Research project september 2009- july 2010

Evaluation of different candidate genes as a cause of chondrodysplasia in Labrador Retrievers and Bouviers des Flandres

Drs. Cathelijn Quirine Hoelen

Utrecht University, Faculty of Veterinary Medicine
Department of Clinical Sciences of Companian animals

Supervisors
Dr. P.A.J. Leegwater
Prof. Dr. H.A.W. Hazewinkel

Date: 22-07-2010
Email-address: C.Q.Hoelen@students.uu.nl

Abstract

Chondrodysplasia is characterized by a disturbed cartilage growth, endochondral ossification or both, leading to short stature. Besides disproportionately short front legs there is malformation of the elbow joint and carpus, which reduces the range of motion and ultimately leads to osteoarthrosis of the joints. It is a hereditary disease but the mode of inheritance is still unknown. In the Bouvier des Flandres a genetically interesting phenomenon exists in that several litters are completely affected, while both parents are healthy. The well-being of affected animals is often severely compromised, making it essential to diminish the prevalence of this disease through specific breeding advice. The aim of this study was to investigate the role of candidate genes as cause of chondrodysplasia in the Labrador Retriever and Bouvier des Flandres. In 2009 a fibroblast growth factor retrogene was demonstrated by Parker et al. to be strongly associated with chondrodysplasia in chondrodysplastic breeds, like the Basset Hound and Pembroke Welsh Corgi. However, here it was found that affected Labradors (n=13) and Bouviers (n=24) of the research population did not exhibit the specific retrogene. Linkage analysis using microsatellite markers was then conducted on a number of candidate genes. Based on LOD-scores, most of these candidate genes were excluded or were highly unlikely to play a role in this disease. Bouvier patients display an extraordinary phenotype that differs from that of Labrador patients. It resembles the phenotype of humans with Leri-Weill dyschondrosteosis and Langer Mesomelic dysplasia, two types of chondrodysplasia caused by mutations or deletions of the SHOX-gene. Therefore, the SHOX gene was denominated as an important candidate gene and was investigated, performing linkage analysis, gene sequencing and determination of the degree of CpG-methylation to check for epigenetic silencing. Further research of the SHOX-gene needs to be conducted in the future.

Author keywords: chondrodysplasia, short stature, endochondral ossification, fgf4 retrogene, *FGF4*, *FGFR3*, *IHH*, *ILK*, *NPR2*, *DDR2*, *SPRED2*, *SHOX*, linkage analysis, gene sequencing, downstream enhancer element, CpG-methylation, X-inactivation, CGG-repeat, SNPs, canine, human.

Introduction

Endochondral ossification is a process of bone formation and elongation which takes place at the epiphysial growth plates of long bones. The growth plate is a cartilaginous tissue consisting of one cell type, the chondrocyte. Chondrocytes are aligned in columns and subsequently progress from their resting stage through the proliferative, maturation, and hypertrophic stage. Hypertrophic chondrocytes eventually undergo apoptosis and the resulting blank spots are invaded by vascular structures and osteoblasts. Bone marrow arises and mineralization takes place resulting in the formation of trabecular bone (Naski et al., 1998; Newman, Wallis, 2003; Mundlos, Olsen, 1997; Munns et al., 2004). A delicate balance exists between all steps of endochondral bone formation, making a tight regulation crucial. Regulating factors in longitudinal bone growth, shown in figure 1, can be subdivided in systemic factors like growth hormone, thyroid hormone, estrogens, and androgens and locally produced factors for instance fibroblast growth factors (FGF), parathyroid hormone related peptides (PTHrP), bone morphogenetic proteins (BMP), and hedgehog proteins like Indian hedgehog (IHH) (Naski et al., 1998). Besides different molecular factors, numerous environmental factors seem to contribute to bone formation.

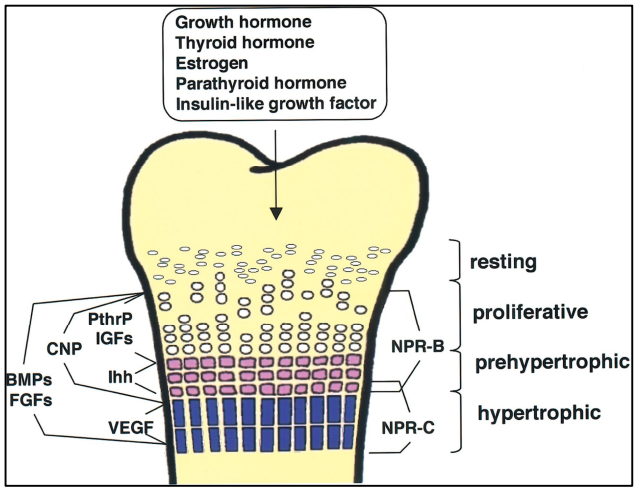


Figure 1.

Schematic illustration of endochondral ossification with important regulators. (Reprinted from The American Journal of Human Genetics, 75, Bartels et al., Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux, 27-34, copyright 2004, with permission from Elsevier.)

Skeletal dysplasia in humans refers to a diverse category of more than 150 heritable disorders affecting two to five per 10.000 live born (Rasmussen et al., 1996; Spranger, J., 1992). These

disorders can be subdivided in two main groups: osteodysplasia and chondrodysplasia (Dreyer et al., 1998). The former is characterized by increased bone deposition or decreased bone mineral density, whereas the latter is characterized by a disturbed cartilage growth, endochondral ossification or both, leading to short stature. Premature ossification of the physes impedes longitudinal growth leading to dwarfism (Munns et al., 2004; Newman, Wallis, 2003). Many types of human chondrodysplasia exist, of which achondroplasia is most frequently seen (Richette et al., 2008; Shiang et al., 1994). They differ in grade of severity, for example ranging from a sometimes nearly undetectable hypochondroplasia to inevitable perinatal lethality as is the case in thanatophoric dysplasia type I and II (Horton, 1997; Horton, Lunstrum, 2002). Chondrodysplasia is also frequently seen in dogs and characterizes the group of chondrodysplastic breeds, like the Basset Hound, Dachshund, Pembroke Welsh Corgi and Norwich Terrier, which are considered to be fixed for chondrodysplasia because there is no segregation of the phenotype (Parker et al., 2009, Young et al., 2006; www.raadvanbeheer.nl; www.nbhc.nl). Furthermore, chondrodysplasia has been reported to occur within non-chondrodysplastic breeds, like the Labrador Retriever, Bouvier des Flandres, Alaskan Malamute and Samoyeed (Brocks, Hazewinkel, 2004; Hazewinkel, unpublished observations; Meyers et al., 1983; Newton, Nunamaker, 1985; Sande et al., 1982; Smart, Fletch, 1971; Smit et al., 2009) This disease is believed to be hereditary but at present it is unclear whether it is a monogenic or polygenic disease and the mode of inheritance is still unknown. It is characterized by a disorganized and irregular cartilage growth and endochondral ossification which can be revealed by microscopy. Dogs clinically show short front legs with radius curvus syndrome, depicted in figure 2 (Temwichitr et al., 2009). Patients have an aberrant gait and osteoarthrosis develops, leading to a diminished wellbeing.

Figure 2. Labrador pups with chondrodysplasia

Labrador pups of 3,5 (L) and 9 (R) months old. Notice the curving of the lower front legs and the valgus deformity of the carpus.

In the past decades, much research has been conducted in man and dog aiming to find the cause of different types of chondrodysplasia. A whole range of candidate genes that play an important role in endochondral ossification have been studied (Kant et al.,2003). For example, genes coding for collagen type II, IX, X, and XI have been investigated in Labrador Retriever patients, because they contribute to a major part of the extracellular matrix of the physes of long bones and mutations of these genes often cause chondrodysplasia in humans. Furthermore, *COMP*, *MATN3* and *SLC26A2* were investigated by the same research group, but in dogs all these genes appear not to be involved in the disease (Young et al., 2006; Smit et al., 2009).

Another type of chondrodysplasia exists, in which dogs with chondrodysplasia share an extraordinary phenotype with litter mates, but have healthy parents. This type of chondrodysplasia is, in addition to the Bouvier des Flandres, known to exist in several dog breeds (including the Labrador Retriever, Alaskan Malamute and Samoyeed) and also in the cat (Brocks, Hazewinkel, 2004; Hazewinkel, unpublished observations; Meyers et al., 1983; Newton, Nunamaker, 1985; Sande et al., 1982; Smart, Fletch, 1971). Temwichitr described in detail the clinical and radiological appearance of Bouviers with this type of chondrodysplasia (Temwichitr et al., 2009). The most remarkable feature is mesomelic shortening, in which the middle part of the front legs, id est radius and ulna, is disproportionally short. There is marked curving of the radius, known as Madelung deformity, and valgus deformity of the carpi. The radial head is caudolaterally (sub-) luxated and a cartilage core is present in the distal radial and ulnar metaphyses. Figures 3 and 4 give an impression of the phenotype and radiological characteristics of these animals. Malformation of the elbow joint reduces the range of motion and ultimately leads to osteoarthrosis of the joint. The well-being of the animal is severely compromised and euthanasia sometimes is inevitable.

Figure 3.
Bouvier with a specific type of chondrodysplasia.
The picture shows noticeable dwarfism, bowing of the lower front legs and valgus deformity of the carpi.

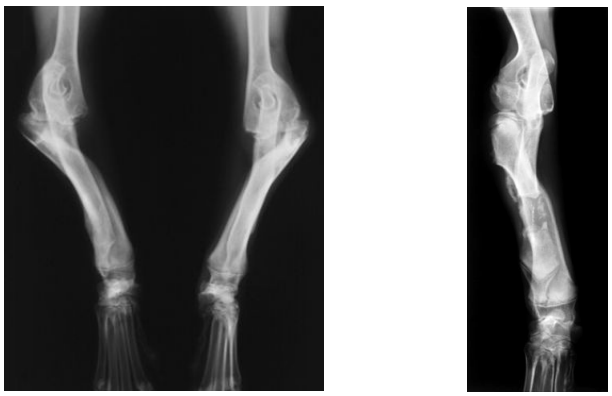


Figure 4.

Radiographs of the lower front legs of a Bouvier with a specific type of chondrodysplasia. Notice the bowing of the radius and the (sub)luxation of the radial head.

Considering the distress that the animals suffer from, it is essential to diminish the prevalence of this disease through specific breeding advice. In Bouviers with this specific type of chondrodysplasia, affected litters were all out of normal parent dogs and appeared suddenly in the population (figure 5). Therefore, genetic screening of dogs at risk before entering any breeding program is necessary. This is only possible when the genetic cause and the inheritance pattern of the disease are fully understood. As a consequence, the objective of this research was to evaluate certain candidate genes as a cause of chondrodysplasia in Labrador Retriever, Bouvier des Flandres or both and to develop a genetic test based on the causative mutation.

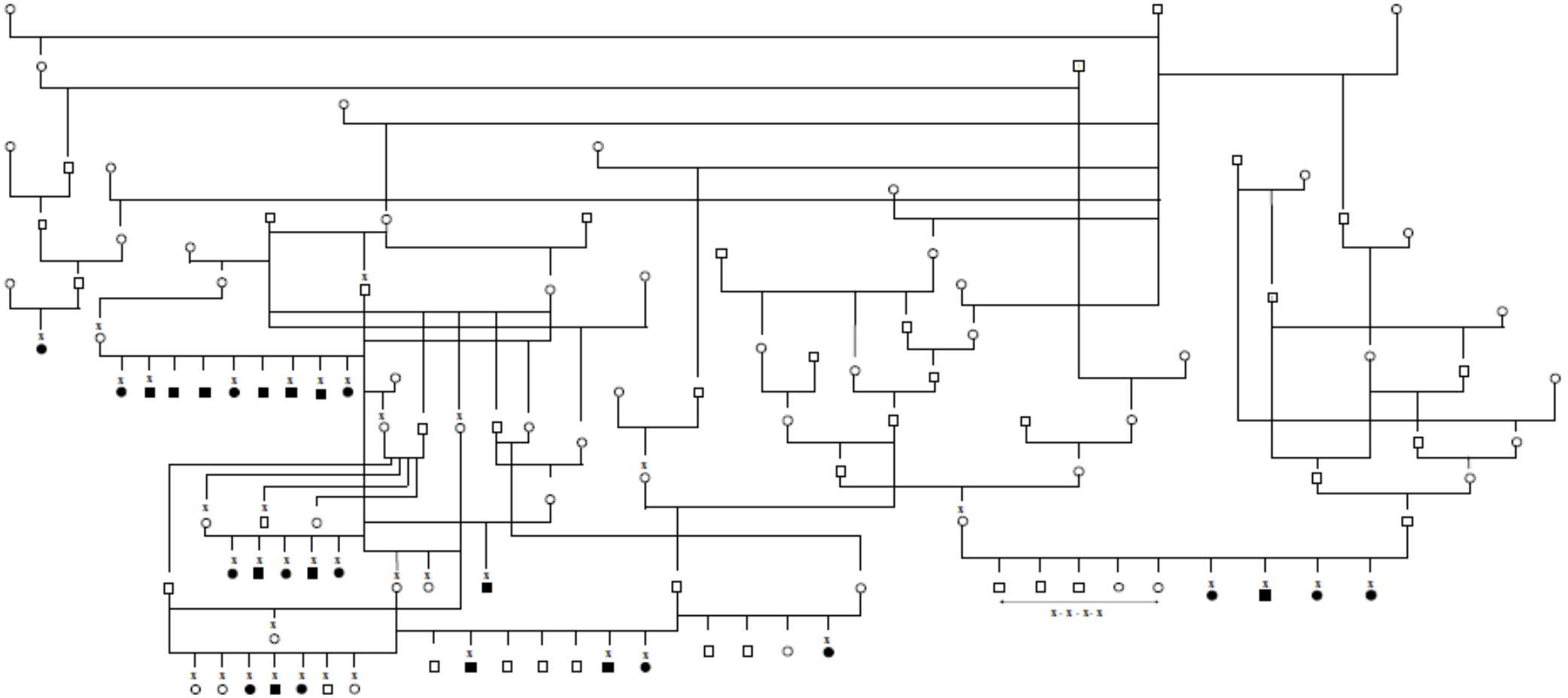


Figure 5. Pedigree of Bouviers des Flandres

Squares are males and circles are females.

Open and filled symbols are healthy and affected animals, respectively.

A cross means DNA is available at the Utrecht University Clinic for Companion Animals

1. fgf4 retrogene

Recently it was described that a retrogene of fibroblast growth factor 4 (*FGF4*) is strongly associated with chondrodysplasia in aforementioned 'chondrodysplastic' breeds (Parker et al., 2009). This retrogene, which is expressed, is a cDNA copy of *FGF4*, a gene encoding for fibroblast growth factor 4. The first part of this study focuses on the FGF4 retrogene as a possible cause of chondrodysplasia in the non-chondrodysplastic breeds, Labrador Retriever and the Bouvier des Flandres.

2. Linkage analysis on different candidate genes

The second part of this study concerns a linkage analysis on a couple of candidate genes associated with chondrodysplasia that were selected from published reports regarding mice, humans or both. With the help of microsatellite markers the involvement of the following genes in chondrodysplasia in Labradors and Bouviers is being investigated: *FGF4*, Fibroblast Growth Factor Receptor 3 (*FGFR3*), Indian HedgeHog (*IHH*), Integrine Linked Kinase (*ILK*), Natriuretic Peptide Receptor B (*NPR2*), Dicoidin Domain Receptor tyrosine kinase 2 (*DDR2*), and SProuty-Related EHV1 Domain containing 2 (*SPRED2*). These genes will be reviewed shortly. The rationale for testing these genes is depicted in table 1 of appendix 1. Figure 1 shows a global schematic illustration of the various genes involved in long bone development.

The Fibroblast Growth Factor family (FGF) consists of approximately 22 members in humans, all mediating their effects through seven different FGF receptors, encoded by four *FGFR* genes (Coumoul, Deng, 2003; Itoh, Ornitz, 2008). FGF4 is an important mitogen for a variety of cell types. It is essential in embryonic development and plays an important role in early murine limb development (Feldman et al., 1995; Niswander et al., 1994; Niswander, Martin., 1992; Ochiya et al., 1995). One of the FGF receptors is FGFR3, known to inhibit chondrocyte proliferation and retard chondrocyte differentiation, thereby negatively regulating bone growth. These effects can be direct, via activation of the MAPK pathway (mitogen-activated protein kinase), or indirect, via down-regulation of Bone Morphogenetic Protein 4 (BMP4) or IHH/PTHrP. (Chen et al., 2001; Coumoul, Deng, 2003; Deng et al., 1996; Hart et al., 2000; Matsushita et al., 2009; Naski et al., 1998; Richette et al., 2008; St-Jacques et al., 1999).

IHH has an important role in different regulatory pathways of long bone development. It is essential for proliferation and differentiation of chondrocytes and its expression in mainly prehypertrophic chondrocytes is partly regulated by RUNX2. IHH is required to maintain the expression of PTHrP in the periarticular perichondrium, which successively delays progression of chondrocytes through the prehypertrophic and hypertrophic state, thereby creating a negative feedback loop with IHH (Chung et al., 2001; Jüppner, 2000; Lanske et al., 1996; Mak et al., 2008; Shimizu et al., 2007; St-Jacques et al., 1999; Vortkamp et al., 1996). IHH can also directly stimulate chondrocyte proliferation independent of PTHrP. Furthermore, there is a PTHrP-independent role of IHH in regulating chondrocyte hypertrophy in which BMPs and Wnt/β-catenin may be mediators (Mak et al., 2008). IHH and PTHrP signals seem to become down-regulated by effects of FGFR3 (Chen et al., 2001). IHH also is imperative for normal skeletal angiogenesis during endochondral ossification (Colnot et al., 2005).

Integrins are cell adhesion molecules that are expressed on every cell type, mediating adhesion to the extracellular matrix. Attachment of chondrocytes to the collagen-rich bone matrix requires interaction with these integrins and their cytoplasmic effectors like integrin-linked kinase (ILK). ILK regulates actin reorganization and thus determines the architecture of chondrocytes but it also affects chondrocyte proliferation (Grashoff et al., 2003; Radeva et al., 1997; Terpstra et al., 2003).

The gene *NPR2* encodes a transmembrane receptor Natriuretic Peptide Receptor B (NPR-B) that has a broad pattern of expression, with a principal role in longitudinal skeletal growth. The C-type Natriuretic Peptide (CNP), encoded by *NPPC*, binds to NPR-B and positively regulates chondrocyte proliferation, matrix synthesis, and cell hypertrophy in the growth plate, thereby stimulating longitudinal growth (Bartels et al., 2004). The signaling pathways of CNP/NPRB and FGFR3 partly overlap (Tsuji, Kunieda, 2005).

The Discoidin Domain Receptor 2 (DDR2) is a fibrillair collagen receptor that is expressed in various postnatal tissues. It binds and becomes activated by these types of fibrillair collagen (I, II, III and IV) and modulates chondrocyte proliferation (Kano et al., 2008; Labrador et al., 2001; Shrivastava et al., 1997). Targeted deletion of *DDR2* in mice results in reduced chondrocyte proliferation leading to dwarfism and shortening of long bones (Labrador et al., 2001).

The protein SPRED2 (Sprouty-related EHV1 (Ena/VASP homology 1) domain) has been implicated to be an antagonist of growth factor signaling pathways and comes to expression in a variety of tissues. Like Sprouty, it induces inhibition of MAPK pathways, such as the FGFR3/MAPK signaling pathway, and thereby has a negative effect on bone growth. SPRED2 can for example block the activation of MAPK by suppressing phosphorylation and activation of Raf, one of the signal transduction pathways, but it can also redirect activated receptors to a lysosomal degradation pathway, thereby attenuating signaling of mediators like FGF. (Bunschu et al., 2005; King et al., 2005; Mardakheh et al., 2009; Wakloka et al., 2001)

3. *SHOX*-gene

The third part of this study focuses on the specific type of chondrodysplasia in which dogs with chondrodysplasia share an extraordinary phenotype with litter mates, but have healthy parents. The Bouvier des Flandres is selected as a model, because at the Utrecht University Clinic for Companion Animals, extensive information is present concerning their pedigrees (figure 5) and 43 DNA-samples of patients, healthy siblings, and parents have been collected. Pedigree analysis of the Bouvier des Flandres (figure 4) shows that dogs with chondrodysplasia are often closely related, confirming the conception that chondrodysplasia is hereditary. The third part of this study investigates the genetically interesting phenomenon that in several average sized litters all animals have a chondrodysplastic phenotype while both parents are healthy.

For the specific type of chondrodysplasia seen in Bouviers des Flandres the Short Stature Homeobox-containing Gene on chromosome X (*SHOX*) has been selected in this study as a strong candidate gene. *SHOX* is a member of the paired-related homeobox family, extremely conserved among vertebrate species, but absent in rodent species (Blaschke, Rappold, 2006; Clement-Jones et al., 2000). At the ends of the short and the long arm of the X- and Y-chromosomes regions of identity exist, to which pairing and recombination between sex chromosomes in male meiosis is restricted. Because of the obligatory recombination, genes in these regions are only partially sex linked, hence the designation PseudoAutosomal Regions (PAR). The *SHOX*-gene is one of the many genes located on PAR1, a pseudoautosomal region which resides on the short arm of the chromosomes. PAR2 is a pseudoautosomal region that consists of only a few genes and is located on the long arm of the chromosomes.

Genes in PAR1 generally escape the process of X-inactivation. Of the entire human genome, PAR1 has the highest recombination frequencies, resulting in a significantly faster evolution of this region of the genome. A linear gradient of recombination frequencies was detected, ranging from approximately 50% at the telomeric ends to 0% at the pseudoautosomal boundary. Presumably, the large number of microsatellite- and other types of relatively short repeats contribute to this high recombination frequency. Besides a high recombination frequency, human PAR1 has an exceptionally high GC content of >>>48%, while the rest of the X-chromosome displays a GC content of approximately 39% (Belin et al., 1998; Benito-Sanz et al., 2005; Binder et al., 2004; Blaschke, Rappold, 2001; Blaschke, Rappold, 2006; Chen, 2006; Chen et al., 2009; Ezquieta et al., 2002; Goldberg, 1999; Grigelioniene et al., 2001; Mangs, Morris, 2007; Ogata, 1999; Palka et al., 2000; Rappold, 1993; Sabherwal et al., 2007; Shears et al., 1998; Thomas et al., 2009; Young et al., 2008)

The human *SHOX*-gene (figure 6 and 7), possesses six exons, of which five are coding (Blaschke, Rappold, 2006; Marchini et al., 2007a; Rao et al., 1997a). Alternative splicing of *SHOX* results in different mRNA products leading to the formation of two protein isoforms, that are both thought to be located in the nucleus of cells (Rao et al., 2001; Sabherwal et al., 2004). The isoforms SHOXa and SHOXb differ from each other from the end of exon 4, with SHOXa displaying exon 5a and SHOXb showing a shorter exon 5b. The entire C-terminal domain is absent in SHOXb. (Mangs, Morris, 2007; Rao et al., 1997a and 2001).

