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THESIS

QUANTITATIVE TRAIT LOCI MAPPING FOR CANINE HIP DYSPLASIA

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Canine hip dysplasia is a common complex disease, the secondary effects of which cause debilitating hip osteoarthritis. To locate the quantitative trait loci (QTL) that contribute to canine hip dysplasia, 159 crossbred and 192 purebred Labrador Retrievers were analyzed. Hip trait was measured using 4 radiographic methods: the Norberg angle, the dorsolateral subluxation score, the distraction index and OFA score. A genome-wide screening was undertaken at 428 and 284 unique microsatellite loci in crossbred and purebred Labrador Retrievers, respectively. The results from the genome-wide screening identified 11 regions i.e. CFA02, 03, 04, 05, 06, 09, 10, 11, 16, 29 and 37 in crossbred and 6 regions i.e. CFA01, 02, 10, 20, 22 and 32 in purebred Labrador Retrievers that harbored significant ($p<0.05$) putative QTLs associating with hip dysplasia at LOD scores > 2.0. Two chromosomal regions (CFA11 and 29) from the genome-wide screening were chosen for fine mapping with SNP markers to narrow down the QTL position. The analysis result revealed QTL at 19.7 cM and 19.6 cM for DIL and DIR on CFA11 and at 20.3 cM for DIL and 2 QTL for DIR at 20.3 and at 21 cM in CFA29, respectively. The aim of QTL mapping is to apply genetic testing and marker-assisted selection that may improve susceptibility of hip trait screening at a very young age. Genetic testing should assist in preventing carriers with mutant alleles from entering the genetic pool before breeding time and thus decrease the incidence of the disease.

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Student's signature Thesis Advisor's signature

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Janjira Phavaphutanon

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LIST OF ABBREVIATIONS

a	=	additive effect
ABI	=	Appiled Biosystems
BCG	=	backcross to Greyhound
BCL	=	backcross to Labrador Retriever
BF	=	Bayes factor
bp	=	base pair
BRC	=	Cornell Bioresource Center
ºC	=	Degree Celsius
CFA	=	*Canis familiaris*
CHD	=	Canine hip dysplasia
CIM	=	composite interval mapping
cM	=	centiMorgans
d	=	dominance effect
DBCL	=	double backcross of Labrador Retriever
DI	=	Distraction index
DLS	=	Dorsolateral Subluxation Score
DNA	=	Deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
E	=	environmental effect
F_1	=	F_1 offsprings
F_2	=	F_2 offsprings
G	=	genotypic value
GD	=	Golden Retriever dog
Grey	=	Greyhound dog
GSD	=	German Shepherd dog
Het	=	heterozygosity
HWE	=	Hardy Weinberg Equilibrium
IBD	=	Identity-By-Descent
IM	=	interval mapping
Kb	=	kilobase

LIST OF ABBREVIATIONS (Continued)

L	=	left side
Lab	=	Labrador Retriever dog
LD	=	linkage disequilibrium
LOD	=	logarithm of the odds
LR	=	likelihood ratio
M	=	molarity, mole/liter
Mb	=	megabase
MCMC	=	Markov Chain Monte Carlo
mg	=	milligram
$MgCl_2$	=	Magnesium chloride
ml	=	millitre
mM	=	milimolar
MSS-1	=	Minimal screening set-1
MSS-2	=	Minimal screening set-2
na	=	not applicable
NA	=	Norberg angle
ng	=	nanogram
Ɵ	=	recombination frequency
OA	=	osteoarthritis
OFA	=	Orthopedic Foundation for Animals
P	=	phenotypic value
PC	=	principle component
PC1	=	first principle component
PC2	=	second principle component
PCR	=	Polymerase Chain Reaction
pg	=	picogram
PIC	=	Polymorphic Information Content
QTL	=	Quantitative Trait Loci
R	=	right side
RAPD	=	Randomly Amplified Polymorphic DNA

LIST OF ABBREVIATIONS (Continued)

RBC	=	red blood cell
RFLP	=	Restriction Fragment Length Polymorphism
rpm	=	rotations per minute
SD	=	standard deviation
SE	=	standard error
SNP	=	Single Nucleotide Polymorphism marker
TBE	=	Tris-borate EDTA electrophoresis buffer
TE	=	Tris-EDTA electrophoresis buffer
µg	=	microgram
µl	=	microlitre
µM	=	micromole
V_A	=	additive genetic variance
V_D	=	dominance genetic variance
V_e	=	environmental variance
V_G	=	genetic variance
V_I	=	epistatic variance
V_P	=	phenotypic variance
VCA	=	variance-component approach
VNTR	=	Variable Number of Tandem Repeats

QUANTITATIVE TRAIT LOCI MAPPING FOR CANINE HIP DYSPLASIA

INTRODUCTION

Recently technology has become available to study the extent and pattern of genetic variations, and to use these variations to find the complex traits that contribute to many genetic diseases. Diseases known as complex traits or quantitative traits are due to an interaction of many genes and environmental effects, which each of these genes contributes only a little phenotypic range. Quantitative trait loci associated with the genetic variance of complex trait has studied in various kinds of animal especially in livestock animals (Spelman *et al.*, 1996; Rodriguez-Zas *et al*., 2002; de Koning *et al*., 2003; Li *et al*., 2003; Rowe *et al*., 2006). In companion animal, canine hip dysplasia (CHD) is one of the most common, inherited, orthopedic traits in dogs. It is marked by hip laxity and subluxation that leads to osteoarthritis (OA), pain and disability in affected hips (Todhunter and Lust, 2003). It decreases the capacity of dogs to engage actively with their owners and limit their qualities of lives. It may affect any dogs; small, medium or large breed, however it is estimated that more than 50 percent of some breeds are affected (Kaneene *et al*., 1997), making it far more common in these animals than in smaller dogs.

