGGCCCCTC
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CCTCCAAGGACCACAAGGCGTGCCTGGCGTTAGCAAAGACGGCCGCGACGGGGCCAACGGCGAG
CCCGGGCCTCCAGGCGATCCTGGCCTCCCCGGTGCTGTGGGTGCTCAGGGGACACCCGGCATCT
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Figure 4B. *(SEQ ID NO:4)*

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AGAGGGGCCCAGAGGGTTTCCGCGGCCCCAAGGGTGACCTTGGCAGACCCGGCCTCAAGGGA
GTCCCCGGGATGGCCGGGCCGGGCGGGGAGCCGGGAATGCCAGGCAAGGACGGCCGGGATGG
CGTGCCGGGACTGGACGGCGAGAAGGGAGAGGCCGGTCGCAACGGTGCCCCAGGAGAGAAGG
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TGCGACACCTCGGCCTGCCAAGGAGCTGTGATGGGAGGCGGCGGGGAAAAGTCAGGTTCTAG
AAGCTTCTAA

Figure 4C. *(SEQ ID NO:6)*

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Figure 4D. *(SEQ ID NO:7)*

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Figure 5A.

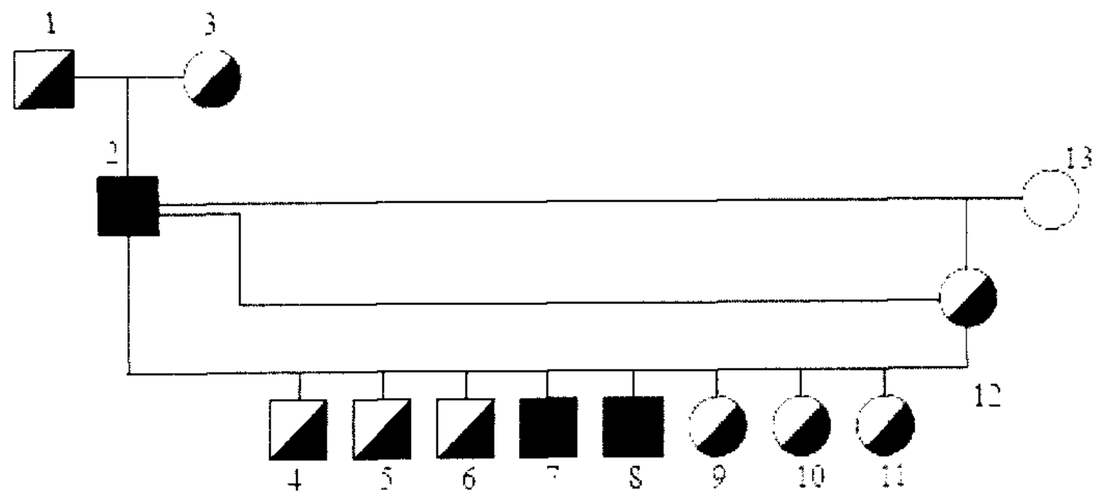

Figure 5B.

Figure 5C.
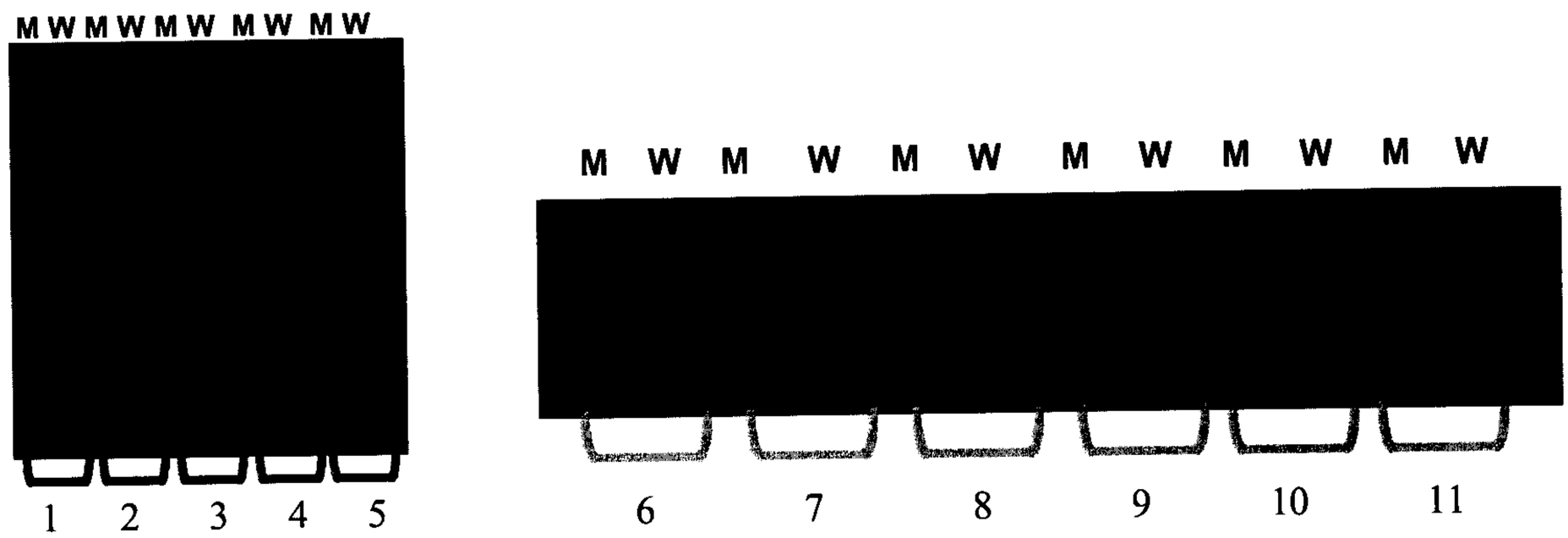
Figure 6.