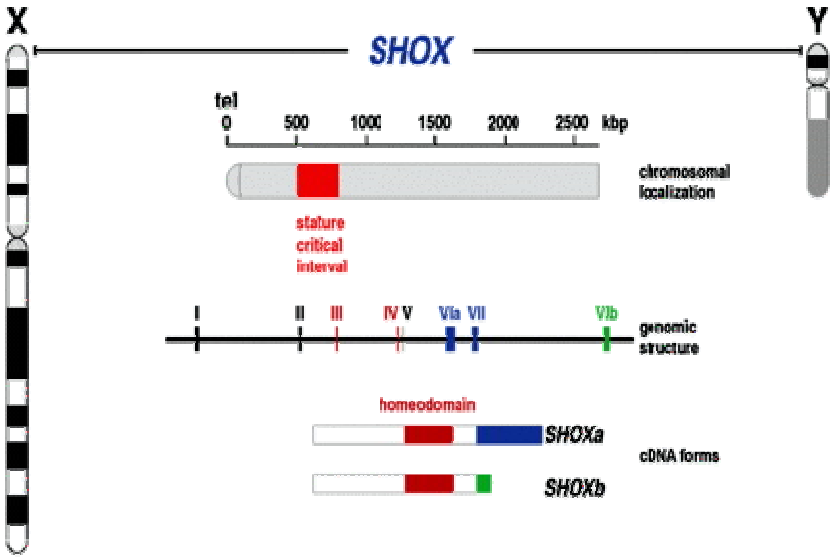


Figure 6: Illustration of *SHOX*

Position of the human *SHOX*-gene on both sex chromosomes, the different exons and the two isoforms SHOXa and SHOXb. This figure is adopted from the department of human molecular genetics/ SHOX research group Rappold, University of Heidelberg, Germany. www.med.uni-heidelberg.de/SHOX-Database.1201.0.html?&L=ar

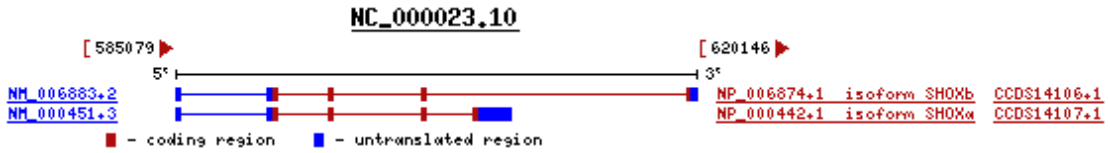


Figure 7: Human *SHOX*-gene

The human *SHOX*-gene has 6 exons that are alternatively spliced into two isoforms, SHOXa and SHOXb. www.ncbi.nlm.nih.gov/gene/6473

So far little is known about the exact role of these two transcripts. *SHOX* in general is predominantly expressed in bone marrow fibroblasts and skeletal muscle and in the chondrocytes of the reserve, proliferative, and hypertrophic zone of growth plates from twelve weeks of gestation until the time human growth plates fuse (Mangs, Morris, 2007; Marchini et al., 2004; Marchini et al., 2007a; Munns et al., 2004; Rao et al., 1997a). It appears not to be present in osteoblasts or osteoclasts (Marchini et al., 2004). Munns et al. suggested SHOX to exert a repressor function on chondrocyte differentiation, keeping chondrocytes in the reserve zone and thereby causing a delay in the progression of chondrocytes to a proliferative state (Kosho et al., 1999; Marchini et al., 2004; Munns et al., 2004). Overexpression of *SHOX* is found to induce cell cycle arrest and apoptosis (Marchini et al., 2004; Marchini et al., 2007a; Rao et al., 2001). It encodes a cell-specific homeodomain protein that is likely to be involved in chondrocyte stacking in the proliferative zone and differentiation of chondrocytes into hypertrophic cells. SHOX-haploinsufficiency could thus result in premature differentiation of proliferative chondrocytes into the hypertrophic state, thereby stimulating early growth plate fusion. The expression of SHOX in the developing upper and lower/hind limb shows a comparable pattern. From an early stage of embryogenesis, expression is predominantly present in the middle part of the limb, in the forelimb confined to the distal humerus, elbow, radius, and ulna and sometimes extending into bones of the carpus. Besides its expression in limbs, SHOX is also detectable in the first and second pharyngeal arches that will among others develop in the maxilla and mandible. The expression profile is somewhat different for the two isoforms, with SHOXb expression being more restricted to bone marrow fibroblasts and skeletal muscle and SHOXa being more widely expressed, suggesting SHOX to be also important in angiogenesis, myogenesis, and neural development (Marchini et al., 2007; Rao et al., 1997a). Rao et al predicted SHOXb to be important in modulating SHOXa activity and not to have a transcriptional activating function because of the absence of the C-terminal portion. The transcriptional activating capacity of SHOXa homodimers therefore is expected

to be different from SHOXa/SHOXb heterodimers or SHOXb homodimers (Rao et al., 2001). SHOX has been proposed to interact with estrogen and FGFR 3 in the process of chondrocyte differentiation (Kosho et al., 1999). The signalling pathways comprising SHOX are currently not defined, but recently SHOX has been found to interact with *NPPB*, a gene that encodes for Brain Natriuretic Peptide (BNP). The promoter region of *NPPB* contains SHOX responsive elements, making this gene a direct target for transactivation by SHOX. Co-expression of BNP and SHOX has been identified in late proliferative, prehypertrophic, and hypertrophic chondrocytes, suggesting BNP like SHOX to play a role in chondrocyte maturation (Marchini, A. et al., 2007b).

The effects of SHOX are complicated, but mutations give a more clear insight into the function of this important protein. Dysfunction of SHOX is likely to result in abnormal proliferation and differentiation of chondrocytes, or even complete absence of these processes, leading to a retarded longitudinal growth, causing short stature (Marchini et al., 2007a). The small C-terminal portion of SHOXa seems to be a transactivating domain responsible for activating gene transcription in osteogenic cells. Nonsense mutations leading to C-terminal SHOX truncation result in short stature (Rao et al., 2001). A patient with a Ser106A *SHOX* mutation revealing defective phosphorylation showes absence of transcriptional activation and therefore cell-cycle arrest and apoptosis do not take place, leading to the conclusion that phosphorylation has shown to be of clinical importance in regulating SHOX biological activity (Marchini et al., 2006). Furthermore, missense mutations leading to alterations in the biological function of SHOX by impairment of nuclear translocation, loss of DNA binding or reduced dimerization ability are demonstrated to result in Idiopathic Short Stature and Leri-Weill disease, two human types of chondrodysplasia (Sabherlwal et al., 2004; Schneider et al., 2005a). Zinn et al. found a mutation in exon 6a in a patient with Langer mesomelic dysplasia, leading to the conclusion that the SHOXa isoform is necessary for normal skeletal development (Zinn et al., 2002). RNA expression analysis conducted by Flanagan et al. though revealed monoallelic expression of the SHOXb transcript in two chondrodysplastic patients, suggesting at least this isoform to be required for development of normal stature (Flanagan et al., 2002).

The canine SHOX-gene, shown in figure 8 probably contains five exons and forms only one isoform.

Figure 8: Canine *SHOX*-gene

The canine *SHOX*-gene has five predicted exons and only one isoform.

www.ncbi.nlm.nih.gov/gene/491706

In patients with SHOX abnormalities phenotype can be very variable (Jorge et al., 2007). Benito-Sanz et al., 2005, described a variable degree of affectation in patients with Leri-Weill Dyschondrosteosis caused by deletions downstream of the *SHOX*-gene, Grigelioniene et al., 2001, confirmed previously reported phenotype heterogeneity and Huber et al., 2006, reported patients with SHOX deficiency and normal stature. Blaschke et al. investigated a possible explanation for this phenotypic heterogeneity observed in SHOX-deficient patients. They discovered an alternative, intragenic promoter, located in exon 2 of the *SHOX*-gene, of which the transcribed mRNA, encoding an identical SHOX-protein, is translated much more efficiently than that of promoter 1. Apparently transcriptional and translational control mechanisms exist that can regulate functional SHOX protein levels (Blaschke et al. 2003).

The typical phenotype of the affected Bouviers is very similar to a type of human chondrodysplasia, for which mutations and deletions in the SHOX-gene or in flanking regions of this gene are responsible (Rappold et al., 2002; Schneider et al., 2005b). Since *SHOX*, located within PAR1, normally escapes X-inactivation, it exhibits dosage-sensitive functions, meaning two active copies are required for normal functioning. SHOX-haploinsufficiency due to these kinds of mutations results in a reduced production of SHOX, leading to structural changes in the growth plate. Premature growth plate fusion and consequently early cessation of endochondral ossification finally results in disproportionate short stature. (Blaschke, Rappold, 2006; Thomas et al., 2009). Heterozygous and homozygous mutations in human *SHOX* have been demonstrated in approximately 50-70% of the Léri-Weill Dyschondrosteosis (LWD) cases and in Mesomelic Dysplasia Langer Type, respectively (Benito-Sanz et al., 2005; Blaschke, Rappold, 2006; Flanagan et al., 2002; Grigelioniene et al., 2000 and 2001; Marchini et al., 2007a; Schiller et al., 2000). In these syndromes females are generally more severely affected than males and the severity seems to increase with age. So far, there is no explanation for this end (Benito-Sanz et al. 2005; Kosho et al., 1999). The heterozygous LWD shows short stature because of mesomelic shortening of the forelimb and Madelung

deformity, characterized by shortening of the forearm with radial curving, dorsal dislocation of the distal ulna, and wedging of carpal bones between the distorted radius and ulna (Belin et al., 1998; Shears et al., 1998). The homozygous Langer Mesomelic Dysplasia shows resemblance to LWD but is much more severe. There is severe shortening of the extremities, Madelung deformity, varus deformity of the humeral head, curving of the radius, carpal deformation and hindlimb impairment consisting of a short femoral collum and a hypoplastic or absent proximal half of the fibula (Zinn et al., 2002). It is genetically relevant to investigate whether Bouviers like humans display a similar difference in severity of the complaint, depending on the type of mutation (heterozygous or homozygous). In Turner syndrome, in which females only have one X-chromosome (45X), SHOX-haploinsufficiency is responsible for a major part of the characteristic growth retardation and other skeletal defects seen in this disease, because two active copies of the SHOX-gene are required for normal function in both genders (Benito-Sanz et al., 2005; Müsebeck et al., 2001; Ogata et al., 2001; Zinn et al., 2002). Short stature with cubitus valgus, Madelung deformity, short metacarpals, short neck, high arched palate, and micrognathia seen in Turner syndrome are, taking into account the expression pattern of SHOX described earlier, presumably all symptoms attributable to SHOX-haploinsufficiency (Marchini et al., 2007a; Zinn et al., 1993). The fact that rodents do not have an X-linked *SHOX*-gene might explain the lack of short stature in X0-mice, having only one X chromosome (Rao et al., 1997a). Besides the reduced expression of *SHOX* in humans when there is one less X-chromosome, the expression of all the other genes on PAR1 is decreased, since normally these genes escape X-inactivation, resulting in two active copies. This leads to a whole spectrum of somatic clinical symptoms like heart- and renal abnormalities and infertility because of compromised ovarian development (Blaschke, Rappold, 2001; Mangs, Morris, 2007; Rappold et al., 1993; Zinn, 1993). In contrary to what the name of the disease implies patients with Idiopathic Short Syndrome are also often found to have mutations in *SHOX* or its flanking regions (Chen et al., 2009).

In the current study the *SHOX*-gene is being investigated by performing a combination of molecular genetic techniques. First, linkage analysis using polymorphic microsatellite markers has been performed to verify whether patients had an allele of the SHOX-gene in common, which would have been strongly indicative for the involvement of this gene in the disease. Here several possible changes in *SHOX* and flanking regions of this gene are described that may be responsible for chondrodysplasia and which are being investigated in this study.

a) Downstream enhancer-element deletion

The existence of "gene deserts" in the human genome has been described, gene-poor regions greater than 500 kb in length. Gene regulatory elements can be situated in these regions, modulating gene expression over very long distances (Nobrega et al., 2003). In man there appears to be such Conserved Non-coding DNA Elements (CNE) downstream of the SHOX-gene, but still in PAR1, some of which express enhancer-like activities and are also known as "long-range-cis-regulatory elements" (Benito-Sanz et al., 2005; Chem et al., 2009; Fukami et al., 2006; Sahberwahl et al., 2007). Deletions of varying size in these regions are reported to cause chondrodysplasia, resembling the chondrodysplasia occurring with SHOX-haploinsufficiency and *SHOX*-mutations (Benito-Sanz et al., 2005 and 2006; Bertorelli et al., 2007; Blaschke, Rappold, 2006; Chen, 2009; Flanagan et al., 2002; Fukami et al., 2006; Huber et al., 2006; Rao et al., 1997a and 1997b; Sabherwal et al., 2007). For example, Benito-Sanz et al., 2005, found deletions, located 30-530 kb downstream of the SHOX-gene and ranging from 81-501 kb in size, in 15% of patients with Léri-Weill Dyschondrosteosis that were tested negative for mutations or deletions of *SHOX* itself. A region of 29 kb, defined by specific microsatellite markers and Single Nucleotide Polymorphisms (SNPs), was found to be deleted in all twelve reported cases of enhancer-element deletion. Other researchers found commonly shared deletion intervals or part of it (Fukami et al., 2006; Huber et al., 2006; Sabherwal, 2007). The presence of hotspots for deletion breakpoints seems to be a pliable explanation (Benito-Sanz et al., 2005; Schneider et al., 2005b). By using microsatellite markers the current study investigates whether similar deletions might be causative for chondrodysplasia in the Bouvier des Flandres.

b) Inactivation of *SHOX*

X-inactivation, a process exhibited by mammalian species, equalizes the gene dosage difference that arises from the different numbers of X chromosomes between males (XY) and females (XX), by silencing one of the two X chromosomes (Ng et al., 2007). As previously stated, genes in PAR1 are normally not inactivated in the process of X-inactivation. When mutations should arise resulting in genes failing the escape of X-inactivation, expression of these genes is reduced. The population of Bouviers in this research displays skeletal dysplasia as the main complication, suggesting that only *SHOX* on PAR1 may be inactivated and not the entire PAR1. In case the entire PAR1 would be inactivated other additional clinical symptoms are expected, like the somatic complaints seen with Turner syndrome. Two important epigenetic gene silencing mechanisms are DNA methylation and histone

modification. The first type of methylation occurs at the site of CpG islands, where many cytosine residues directly followed by guanines can be converted to 5-methyl cytosines. These CpG islands are normally hypomethylated and are located upstream of the 5' end of active genes, often linked to promoter regions of these genes. A high degree of this type of methylation results in inactivation of those regions. Even when enhancer elements upstream or downstream of the gene are not inactivated, gene expression might be reduced if promoter regions are embedded in heterochromatin, making them less accessible to the enhancer elements. (Disteche et al., 2002; Heard, Disteche, 2006; Li, Dahiya, 2002; Moss, Wallrath, 2007; Prothero et al., 2009) In the current study the hypothesis of aberrant inactivation of the *SHOX*-gene is being investigated by determining the level of methylation in this region on PAR1 where *SHOX* is located.

c) Expansion of a special microsatellite repeat

The canine species is extremely susceptible to selection based on morphological characteristics. A variation in the length of microsatellite repeats in genes involved in developmental processes of the animal might play an important role in this enormous morphological variation. Microsatellites are tandemly repeated sequences, consisting of less than seven nucleotides per sequence motif. Gene-associated repeating microsatellite elements can lead to polymorphism because of slipped-strand mispairing or uneven recombination, resulting in expansion or contraction of the repeat (Fondon, Garner, 2004; Wren et al., 2000). Due to a genome-wide elevated slippage mutation rate in dogs, tandem repeats in genes responsible for development are far less stable and therefore much more polymorphic in canine than in human species. Furthermore, tandem repeats in dogs are less frequently interrupted by dissonant nucleotides than is the case in humans (Fondon, Garner, 2004; Laidlaw et al., 2007). Special tandem repeats exist, consisting of three nucleotides per repeat, an example of such a trinucleotide repeat is the CGG-repeat. In human, massive expansion of a CGG-repeat in successive generations can lead to fracturing of an X-chromosome at the site of the repeat. This disables the expression of the nearby *FMR-1* gene (Fragile X Mental Retardation-1), resulting in "fragile X-syndrome", one of the most important causes of human hereditary mental retardation (Jin, Warren, 2000; Kremer et al., 1991; Syrrou et al., 1996; Wren et al., 2000). The coding region of the *SHOX*-gene of dogs also displays a CGG-repeat, with eight uninterrupted repeats coding for glycin residues. Eight glycin codons are conserved in the human gene, but in man different glycin codons alternate. This CGG-repeat by itself is harmless. The hypothesis is that when the repeat expands in offspring, it might cause a

disturbance of the SHOX-gene, leading to chondrodysplasia. This could explain the phenomenon that two Bouvier litters are completely affected, while their parents are healthy.

d) DNA sequence variations in *SHOX* and flanking regions

A whole spectrum of different mutations in or around *SHOX* could be responsible for the gene to dysfunction (Rappold et al., 2002). A human database exists in which almost all published mutations are reported (Niesler et al., 2002; www.shox.uni-hd.de). The *SHOX*-gene has already been sequenced in three chondrodysplastic (Basset Hound, Dachshund, Pembroke Welsh Corgi) and three non-chondrodysplastic unaffected dog breeds. In this research SNPs were identified in the promoter region and in exon 2, but they were found not to be associated with that type of canine chondrodysplasia (Young, Bannasch, 2008). Sequencing of the coding regions, the exon-intron bounderies and the promoter region(s) of *SHOX* in Bouviers has been performed in the current study to investigate the possible presence of SNPs and mutations which might be causative for this specific type of chondrodysplasia in the Bouvier.

Materials & Methods

Subjects

The Labrador Retriever population consisted of 13 dogs with chondrodysplasia and 20 healthy dogs (figure 9). The population of Bouviers des Flandres consists of 43 dogs originating from 9 litters of which 24 animals were affected (figure 5). (Smit et al., 2009; Temwichitr et al., 2009)

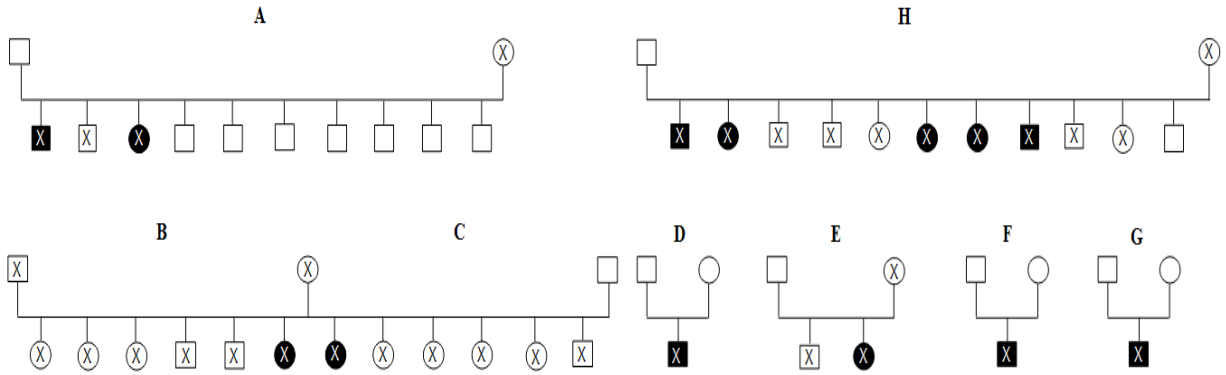


Figure 9: Labrador Retriever population

Research population of Labrador Retrievers. Squares are males and circles are females. Open and filled symbols are healthy and affected animals, respectively. A cross means DNA is available at the Utrecht University Clinic for Companion Animals. Depicted litters A, B-C and H are complete as opposed to litters D, E, F and G, of which little information was available. All of these litters share a common ancestor which is not illustrated in this picture. Smit et al. 2009 showed more detailed pedigrees of this research population.

Following informed consent, blood samples were taken as part of a previous study after which DNA was isolated (Temwichitr et al., 2009). DNA from chondrodysplastic dog breeds and from unaffected, unrelated dogs of various breeds served as a positive or negative control, respectively. Radiographs of the front legs of various (n=15) affected as well as unaffected Labradors and Bouviers were available at the Utrecht University Clinic for Companion Animals.

1. fgf4 retrogene

To examine whether a retrogene of *FGF4* on chromosome 18 is the cause of chondrodysplasia in Labradors and Bouviers, PCR (Polymerase Chain Reaction) primers (oligonucleotides) were chosen from the supplementary material of the study of Parker et al. (Parker et al., 2009). The organisation of the *FGF4* gene and its retrogene with selected primers are depicted in figure 10 and table 1.

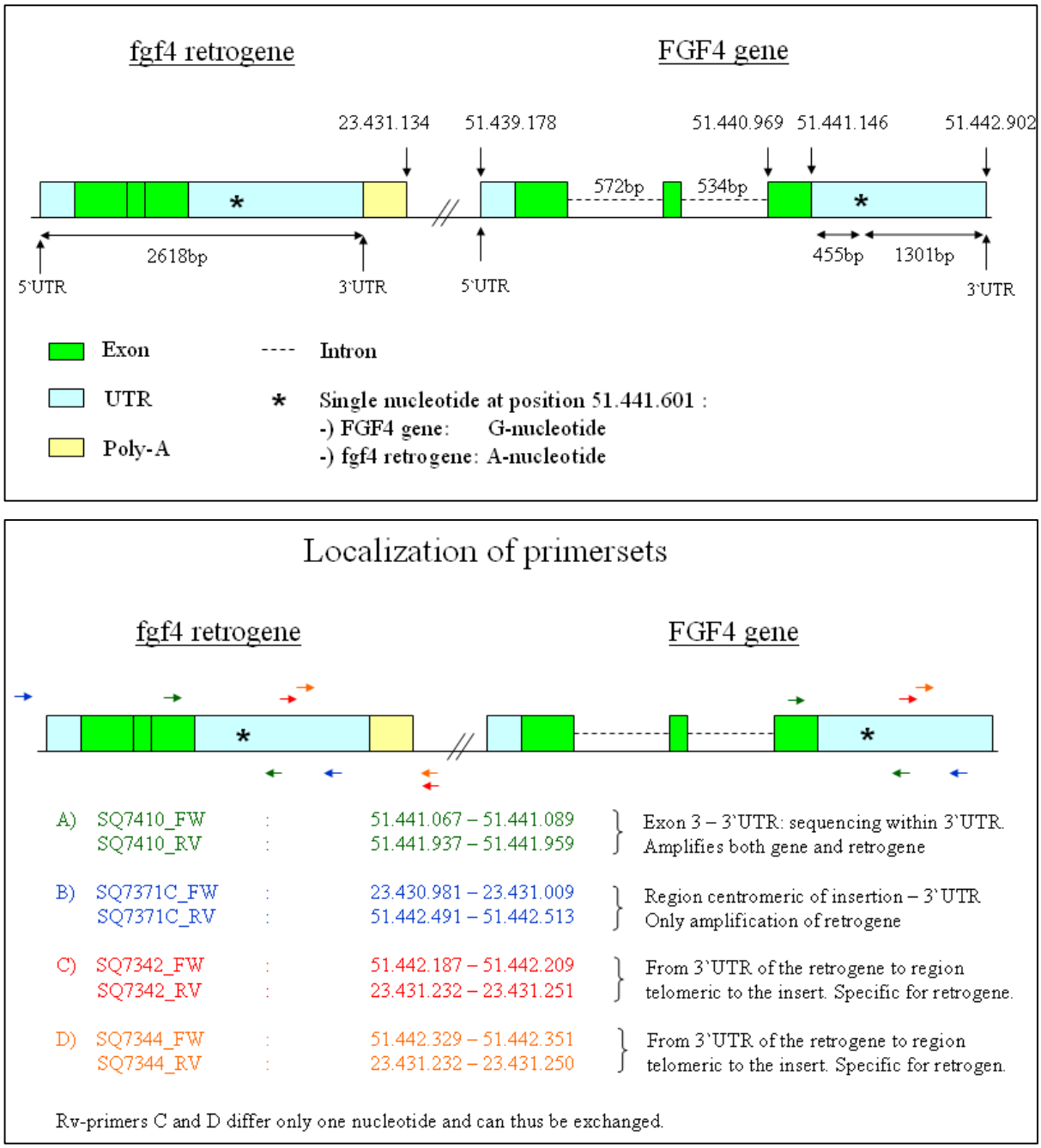


Figure 10.

Schematic drawing of *FGF4* and its retrogene on chromosome 18 and the localization of the primer sets used. Not drawn to scale. Information was collected from website www.ncbi.nlm.gov.

Nº	Gene	Name of primer	Fw-primer	Rv-primer	Product size (bp)
A	*FGF4* + retrogene	SQ7410	GAGCAAGAACGGGAAGACCAAG	TTTATCACTCGGGCTTTGGTTG	850*
B	Only retrogene	SQ7371C	GCAAAGATATGCAAACAACCCAAGTATC	ACATCACCGACCCTGCCTCTTC	2500*
C	Only retrogene	SQ7342	TCGAAACCCTTAACCCACTCATC	CCTTTCCCTCTGGCAACCAC	820*
D	Only retrogene	SQ7344	TCACGTTTGAGCTATCTTTACCC	CTTTCCCTCTGGCAACCAC	680*

Table 1: List of primers used for fgf4 (retro)gene

* PCR product sizes are roughly estimated values.

Primer set A can amplify both *FGF4* gene and the possible retrogene, but primers B, C, and D are retrogene specific, amplifying a DNA fragment including part of the retrogene. Therefore, they will not generate a PCR-product when the fgf4 retrogene is absent. Seven Labrador as well as six Bouvier patients were tested. These patients were selected from various litters throughout the whole Dutch pedigree, with average severity of chondrodysplasia for Bouviers. Two healthy Labradors functioned as negative controls and DNA of three chondrodysplastic dog breeds (Basset Hound, Welsh Corgi and Norwich terrier) was used as a positive control, since the presence of such a retrogene has been demonstrated in these breeds (Parker et al., 2009). PCR reactions have been conducted using protocols illustrated in table 2.1 and 2.2, using Platinum Taq instead of Recombinant Taq. The optimal annealing temperature of 72°C for primer sets A, C and D was established by performing a temperature gradient on a C1000 thermal cycler (Bio-Rad; The Netherlands). For primer B an annealing time of 2 minutes was used; 1 minute was sufficient for the other three primer sets.

Product	Quantity (µl)
mQ	4,7
dNTPs (1mM)	5,0
10x PCR buffer	2,5
$MgCl_2$ (50mM) *	1,25
Fw-primer (10µM)	1,25
Rv-primer (10µM)	1,25
Recombinant Taq (5U/µl) **	0,2
DNA (5-25ng/µl)	5,0

Temperature (°C)	Time	Cycles
95	5 min.	
95	30 sec.	
72 (1)	30 sec.	35x (3)
72	1-2 min. (2)	
72	10 min.	
20	∞	

Table 2.1 and 2.2: PCR-protocol

10x PCR buffer, 50mM $MgCl_2$ and Taq DNA polymerase (Recombinant or Platinum): Invitrogen, The Netherlands. Primers: Eurogentec, The Netherlands

* $MgCl_2$ concentration of 2.0mM was found to make primers A,C and D (especially C) more specific.

** Platinum Taq can be used instead

(1) Annealing temperature is based on Tm of the primer set and can therefore be varied.

(2) Extension time can be varied. A product of 1kb can be amplified within 60 seconds.

(3) The amount of cycles necessary can be determined based on the efficiency of the PCR, as seen on gel electrophoresis after a test-PCR.

With the aforementioned protocol primer B failed to work, therefore a Phusion protocol shown in tables 3.1 and 3.2 was performed, using 5x Phusion HF buffer and no DMSO and selecting an annealing temperature and time of 69°C and 20 seconds respectively, an extension time of 1,5 minutes and 35 cycles. PCR-products were stored at 8-10°C.

Product	Quantity (µl)
mQ	7,8
5x Phusion buffer*	4,0
Fw-primer (10µM)	1,0
Rv-primer (10µM)	1,0
DMSO**	0,6
dNTPs (10mM)	0,4
Phusion Hot Start polymerase (2U/µl)	0,2
DNA (5-25ng/µl)	5,0

Temperature (°C)	Time	Cycles
98	30 sec.	
98	10 sec.	
60-72 (1)	10-30 sec. (2)	25-40x (4)
72	15-30 sec/kb. (3)	
72	5-10 min.	
12	∞	

Table 3.1 and 3.2: Phusion PCR-protocol

Phusion buffer, DMSO and Hot Start polymerase: Finnzymes, Finland.

Primers: Eurogentec, The Netherlands

* HF buffer or GC buffer; the last one is used for PCR-products with a very high GC content.

** The use of DMSO is recommended for GC-rich PCR-products.

(1) Annealing temperature is based on Tm of the primer set and can therefore be varied. Suggested annealing temperature for primers >20bp is 3°C above the lowest Tm-value of the primer set.

(2) Annealing time can be varied, with shorter annealing temperatures resulting in higher specificity. Suggested annealing time for primers >20bp is 10-30 sec.

(3) Extension time can be varied. A product of 1kb can be amplified within 15-30 seconds.

(4) The amount of cyli necessary can be determined based the efficiency of the PCR, as seen on gel electrophoresis after a test-PCR

Gel electrophoresis was performed to analyse the presence or absence of PCR-products and their respective sizes. A 1% agarose-gel (Promega) containing EtBr (1:25000) was used, with a 1kb ladder of Promega as reference and a loading dye with brominephenolblue and glycerol.

Sequencing was then performed for primers A, C en D. Parker et al. (2009) described a single base at position 51441601 in the UTR adjacent to exon 3 (see figure 10) that differed between retrogene and source gene. An A-nucleotide was present in the retrogene, while the FGF4 gene displayed a G-nucleotide. Sequencing of DNA-fragments produced with primer set A was therefore required to distinguish between retrogene and source gene based on this single

nucleotide. PCR-products obtained with primer A consisted of DNA from seven affected Labradors, six affected Bouviers, 3 chondrodysplastic dogs and one test-DNA. The retrogene specific primers C and D only resulted in PCR-products for two chondrodysplastic breeds and a test-DNA. The PCR-products underwent a SAP-Exo purification step, shown in tables 4.1 and 4.2, to remove excess primers and dNTPs. Afterwards a Tercycle-reaction, without the use of DMSO, was performed, with annealing temperatures of 55°C and 50°C for primer set A respectively C and D, and with an annealing time of 2 minutes for all of the primers (table 5.1 and 5.2).

Product	Quantity (µl)
PCR-product	7,5
Shrimp Alkaline Phosphatase (SAP) (1U/µl)	2,5
Exonuclease 1 (20U/µl)	0,05
mQ	0,45

Temperature (°C)	Time
37	60 min
75	20 min.
12	∞

Table 4.1 and 4.2: SAP-Exo-protocol

SAP: Promega, The Netherlands; Exonuclease 1: New England BioLabs

Product	Quantity (µl)
mQ	5,0
5x Sequencing buffer	2,0
Fw- or Rv- primer (3,2µM)	1,0
Big Dye Terminator	1,0
DMSO *	1,0
SAP-Exo-product	1,0

Temperature (°C)	Time	Cycles
96	30 sec.	
50-55 (1)	15 sec.	25x
60	1-2 min. (2)	
4-12	∞	

Table 5.1 and 5.2: Tercycle-protocol

Big Dye Terminator v 1.1, v 3.1 - 5x Sequencing Buffer: Applied Biosystems, USA

Big Dye Terminator v 3.1 - Sequencing RR-100: Applied Biosystems, USA

Primers: Eurogentec; DMSO: Finnzymes, Finland

* The use of DMSO is optional but recommended when a high GC content is present

(1) Can be varied depending on the Tm of the primer set.

(2) This time is dictated by the length of the PCR-product.

After thermal cycling the 10µl Tercycle-product was centrifuged for a couple of seconds at 1900rpm and then together with 10µl of mQ loaded onto a Sephadex plate (Multi Screen 96-well sterile filtration plate, Millipore, Ireland), that was prepared according to corresponding protocol and placed on a sequence plate. Five minutes of centrifuging at 1900rpm followed, to filter the Tercycle-products through the Sephadex plate. Capillary electrophoresis and automatic detection of reaction products was performed by an automated DNA sequencer (3130 xl Genetic Analyzer of Applied Biosystems, USA). DNASTAR Lasergene sequence analysis software was used for analysis of all obtained sequence data.

2. Linkage analysis of various candidate genes

All Labrador Retrievers patients of nest A, B/C and H (see figure 9), almost all Bouviers des Flandres patients (see figure 5) and various healthy relatives of both breeds were included in this linkage analysis. Microsatellite markers located as close as possible to the genes of interest, with a maximum distance of 1,5 Mb, were selected using following criteria: dinucleotide-repeat markers were preferred, since this type of repeat is much more stable than a tetranucleotide-repeat; a number of repeats between 20 and 30 is considered optimal, but a 100% match is more important than repeat length. Table 6 shows the different microsatellite markers, their distance to the corresponding candidate genes and the selected primers used for amplification of the markers. Of the ten PCR-primer sets, six were fluorophore-labeled. The primer set for the FGF4-gene was selected from the NCBI-UniSTS website, the other three primer sets were designed with either Primer3 software or with the UC Davis website (www.ncbi.nlm.nih.gov/sites/entrez; http://frodo.wi.mit.edu/primer3/; www.vgl.ucdavis.edu/dogset/). Criteria used for primerdesign for fragment analysis are demonstrated in table 7.

Criteria for primerdesign (fragment analysis)	
Primerlength (bp)	20-24
PCR-product size (bp)	300-500
GC%	50-60
Tm (°C)	55-75
Hairpins	0
Dimers	Reject if $\geq$ 3 matches at 3´end
	Reject if $\geq$ 7 adjacent homologous bases
Runs	Reject if $\geq$ 3 bases
	Reject if $\geq$ 3 G or C at 3´end

Table 7: Criteria used for designing primers for linkage analysis

Before ordering these last 4 primers at Eurogentec, a M13-taq was added to the 5' end of the forward primer and an A-tail was added to the 5' end of the reverse primer. These M13-taqqed primers require a 3-primer PCR-protocol in which a M13-label primer is used that contributes to creating fluorophore-labeled products. DNA fragments containing the microsatellite repeats were amplified using selected primers in a PCR-protocol for fragment analysis. Normal as well as 3-primer PCR-protocols are depicted in tables 8 and 9, with thermal cycling being identical for both protocols. Annealing temperatures varied between different markers: 50°C for marker 5 and 7; 55°C for markers 2, 8 and 10 an 60°C for markers 1, 3, 4, 6 and 9 (table 6). Amplification products were kept out of the light an stored at 8-10°C.

Product	Quantity (µl)
mQ	3,0
dNTPs (1mM)	3,0
10x PCR gold buffer	1,5
$MgCl_2$ gold (25mM)	1,5
Fw-primer (10µM)	0,5
Rv-primer (10µM)	0,5
Ampli Taq gold (5U/µl)	0,06
DNA (5-25ng/µl)	5,0

Table 8.1: PCR-protocol for fragment analysis

Product	Quantity (µl)
mQ	5,75
dNTPs (1mM)	5,0
10x PCR gold buffer	2,5
MgCl2 gold (25mM)	2,5
Fw-primer (+ M13 tail) (1µM)	1,25
Rv-primer (+ A) (10µM)	1,25
M13-lable primer (10µM)	1,25
Ampli Taq gold (5U/µl)	0,25
DNA (5-25ng/µl)	5,0

Table 8.2: 3-Primer PCR-protocol for fragment analysis

10x PCR gold buffer, 50mM $MgCl_2$ gold and Ampli Taq DNA polymerase: Applied Biosystems, USA

Primers: Eurogentec, The Netherlands

Temperature (°C)	Time	Cycles
95	5 min.	
95	30 sec.	
50-65 (1)	15 sec.	10x
72	30 sec.	
92	30 sec.	
50-65 (1)	15 sec.	25x
72	30 sec.	
72	10 min.	
10	∞	

Table 9: Thermal cyling

Thermal cycling is identical for both normal and 3-Primer PCR-protocols.

(1) Annealing temperature depends on the Tm of the primer set.

Gel electrophoresis on a 1,5% agarose-gel, using a 100bp ladder (Promega) was performed for a couple of samples after PCR, to ascertain amplification succeeded. Amplification products were then diluted 10 times with mQ followed by the protocol shown in table 10.

Product	Quantity (µl)
Hi-Di Formamidine	10,0
GeneScan 500-LIZ Size Standard	0,2
10x mQ diluted PCR-product	2,0
5 Min. denaturation on 95°C	
Cooling on ice until room temperature is reached	
Centrifuging at 1900rpm for a couple of seconds	

Table 10: Protocol for preparing PCR-samples for fragment analysis

Formamidine and Size Standard: Applied Biosystems, USA

For reproducible sizing of fragment analysis data the 500-LIZ size standard was chosen because of the expected size of the amplification products of 100-400bp . After 5 minutes of denaturing on 95°C the samples were cooled on ice until room temperature was reached after which the samples were centrifuged at 1900rpm for a couple of seconds. Electrophoresis was

then performed using an automated DNA sequencer (3130 xl Genetic Analyzer of Applied Biosystems, USA). The size of the DNA products was established and alleles were assigned using GeneMapper software of Applied Biosystems. Alleles were then depicted in pedigrees manually designed using Microsoft Office Excel software, at the same time displaying phenotype of the dogs. It was verified whether patients had alleles of the candidate genes in common, which would be strongly indicative for the involvement of those genes in the disease. Markers and their genes were excluded based on a recessive inheritance pattern, a dominant pattern or both in case this type of chondrodysplasia appears to be a polygenic disease, by studying these pedigrees. Affected siblings had to display the exact same combination of alleles for inheritance to be recessive. For a dominant inheritance pattern affected siblings had to have one allele in common. Because all parents of the patients were healthy, incomplete penetrance was expected. Furthermore, pedigree files were conducted to enable LOD-scores (Logarithm Of Odds) to be calculated for these different possible modes of inheritance. The MLINK program of Linkage in MS DOS was used to compute two-point LOD-scores for different recombination values. An online SUPERLINK program conducted two-point as well as multipoint analyses for all of the markers with various penetrance-values for the different modes of inheritance and for recombination values of between 0,01-0,02 (http://bioinfo.cs.technion.ac.il/superlink-online/). Haplotypes were constructed for marker combinations that showed positive LOD-scores.

N°	Gene	Chromosome	Micosatellite marker	Type of repeat	Fw-Primer	Rv-Primer	M13- + A-tail	Type of Label	Distance to gene (bp)	Product size (bp)
1	FGF4	CFA18	REN266I17	2n	CCGGTTGTATGAGCCTTTGT	TCACACAGACCAATCCCAGA	+	-	106.792	132
2	FGFR3	CFA3	REN260I04	2n	CTGTCAGATGCTCTGTCCCA	CCACACAAAGACACACACCC	-	VIC	1.403.277	185
3	FGFR3	CFA3	Cfa3_65M	2n	AAACCCAGCCTTCTATCCACA	GAAGTGATAGCAGGGGAAGCA	+	-	85.343	332
4	FGFR3	CFA3	Cfa3_65.2M	2n	TCCAACCTGGATGATTGAGC	GAACAATCTGGCCCTGCTTC	+	-	69.176	356
5	FGFR3	CFA3	CFA03:65M_FGFr3	2n	CCCGGCTGGCTATACCATTA	GGTCTGTGGCCTTCTGAACC	+	-	106.590	334
6	Ihh	CFA37	FH2587	4n	GGCATGAACAAATCAGTGGA	TTTGCTGTTTAATCCATCTGG	-	NED	1.127.021	198
7	Ilk	CFA21	FH2441	4n	TAGTTGTGTGCATGATCTCG	TGGAGAAAGTTCCATGTGCA	-	NED	625.687	129
8	NPR2	CFA11	REN194N17	2n	GGTGGAGAAACTTGATGGGA	CCCCATGGAGACCATTCTTA	-	VIC	988.081	271
9	DDR2	CFA38	REN164E17	2n	GGTCTTCACCCATCACCATT	TTAGATGGAAAATGTGGCCC	-	VIC	103.115	138
10	Spred2	CFA10	FH3381	4n	CCCAGAAACTCAACTGATGC	AGCTCTTACACGCATTGAGG	-	PET	892.789	283

Table 6: Microsatellite markers

Summary table of microsatellite markers used for linkage analysis.

M13-taq 5’ → 3’: GTTTTCCCAGTCACGAC; A-tail 5’ → 3’: GTGTCTT.

Different labels, encoding for different colours, exist for the fluorophore-labeled markers, indicated by the type of label shown in this table.

VIC-, NED- and PET-labels result in respectively green, black/yellow and red fluorescence. The M13-label-primer required for conducting a fragment analysis-PCR using primers taqqed with M13 lead to blue fluorescence.

3. *SHOX*-gene

This part of the study focussed on the *SHOX*-gene located on the X-chromosome of Bouviers des Flandres. Radiographs of the extremities of patients were studied to determine whether Bouviers show a similar dichotomy regarding the grade of severity of the complaint as is seen in humans. Linkage analysis, determination of the degree of CpG-methylation, investigation of the CGG-repeat and gene sequencing were performed on all Bouviers in the population in order to examine whether *SHOX* might be responsible for causing this specific type of chondrodysplasia. In addition to carrying out these last two tests on the whole research population of Bouviers, DNA of a (couple of) healthy non-chondrodysplastic dog breeds and of a (few) chondrodysplastic dog breeds was most of the time included as comparison.

a) Assessment of radiological features

Radiographs of the front limbs of 15 affected Bouviers were available at the Utrecht University Clinic for Companion Animals, as part of the research of Temwichitr (Temwichitr et al., 2009). Based on radiological features, the animals were assigned to one of four different classes, ranging in severity of the disease. Degree of radial curvature and absence or presence of (sub)luxation of the radial head were the main criteria for classification.

b) Linkage analysis

Linkage analysis using microsatellite markers was conducted to verify whether patients have an allele of the SHOX-gene in common, which would be strongly indicative for the involvement of this gene in the disease. The same microsatellite markers were also used for investigating whether downstream enhancer-element deletions might be causative for chondrodysplasia. The canine genome was searched for regions showing similarity to the 29 kb overlapping downstream deletion region described by Benito-Sanz et al., 2005. Alignments made in Ensembl revealed similarities of a 17 kb region on the canine X-chromosome, located at a distance of approximately 140 kb downstream of *SHOX*, to the human 29 kb region (www.ensembl.org/Homo_sapiens/Location/Compara_Alignments).

A region on chromosome X containing the *SHOX*-gene and flanking regions of approximately 1 Mb were searched for microsatellite markers. The use of NCBI uniSTS resulted in only two optional markers (REN44K10; REN147N10), residing at a relatively large distance of almost 3 Mb to the SHOX-gene (www.ncbi.nlm.nih.gov/unists). Therefore markers were searched

using the following website: http://www.vgl.ucdavis.edu/dogset/. Appropriate markers were selected, applying the same selection criteria as with linkage analysis of the other candidate genes. Primers that could amplify DNA fragments encompassing the dinucleotide markers were designed with Primer3 (http://frodo.wi.mit.edu/primer3/), using criteria specific for linkage analysis with fragment analysis depicted in table 7. By blasting selected primers in Ensembl, they were audited for the existing amount of competition resulting from binding to alternative spots on the canine genome (www.ensembl.org/Canis_familiaris/blastview). Chosen primers received a M13-taq and an A-tail and are depicted in table 11. Polymerase chain reactions were conducted following a 3-Primer PCR protocol shown in table 8.2 and 9, with an annealing temperature of 59°C. Gel electrophoresis on a 2% agarose-gel was used to test whether the amplification succeeded, and amplification products were kept out of the light and stored at 8-10°C. PCR-products were prepared for fragment analysis following the protocol of table 10.

Micosatellite marker	Fw-Primer	Rv-Primer	Distance to gene (bp)	Distance to RR (Kb)	Product size (bp)
SHOX-0.4M	AGGGAGTAATCACCGCTTGG	TGTAGGGATCTTGGCTGAGG	102.934	37	416
SHOX-0.5M	AGCCGAAAACGTGACACAGG	TTTCTAAGCACGGAGAGGATGC	123.504	16,5	391

Table 11: Primers used for linkage analysis of the SHOX-gene

The following website is used for primerdesign: http://frodo.wi.mit.edu/primer3/

RR: regulatory region; this value is a rough estimate of the distance between marker and supposed regulatory region downstream of the SHOX-gene.

Capillary electrophoresis and automated detection of products was achieved by the 3130 xl Genetic Analyzer of Applied Biosystems and GeneMapper software was used for allelecalling of samples (Applied Biosystems, USA). Alleles were clearly defined in pedigrees and it was verified whether patients had joint alleles of the SHOX-gene.

c) Determination of degree of CpG-methylation

Possible inactivation of the *SHOX*-gene was investigated by determining the level of CpG-methylation of this region on PAR1. Conversion of DNA was performed, using a sodium bisulphite kit (EZ DNA Methylation – Gold Tm Kit Catalog Nos. D5005 & 5006) following the prescribed protocol of this kit. Primers specific for amplifying bisulphite modified DNA were designed following published directions on primer design for methylation PCRs (Clark et al., 1994; Li, Dahiya, 2002). Selected primers are depicted in table 12.

Name of primer	Fw-primer	Rv-primer	Product size (bp)
SHOX-CpG-1	TGAAATGAAAAGAAAGTTAATTG	TATCCTACAAACTAAATTCC	463
SHOX-CpG-2	TAGATTAAGTTGAAGTAGAGGT	AAAAAACTAAAAACCTAAAC	542

Table 12: Bisulphite primers used for determining the degree of methylation

Primer sets were designed with http://bisearch.enzim.hu/

Thermal cycling was performed making use of gold taq polymerase in an adjusted 3-Primer protocol of table 8.2, in which the amount of bisulphite converted DNA was 2µl, the M13-label primer was replaced by mQ and a normal concentration of 10µM fw-primer was used. A normal thermal cycling program was used, shown in table 2.2, with a temperature gradient of 45-52,3°C carried out on a MyIQ thermal cycler (Bio-Rad, The Netherlands), using 40 cycles with annealing and extension times of both 30 seconds. The efficiency of amplification was checked by gel electrophoresis. This experiment was not completed and is still in progress.

d) Fragment analysis of CGG-repeat

The possibility of expansion of the CGG repeat, located within exon 1, as cause of chondrodysplasia was investigated by performing fragment analysis and by conducting DNA-sequencing on it. All of the Bouviers, one healthy Labrador and two chondrodysplastic dog breeds were used in these experiments. Microsatellite markers SHOX0.4M and SHOX0.5M, used for accomplishing linkage analysis, could not be used to detect the possible repeat expansion. Because when the CGG-repeat changes fast among generations, causing chondrodysplasia, while the microsatellite markers are more stable, linkage analysis with these microsatellite markers will not detect linkage. Primers, shown in table 13, were therefore designed surrounding the repeat and labelled with a M13-taq and A-tail, enabling fragment analysis to be performed. Only primer set 1 (SHOX-CGG-1) was used for fragment analysis, sequencing of the repeat was accomplished using the other primer set.

Name of primer	Fw-primer	Rv-primer	Product size (bp)
SHOX-CGG-1	GGAACTGACGGCTTTTGTATCC	GCCGAACTCTTTCAGTTTGTCC	252
SHOX-CGG-2	ATAACAGCAGGAGGGACCTACCC	CCTGTCCTTCCTTTCCTTTGC	541

Table 13: Primers used for investigating CGG-repeat of *SHOX*

Primer sets were selected with http://frodo.wi.mit.edu/primer3/

To perform fragment analysis, thermal cycling with primer set 1 to amplify the concerning DNA-fragment was carried out using the 3-Primer protocol of table 8.2 and 9, with an annealing temperature of 60°C, determined by a temperature gradient. Despite the adjustments that were made, using 7µl of DNA instead of 5µl and 35 cycles instead of 25 cycles, gel electrophoresis showed inefficient amplification. Therefore it was decided to use a combination of a 3-Primer PCR protocol and a Phusion PCR protocol because of the high GC content in that region. The Phusion protocol with GC buffer and DMSO of table 4.1 and 4.2 was used, substituting 1µl of mQ by 1µl of M13-label primer and using a concentration of 1µM for the forward-primer. The annealing step of thermal cycling was conducted at a temperature of 59°C for 10 seconds, followed by extension of 15 seconds, 35 cycles were used. After amplification was accomplished and the efficiency verified by gel electrophoresis (1,5% agarose-gel), samples were prepared for GeneScan with the protocol shown in table 10. Capillary electrophoresis and automated detection of products was then achieved by the 3130 xl Genetic Analyzer (Applied Biosystems) and GeneMapper software was used for assigning alleles (Applied Biosystems). Alleles were studied to determine whether an expansion of the CGG-repeat was present in patients.

For sequencing of the DNA-fragment conducted with primer set 2 (SHOX-CGG-2), the first part of the 3-Primer-protocol was used (table 8.2), substituting the M13-label primer for mQ. Normal thermal cycling was carried out (table 2.2), with an annealing temperature of 55°C (established by a temperature gradient) and an extension time of 30 seconds. Gel electrophoresis revealed only faint bands and sequence data of these products were unusable, despite using 5µl SAP-Exo-product instead of 1µl, therefore it was decided to use a Phusion PCR protocol (table 3.1 and 3.2) with GC buffer and DMSO because of the already mentioned high GC content. Annealing time and temperature of 56°C for 10 seconds, an extension time of 15 seconds and a total of 40 cycles were performed, followed by SAP-Exo purification (table 4.1, 4.2) after the amplification efficiency was verified by gel electrophoresis. A Tercycle reaction, shown in table 5.1 and 5.2 using no DMSO was performed, cycling with an annealing temperature of 50°C and an extension time of 1 minute. Further steps in the sequence protocol and subsequent data analysis are the same as described for sequencing of *FGF4*.

e) DNA-sequencing

Sequencing of the coding regions, the exon-intron bounderies and the promoter region(s) of *SHOX* was performed to investigate the possible presence of DNA sequence variations that might be causative for this specific type of chondrodysplasia in the Bouvier. Table 14 shows eight primer sets that were developed using Primer3 or a software program Primer.exe, but because of the high GC content in the entire region, selection criteria had to be adjusted, allowing primers to have a PCR-product size of 300-550bp, a primer Tm of 55-80°C and a primer GC% of 20-80%. The forward primer of primer set 1 differs only one base pair from a primer used by Young and Bannasch (Young, Bannasch, 2008). Primer sets 1 and 2 could encompass the possible promoter region located upstream of SHOX and primer set 3 amplifies the entire exon 1 including exon/intron bounderies. Primer sets 4, 5 and 8 together are enclosing exon 2, with primer set 8 being developed at a later stage mainly to investigate the possible existence of a SNP in one of the primer sequences of primer set 4. Primer 6 comprises both exon 3 and 4 and primer 7 can amplify exon 5.

Nº	Name of primer	Fw-primer	Rv-primer	Product size (bp)
1	SHOX-PromoterA	GGGTCCTGGAGCTGTTCACC	CCTGTCTGCAGCAATTAGGG	539
2	SHOX-PromoterB	TCTCTCTTCAAAAAGCTGGATGC	GGATACAAAAGCCGTCAGTTCC	382
3	SHOX-Exon1	TCACACGGACCGTCCTCTCC	CCTGTCCTTCCTTTCCTTTGC	516
4	SHOX-Exon2a	AGAGTGAGGAGACCGGCTAGG	GTAGTGGGTCTCGTCGAAAAGC	488
5	SHOX-Exon2b	AAGCGCGAGGACGTGAAGTCG	GGGTGGGCACTCGCCCTTCG	488
6	SHOX-Exon3,4	ACTTAGGGCCTGGCTGGGTTGC	TAAAAGCGGCAGCCTTTCTTCC	535
7	SHOX-Exon5	GGCATCCAGAGGACAGTTGC	CTCCAGGGCGTCCGTCAGTGC	502
8	SHOX-Exon2a(2)	AACAGACACCCGGGCTATTC	CTGAGCTCCTCCCGCATGAA	589

Table 14. Primers used for sequencing of the SHOX-gene

Primer set 5 was developed with software Primer.exe

The other primers were selected with http://frodo.wi.mit.edu/primer3/

For thermal cycling of all primer sets a Phusion PCR protocol was performed (table 3.1 and 3.2) using DMSO and GC-buffer. Forty cycles were carried out on a C1000 thermal cycler (Bio-Rad, The Netherlands), with annealing and extension times of 15-30 seconds and 10-15 seconds, respectively. A temperature gradient revealed the following optimal annealing temperatures: 55°C for primer 8, 57°C for primer sets 1 and 2, 61°C for primer set 4 and 67°C for primer 6. Different annealing temperatures were tried for primer sets 3, 5 and 7, but they failed to produce amplification products ready for sequencing. After checking the results of

amplification by performing gel electrophoresis, SAP-Exo purification and Tercycle reaction were applied to PCR-products, following protocols illustrated in tables 4 and 5. In the Tercycle protocol DMSO, an annealing temperature of 50°C and an extension time of 1 minute were used. Further steps in the sequence protocol and subsequent data analysis are the same as described for sequencing of *FGF4*. SNPs and other sequence variations were mapped for the population of Bouviers and haplotypes were determined.

Results

1. fgf4 retrogene

The first part of this study focussed on the fgf4 retrogene as a possible cause of chondrodysplasia in Labrador Retrievers and Bouvier de Flandres. Primer set A amplified the right DNA fragments in all tested DNA samples, including affected Labradors and Bouviers, healthy Labradors and chondrodysplastic dog breeds. Sequence data of these fragments, required to distinguish between retrogene and source gene based on a single nucleotide, were not yet analysed. Primer B failed to work properly. Primer sets C and D, also retrogene-specific primers, were then ordered to investigate the existence of a retrogene. They gave PCR-products of expected size for the three chondrodysplastic breeds, while none of the 7 respectively 6 chondrodysplasia affected Labradors and Bouviers showed PCR-products on gel electrophoresis (figure 11).

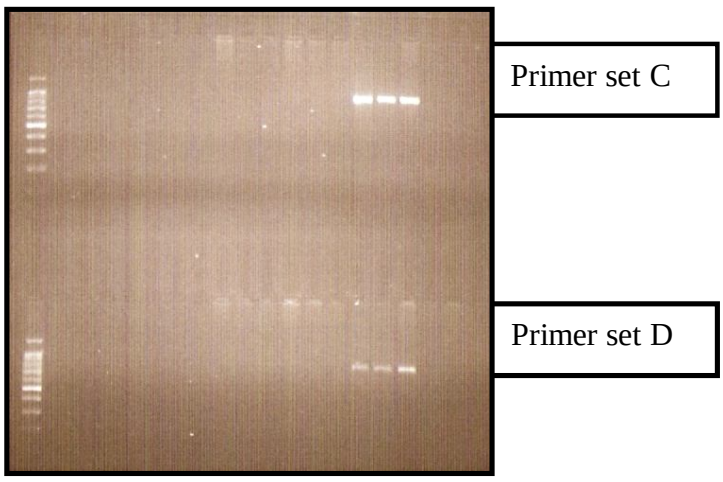


Figure 11. Gel electrophoresis for PCR with primer sets C and D

On the left, there is a 100bp ladder (Promega). The following samples were loaded onto the gel from left to right: thirteen Labrador and Bouvier patients, three chondrodysplastic breeds, two healthy Labradors, mQ. For the three chondrodysplastic breeds, PCR-products of expected size indicate the presence of a fgf4 retrogene. No bands are visible for the other samples, therefore a fgf4 retrogene is absent in these animals.

This leads to the conclusion that the fgf4 retrogene is only present in chondrodysplastic dog breeds and that it is not responsible for causing this type of chondrodysplasia in these populations of Labradors and Bouviers.

2. Linkage analysis of various candidate genes

Linkage analysis using polymorphic microsatellite markers was performed on a couple of candidate genes to investigate their roles in chondrodysplasia in the Labrador Retriever and Bouvier des Flandres. The number of alleles displayed by markers 1-10 is depicted in table 15. The informativity of the markers is also shown, based on reliability of allele-calling and presence or absence of polymorphism. A marker was denominated as non-informative when LOD-scores were around 0. LOD-scores above 3.0 were considered evidence for linkage, because then there is a chance of 1000 to 1 that data is not obtained by mere coincidence. Linkage was concluded to be absent when LOD-scores were below -2.0. Most of the candidate genes can be excluded or are highly unlikely to play a role in chondrodysplasia in both Labradors and Bouviers, based on LOD-scores for recombination values ranging from 0.01 to 0.02 calculated in two-point and multipoint analyses. LOD-scores are illustrated in Appendix 2.

N°	Gene	Micosatellite marker	Number of alleles	Quality of marker
1	FGF4	REN266I17	7 (Labradors); 2 (Bouviers)	Non-informative in Bouviers (X)
2	FGFR3	REN260I04	2	Non-informative (R,D)
3	FGFR3	Cfa3_65M	5	Non-informative (R,D)
4	FGFR3	Cfa3_65.2M	5 (or 4)	Non-informative (R,D)
5	FGFR3	CFA03:65M_FGFr3	1	Unreliable
6	Ihh	FH2587	5	
7	Ilk	FH2441	6	
8	NPR2	REN194N17	4	
9	DDR2	REN164E17	5	Non-informative (D)
10	Spred2	FH3381	11	Unreliable

Table 15. Number of alleles and quality depicted for markers 1-10.

When calculated LOD-scores are around 0, the marker is non-informative. This can be for a recessive inheritance pattern (R), a dominant one (D) or both (R,D).

Evaluating LOD-scores from the two-point and multi-point analysis demonstrated following results: In Labradors this type of chondrodysplasia is definitely not monogenically caused by *IHH* or *ILK* (both with a recessive (R) and a dominant (D) pattern) or by *DDR2* or *SPRED2* (following a recessive pattern). Furthermore it is highly unlikely to be monogenically caused by *NPR2* or *FGF4* (R,D) or by *SPRED2* (R). In Bouviers this type of chondrodysplasia is definitely not monogenically caused by *ILK*, *SPRED2* or *FGFR3* (R,D) or by *NPR2*, *DDR2* or *IHH* (R) or by *FGF4* (D). Furthermore it is highly unlikely to be monogenically caused by *NPR2* or *IHH* (D).

Because of the curious inheritance pattern in Bouviers of (almost) completely affected litters being born out of normal parent dogs, it is more likely for this type of chondrodysplasia to be polygenic. Results of the therefore performed two-loci two-point and multi-point analysis were as follows: In Labradors, the only positive LOD-scores of above 1.000 (but all below 1.800) for recombination frequencies of 0.00 and 0.01 were in nest H for marker combinations including marker 1. Though when studying alleles, it was found that the other two litters showed completely different patterns, therefore the positive LOD-scores are not really important. In Bouviers LOD-scores could only be calculated for a recessive inheritance pattern, because calculations within this extensive pedigree were too time-consuming to be performed by the Superlink website. Only for marker combinations including marker 1 positive LOD-scores of above 1.000 were calculated. The combination of marker 1 and marker 10 revealed a LOD-score of 1.57 and 1.60 calculated for recombination fractions of 0.00 and 0.01 respectively. One-locus analysis for a recessive inheritance pattern showed LOD-scores of $-\infty$ with θ 0.001 for marker 1 and -13.4 with θ 0.01 for marker 10. Especially this great increase in LOD-scores of these markers from a one-locus analysis to a two-locus analysis was thought to be interesting, but important remarks have to be made: a LOD-score of 1.57 is not really high, marker 1 is not polymorphic and marker 10 displays different marker patterns for every allele, thereby making allele calling less reliable. The demonstrated positive LOD-scores are therefore not of great importance.

Altogether, relevance for further testing of these candidate genes seems negligible.

3. SHOX-gene

In the third part of this study *SHOX* was investigated, because it was considered to be an important candidate gene for the specific type of chondrodysplasia seen in Bouvier des Flandres.

a) Assessment of radiological features

Table 16 shows a classification of radiological features for 15 Bouviers. Animals depicted in identical colours are from the same litter. Within litters there is no distinct dichotomy regarding severity of the disease. Furthermore, there appears to be no difference in severity between males and females. Based on radiological features, the phenotype of the affected

Bouviers is remarkably similar to the relatively mild Léri-Weill Dyschondrosteosis and the more severe Langer Mesomelic Dysplasia in men.

+	++	+++	++++
A (♂)	C (♀)	J (♀)	L (♂)
B (♂)	D (♀)	K (♂)	M (♀)
	E (♂)		N (♂)
	F (♀)		O (♀)
	G (♀)		
	H (♀)		
	I (♂)		

Table 16. Radiological classification of 15 affected Bouviers

It ranges from + which is characterized by only slight bowing of the radius to ++++ revealing severe curving of the radius and luxation of the radial head.

b) Linkage analysis

Microsatellite marker SHOX0.5M appeared to be non-polymorphic and allele-calling was unreliable, because all Bouviers showed one peak of a length varying between 409 bp and 411 bp. Therefore only one marker (SHOX0.4M) was used to detect the presence or absence of linkage. To exclude SHOX as a candidate gene based on the results of this single marker seems to be inappropriate, but when the distance between marker and gene is minor (less than 1Mb) and the marker is polymorphic, one marker suffices to reliably confirm or reject linkage. Marker SHOX0.4M is located at the relatively small distance of 102.9 kb and approximately 30-40 kb to the gene and the possible regulatory region respectively and with exhibiting 4 alleles it is a polymorphic marker. There is no linkage demonstrated, leading to the conclusion that mutations or deletions of the SHOX-gene or regulatory region are unlikely to be responsible for causing chondrodysplasia in the Bouvier. Further on, remarks are made on this topic.

c) Determination of degree of CpG-methylation

Primer set 2 failed to produce DNA fragments visible with gel electrophoresis, but primer set 1 showed a faint band on gel electrophoresis, using a 1,5% agarose gel, with correct EtBr concentrations and a 100bp ladder (Promega), 2µl loading dye, 4µl mQ and 5µl PCR-product. This product was generated using the last described thermal cycling protocol at an annealing temperature of 52,3°C performed on the MyIQ thermal cycler (Bio-Rad, The Netherlands). Lower temperatures revealed no amplification products. Optimizing amplification needs to be

carried out followed by sequencing of the PCR-product to determine the degree of methylation.

d) Fragment analysis of CGG-repeat

All 43 Bouviers, one healthy Labrador and two chondrodysplastic dog breeds showed the same allele with fragment analysis and sequencing revealed that they all had the same number of CGG-repeats, eight in total. From this, it can be concluded that this type of chondrodysplasia in the Bouvier is not caused by an expansion of the CGG-repeat.

e) DNA sequencing

Notwithstanding the fact that linkage has not been demonstrated with a polymorphic microsatellite marker, mutations in the SHOX-gene or in flanking regions are still a possible cause of chondrodysplasia in the Bouvier. The percentage of recombination in PAR1 is extremely high, increasing the chance of recombination to occur in the DNA fragment of 102,9 kb between marker and gene, hence abolishing linkage. Therefore gene sequencing was performed.

Primer sets 3, 5 and 7 were not ready for sequencing, mainly because especially primers 3 and 7 produced multiple amplification products, requiring the correct bands to be eluted from the gel. Excision of the right products from the gel and eluting it overnight, after which eluate was used in a Tercycle reaction followed by sequencing, failed to result in accurate sequence data because input was to marginal. A Phusion PCR-protocol (table 3.1, 3.2) on the eluate was therefore performed on the C1000 thermal cycler using 40 cycles with an annealing temperature and time of 60°C for 15 seconds followed by an extension time of 10 seconds. This failed to produce products visible with gel eclectrophoresis. Primer 5 showed distinct products of the right length on gel electrophoresis, except for one unaffected Bouvier sample, that revealed one product causing a strong band of a slightly smaller length. This could be interesting and needs to be examined by carrying out gene sequencing. Thermal cycling needs yet to be optimized for these three primers, enabling sequencing to be performed.

Sequencing DNA-fragments produced by the other primer sets revealed the presence of multiple alterations, shown in table 17. The sequence of *SHOX*, including 1000 bp directly in front of exon 1, was used as reference, therefore deletion 481 for example is located 519 bp ahead of the start of exon 1.

Type	Localization		Amplified by primerset
Deletion of G-nucleotide	481	Possible promoter region	*1*
SNP	650	Possible promoter region	*1*
SNP	4027	Primer sequence of primer set 4	*8*
SNP	4043	Primer sequence of primer set 4	*8*
Insertion of G-nucleotide	4221	Intron 1	*4*
SNP	4290	Intron 1	*4*
SNP	8600	Intron 3	*6*

Table 17. SNPs, a deletion and an insertion demonstrated in or nearby the *SHOX*-gene.

Localization is counted from 1000 bp ahead of the beginning of exon 1 and the place where these changes reside is shown.

The insertion at position 4221 and SNP 4290 were both discovered by primer set 4. When they were clearly defined in pedigrees, it was noticed that the alleles did not segregate, offspring showed for example only the A-allele for SNP 4290 while both parents revealed just the G-allele. Furthermore, for SNP 4290 there are 28 homozygote G, while only 17 homozygote A is present and except for one sample (a Bouvier that was born a couple of years earlier than the rest of the dogs) there are no heterozygous animals for SNP 4290. This is genetically highly unlikely when a population of 46 animals is tested. A SNP in the forward or reverse primer sequence of primer set 4 in one allele would explain this peculiar phenomenon. The SNP makes thermal cycling less efficient for that allele, resulting in the formation of a 'null'-allele, because that allele is not revealed in the sequence data. The hypothesis was that a SNP existed in the primer sequence of the A-allele of SNP 4290, because in that case the A-allele comes to expression only when there are two A-alleles with this primer SNP, both with equally inefficient thermal cycling. The combination of an A-allele with primer SNP and a G-allele then results in almost exclusive amplification of the G-allele, resulting in only G peaks in sequence data, explaining the skewed proportions between G and A. The insertion at position 4221 is amplified by the same primer set, therefore the primer SNP will also influence the amplification of this insertion, leading to a comparable distorted proportion of alleles. The alleles of insertion 4221 numbered as 5 (G-nucleotides) or 6 (G-nucleotides) and SNP 4290 shown as G or C, based on sequence data are depicted in pedigrees in figure 1 of appendix 3. Table 18 shows what sequence data could be expected for different genotypes when a primer SNP in allele A is present. From this, real genotypes could be (partly) concluded.

Genotype		Sequence data
5A	5A	5A
5A	5G	5G
5A	6A	5,6A
5A	6G	6G
5G	5G	5G
5G	6A	5G
5G	6G	5,6G
6A	6A	6A
6A	6G	6G
6G	6G	6G

Table 18. Possible genotypes belonging to sequence data with insertion 4221(5 or 6) and SNP4290(G or C)
Sequence data obtained when a SNP is present in one of the primer sequences of allele A for SNP 4290. The accurate genotypes are shown for all sequence results.

To test the hypothesis of this primer SNP, primer set 8 was designed encompassing both primer sequences of primer set 4. Two SNPs were found to be present in the primer sequence of forward primer 4 (ranging from 4023-4044), one at position 4027, the other at position 4047. These SNPs are illustrated in figure 12.

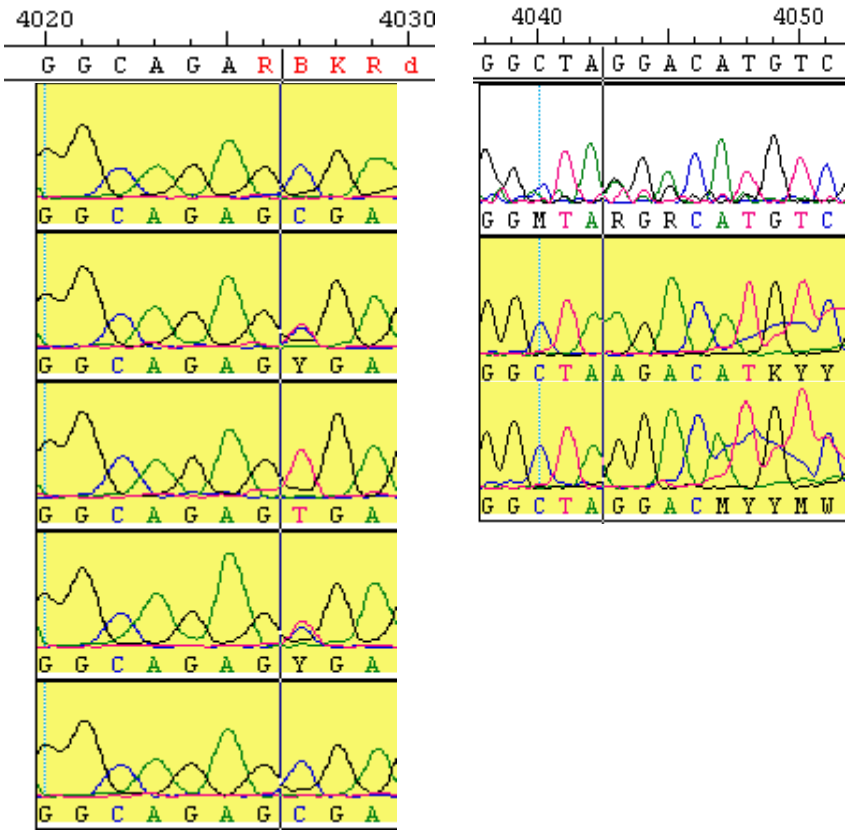


Figure 12. SNPs in primer sequence of forward primer 4
Two SNPs present in the primer sequence of the forward primer of primer set 4.

SNPs at the 5' end of a primer sequence usually do not have a great influence on efficiency of amplification. On the other hand, SNPs at the 3'end of a primer often result in a markedly reduced amplification efficiency. The actual alleles for insertion 4221 and SNP 4290 were revealed by this new primer set, two of the twelve tested samples were heterozygous (5,6) for the insertion instead of the homozygous absence of insertion (5) and two of the nine tested samples revealed A,G instead of GG. This proved table 18 to be accurate, leading to the conclusion that the hypothesis was correct. Table 19 shows which alleles were linked to each other in the DNA-fragment amplified by primer set 8. Therefore in this study, it is a SNP at position 4043 that causes the 'null' allele.

	SNP 4027	SNP 4043	Insertion 4221	SNP 4290
"Normal"	T	G	5	G
"Changed"	C	A	6	A

Table 19. Report of which alleles appeared to be linked

There appears to be no causal relationship between the SNPs, the insertion or the deletion found in the SHOX-gene and chondrodysplasia in Bouviers, since affected as well as unaffected animals show all different types of alleles for every variation. Although *SHOX*-mutations are an important cause of chondrodysplasia in humans resembling the specific canine type of chondrodysplasia seen in Bouviers, they do not seem to be correlated to this type of skeletal dysplasia in this particular dog breed.

Discussion

1. fgf4 retrogene

The number of Labrador and Bouvier patients (n=7 and n=6, respectively) might be considered too low, but patients were selected from different litters throughout the whole population and animals displayed varying degrees of severity, making it a sufficient sample size. Performing a normal PCR-protocol on primer B revealed that lower annealing temperatures were able to generate product, though with low specificity, while higher temperatures, according to Parker et al. (2009) believed to be required for accurate amplification with primer B, failed to efficiently amplify the desired DNA-fragment. When there is a fgf4 retrogene present, primer set B amplifies a DNA-fragment encompassing all three exons. A N-stretch is located between exon 1 and 2 of the *FGF4* gene (Genebank – accession XM_540801+2) that could result in a longer PCR-product than the expected fragment size of 2500 bp. Therefore possibly explaining the fact that amplification with an extension time of 2 minutes in a normal PCR-protocol failed to work properly, because approximately 1000 bp can be formed per minute of extension. However, this theory can be rejected, because introns were already removed by splicing before the cDNA copy was inserted into the chromosome. It is still possible that extension time was too short for the DNA-fragment to be fully amplified, but a Phusion PCR-protocol with an extension time of 90 seconds has also been performed, amplifying about 1000 bp per 30 seconds, meaning that thermal cycling should in theory have been able to amplify this product of 2500 bp. Altogether thermal cycling with longer extension times could have been tried for primer set B. $MgCl_2$-concentrations could also have been varied to try to achieve a higher specificity.

2. Linkage analysis of various candidate genes

Dogs and humans display a certain resemblance regarding phenotypic features of various diseases, therefore studying grounds for any human disease can be helpful in selecting candidate genes for dogs with a disease showing similar characteristics to that of humans. Variation in phenotypic expression though complicates classification of dogs in a research population investigating a particular type of disease. Furthermore, clinical features of a

disease are never identical for various individuals of the same, let alone different, species. With the same disease, dogs can exhibit a phenotype remarkably distinct from that of humans. This is the reason that, despite the absence of truly similar clinical features of chondrodysplasia between dogs and humans, some of the candidate genes in this study were tested. For a couple of selected candidate genes though, the test rationale was not strong enough and when clinical features were more clear at the beginning of this study, these genes would not have been selected for this research. For example the Acromesomelic Dysplasia, type Maroteaux caused by *NPR2* mutations is characterized by distal shortening of the extremities, resulting in shortened and widened metacarpalia and phalanges. Another illustration is the achondroplasia caused by mutations in *FGFR3*, which is characterized mainly by proximal shortening of the extremities, known as rhizomelic dwarfism. The features of these two diseases were not shown by the Labradors and Bouviers in this study. Although certain characteristics may vary between different species, the specific phenotype brought on by mutations of several of the investigated candidate genes differs too much from that seen in humans and mice, making them less valuable as candidate genes. According to Smit et al. the type of chondrodysplasia in the Labrador population might display parallels with spondyloepiphyseal dysplasia and multiple epiphyseal dysplasia in humans (Smit et al., 2009). This information was not known beforehand, otherwise it could have been used to select more appropriate candidate genes.

Microsatellite markers need to be in close proximity to the gene of interest, thereby reducing the possibility of recombination between marker and gene. Marker 2 and marker 6 were located at a considerable distance to their genes, leaving room for recombination to occur. A distance of 1 Mb corresponds with 1 cM (centiMorgan), leading to a recombination frequency of 1%, meaning crossing over occurs in 1 out of *IHH*, with a distance of more than 1.1 Mb to the gene, demonstrating absence of linkage is questionable. Dinucleotide REN microsatellite markers are believed to be more stable than the tetranucleotide FH repeats and are therefore more reliable in linkage analysis. The only reason for still using FH markers was that they were already on hand in the laboratory. Besides their essential stability and localization close to a candidate gene, microsatellite markers are obliged to be polymorphic for them to be valuable in linkage analysis. When only one or two alleles exist for a specific marker (like the two alleles of marker 2), concluding whether linkage is absent or present is impossible. Furthermore, markers can be officially excluded based on a LOD-score of less than -2.0 for a recombination value θ of 0.05 or higher, but these values were thought to be too stringent. A

recombination value of 0.01 equals both 1 cM and 1 Mb and the maximum distance between markers and candidate genes in this study was 1.5 Mb. LOD-scores below -2.0 were therefore considered to exclude linkage for recombination values ranging from 0.01 to 0.02.

3. *SHOX*-gene

a) Assessment of radiological features

It would have been best if the animals were classified in two groups instead of four, making comparison between this type of canine chondrodysplasia and the two humane forms of chondrodysplasia (LWD and LMD) more distinct. Furthermore, classification in Bouviers was found to be challenging, because this disease seemed to exhibit a sliding scale of severity instead of showing a couple of separate subtypes, markedly differing from each other.

b) Linkage analysis

Notwithstanding the fact there was no linkage demonstrated using a polymorphic microsatellite marker located at a relatively small distance to *SHOX* and the proposed enhancer region, mutations in this gene or in its regulatory regions could still be accountable for causing chondrodysplasia in Bouviers. The reason for questioning linkage analysis in this study is that recombination frequency of PAR1 is exceptionally high (Blaschke, Rappold, 2006). This increases the chance of recombination to take place between marker and gene, making negative results of linkage analysis less reliable. Gene sequencing to detect any mutations or deletions was therefore subsequently performed. The canine genome was searched for regions that showed similarity to the reported 29 kb overlapping downstream deletion region (Benito-Sanz et al., 2005). As previously stated, other researchers found overlapping deletions together with slightly different regions. Combining information of the various studies on enhancer regions of *SHOX* reveals that a downstream flanking region of maximal 550 kb encompasses (almost) every deletion reported to result in idiopathic short stature, Léri-Weill Disease and Langer Mesomelic Dysplasia. The 29 kb region was chosen based on the results of multiple studies encompassing a large group of patients (Benito-Sanz et al., 2005; Fukami et al., 2006; Huber et al., 2006). Further investigation is required to examine whether other parts of the 550 kb flanking region are also present adjacent to the canine *SHOX*-gene. In humans multiple enhancers probably exist, all located downstream of *SHOX*. It would be interesting to investigate whether dogs exhibit the same enhancers as

humans and if chondrodysplasia is indeed caused by deletions of these enhancer regions. Especially because of the fact that PAR1 deletions have been reported to represent a high proportion of identified mutations compared to *SHOX* mutations and deletions in LWD (Benito-Sanz, 2006; Chen et al., 2009). MLPA (Multiplex Ligand dependant Probe Amplification), FISH analysis (Fluorescent In Situ Hybridization) or SNP analysis could be used to screen for these enhancer deletions.

c) Determination of degree of CpG-methylation

Different thermal cycling protocols were tried, but both primer sets failed to work properly. Primer set 2 can be withdrawn from the experiment, because it failed to work at all, but amplification with primer set 1 can probably be optimized resulting in amplification products ready to be sequenced. This work needs to be conducted in the future. A few remarks need to be made on primer selection. According to Li and Dahiya, the advised PCR-product length is approximately 100-300 bp, product sizes greater than 300 would be difficult to amplify (Li, Dahiya, 2002). Primer set 1 and 2 result in PCR-product sizes of 463 bp and 543 bp respectively, thereby possibly explaining the dysfunctioning of both primer sets. Furthermore, primers selected from http://bisearch.enzim.hu/ have a length of 20-23 bp, al lot shorter than suggested to be advisable (Clark et al., 1994; Li, Dahiya, 2002). Bisulphite conversion leads to a decreased GC content, especially when the original sequence had a high GC content, as is the case in these regions of the *SHOX*-gene. Long stretches of T's therefore appear in the sequence, illustrated by the high numbers of A-mononucleotide repeats seen in selected primer sets 1 and 2, making primer selection with appropriate stability and Tm values difficult. Efficiency of bisulphite primers in general seems to be quite variable making it important to design multiple primer sets to any sequence (Clark et al., 1994). Preferably, primers would be selected to lie exactly in the (CpG islands linked to the) suspected promoter region of *SHOX*, located in a region of approximately 800 bp adjacent to the 5' end of *SHOX*. Therefore primers had to be designed encompassing these regions, but no acceptable primer sets were found to match the above described criteria, hence the selection of primer set CpG1 with its reverse primer located in exon 1 and its forward primer more than 400 bp upstream. It is possible that this primer set does not include CpG islands linked to the promoter region of *SHOX*.

Further research of CpG methylation is thus required to investigate whether there is epigenetic silencing of the *SHOX*-gene. It seems advisable to design another one or two

primer sets, precisely following criteria for primer selection, because as stated, efficiency in general can sometimes be very low.

d) Fragment analysis of CGG-repeat

Performing fragment analysis on the DNA-fragment encompassing the CGG-repeat with primer set 1 was challenging. Normal protocols were adjusted because results were disappointing and unreliable for many samples. A possible explanation is that amplification was not efficient due to the exceptionally high GC content in the DNA sequence (Blaschke, Rappold, 2006). The use of a combined Phusion/3-Primer PCR-protocol, much more capable of amplifying DNA-fragments with a high GC content, offered a solution. Sequence data of the forward primer of primer set 2 revealed an abnormal pattern, formed by background as a result of a shift of one base pair. This could have been due to a certain percentage of the produced forward primer missing one base. As a consequence, sequence data could not be thoroughly examined, because studying of both forward and reverse primer data was necessary. Forward primer 1 could have been used instead to enable sequencing, but this was not performed, because there already was intelligibility regarding the CGG-repeat. Since absence of linkage revealed by SHOX0.4M was not reliable and sequencing of SHOX was planned, the CGG-primer sets could have been used for this.

e) DNA sequencing

Various parts of the SHOX-gene and flanking regions exhibit an exceptionally high GC content, previously reported by Blaschke and Rappold (Blaschke, Rappold, 2006). Due to this, creating primer sets for several regions on the *SHOX*-gene was found to be challenging. Schiller et al. were found to have experienced the same problems with primer design in these regions (Schiller et al., 2000). In the future, sequencing of the coding regions of *SHOX*, its flanking regions and promoter region and also the possible long-range regulatory enhancer needs to be completed.

Overview of candidate genes

Much research has been performed on canine chondrodysplasia in Labrador Retrievers and Bouviers des Flandres by the staff of the Department of Clinical Sciences of Companian animals at the Utrecht University, Faculty of Veterinary Medicine (Smit et al., 2009; Temwichitr et al., 2009). Here an overview is given on investigated candidate genes that were demonstrated not to play a role in this type of skeletal disease.

Excluded as candidate genes in previous studies:

- *Col 2a1* (Collagen 2a1)
- *Col 9a1* (Collagen 9a1)
- *Col 9a2* (Collagen 9a2)
- *Col 9a3* (Collagen 9a3)
- *Col 11a1* (Collagen 11a1)
- *Col 11a2* (Collagen 11a2)
- *COMP* (Cartilage Oligomeric Matrix Protein)
- *MATN3* (Matrilin 3)
- *SLC26A2* (Sulphate transporter glycoprotein)

Excluded as candidate genes in the present study:

- *FGF4* (Fibroblast Growth Factor 4)
- *FGFR3* (Fibroblast Growth Factor Receptor 3)
- *IHH* (Indian Hedgehog)
- *NPR2* (Natriuretic Peptide Receptor B)
- *ILK* (Integrin Linked Kinase)
- *DDR2* (Discoidin Domain Receptor 2)
- *SPRED 2* (Member of the Sprouty/Spred family of proteins that regulate growth factor induced activation of the MAP-kinase pathway)

Future research

- ✧ The downstream regulatory region of *SHOX* needs to be further examined, because in man, deletions in this part of PAR1 are almost a more common cause of chondrodysplasia than mutations in the *SHOX*-gene itself. Within these regulatory regions, regions of homology between man and canine species should be detected, followed by sequencing of these parts of the canine X-chromosome.
- ✧ It should be investigated whether gene conversion occurs at PAR1 of the X-chromosome of Bouviers. This would have the same implications as a high recombination rate, making linkage analysis unusable to examine a possible association between *SHOX* and chondrodysplasia in the Bouvier.
- ✧ Determination of the degree of CpG-methylation is required to confirm or reject epigenetic silencing of the SHOX-gene as a cause of chondrodysplasia.
- ✧ Expansion of the CGG-repeat in the SHOX-gene was not responsible for causing chondrodysplasia in the Bouvier, but this theory should also be investigated in the research population of Labrador Retrievers.
- ✧ Sequencing of all of the exons, exon/intron bounderies and promoter regions needs to be completed, because in man there are numerous mutations in the *SHOX*-gene known to cause chondrodysplasia with phenotypic features remarkably similar to that of the Bouvier patients.
- ✧ SHOX-protein in dogs is most likely located in bone marrow fibroblasts, skeletal muscle and in the chondrocytes of the reserve, proliferative, and hypertrophic zone of growth plates until the time growth plates fuse. Immune histochemistry could be performed to determine whether SHOX is present in these tissues. Microscopic evaluation of cartilage might reveal important information on its architecture and on the process of endochondral ossification, so that it can be determined exactly when and where disturbances take place.
- ✧ Recently SHOX has been found to interact with *NPPB*, a gene that encodes for Brain Natriuretic Peptide (BNP). BNP like SHOX was suggested to play a role in chondrocyte maturation, therefore BNP might be an important candidate gene for chondrodysplasia in the Bouvier des Flandres, that needs to be investigated in the future.

Acknowledgements

The author sincerely thanks Prof. Dr. Hazewinkel, Dr. Leegwater and Dr. Tryfonidou for their great support and their insightful comments on the manuscript. Furthermore, the author wishes to show her gratitude to Ineke Lavrijsen, Frank van Steenbeek, Manon Vos-Loohuis and Ellen Martens who's help with all kinds of laboratory work and data-analysis was really fantastic and indispensable. This work is supported in part by the Van der Hucht de Beukelaar Foundation and by Hill's Pet Nutrition.

References

Bargal, R. et al. (2009). Mutations in DDR2 gene cause SMED with short limbs and abnormal calcifications. *The American Journal of Human Genetics*, 84: pp.80-84

Bartels, C.F. et al. (2004). Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux. *The American Journal of Human Genetics*, 75: pp.27-34

Belin, V. et al. (1998). SHOX mutations in dyschondrosteosis (Leri-Weill syndrome). *Nature Genetics*, 19: pp.67-69

Bellus, G.A. et al. (1995). A recurrent mutation in the tyrosine kinase domain of fibroblast growth factor receptor 3 causes hypochondroplasia. *Nature Genetics*, 10: pp.357-359

Benito-Sanz, S. et al. (2005). A novel class of pseudoautosomal region 1 deletions downstream of SHOX is associated with Léri-Weill dyschondrosteosis. *The American Journal of Human Genetics*, 77: pp.533-544

Benito-Sanz, S. et al. (2006) PAR1 deletions downstream of SHOX are the most frequent defect in a Spanish cohort of Leri-Weill dyschondrosteosis (LWD) probands. *Human Mutation*, 10: pp.1062

Bertorelli, R. et al. (2007). The homozygous deletion of the 3' enhancer of the SHOX gene cause Langer mesomelic dysplasia. *Clinical Genetics*, 72: pp.490-491

Binder, G. et al. (2004). SHOX haploinsufficiency and Leri-Weill dyschondrosteosis: prevalence and growth failure in relation to mutation, sex and degree of wrist formation. *The Journal of Clinical Endocrinology & Metabolism*, 89(9): pp.4403-4408

Blaschke, R. J. et al. (2003). Transcriptional and translational regulation of the Léri-Weill and Turner syndrome homeobox gene SHOX. *The Journal of Biological Chemistry*, 278(48): pp.47820-47826

Blaschke, R.J., Rappold, G.A. (2001). SHOX in short stature syndrome. *Hormone Research*, 55(suppl.1): pp.21-23

Blaschke, R.J., Rappold, G.A. (2006). The pseudoautosomal regions, SHOX and disease. *Current Opinion in Genetics & Development*, 16: pp.233-239

Brocks, B.A.W., Hazewinkel, H.A.W. (2004). Abnormal skeletal growth in the Labrador Retriever: a case report. *12th ESVOT Congress, Munich, 2004.*

Bunschu, K. et al. (2005). Gene disruption of Spred-2 causes dwarfism. *The Journal of Biological Chemistry*, 280(31): pp.28572-28580

Chen, L. et al. (1999). Gly369Cysmutation in mouse FGFR3 causes achondroplasia by affecting both chondrogenesis and osteogenesis. *The Journal of Clinical Investigation*, 104: pp.1517-1525

Chen, L. et al. (2001). A Ser(365)→ Cys mutation of fibroblast growth factor receptor 3 in mouse downregulates Ihh/PTHrP signals and causes severe achondroplasia. *Human Molecular Genetics*, 10: pp.457-465

Chen, H. (2006). Atlas of Genetic Diagnosis and Counseling. Chapter: Dyschondrosteosis (Leri-Weill Syndrome) and Langer Mesomelic Dysplasia. pp.305-311. Totowa, New Jersey: Humana Press. ISBN: 978-1-60327-161-5 (online)

Chen, J. et al. (2009). Enhancer deletions of the SHOX gene as a frequent cause of short stature: the essential role of a 250 kb downstream regulatory domain. *Journal of Medical Genetics*, 46: pp.834-839

Chung, U. et al. (2001). Indian hedgehog couples chondrogenesis to osteogenesis in enchondral bone development. *The Journal of Clinical Investigation*, 107(3): pp.295-304

Chusho, H. et al. (2001). Dwarfism and early death in mice lacking C-type natriuretic peptide. *Proceedings of the National Academy of Sciences, Society* 98(7): pp.4016-4021

Clark, S.J. et al. (1994). High sensitivity mapping of methylated cytosines. *Nucleic Acids Research*, 22(15): pp.2990-2997

Clement-Jones, M. et al. (2000). The short stature homeobox gene SHOX is involved in skeletal abnormalities in Turner syndrome. *Human Molecular Genetics*, 9(5): pp.695-702

Colnot, C. et al. (2005). Indian hedgehog synchronizes skeletal angiogenesis and perichondral maturation with cartilage development. *Development*, 132: pp.1057-1067

Coumoul, X., Deng, C. (2003). Roles of FGF receptors in mammalian development and congenital diseases. *Birth Defects Research part C*, 69: pp.286-304

Deng, C. et al. (1996). Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell*, 84: pp.911-921

Disteche, C.M. et al. (2002). Escape from X inactivation. *Cytogenetic and Genome Research*, 99: pp.36-43

Dreyer, S.D. et al. (1998). The long and the short of it: developmental genetics of the skeletal dysplasias. *Clinical Genetics*, 54: pp.464-473

Ezquieta, B. et al. (2002). SHOX intragenic microsatellite analysis in patients with short stature. *Journal of Pediatric Endocrinology & Metabolism*, 15(2): pp.139-148

Feldman, B. et al. (1995). Requirement of FGF-4 for postimplantation mouse development. *Science*, 167(5195): pp.246-249

Flanagan, S.F. et al. (2002). Prevalence of mutations in the short stature homeobox containing gene (SHOX) in Madelung deformity of childhood. *Journal of Medical Genetics*, 39: pp.758-763

Fondon, J.W. (2004). Molecular origins of rapid and continuous morphological evolution. *Proceedings of the National Academy of Sciences*, 101(52): pp.18058-18063

Fukami, M. et al. (2006). Transactivation function of an ~800-bp evolutionarily conserved sequence at the SHOX 3' region: implication for the downstream enhancer. *The American Journal of Human Genetics*, 78: pp.167-170

Goldberg, P. (1999). A SHOXing new kind of inheritance. *Clinical Genetics*, 56(4): pp.263-266

Grashoff, C. et al. (2003). Integrin-linked kinase regulates chondrocyte shape and proliferation. *European Molecular Biology Organization reports*, 4(4): pp.432-438

Grigelioniene, G. et al. (2000). Mutations in short stature homeobox containing gene (SHOX) in dyschondrosteosis but not in hypochondroplasia. *Human Genetics*, 107:pp.145-149

Grigelioniene, G. et al. (2001). Analysis of short stature homeobox-containing gene (SHOX) and auxological phenotype in dyschondrosteosis and isolated Madelung deformity. *Human Genetics*, 109: pp.551-558

Guo, C. et al. (2008). Sprouty 2 disturbs FGFR3 degradation in thanatophoric dysplasia type II: A severe form of human achondroplasia. *Cellular Signaling*, 20: pp.1471-1477

Hart, K.C. et al. (2000). Transformation and Sts activation by derivatives of FGFR1, FGFR3 and FGFR4. *Oncogene*, 19(29): pp.3309-3320

Heard, E., Disteche, C.M. (2006). Dosage compensation in mammals: fine-tuning the expression of the X chromosome. *Genes & Development*, 20: pp.1848-1867

Horton, W.A. (1997). Fibroblast growth factor receptor 3 and the human chondrodysplasias. *Current Opinion in Pediatrics*, 9: pp.437-442

Horton, W.A., Lunstrum, G.P. (2002). Fibroblast growth factor receptor 3 mutations in achondroplasia and related forms of dwarfism. *Reviews in Endocrine & Metabolic Disorders*, 3: pp.381-385

Huber, C. et al. (2006). High incidence of SHOX anomalies in individuals with short stature. *Journal of Medical Genetics*, 43: pp.735-739

Itoh, N., Ornitz, .M. (2008). Functional evolutionary history of the mouse Fgf gene family. *Developmental Dynamics*, 237: pp.18-27

Jin, P., Warren, S.T. (2000). Understanding the molecular basis of fragile X syndrome. *Human Molecular Genetics*, 9(6): pp.901-908

Jorge, A.A.L. et al. (2007). SHOX mutations in idiopathic short stature and Leri-Weill dyschondrosteosis: frequency and phenotypic variability. *Clinical Endocrinology*, 66: pp.130-135

Jüppner, H. (2000). Role of parathyroid hormone-related peptide and Indian hedgehog in skeletal development. *Pediatric Nephrology*, 14: pp.606-611

Kano, K. et al. (2008). A novel dwarfism with gonadal dysfunction due to loss-of-function allele of the collagen receptor gene, ddr2, in the mouse. *Molecular Endocrinology*, 22(8): pp.1866-1880

Kant, S.G. et al. (2003). Genetic analysis of short stature. *Hormone Research*, 60: pp.157-165

King, J.A.J. et al. (2005). Distinct requirements for the Sprouty domain for functional activity of Spred proteins. *Biochemical Journal*, 388: pp.445-454

Kosho, T. et al. (1999). Skeletal features and growth patterns in 14 patients with haploinsufficiency of SHOX: Implications for the development of Turner syndrome. *The Journal of Clinical Endocrinology & Metabolism*, 84: pp.4613-4621

Kremer, E.J. et al. (1991). Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence $p(CCG)_n$. *Science, New Series*, 252(5013): pp.1711-1714

Labrador, J.P. et al. (2001). The collagen receptor DDR2 regulates proliferation and its elimination leads to dwarfism. *European Molecular Biology Organization reports*, 2(5): pp.446-452

Laidlaw, J. et al. (2007). Elevated basal slippage mutation rates. *Journal of Heredity*, 98(5): pp.452-460

Lanske, B. et al. (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science*, 273: pp.663-666

Li, C. et al. (1999). A Lys644Glu substitution in fibroblast growth factor receptor 3 (FGFR3) causes dwarfism in mice by activation of STATs and ink4 cell cycle inhibitors. *Human Molecular Genetics*, 8(1): pp.35-44

Li, L., Dahiya, R. (2002). MethPrimer: designing primers for methylation PCRs. *Bioinformatics*, 18(11): pp.1427-1431

Liu, Z. et al. (2002). Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. *Genes & Development*, 16: pp.859-869

Mak, K.K. et al. (2008). Indian hedgehog signals independently of PTHrP to promote chondrocyte hypertrophy. *Development*, 135: pp.1947-1956

Mangs, A.H., Morris, B.J. (2007). The human pseudoautosomal region (PAR): origin, function and future. *Current Genomics*, 8: pp.129-136

Marchini, A. et al. (2004). The short stature homeodomain protein SHOX induces cellular growth arrest and apoptosis and is expressed in human growth plate chondrocytes. *The Journal of Biological Chemistry*, 279(35): pp.37103-37114

Marchini, A. et al. (2006). Phosphorylation on Ser106 modulates the cellular functions of the SHOX homeodomain protein. *Journal of Molecular Biology*, 355: pp.590-603

Marchini, A. et al. (2007a). SHOX at a glance: from gene to protein. *Archives of Physiology and Biochemistry*, 113(3): pp.116-123

Marchini, A. et al. (2007b). BNP is a transcriptional target of the short stature homeobox gene SHOX. *Human Molecular Genetics*, 16(24): pp.3081-3087

Mardakheh, F.K. et al. (2009). Spred2 interaction with the late endosomal protein NBR1 down-regulates fibroblast growth factor receptor signaling. *Journal of Cell Biology*, 187(2): pp.265-277

Matsushita, T. et al. (2009). FGFR3 promotes synchondrosis closure and fusion of ossification centers through the MAPK pathway. *Human Molecular Genetics*, 18(2): pp.227-240

Meyers, V.N. et al. (1983). Short-limbed dwarfism and ocular defects in the Samoyed dog. *Journal of the American Veterinary Medical Association*, 183(9): pp.975-979

Minowada, G. et al. (1999). Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development*, 126: pp.4465-4475

Moss, T.J., Wallrath, L.L. (2007). Connections between epigenetic gene silencing and human disease. *Mutation Research*, 618(1-2): pp.163-174

Mundlos, S., Olsen, B. (1997). Heritable diseases of the skeleton. Part 1. Molecular insights into skeletal development – transcription factors and signaling pathways. *FASEB Journal*, 11: pp.125-132

Munns, C.J.F. et al. (2004). Expression of SHOX in human fetal an childhood growth plate. *The Journal of Clinical Endocrinology & Metabolism*, 89(8): pp.4130-4135

Müsebeck, J. et al. (2001). Short stature homeobox-containing gene deletion screening by fluorescence in situ hybridisation in patients with short stature. *European Journal of Pediatrics*, 160: pp.561-565

Naski, M.C. et al. (1998). Repression of hedgehog signaling and BMP4expression in growth plate cartilage by fibroblast growth factor receptor 3. *Development*, 125: pp.4977-4988

Newman, B., Wallis, G.A. (2003). Skeletal dysplasias caused by a disruption of skeletal patterning and endochondral ossification. *Clinical Genetics*, 63: pp.241-251

Newton, C.D., Nunamaker, D.M. (1985). Textbook of Small Animal Orthopaedics. Chapter 57: Jezyk, P.F. Constitutional disorders of the skeleton in dogs and cats. pp.637-654. Philadelphia, Pennsylvania: J.B.Lippincott Company. ISBN: 0-397-52098-0.

Ng, K. et al. (2007). Xist and the order of silencing. *European Molecular Biology Organization reports*, 8(1): pp.34-39

Niesler, B. et al. (2002) The human SHOX mutation database. *Human Mutation*, 20: pp.338-341

Niswander, L., Martin, G.R. (1992). FGF-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development*, 114: pp.755-768

Niswander, L. et al. (1994). Function of FGF-4 in limb development. *Molecular Reproduction and Development*, 39: pp.83-89

Nobrega, M.A. et al. (2003). Scanning human gene deserts for long-range enhancers. *Science*, 302: pp. 413

Ochiya, T. et al. (1995). Hst-1 (FGF-4) antisense oligonucleotides block murine limb development. *The Journal of Cell Biology*, 130: pp.997-1003

Olney, R.C. et al. (2006). Heterozygous mutations in natriuretic peptide receptor-B (NPR2) are associated with short stature. *The Journal of Clinical Endocrinology & Metabolism*, 91(4): pp.1229-1232

Ogata, T. (1999). SHOX: pseudoautosomal homeobox containing gene for short stature and dyschondrosteosis. *Growth Hormone & IGF Research*, 9: pp.53-58

Ogata, T. (2001). SHOX haploinsufficiency and overdosage: impact of gonadal function status. *Journal of Medical Genetics*, 38: pp.1-6

Palka, G. et al. (2000). Short arm rearrangements of sex chromosomes with haploinsufficiency of the SHOX gene are associated with Leri-Weill dyschondrosteosis. *Clinical Genetics*, 57: pp.449-453

Parker, H.G. et al. (2009). An expressed *fgf4* retrogen is associated with breed-defining chondrodysplasia in domestic dogs. *Science*, 325(5943): pp.995-998

Prothero, K.E. et al. (2009). Dosage compensation and gene expression on the mammalian X chromosome: one plus one does not always equal two. *Chromosome Research*, 17: pp.637-648

Radeva, G.T. et al. (1997). Overexpression of the integrin-linked kinase promotes anchorage-independent cell cycle progression. *Journal of Biological Chemistry*, 272: pp.13937-13944

Rao, E. et al. (1997a). Pseudoautosomal deletions encompassing a novel homeobox gene cause growth failure in idiopathic short stature and Turner syndrome. *Nature Genetics*, 16(1): pp.54-63

Rao, E. et al. (1997b). FISH-deletion mapping defines a 270-kb short stature critical interval in the pseudoautosomal region PAR1 on human sex chromosomes. *Human Genetics*, 100: pp.236-239

Rao, E. et al. (2001). The Leri-Weill and Turner syndrome homeobox gene SHOX encodes a cell-type specific transcriptional activator. *Human Molecular Genetics*, 10(26): pp.3083-3091

Rappold, G.A. et al. (1993). The pseudoautosomal regions of the human sex chromosomes. *Human Genetics*, 92: pp.315-324

Rappold, G.A. et al. (2002). Deletions of the homeobox gene SHOX (short stature homeobox) are an important cause of growth failure in children with short stature. *The Journal of Clinical Endocrinology & Metabolism*, 87(3): pp.1402-1406

Rasmussen, S.A. et al. (1996). Epidemiology of osteochondrodysplasias: changing trends due to advances in prenatal diagnosis. *American Journal of Medical Genetics*, 61A: pp.49-58

Richette, P. et al. (2008). Achondroplasia: From genotype to phenotype. *Joint Bone Spine*, 75: pp.125-130

Sabherwal, N. et al. (2004). Impairment of SHOX nuclear localization as a cause for Leri-Weill syndrome. *Journal of Cell Science*, 117: pp.3041-3048

Sabherwal, N. et al. (2007). Long-range conserved non-coding SHOX sequences regulate expression in developing chicken limb bud and are associated with short stature phenotypes in human patients. *Human Molecular Genetics*, 16(2): pp.210-222

Sande, R.D. et al. (1982). Dwarfism in Alaskan Malamutes, a disease resembling metaphyseal dysplasia in human beings. *American Journal of Pathology*, 106: pp.224-236

Schiller, S. et al. (2000). Phenotypic variation and genetic heterogeneity in Léri-Weill syndrome. *European Journal of Human Genetics*, 8: pp.54-62

Schneider, K.U. et al. (2005a). Alteration of DNA binding, dimerization, and nuclear translocation of SHOX homeodomain mutations identified in idiopathic short stature and Leri-Weill dyschondrosteosis. *Human Mutation*, 26: pp.44-52.

Schneider, K.U. et al. (2005b). Identification of a major recombination hotspot in patients with short stature and SHOX deficiency. *American Journal of Human Genetics*, 77: pp.89-96

Shears, D. et al. (1998). Mutation and deletion of the pseudoautosomal gene SHOX cause Leri-Weill dyschondrosteosis. *Nature Genetics*, 19: pp.70-73

Shiang, R. et al. (1994). Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. *Cell*, 78: pp.335-342

Shimizu, H. et al. (2007). Growth and differentiation of the developing limb bud from the perspective of chondrogenesis. *Development, Growth & Differentiation*, 49: pp.449-454

Shrivastava, A. et al. (1997). An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptor. *Molecular cell*, 1: pp.25-34

Smart, M.E.; Fletch, S. (1971). A hereditary skeletal growth defect in purebred Alaskan Malamutes. *Canadian Veterinary Journal*, 12(1): pp.31-32

Smit, J.J, et al. (2009). Evaluation of candidate genes as cause of chondrodysplasia in Labrador retrievers. In: Temwichitr J. (2009) The genetic defect of fragmented coronoid process in Labrador retrievers and other skeletal diseases in dogs. Thesis Utrecht University pp.85-92

Sogawa, C. et al. (2007). Short-limbed dwarfism: *slw* is a new allele of *npr2* causing chondrodysplasia. *Journal of heredity*, 98(6): pp.575-580

Spranger, J. (1992). International classification of osteochondrodysplasias. *European Journal of Pediatrics*, 151: pp 407-415

St-Jacques, B. et al. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes & Development*, 13: pp.2072-2086

Syrrou, M. et al. (1996) Evidence for high-risk haplotypes and $(CGG)_n$ expansion in Fragile X Syndrome in the hellenic populations of Greece and Cyprus. *American Journal of Medical Genetics*, 64: pp.234-238

Temwichitr, J. (2009). Radiographic and genetic study of a hereditary subluxation of the radial head in Bouvier des Flandres. In: Temwichitr J. (2009) The genetic defect of fragmented coronoid process in Labrador retrievers and other skeletal diseases in dogs. Thesis Utrecht University pp.93-108

Terpstra, L. et al. (2003). Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the integrin-linked kinase in chondrocytes. *The Journal of Cell Biology*, 162(1): pp.139-148

Thomas, N. et al. (2009). Clinical and molecular characterization of duplications encompassing the human SHOX gene reveal a variable effect on stature. *American Journal of Medical Genetics Part A*, 149A: pp.1407-1414

Tsuji, T., Kunieda, T. (2005). A loss-of-function mutation in natriuretic peptide receptor 2 (Npr2) gene is responsible for disproportionate dwarfism in cn/cn mouse. *The Journal of Biological Chemistry*, 280(14): pp.14288-14292

Tsuji, T et al. (2008). Hypomorphic mutation in mouse Nppc gene causes retarded bone growth due to impaired endochondral ossification. *Biochemical and Biophysical Research Communications*, 376: pp.186-190

Vortkamp, A. et al. (1996). Regulation of rate of cartilage differentiation by Indian Hedgehog and PTH-Related Protein. *Science*, 273: pp.613-622

Wakloka, T. et al. (2001). Spred is a Sprouty-related suppressor of Ras signalling. *Nature*, 412: pp.647-651

Wang, Y. et al. (1999). A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. *Developmental Biology*, 96: pp.4455-4460

Wren, J.D. et al., (2000). Repeat polymorphisms within gene regions: phenotypic and evolutionary implications. *The American Journal of Human Genetics*, 67: pp.345-356

Young, A.E., Bannasch, D.L. (2008). SNPS in the promoter region of the canine RMRP and SHOX genes are not associated with canine chondrodysplasia. *Animal Biotechnology*, 19(1): pp.1-5

Young, A.E., et al. (2006). Deletions in the COL10A1 gene are not associated with skeletal changes in dogs. *Mammalian Genome*, 17(7): pp.761-768

Young, A.E. et al. (2008). Tackling the characterization of canine chromosomal breakpoints with an integrated in-sit/in-silico approach: the canine PAR and PAB. *Chromosome Research*, 16: pp.1193-1202

Zinn, A.R. et al. (1993). Turner syndrome: the case of the missing sex chromosome. *Trends in Genetics*, 9(3): pp. 90-93

Zinn, A.R. et al. (2002). Complete SHOX deficiency causes Langer mesomelic dysplasia. *American Journal of Medical Genetics*, 110(2): pp.158-163

Websites

Ensembl

Blast:
http://www.ensembl.org/Canis_familiaris/blastview
Compare Alignments:
www.ensembl.org/Homo_sapiens/Location/Compara_Alignments?align=404&r=X%3A817235-846074&r1=X%3A523623-552462%3A1&s1=Canis_familiaris

Human SHOX database
www.shox.uni-hd.de
www.med.uni-heidelberg.de/SHOX-Database.1201.0.html?&L=ar

National Center for Biotechnology Information
www.ncbi.nlm.gov
www.ncbi.nlm.nih.gov/gene/6473
www.ncbi.nlm.nih.gov/gene/491706
Genebank – accession XM_540801+2
www.ncbi.nlm.nih.gov/nuccore/74032563/?from=51439420&to=51441146&report= fasta
Genebank - accession XM_850533:
position 57-80
www.ncbi.nlm.nih.gov/nuccore/74006420?report=genbank

Primerdesign
Primers for microsatellite analysis:
www.vgl.ucdavis.edu/dogset/
www.ncbi.nlm.nih.gov/unists
http://frodo.wi.mit.edu/primer3/
Bisulphite primers for CpG methylation:
http://bisearch.enzim.hu/

Raad van Beheer op kynologisch gebied in Nederland
www.raadvanbeheer.nl/

Rasvereniging Nederlandse Basset Hound Club
www.nbhc.nl/ras.php

Superlink – LOD-scores
http://bioinfo.cs.technion.ac.il/superlink-online/

Appendix 1: Rationale for testing different candidate genes

Gene	Test rationale
FGF4	o FGF4 antisense oligonucleotides inhibit murine limb development (Ochiya et al., 1995). o Increased/atypical expression of FGF4 leads to activation of FGF receptors like FGFR3, resulting in different types of chondrodysplasia (Minowada et al., 1999; Parker et al., 2009). o Increased/atypical expression of FGF4 leads to (over)expression of sprouty genes. These genes can interfere with ubiquitin mediated degradation of FGF receptors like FGFR3, resulting in chondrodysplasia in mice and men (Minowada et al., 1999; Parker et al., 2009).
FGFR3	o Mutations of FGFR3 predominantly affect the distal skeleton and/or the long bones (Liu et al., 2002) o Many cases (more than 95% and 60-65% respectively) of achondroplasia and hypochondroplasia in humans are caused by FGFR3 gain-of-function mutations that ultimately leads to activation of cell cycle inhibitors (Chen et al., 1999; Li et al., 1999; Minowada et al., 1999; Naski et al., 1998; Parker et al., 2009). o Reduced ubiquitin mediated degradation of FGF receptors caused by certain sprouty genes results in overexpression of FGFR3 leading to chondrodysplasia in mice and men (Guo et al., 2008; Parker et al., 2009). o In humans amino acid changes in transmembrane domain result in achondroplasia and mutations in tyrosine kinase domain cause hypochondroplasia or thanatophoric dysplasia (Bellus et al., 1995; Deng et al., 1996; Horton, 1997; Horton, Lunstrum, 2002; Shiang et al., 1994). o Interference of FGFR3 in mice leads to prolonged endochondral ossification, therefore causing tall stature (Deng et al., 1996). o Achondroplasia, hypochondroplasia and thanatophoric dysplasia share a common phenotype characterized by short extremities, mostly caused by proximal shortening, a long trunk and craniofacial abnormalities consisting of a large head. Especially the autosomal dominant hypochondroplasia has similarities to the canine type of chondrodysplasia. (Coumoul, Deng, 2003; Bellus et al., 1995; Horton, 1997)
Ihh	o Ihh -/- mutations in an embryonic stage are nearly always lethal, perinatal mortality is high, as a result of respiratory failure due to severe shortening of the ribs and extremities can be devoid of bone (Colnot et al., 2005; St-Jacques et al., 1999). o Ihh -/- mutations can lead to a reduced proliferation of chondrocytes, ectopic chondrocyte maturation and impairment of osteoblasts to develop in endochondral ossification, resulting in severe dwarfism of all appendicular and axial skeletal elements (Chung et al., 2001; St-Jacques et al., 1999). o Skeletons of Ihh -/- mice resemble human skeletons with thanatophoric dysplasia , which is caused by activating FGFR3 mutations (St-Jacques et al., 1999) o Mutations in FGFR3 in mouse downregulate Ihh/PTHrP signals and causes severe achondroplasia (Chen et al., 2001).
Ilk	o Shape and proliferation of chondrocytes are influenced by growth plate chondrocyte-specific deletion of the Ilk gene, leading to dwarfism (Grashoff et al., 2003; Terpstra et al., 2003).
NPR2	o Acromesomelic Dysplasia, type Maroteaux (AMDM), an autosomal recessive skeletal dysplasia in humans, maps to an interval that contains NPR2 and different loss-of-function mutations in NPR-B have been demonstrated to cause AMDM. (Bartels et al., 2004; Olney et al., 2006). o A loss-of-function mutation of the NPR2 gene is causative for disproportionate dwarfism in cn/cn mutant achondroplastic mice (Tsuji, Kunieda, 2005). o The new mutant short-limbed dwarfism (SLW) mouse, displaying significantly reduced longitudinal growth of extremities, possesses a mutant allele of NPR2 (Sogawa et al., 2007). o Mice with targeted disruption or spontaneous mutation of CNP (NPPC-/-) or NPR2 (NPR-B-/-) show severe dwarfism as a result of impaired endochondral ossification (Bartels et al., 2004; Chusho et al., 2001; Olney et al., 2006; Tsuji et al., 2008)

DDR2	o Targeted deletion of Ddr2 in mice results in reduced chondrocyte proliferation leading to dwarfism and shortening of long bones (Labrador et al., 2001). o Mutations in the DDR2 gene in men cause Spondylo-Meta-Epiphyseal Dysplasia, Short Limb-abnormal calcification type syndrome (SMED-SL) (Bargal et al., 2009). o Growth retardation and gonadal dysfunction in *slie* mice, mutant mice with a spontaneous, autosomal recessive mutation, are caused by absence of DDR2 (Kano et al., 2008).
Spred2	o Spred2-/- mice display dwarfism, similar to achondroplasia (Bundschu et al.,2005) o Up-regulation of the MAPK pathway by lack of functional Spred2 protein inhibits chondrocyte proliferation, thereby restricting bone growth (Bundschu et al., 2005).

Table 1: Test rationale for candidate genes
Rationale for testing based on published data regarding mutations of these candidate genes.

Appendix 2: LOD-scores for different candidate genes

One-locus Two-point analysis

Breed	Litter	Recessive (R)/ Dominant(D)	Marker name	Recombination fraction 0.00	0.01	0.05	0.10	0.20	0.30	0.40
Labrador	A	R	7	-Infinity	-1,1874	-0,5337	-0,2894	-0,1004	-0,0317	-0,0063
Labrador	B/C	R	7	-0,5056	-0,4782	-0,3825	-0,2869	-0,1501	-0,0638	-0,0156
Labrador	H	R	7	-Infinity	-5,6092	-2,8850	-1,7748	-0,7753	-0,3029	-0,0709
Labrador	**Total**	**R**	7	**-Infinity**	**-7,2748**	**-3,8012**	**-2,3511**	**-1,0258**	**-0,3984**	**-0,0928**
Labrador	A	D	7	-0,0754	-0,0721	-0,0599	-0,0466	-0,0255	-0,0112	-0,0028
Labrador	B/C	D	7	-0,0107	-0,0107	-0,0103	-0,0097	-0,0073	-0,004	-0,0011
Labrador	H	D	7	-3,4449	-2,6403	-1,4212	-0,8791	-0,3851	-0,1506	-0,0353
Labrador	**Total**	**D**	7	**-3,531**	**-2,7231**	**-1,4914**	**-0,9354**	**-0,4179**	**-0,1658**	**-0,0392**
Labrador	A	R	8	-Infinity	-1,2415	-0,5820	-0,3302	-0,1264	-0,0444	-0,0097
Labrador	B/C	R	8	-Infinity	-1,2633	-0,6135	-0,3682	-0,1617	-0,0650	-0,0156
Labrador	H	R	8	0,5274	0,5112	0,4467	0,3681	0,2229	0,1048	0,0271
Labrador	**Total**	**R**	8	**-Infinity**	**-1,9936**	**-0,7488**	**-0,3303**	**-0,0652**	**-0,0046**	**0,0018**
Labrador	A	D	8	-0,1855	-0,1764	-0,1435	-0,1091	-0,0579	-0,0248	-0,0061
Labrador	B/C	D	8	-1,3940	-1,3498	-1,1175	-0,8252	-0,4089	-0,1673	-0,0399
Labrador	H	D	8	0,3380	0,3256	0,2773	0,2210	0,1250	0,0553	0,0137
Labrador	**Total**	**D**	8	**-1,2415**	**-1,2006**	**-0,9837**	**-0,7133**	**-0,3418**	**-0,1368**	**-0,0323**
Labrador	A	R	1	-Infinity	-2,8046	-1,4425	-0,8874	-0,3876	-0,1514	-0,0355
Labrador	B/C	R	1	-Infinity	-1,9846	-1,1155	-0,7035	-0,3110	-0,1218	-0,0286
Labrador	H	R	1	1,5236	1,4888	1,3484	1,1703	0,8079	0,4486	0,1381
Labrador	**Total**	**R**	1	**-Infinity**	**-3,3004**	**-1,2096**	**-0,4206**	**0,1093**	**0,1754**	**0,074**
Labrador	A	D	1	-1,3670	-1,0908	-0,6461	-0,4099	-0,1824	-0,0717	-0,0168
Labrador	B/C	D	1	-2,4434	-1,7473	-1,0405	-0,6780	-0,3107	-0,1239	-0,0293
Labrador	H	D	1	0,8926	0,8734	0,7952	0,6944	0,4830	0,2672	0,0806
Labrador	**Total**	**D**	1	**-2,9178**	**-1,9647**	**-0,8914**	**-0,3935**	**-0,0101**	**0,0716**	**0,0345**
Labrador	A	R	10	-Infinity	-2,8046	-1,4425	-0,8874	-0,3876	-0,1514	-0,0355
Labrador	B/C	R	10	0	0	0	0	0	0	0
Labrador	H	R	10	-Infinity	0,3690	0,9068	0,9970	0,8442	0,5304	0,1801
Labrador	**Total**	**R**	10	**-Infinity**	**-2,4356**	**-0,5357**	**0,1096**	**0,4566**	**0,379**	**0,1446**
Labrador	A	D	10	-1,2876	-1,0503	-0,6354	-0,4060	-0,1816	-0,0716	-0,0168
Labrador	B/C	D	10	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Labrador	H	D	10	0,9010	0,8836	0,8120	0,7183	0,5156	0,2968	0,0936
Labrador	**Total**	**D**	10	**-0,3866**	**-0,1667**	**0,1766**	**0,3123**	**0,334**	**0,2252**	**0,0768**
Labrador	A	R	9	0	0	0	0	0	0	0
Labrador	B/C	R	9	0	0	0	0	0	0	0
Labrador	H	R	9	-Infinity	-2,8046	-1,4425	-0,8874	-0,3876	-0,1514	-0,0355
Labrador	**Total**	**R**	9	**-Infinity**	**-2,8046**	**-1,4425**	**-0,8874**	**-0,3876**	**-0,1514**	**-0,0355**
Labrador	A	D	9	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Labrador	B/C	D	9	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Labrador	H	D	9	-0,2994	-0,2986	-0,2839	-0,2466	-0,1511	-0,0688	-0,0173
Labrador	**Total**	**D**	9	**-0,2994**	**-0,2986**	**-0,2839**	**-0,2466**	**-0,1511**	**-0,0688**	**-0,0173**
Labrador	A	R	6	-Infinity	-1,4023	-0,7212	-0,4437	-0,1938	-0,0757	-0,0177
Labrador	B/C	R	6	-Infinity	-1,4023	-0,7212	-0,4437	-0,1938	-0,0757	-0,0177
Labrador	H	R	6	-Infinity	-2,8046	-1,4425	-0,8874	-0,3876	-0,1514	-0,0355
Labrador	**Total**	**R**	6	**-Infinity**	**-5,6092**	**-2,8849**	**-1,7748**	**-0,7752**	**-0,3028**	**-0,0709**
Labrador	A	D	6	-0,2798	-0,2645	-0,2109	-0,1574	-0,0814	-0,0343	-0,0083
Labrador	B/C	D	6	-1,6062	-1,1980	-0,6777	-0,4250	-0,1878	-0,0737	-0,0173
Labrador	H	D	6	-2,4047	-1,3623	-0,7136	-0,4404	-0,1927	-0,0753	-0,0176
Labrador	**Total**	**D**	6	**-4,2907**	**-2,8248**	**-1,6022**	**-1,0228**	**-0,4619**	**-0,1833**	**-0,0432**

Labrador	A	R	3	0,0458	0,044	0,0374	0,0298	0,017	0,0077	0,0019
Labrador	B/C	R	3	-0,1429	-0,1366	-0,1134	-0,088	-0,0481	-0,0209	-0,0052
Labrador	H	R	3	0,0019	0,0019	0,0018	0,0017	0,0012	0,0007	0,0002
Labrador	**Total**	**R**	3	**-0,0952**	**-0,0907**	**-0,0742**	**-0,0565**	**-0,0299**	**-0,0125**	**-0,0031**
Labrador	A	D	3	-0,0468	-0,0448	-0,0375	-0,0293	-0,0163	-0,0072	-0,0018
Labrador	B/C	D	3	-0,0091	-0,0089	-0,0081	-0,0069	-0,0042	-0,002	-0,0005
Labrador	H	D	3	-0,0008	-0,0008	-0,0008	-0,0008	-0,0007	-0,0004	-0,0001
Labrador	**Total**	**D**	3	**-0,0567**	**-0,0545**	**-0,0464**	**-0,037**	**-0,0212**	**-0,0096**	**-0,0024**
Labrador	A	R	4	0,0458	0,044	0,0374	0,0298	0,017	0,0077	0,0019
Labrador	B/C	R	4	-0,5056	-0,4782	-0,3825	-0,2869	-0,1501	-0,0638	-0,0156
Labrador	H	R	4	0,0019	0,0019	0,0018	0,0017	0,0012	0,0007	0,0002
Labrador	**Total**	**R**	4	**-0,4579**	**-0,4323**	**-0,3433**	**-0,2554**	**-0,1319**	**-0,0554**	**-0,0135**
Labrador	A	D	4	-0,0468	-0,0448	-0,0375	-0,0293	-0,0163	-0,0072	-0,0018
Labrador	B/C	D	4	-0,0107	-0,0107	-0,0103	-0,0097	-0,0073	-0,0040	-0,0011
Labrador	H	D	4	-0,0008	-0,0008	-0,0008	-0,0008	-0,0007	-0,0004	-0,0001
Labrador	**Total**	**D**	4	**-0,0583**	**-0,0563**	**-0,0486**	**-0,0398**	**-0,0243**	**-0,0116**	**-0,003**
Labrador	A	R	2	0	0	0	0	0	0	0
Labrador	B/C	R	2	0	0	0	0	0	0	0
Labrador	H	R	2	0,2499	0,2412	0,2076	0,168	0,0984	0,0451	0,0115
Labrador	**Total**	**R**	2	**0,2499**	**0,2412**	**0,2076**	**0,168**	**0,0984**	**0,0451**	**0,0115**
Labrador	A	D	2	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Labrador	B/C	D	2	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Labrador	H	D	2	0,1420	0,1365	0,1156	0,0916	0,0517	0,0230	0,0058
Labrador	**Total**	**D**	2	**0,1420**	**0,1365**	**0,1156**	**0,0916**	**0,0517**	**0,0230**	**0,0058**

Table 1. LOD-scores for One-locus Two-point analysis in Labradors

One-locus Multi-point analysis (marker 2+3+4)

Breed	Litter	Recessive(R)/	Recombination fraction		
		Dominant(D)	0.0000	0.1668	15.005
Labrador	A	R	0,087	0,087	0,0828
Labrador	B/C	R	0,5823	0,5842	0,5382
Labrador	H	R	0,2396	0,241	0,2523
Labrador	**Total**	**R**	**0,9089**	**0,9122**	**0,8733**
Labrador	A	D	0,0989	0,0989	0,0931
Labrador	B/C	D	0,0106	0,0106	0,0105
Labrador	H	D	0,1268	0,1276	0,1348
Labrador	**Total**	**D**	**0,2363**	**0,2371**	**0,2384**

Table 2. LOD-scores for One-locus Two-point analysis in Labradors

One-locus Two-point analysis

Breed	Recessive(R) /	Marker	Recombination fraction						
	Dominant(D)	name	0.00	0.01	0.05	0.10	0.20	0.30	0.40
Bouvier	R	7	-Infinity	-9,2220	-4,0983	-2,0957	-0,5842	-0,0976	0,0154
Bouvier	D	7	-1,9266	-1,7352	-1,2245	-0,8606	-0,4024	-0,1281	-0,0203
Bouvier	R	8	-Infinity	-9,5208	-4,9760	-3,0000	-1,2389	-0,4545	-0,0975
Bouvier	D	8	-2,6554	-1,6658	-0,9372	-0,5147	-0,1039	0,0329	0,0438
Bouvier	R	1	-Infinity	-1,6536	0,0495	0,4931	0,5649	0,3730	0,1465
Bouvier	D	1	-0,0276	-0,0250	-0,0163	-0,0092	-0,0027	-0,0010	-0,0004
Bouvier	R	10	-Infinity	-13,3972	-5,9651	-3,0778	-0,8132	-0,0786	0,0157
Bouvier	D	10	-2,5432	-2,3122	-0,7554	-0,0181	0,3922	0,2864	0,0295
Bouvier	R	9	-Infinity	-4,1620	-1,8495	-0,9394	-0,2454	-0,0256	0,0196
Bouvier	D	9	-0,6874	-0,6916	-0,6675	-0,5686	-0,3207	-0,1357	-0,0315
Bouvier	R	6	-Infinity	-2,7135	-0,9379	-0,3939	-0,0670	0,0283	0,0439
Bouvier	D	6	-3,5532	-1,8254	-1,086	-0,7392	-0,3782	-0,1778	-0,0596
Bouvier	R	3	-Infinity	-8,7927	-3,7447	-1,8655	-0,4603	-0,0218	0,0449
Bouvier	D	3	-1,3829	-1,3588	-0,9695	-0,5591	-0,1664	-0,0615	-0,0498
Bouvier	R	4	-Infinity	-9,9840	-4,1019	-1,9036	-0,3001	0,1237	0,1068
Bouvier	D	4	-1,0400	-1,0467	-1,0544	-0,8623	-0,2895	-0,0582	-0,0239
Bouvier	R	2	-Infinity	-3,1638	-1,3798	-0,7257	-0,2477	-0,0844	-0,0226
Bouvier	D	2	-0,0155	-0,0152	-0,0137	-0,0115	-0,0068	-0,0027	-0,0001

Table 3. LOD-scores for one-locus two-point analysis in Bouviers

One-locus Multi-point analysis (2+3+4)

Breed	Recessive(R) /	Recombination fraction		
	Dominant(D)	0.0000	0.1668	15.005
Bouvier	R	-Infinity	-Infinity	-Infinity
Bouvier	D	-2,4751	-2,4763	-2,4728

Table 4. LOD-scores for one-locus multi-point analysis in Bouviers

Two-Locus Two-point analysis – Recessive

Breed: Bouvier

Marker information		Recombination fraction	
First Disease Locus	Second Disease Locus	LOD-SCORE on Markers	LOD-SCORE near Markers (+1cM)
	7	LINKED	LINKED
	8	-0,9925	-0,9895
	1	0,2854	0,2582
	10	-Infinity	-0,6215
	9	-Infinity	-3,5028
	6	-Infinity	-3,7707
	3	-0,9925	-0,9895
	4	-0,9925	-0,9895
7	2	-0,9925	-0,9895
	7	-0,9925	-0,9895
	8	LINKED	LINKED
	1	1,0049	0,9802
	10	0,5692	0,6632
	9	-0,4852	-0,4838
	6	-0,9925	-0,9204
	3	0,0000	0,0000
	4	0,0000	0,0000
8	2	0,0000	0,0000
	7	0,2854	0,2582
	8	1,0049	0,9802
	1	LINKED	LINKED
	10	1,5681	1,6036
	9	0,6150	0,5886
	6	0,2854	0,3018
	3	1,0049	0,9802
	4	1,0049	0,9802
1	2	1,0049	0,9802
	7	-Infinity	-0,6215
	8	0,5692	0,6632
	1	1,5681	1,6036
	10	LINKED	LINKED
	9	-Infinity	-0,2643
	6	-Infinity	-0,4908
	3	0,5692	0,6632
	4	0,5692	0,6632
10	2	0,5692	0,6632

	7	-Infinity	-3,5028
	8	-0,4852	-0,4838
	1	0,6150	0,5886
	10	-Infinity	-0,2643
	9	LINKED	LINKED
	6	-Infinity	-2,3454
	3	-0,4852	-0,4838
	4	-0,4852	-0,4838
9	2	-0,4852	-0,4838
	7	-Infinity	-3,7707
	8	-0,9925	-0,9204
	1	0,2854	0,3018
	10	-Infinity	-0,4908
	9	-Infinity	-2,3454
	6	LINKED	LINKED
	3	-0,9925	-0,9204
	4	-0,9925	-0,9204
6	2	-0,9925	-0,9204
	7	-0,9925	-0,9895
	8	0,0000	0,0000
	1	1,0049	0,9802
	10	0,5692	0,6632
	9	-0,4852	-0,4838
	6	-0,9925	-0,9204
	3	LINKED	LINKED
	4	0,0000	0,0000
3	2	0,0000	0,0000
	7	-0,9925	-0,9895
	8	0,0000	0,0000
	1	1,0049	0,9802
	10	0,5692	0,6632
	9	-0,4852	-0,4838
	6	-0,9925	-0,9204
	3	0,0000	0,0000
	4	LINKED	LINKED
4	2	0,0000	0,0000
	7	-0,9925	-0,9895
	8	0,0000	0,0000
	1	1,0049	0,9802
	10	0,5692	0,6632
	9	-0,4852	-0,4838
	6	-0,9925	-0,9204
	3	0,0000	0,0000
	4	0,0000	0,0000
2	2	LINKED	LINKED

Table 5. LOD-scores for two-locus two-point analysis in Bouviers

Based on a recessive inheritance pattern.

Two-Locus Two-point analysis – Recessive

Breed: Labrador

Litter A				Litter B/C				Litter H			
Marker		Recombination fraction		Marker		Recombination fraction		Marker		Recombination fraction	
First Disease Locus	Second Disease Locus	LOD-SCORE on Markers	LOD-SCORE near Markers (+1cM)	First Disease Locus	Second Disease Locus	LOD-SCORE on Markers	LOD-SCORE near Markers (+1cM)	First Disease Locus	Second Disease Locus	LOD-SCORE on Markers	LOD-SCORE near Markers (+1cM)
	7	LINKED	LINKED		7	LINKED	LINKED		7	LINKED	LINKED
	8	-1,7017	-1,3659		8	-1,1802	-0,9974		8	-0,9925	-0,9895
	1	-Infinity	-2,5041		1	-1,4265	-1,2018		1	0,2854	0,2582
	10	-Infinity	-2,5041		10	-0,0819	-0,0785		10	-Infinity	-0,6215
	9	-0,6857	-0,6001		9	-0,0819	-0,0785		9	-Infinity	-3,5028
	6	-1,7017	-1,4453		6	-1,2396	-1,0506		6	-Infinity	-3,7707
	3	-0,6372	-0,5520		3	-0,0452	-0,0452		3	-0,9925	-0,9895
	4	-0,6372	-0,5520		4	-0,0908	-0,0897		4	-0,9925	-0,9895
7	2	x	x	7	2	-0,0819	-0,0785	7	2	-0,9925	-0,9895
	7	-1,7017	-1,3659		7	-1,1802	-0,9974		7	-0,9925	-0,9895
	8	LINKED	LINKED		8	LINKED	LINKED		8	LINKED	LINKED
	1	-Infinity	-2,5041		1	-Infinity	-2,1204		1	1,0049	0,9802
	10	-Infinity	-2,5041		10	-1,0160	-0,8713		10	0,5692	0,6632
	9	-0,6857	-0,6001		9	-1,0160	-0,8713		9	-0,4852	-0,4838
	6	-1,7017	-1,4453		6	-Infinity	-2,0495		6	-0,9925	-0,9204
	3	-0,6372	-0,5520		3	-1,0483	-0,8993		3	0,0000	0,0000
	4	-0,6372	-0,5520		4	-1,2303	-1,0394		4	0,0000	0,0000
8	2	x	x	8	2	-1,0160	-0,8713	8	2	0,0000	0,0000
	7	-Infinity	-2,5041		7	-1,4265	-1,2018		7	0,2854	0,2582
	8	-Infinity	-2,5041		8	-Infinity	-2,1204		8	1,0049	0,9802
	1	LINKED	LINKED		1	LINKED	LINKED		1	LINKED	LINKED
	10	-Infinity	-3,8280		10	-1,1540	-0,9873		10	1,5681	1,6036
	9	-1,7017	-1,5123		9	-1,1540	-0,9873		9	0,6150	0,5886
	6	-Infinity	-2,6701		6	-Infinity	-2,1859		6	0,2854	0,3018
	3	-1,6736	-1,4770		3	-1,1528	-0,9844		3	1,0049	0,9802
	4	-1,6736	-1,4770		4	-1,2853	-1,0882		4	1,0049	0,9802
1	2	x	x	1	2	-1,1540	-0,9873	1	2	1,0049	0,9802
	7	-Infinity	-2,5041		7	-0,0819	-0,0785		7	-Infinity	-0,6215
	8	-Infinity	-2,5041		8	-1,0160	-0,8713		8	0,5692	0,6632
	1	-Infinity	-3,8280		1	-1,1540	-0,9873		1	1,5681	1,6036
	10	LINKED	LINKED		10	LINKED	LINKED		10	LINKED	LINKED
	9	-1,7017	-1,5123		9	0,0000	0,0000		9	-Infinity	-0,2643
	6	-Infinity	-2,6700		6	-1,0749	-0,9205		6	-Infinity	-0,4908
	3	-1,6736	-1,4770		3	-0,0143	-0,0137		3	0,5692	0,6632
	4	-1,6736	-1,4770		4	-0,0819	-0,0785		4	0,5692	0,6632
10	2	x	x	10	2	0,0000	0,0000	10	2	0,5692	0,6632
	7	-0,6857	-0,6001		7	-0,0819	-0,0785		7	-Infinity	-3,5028
	8	-0,6857	-0,6001		8	-1,0160	-0,8713		8	-0,4852	-0,4838
	1	-1,7017	-1,5123		1	-1,1540	-0,9873		1	0,6150	0,5886
	10	-1,7017	-1,5123		10	0,0000	0,0000		10	-Infinity	-0,2643
	9	LINKED	LINKED		9	LINKED	LINKED		9	LINKED	LINKED
	6	-0,8413	-0,7504		6	-1,0749	-0,9205		6	-Infinity	-2,3454
	3	0,0548	0,0528		3	-0,0143	-0,0137		3	-0,4852	-0,4838
	4	0,0548	0,0528		4	-0,0819	-0,0785		4	-0,4852	-0,4838
9	2	x	x	9	2	0,0000	0,0000	9	2	-0,4852	-0,4838
	7	-1,7017	-1,4453		7	-1,2396	-1,0506		7	-Infinity	-3,7707
	8	-1,7017	-1,4453		8	-Infinity	-2,0495		8	-0,9925	-0,9204
	1	-Infinity	-2,6701		1	-Infinity	-2,1859		1	0,2854	0,3018
	10	-Infinity	-2,6700		10	-1,0749	-0,9205		10	-Infinity	-0,4908
	9	-0,8413	-0,7504		9	-1,0749	-0,9205		9	-Infinity	-2,3454
	6	LINKED	LINKED		6	LINKED	LINKED		6	LINKED	LINKED
	3	-0,7914	-0,7013		3	-1,1073	-0,9459		3	-0,9925	-0,9204
	4	-0,7914	-0,7013		4	-1,2396	-1,0506		4	-0,9925	-0,9204
6	2	x	x	6	2	-1,0749	-0,9205	6	2	-0,9925	-0,9204
	7	-0,6372	-0,5520		7	-0,0452	-0,0452		7	-0,9925	-0,9895
	8	-0,6372	-0,5520		8	-1,0483	-0,8993		8	0,0000	0,0000
	1	-1,6736	-1,4770		1	-1,1528	-0,9844		1	1,0049	0,9802
	10	-1,6736	-1,4770		10	-0,0143	-0,0137		10	0,5692	0,6632
	9	0,0548	0,0528		9	-0,0143	-0,0137		9	-0,4852	-0,4838
	6	-0,7914	-0,7013		6	-1,1073	-0,9459		6	-0,9925	-0,9204
	3	LINKED	LINKED		3	LINKED	LINKED		3	LINKED	LINKED
	4	0,1063	0,1025		4	-0,2307	-0,2149		4	0,0000	0,0000
3	2	x	x	3	2	-0,0143	-0,0137	3	2	0,0000	0,0000
	7	-0,6372	-0,5520		7	-0,0908	-0,0897		7	-0,9925	-0,9895
	8	-0,6372	-0,5520		8	-1,2303	-1,0394		8	0,0000	0,0000
	1	-1,6736	-1,4770		1	-1,2853	-1,0882		1	1,0049	0,9802
	10	-1,6736	-1,4770		10	-0,0819	-0,0785		10	0,5692	0,6632
	9	0,0548	0,0528		9	-0,0819	-0,0785		9	-0,4852	-0,4838
	6	-0,7914	-0,7013		6	-1,2396	-1,0506		6	-0,9925	-0,9204
	3	0,1063	0,1025		3	-0,2307	-0,2149		3	0,0000	0,0000
	4	LINKED	LINKED		4	LINKED	LINKED		4	LINKED	LINKED
4	2	x	x	4	2	-0,0819	-0,0785	4	2	0,0000	0,0000
					7	-0,0819	-0,0785		7	-0,9925	-0,9895
					8	-1,0160	-0,8713		8	0,0000	0,0000
					1	-1,1540	-0,9873		1	1,0049	0,9802
					10	0,0000	0,0000		10	0,5692	0,6632
					9	0,0000	0,0000		9	-0,4852	-0,4838
					6	-1,0749	-0,9205		6	-0,9925	-0,9204
					3	-0,0143	-0,0137		3	0,0000	0,0000
					4	-0,0819	-0,0785		4	0,0000	0,0000
				2	2	LINKED	LINKED	2	2	LINKED	LINKED

Table 6. LOD-scores for two-locus two-point analysis in Labradors

Based on a recessive inheritance pattern.

Two-Locus Two-point analysis – Dominant

Breed: Labrador

Litter A				Litter B/C				Litter H			
Marker		Recombination fraction		Marker		Recombination fraction		Marker		Recombination fraction	
First Disease Locus	Second Disease Locus	LOD-SCORE on Markers	LOD-SCORE near Markers (+1cM)	First Disease Locus	Second Disease Locus	LOD-SCORE on Markers	LOD-SCORE near Markers (+1cM)	First Disease Locus	Second Disease Locus	LOD-SCORE on Markers	LOD-SCORE near Markers (+1cM)
	7	LINKED	LINKED		7	LINKED	LINKED		7	LINKED	LINKED
	8	-0,2369	-0,2249		8	-0,2828	-0,2785		8	-0,7313	-0,7227
	1	-0,9592	-0,8600		1	-0,5572	-0,5342		1	0,1575	0,1445
	10	-0,9395	-0,8453		10	-0,1138	-0,1098		10	0,1682	0,1587
	9	-0,1051	-0,1005		9	-0,1138	-0,1098		9	-1,0295	-1,0192
	6	-0,2835	-0,2730		6	-0,3880	-0,3728		6	-2,9075	-2,0421
	3	-0,1005	-0,0958		3	-0,1601	-0,1538		3	-0,7313	-0,7227
	4	-0,1005	-0,0958		4	-0,2682	-0,2568		4	-0,7313	-0,7227
7	2	x	x	7	2	-0,1138	-0,1098	7	2	-0,7313	-0,7227
	7	-0,2369	-0,2249		7	-0,2828	-0,2785		7	-0,7313	-0,7227
	8	LINKED	LINKED		8	LINKED	LINKED		8	LINKED	LINKED
	1	-0,9592	-0,8600		1	-1,6923	-1,3253		1	0,7178	0,7005
	10	-0,9395	-0,8453		10	-0,2725	-0,2612		10	0,8100	0,7934
	9	-0,1051	-0,1005		9	-0,2725	-0,2612		9	-0,2184	-0,2177
	6	-0,2835	-0,2730		6	-1,5059	-1,1986		6	-0,6775	-0,6173
	3	-0,1005	-0,0958		3	-0,2806	-0,2749		3	0,0000	0,0000
	4	-0,1005	-0,0958		4	-0,2833	-0,2795		4	0,0000	0,0000
8	2	x	x	8	2	-0,2725	-0,2612	8	2	0,0000	0,0000
	7	-0,9592	-0,8600		7	-0,5572	-0,5342		7	0,1575	0,1445
	8	-0,9592	-0,8600		8	-1,6923	-1,3253		8	0,7178	0,7005
	1	LINKED	LINKED		1	LINKED	LINKED		1	LINKED	LINKED
	10	-2,0935	-1,6965		10	-0,5426	-0,5055		10	1,1827	1,1637
	9	-0,8412	-0,7501		9	-0,5426	-0,5055		9	0,3930	0,3776
	6	-1,1197	-1,0055		6	-1,8300	-1,3949		6	0,2021	0,2206
	3	-0,8688	-0,7683		3	-0,5477	-0,5124		3	0,7178	0,7005
	4	-0,8688	-0,7683		4	-0,5538	-0,5261		4	0,7178	0,7005
1	2	x	x	1	2	-0,5426	-0,5055	1	2	0,7178	0,7005
	7	-0,9395	-0,8453		7	-0,1138	-0,1098		7	0,1682	0,1587
	8	-0,9395	-0,8453		8	-0,2725	-0,2612		8	0,8100	0,7934
	1	-2,0935	-1,6965		1	-0,5426	-0,5055		1	1,1827	1,1637
	10	LINKED	LINKED		10	LINKED	LINKED		10	LINKED	LINKED
	9	-0,8231	-0,7364		9	0,0000	0,0000		9	0,3527	0,3449
	6	-1,0970	-0,9890		6	-0,3771	-0,3541		6	0,2196	0,2497
	3	-0,8457	-0,7514		3	-0,0348	-0,0335		3	0,8100	0,7934
	4	-0,8457	-0,7514		4	-0,1138	-0,1098		4	0,8100	0,7934
10	2	x	x	10	2	0,0000	0,0000	10	2	0,8100	0,7934
	7	-0,1051	-0,1005		7	-0,1138	-0,1098		7	-1,0295	-1,0192
	8	-0,1051	-0,1005		8	-0,2725	-0,2612		8	-0,2184	-0,2177
	1	-0,8412	-0,7501		1	-0,5426	-0,5055		1	0,3930	0,3776
	10	-0,8231	-0,7364		10	0,0000	0,0000		10	0,3527	0,3449
	9	LINKED	LINKED		9	LINKED	LINKED		9	LINKED	LINKED
	6	-0,2415	-0,2290		6	-0,3771	-0,3541		6	-0,9731	-0,8986
	3	0,0094	0,0090		3	-0,0348	-0,0335		3	-0,2184	-0,2177
	4	0,0094	0,0090		4	-0,1138	-0,1098		4	-0,2184	-0,2177
9	2	x	x	9	2	0,0000	0,0000	9	2	-0,2184	-0,2177
	7	-0,2835	-0,2730		7	-0,3880	-0,3728		7	-2,9075	-2,0421
	8	-0,2835	-0,2730		8	-1,5059	-1,1986		8	-0,6775	-0,6173
	1	-1,1197	-1,0055		1	-1,8300	-1,3949		1	0,2021	0,2206
	10	-1,0970	-0,9890		10	-0,3771	-0,3541		10	0,2196	0,2497
	9	-0,2415	-0,2290		9	-0,3771	-0,3541		9	-0,9731	-0,8986
	6	LINKED	LINKED		6	LINKED	LINKED		6	LINKED	LINKED
	3	-0,2272	-0,2156		3	-0,3829	-0,3622		3	-0,6775	-0,6173
	4	-0,2272	-0,2156		4	-0,3880	-0,3728		4	-0,6775	-0,6173
6	2	x	x	6	2	-0,3771	-0,3541	6	2	-0,6775	-0,6173
	7	-0,1005	-0,0958		7	-0,1601	-0,1538		7	-0,7313	-0,7227
	8	-0,1005	-0,0958		8	-0,2806	-0,2749		8	0,0000	0,0000
	1	-0,8688	-0,7683		1	-0,5477	-0,5124		1	0,7178	0,7005
	10	-0,8457	-0,7514		10	-0,0348	-0,0335		10	0,8100	0,7934
	9	0,0094	0,0090		9	-0,0348	-0,0335		9	-0,2184	-0,2177
	6	-0,2272	-0,2156		6	-0,3829	-0,3622		6	-0,6775	-0,6173
	3	LINKED	LINKED		3	LINKED	LINKED		3	LINKED	LINKED
	4	0,0177	0,0171		4	-0,1592	-0,1530		4	0,0000	0,0000
3	2	x	x	3	2	-0,0348	-0,0335	3	2	0,0000	0,0000
	7	-0,1005	-0,0958		7	-0,2682	-0,2568		7	-0,7313	-0,7227
	8	-0,1005	-0,0958		8	-0,2833	-0,2795		8	0,0000	0,0000
	1	-0,8688	-0,7683		1	-0,5538	-0,5261		1	0,7178	0,7005
	10	-0,8457	-0,7514		10	-0,1138	-0,1098		10	0,8100	0,7934
	9	0,0094	0,0090		9	-0,1138	-0,1098		9	-0,2184	-0,2177
	6	-0,2272	-0,2156		6	-0,3880	-0,3728		6	-0,6775	-0,6173
	3	0,0177	0,0171		3	-0,1592	-0,1530		3	0,0000	0,0000
	4	LINKED	LINKED		4	LINKED	LINKED		4	LINKED	LINKED
4	2	x	x	4	2	-0,1138	-0,1098	4	2	0,0000	0,0000
					7	-0,1138	-0,1098		7	-0,7313	-0,7227
					8	-0,2725	-0,2612		8	0,0000	0,0000
					1	-0,5426	-0,5055		1	0,7178	0,7005
					10	0,0000	0,0000		10	0,8100	0,7934
					9	0,0000	0,0000		9	-0,2184	-0,2177
					6	-0,3771	-0,3541		6	-0,6775	-0,6173
					3	-0,0348	-0,0335		3	0,0000	0,0000
					4	-0,1138	-0,1098		4	0,0000	0,0000
				2	2	LINKED	LINKED	2	2	LINKED	LINKED

Table 7. LOD-scores for two-locus two-point analysis in Labradors

Based on a dominant inheritance pattern.

Two-Locus Two-point analysis – Dominant/Recessive

Breed: Labrador

Litter A				Litter B/C				Litter H			
Marker		Recombination fraction		Marker		Recombination fraction		Marker		Recombination fraction	
First Disease Locus	Second Disease Locus	LOD-SCORE on Markers	LOD-SCORE near Markers (+1cM)	First Disease Locus	Second Disease Locus	LOD-SCORE on Markers	LOD-SCORE near Markers (+1cM)	First Disease Locus	Second Disease Locus	LOD-SCORE on Markers	LOD-SCORE near Markers (+1cM)
	7	LINKED	LINKED		7	LINKED	LINKED		7	LINKED	LINKED
	8	-1,0315	-0,8686		8	-2,0901	-1,2869		8	-0,4677	-0,4533
	1	-2,8745	-2,1583		1	-2,5195	-1,5090		1	0,7431	0,7294
	10	-2,8745	-2,1583		10	-0,0202	-0,0195		10	-0,2546	0,0781
	9	-0,0912	-0,0871		9	-0,0202	-0,0195		9	-1,4587	-1,4440
	6	-1,3391	-1,1125		6	-2,2075	-1,3608		6	-Infinity	-2,4912
	3	-0,0436	-0,0413		3	-0,0446	-0,0431		3	-0,4677	-0,4533
	4	-0,0436	-0,0413		4	-0,1731	-0,1661		4	-0,4677	-0,4533
7	2	x	x	7	2	-0,0202	-0,0195	7	2	-0,4677	-0,4533
	7	-1,0315	-0,8686		7	-0,6175	-0,5795		7	-2,1654	-2,1350
	8	LINKED	LINKED		8	LINKED	LINKED		8	LINKED	LINKED
	1	-2,8745	-2,1583		1	-2,2284	-1,7452		1	1,1359	1,1094
	10	-2,8745	-2,1583		10	-0,3957	-0,3725		10	0,4109	0,6056
	9	-0,0912	-0,0871		9	-0,3957	-0,3725		9	-0,7763	-0,7730
	6	-1,3391	-1,1125		6	-2,0502	-1,5983		6	-2,1654	-1,6892
	3	-0,0436	-0,0413		3	-0,4490	-0,4231		3	0,0000	0,0000
	4	-0,0436	-0,0413		4	-0,6200	-0,5832		4	0,0000	0,0000
8	2	x	x	8	2	-0,3957	-0,3725	8	2	0,0000	0,0000
	7	-1,7942	-1,4451		7	-0,7884	-0,7269		7	-1,2747	-1,2757
	8	-1,7942	-1,4451		8	-2,1990	-1,6341		8	0,5312	0,5167
	1	LINKED	LINKED		1	LINKED	LINKED		1	LINKED	LINKED
	10	-5,1507	-2,8163		10	-0,4915	-0,4573		10	1,2630	1,3211
	9	-0,5670	-0,5237		9	-0,4915	-0,4573		9	0,0773	0,0604
	6	-1,9702	-1,6121		6	-2,3175	-1,7330		6	-1,2747	-0,9591
	3	-0,5371	-0,4924		3	-0,5116	-0,4776		3	0,5312	0,5167
	4	-0,5371	-0,4924		4	-0,6799	-0,6343		4	0,5312	0,5167
1	2	x	x	1	2	-0,4915	-0,4573	1	2	0,5312	0,5167
	7	-1,7811	-1,4350		7	-0,1656	-0,1584		7	-1,2638	-1,2574
	8	-1,7811	-1,4350		8	-1,8766	-1,2374		8	0,6602	0,6451
	1	-4,6736	-2,8034		1	-2,1693	-1,4285		1	1,7787	1,7383
	10	LINKED	LINKED		10	LINKED	LINKED		10	LINKED	LINKED
	9	-0,5546	-0,5133		9	0,0000	0,0000		9	0,2576	0,2381
	6	-1,9570	-1,6019		6	-1,9927	-1,3100		6	-1,2638	-0,8831
	3	-0,5190	-0,4775		3	-0,0333	-0,0320		3	0,6602	0,6451
	4	-0,5190	-0,4775		4	-0,1656	-0,1584		4	0,6602	0,6451
10	2	x	x	10	2	0,0000	0,0000	10	2	0,6602	0,6451
	7	-0,9567	-0,7920		7	-0,1656	-0,1584		7	-2,4654	-2,4272
	8	-0,9567	-0,7920		8	-1,8766	-1,2374		8	-0,1438	-0,1426
	1	-2,7508	-2,0570		1	-2,1693	-1,4285		1	1,0325	1,0069
	10	-2,7508	-2,0570		10	0,0000	0,0000		10	0,3744	0,5390
	9	LINKED	LINKED		9	LINKED	LINKED		9	LINKED	LINKED
	6	-1,1294	-0,9565		6	-1,9927	-1,3100		6	-2,4654	-1,9180
	3	0,0481	0,0463		3	-0,0333	-0,0320		3	-0,1438	-0,1426
	4	0,0481	0,0463		4	-0,1656	-0,1584		4	-0,1438	-0,1426
9	2	x	x	9	2	0,0000	0,0000	9	2	-0,1438	-0,1426
	7	-1,2935	-1,0600		7	-0,6469	-0,6044		7	-4,7074	-3,3709
	8	-1,2934	-1,0600		8	-2,0373	-1,5654		8	-0,3478	-0,3264
	1	-3,0472	-2,2878		1	-2,3344	-1,7995		1	0,8629	0,8508
	10	-3,0472	-2,2878		10	-0,4350	-0,4066		10	-0,1933	0,1970
	9	-0,1983	-0,1886		9	-0,4350	-0,4066		9	-1,3972	-1,3395
	6	LINKED	LINKED		6	LINKED	LINKED		6	LINKED	LINKED
	3	-0,1542	-0,1459		3	-0,4805	-0,4491		3	-0,3478	-0,3264
	4	-0,1542	-0,1459		4	-0,6469	-0,6044		4	-0,3478	-0,3264
6	2	x	x	6	2	-0,4350	-0,4066	6	2	-0,3478	-0,3264
	7	-0,9384	-0,7734		7	-0,1576	-0,1512		7	-2,1654	-2,1350
	8	-0,9384	-0,7734		8	-1,9352	-1,2555		8	0,0000	0,0000
	1	-2,7795	-2,0497		1	-2,1801	-1,4274		1	1,1359	1,1094
	10	-2,7795	-2,0497		10	-0,0045	-0,0043		10	0,4109	0,6056
	9	0,0211	0,0203		9	-0,0045	-0,0043		9	-0,7763	-0,7730
	6	-1,1273	-0,9479		6	-2,0517	-1,3242		6	-2,1654	-1,6892
	3	LINKED	LINKED		3	LINKED	LINKED		3	LINKED	LINKED
	4	0,0657	0,0633		4	-0,2020	-0,1921		4	0,0000	0,0000
3	2	x	x	3	2	-0,0045	-0,0043	3	2	0,0000	0,0000
	7	-0,9384	-0,7734		7	-0,1720	-0,1651		7	-2,1654	-2,1350
	8	-0,9384	-0,7734		8	-2,1270	-1,2996		8	0,0000	0,0000
	1	-2,7795	-2,0497		1	-2,3508	-1,4656		1	1,1359	1,1094
	10	-2,7795	-2,0497		10	-0,0202	-0,0195		10	0,4109	0,6056
	9	0,0211	0,0203		9	-0,0202	-0,0195		9	-0,7763	-0,7730
	6	-1,1273	-0,9479		6	-2,2075	-1,3608		6	-2,1654	-1,6892
	3	0,0657	0,0633		3	-0,0800	-0,0760		3	0,0000	0,0000
	4	LINKED	LINKED		4	LINKED	LINKED		4	LINKED	LINKED
4	2	x	x	4	2	-0,0202	-0,0195	4	2	0,0000	0,0000
					7	-0,1656	-0,1584		7	-2,1654	-2,1350
					8	-1,8766	-1,2374		8	0,0000	0,0000
					1	-2,1693	-1,4285		1	1,1359	1,1094
					10	0,0000	0,0000		10	0,4109	0,6056
					9	0,0000	0,0000		9	-0,7763	-0,7730
					6	-1,9927	-1,3100		6	-2,1654	-1,6892
					3	-0,0333	-0,0320		3	0,0000	0,0000
					4	-0,1656	-0,1584		4	0,0000	0,0000
				2	2	LINKED	LINKED	2	2	LINKED	LINKED

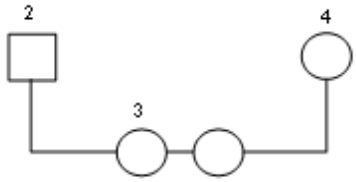
Table 8. LOD-scores for two-locus two-point analysis in Labradors

Based on a dominant inheritance pattern for one and a recessive inheritance pattern for the other gene.

Appendix 3: Sequence data of the SHOX-gene

Combination of Insertion 4221 - SNP 4290

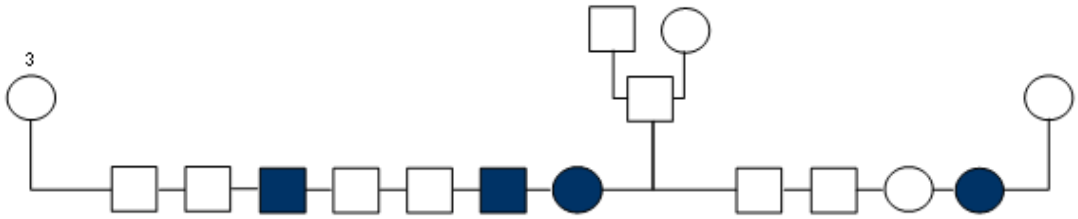
Insertion 4221	5	5	5	5	5	5	5
	5	5	5	5	5	5	5
SNP - 4290	G	G	G	G	G	G	G
	G	G	G	G	G	G	G
Together	5G	5G	5G	5G	5G	5G	5G
	...	...	...	...	...	...	...



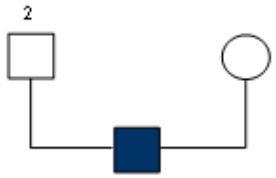
Insertion 4221	5	5	5	5
	5	6	6	5
SNP - 4290	G	A	A	G
	G	A	A	G
Together	5A	5A	5A	5G
	5G	6A	6A	6A

Insertion 4221	5	5	6			5		5	6	6	6
	5	5	6			5		5	6	6	6
SNP - 4290	G	G	A			G		G	A	A	A
	G	G	A			G		G	A	A	A
Together	5G	5G	6A			5G		5G	6A	6A	6A
	6A	6A	6A			6A		6A	6A	6A	6A

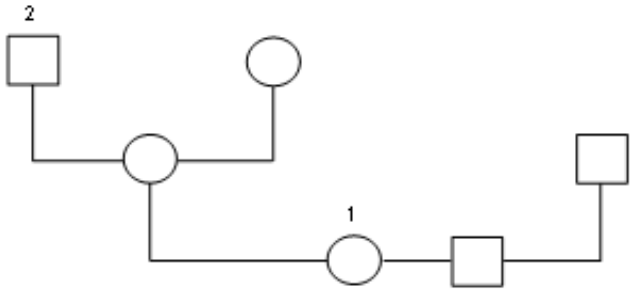
Insertion 4221		5	5	5	5	5	5	5	5
		6	5	5	5	6	5	5	6
SNP - 4290		A	G	A	G	A	G	A	A
		A	G	A	G	A	G	A	A
Together	5A	5A	5G	5A	5G	5A	5G	5A	5A
	5G	6A	5A/6A	5A	5A/6A	6A	5A/6A	5A	6A



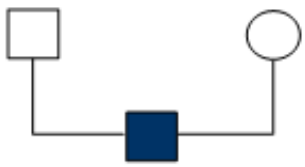
Insertion 4221	5			6			5	5		5				5	
	6			6			6	6		5				5	
SNP - 4290	A			A			A	A		G				G	
	A			A			A	A		G				G	
Together	5A			6A			5A	5A		5G				5G	
	6A			6A			6A	6A		...				...	



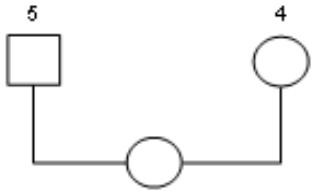
Insertion 4221	5	5	
	5	5	
SNP - 4290	G	G	
	G	G	
Together	5G	5G	
	...	...	



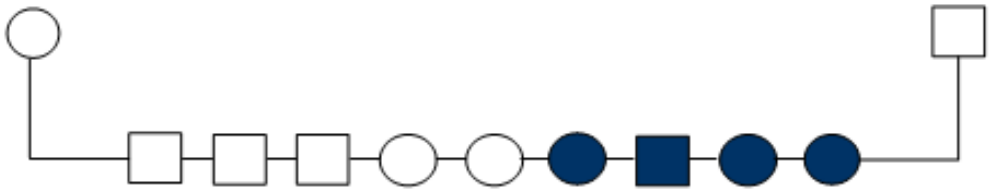
Insertion 4221	5	5		5	5	
	5	5		5	5	
SNP - 4290	G	G		G	G	
	G	G		G	G	
Together	5G	5G		5G	5G	
	...	...		...	...	



Insertion 4221		5	
		6	
SNP - 4290		A	
		A	
Together		5A	
		6A	



Insertion 4221		5	5
		5	5
SNP - 4290		G	G
		G	G
Together		5G	5G
		…	…



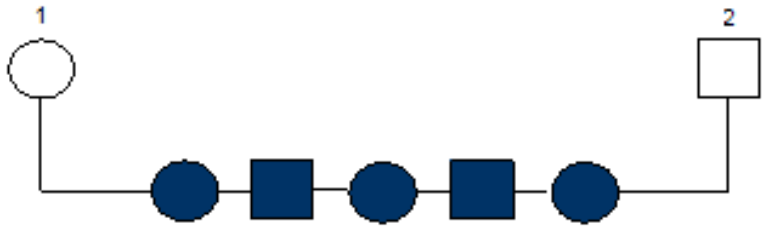
Insertion 4221	5	5	5	6	5		5	5	6	5	
	5	5	5	6	5		5	5	6	5	
SNP - 4290	G	G	G	A	G		G	G	A	G	
	G	G	G	A	G		G	G	A	G	
Together	5G	5G	5G	6A	5G		5G	5G	6A	5G	5G
	6A	…	…	6A	…		…	…	6A	…	6A

!!

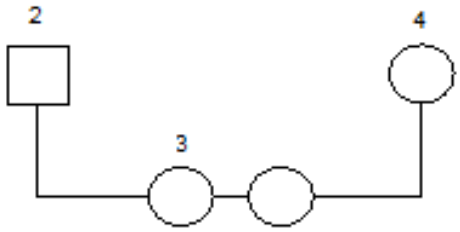
!!

Figure 1. Pedigrees with sequence data for insertion 4221 and SNP 4290

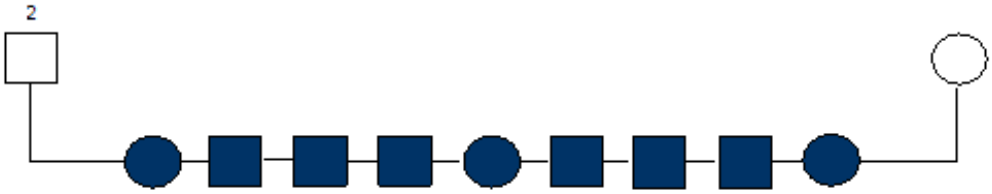
Combination of SNP 4027 - SNP 4043 - Insertion 4221 - SNP 4290



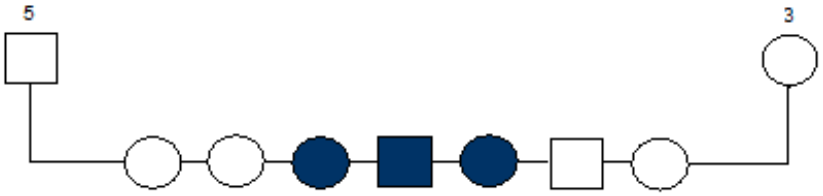
SNP - 4027			T	C			C
			T	T			T
SNP - 4043			G	A			A
			G	G			G
Insertion - 4221	5	5	5	5	5	5	5
	5	5	5	5	5	5	5
SNP - 4290	G	G	G	G	G	G	G
	G	G	G	G	G	G	G
Together	**5G**	**5G**	**5G**	**5G**	**5G**	**5G**	**5G**
	...	...	**5G**	...	...	...	...



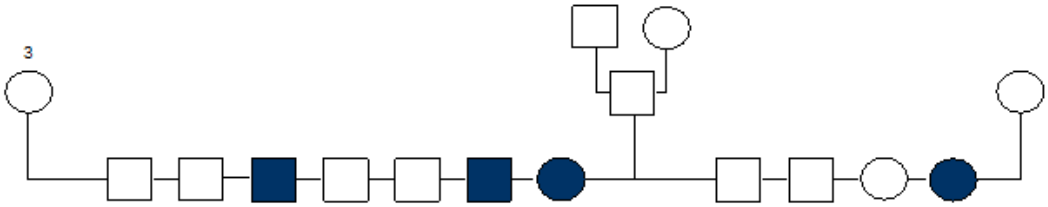
SNP - 4027	C	C		C
	T	C		T
SNP - 4043	A	A		A
	G	A		G
Insertion - 4221	5	5	5	5
	6	6	6	5
SNP - 4290	G	A	A	A
	G	A	A	G
Together	**6A**	**5A**	**5A**	**5G**
	5G	**6A**	**6A**	**5A**



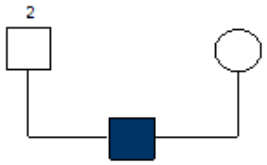
SNP - 4027	C					C			C		C
	T					T			C		C
SNP - 4043	A					A			A		A
	G					G			A		A
Insertion - 4221	5	5	6			5		5	6	6	6
	5	5	6			6		5	6	6	6
SNP - 4290	G	G	A			G		G	A	A	A
	G	G	A			G		G	A	A	A
Together	**5G**	**5G**	**6A**			**5G**		**5G**	**6A**	**6A**	**6A**
	6A	**6A**	**6A**			**6A**		**6A**	**6A**	**6A**	**6A**



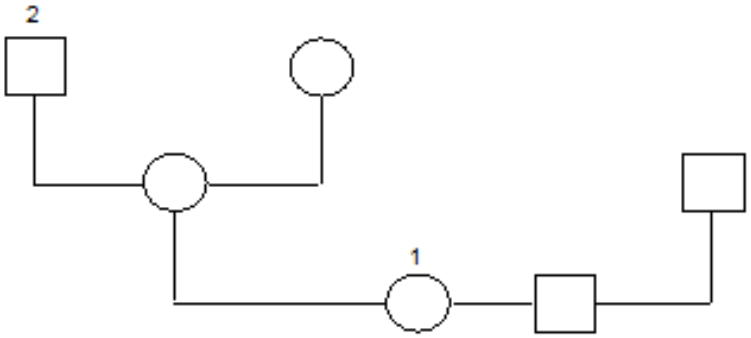
SNP - 4027								C	C
								C	C
SNP - 4043								A	A
								A	A
Insertion - 4221		5	5	5	5	5	5	5	5
		6	5	5	5	6	5	5	6
SNP - 4290		A	G	A	G	A	G	A	A
		A	G	A	G	A	G	A	A
Together	**5A**	**5A**	**5G**	**5A**	**5G**	**5A**	**5G**	**5A**	**5A**
	5G	**6A**	**5A/6A**	**5A**	**5A/6A**	**6A**	**5A/6A**	**5A**	**6A**



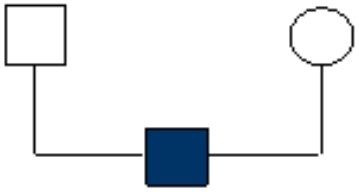
SNP - 4027	C														
	C														
SNP - 4043	A														
	A														
Insertion - 4221	5			6			5	5		5				5	
	6			6			6	6		5				5	
SNP - 4290	A			A			A	A		G				G	
	A			A			A	A		G				G	
Together	**5A**			**6A**			**5A**	**5A**		**5G**				**5G**	
	6A			**6A**			**6A**	**6A**		...				...	



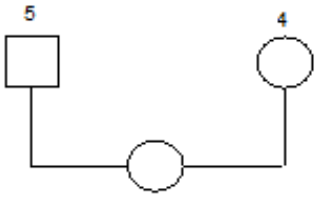
SNP - 4027	C		
	T		
SNP - 4043	A		
	G		
Insertion - 4221	5	5	
	5	5	
SNP - 4290	G	G	
	G	G	
Together	**5G**	**5G**	
	...	...	



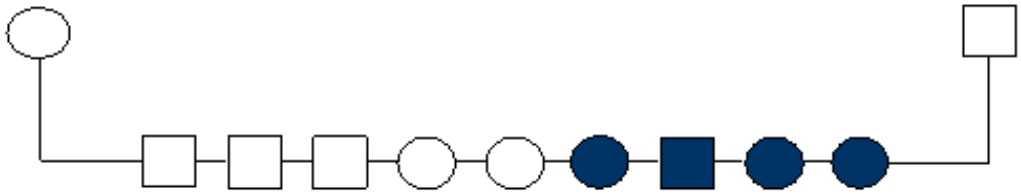
SNP - 4027	C					
	T					
SNP - 4043	A					
	G					
Insertion - 4221	5	5		5	5	
	5	5		5	5	
SNP - 4290	G	G		G	G	
	G	G		G	G	
Together	**5G**	**5G**		**5G**	**5G**	
	...	...		...	...	



SNP - 4027			
SNP - 4043			
Insertion - 4221		5	
		6	
SNP - 4290		A	
		A	
Together		**5A**	
		6A	



SNP - 4027			C
			T
SNP - 4043			A
			G
Insertion - 4221		5	5
		5	5
SNP - 4290		G	A
		G	G
Together		**5G**	**5A**
		...	**5G**



SNP - 4027	C			C					C		
	T			C					C		
SNP - 4043	A			A					A		
	G			A					A		
Insertion - 4221	5	5	5	6	5		5	5	6	5	
	6	5	5	6	5		5	5	6	5	
SNP - 4290	G	G	G	A	G		G	G	A	G	
	G	G	G	A	G		G	G	A	G	
Together	**5G**	**5G**	**5G**	**6A**	**5G**		**5G**	**5G**	**6A**	**5G**	**5G**
	6A	...	...	**6A**	...		...	...	**6A**	...	**6A**

Figure 2. Pedigrees with sequence data for SNP 4027, SNP 4043, Insertion 4221, and SNP 4290