Selective breeding program determined on the basis of phenotypic evaluation can only reduce the incidence but cannot eradicate hip dysplasia within the population. In addition, powerful of screening methods, timing of detection and genetic control programs require many years to be successful. Therefore, early detection by using genetic testing would help breeders and owners in selection and get rid of immature carried dogs that carry susceptibility alleles for canine hip dysplasia.

In the recent decades, there is an advance in knowledge of the genome research both in human and animals. To identify genes influencing quantitative traits, different strategies such as candidate gene approach or whole genome scan approach

were used. The candidate gene approach uses the functions of genes to trace those genes that might be related to the trait of interest. These candidate genes then were used to test for the association between the genes and phenotype. But, it has particularly been successful for simple traits with only few genes involved. In the whole genome scan approach, many genetic markers from across the whole genome were analyzed to observe the segregation of genetic markers and trait of interest. To identify some of the major genes contribute to CHD and reduction the size of significant interval as much as possible, many analytical methods such as genome-wide screening, QTL fine mapping and single nucleotide polymorphism haplotype analysis are very important. The first step is to map the QTL to a broad interval with linkage analysis and microsatellite. Recently, there is an evidence for haplotype blocks in dogs making it is possible to perform fine mapping by using dense SNPs markers. Therefore the next step is to narrow the interval to 1-2 cM by multiple linkage analysis with SNP markers.

Canine hip dysplasia is a model of developmental dysplasia of the human hip. If gene or protein associate with the CHD is identified, it may reveal the same cause of hip dysplasia in human**.** Therefore, early identification of susceptible loci for CHD will play an important role in prevention these carrier alleles to come to genetic pool before breeding time that can apply for marker-assisted selection to improve canine breeding program in the future.

OBJECTIVES

1. To identify the location of quantitative trait loci contributed to canine hip dysplasia using a whole-genome scan.

2. To narrow the Quantitative trait loci interval to 1-2 cM by multiple linkage analysis with Single Nucleotide Polymorphism markers.

LITERATURE REVIEW

1. Canine Hip Dysplasia (CHD)

1.1 Mode of Inheritance

From the Mendelian genetics, inheritance of discrete characters (qualitative) such as seed shape (wrinkled or smooth seeds) or flower color (white or purple) are controlled by genetics factor genes. These genes are transmitted from the parents to offspring and inherited from generation to generation. However, most of the important traits especially that relate to genetic diseases or economical traits are not qualitative in nature. Most of them distribute as quantitative trait value that cannot be divided into several categories. In companion animal, CHD is an inheritance orthopedic disease that is controlled by several genes at many loci. These loci may act in additive or dominant effects. There is a significant of additive effects for age at detection of femoral capital epiphyseal ossification and one additive effect on radiographic method (distraction index). For dorsolateral subluxation score, additive and dominant effects also showed significant effect (Bliss *et al*., 2002). Canine hip dysplasia is characterized by hip joint laxity and abnormal development of the chondroepiphysis of the femoral head and acetabulum that results in hip subluxation and secondary osteoarthritis. It develops during the period of fast-growing especially in large breed. The prevalence in a breed like German Shepherds is about 50 to 55 % (Leighton, 1997). There is a difference of age at onset of capital femoral chondroepiphysis-ossification between dogs with excellent and dysplastic hip conformation (Todhunter *et al*., 1999).

Damage of hip joint in CHD is occurred from the abnormal forces on the cartilage from the abnormal hip socket (Figure 1). During the degenerative process the cartilage that lines the hip joint, called hyaline cartilage, is damaged. Sooner or later, an enzyme is released that degrades the joint further and decreases the synthesis of an important joint protectant called proteoglycans. The result is that the cartilage

becomes thinner and stiffer. As the problem progresses more enzymes are released, which now affect the precursors to proteoglycans, molecules called glycosaminoglycans and hyaluronate. Lubrication is negligible which inflammation occurs, and finally pain occurs. New bone deposited at the joint, both inside and outside, along with some of the ligaments and muscle attachments to the area. This causes thickening and a decrease in the range of motion. At this moment, dogs try to compensate their pain by transferring their body weight to front legs to decrease pain. The result is rear leg muscle atrophy and causing abnormal movement.

Dysplastic parents tend to have dysplastic offspring. However there are some dysplastic dogs that have the normal parents. Dog with nondysplastic hips may have one or more alleles at loci that promote expression of CHD and when they recombine with other alleles from a mate during subsequent meiosis at fertilization. Then CHD will result in the offspring (Todhunter and Lust, 2003)

Some dogs show signs of hip dysplasia at a very young of age, whereas, for many dogs, the clinical signs will develop later. Presently, radiography is the definitive way to diagnose; some dogs can be hip dysplasia free on radiograph (phenotype) but carry the genetic predisposition to the disease. These dogs have the potential to be carriers even though they do not show symptoms themselves and will be transmitted defected gene to their offsprings. There is equal distribution of the disease between male and female dogs. It expresses as a developmental disorder that non-genetic factors such as nutrition, exercise, body weight or environment also have influenced on it. The interactions between genetic and environmental effects play an important role in the development of this disease and determine whether an individual dog will develop CHD. The expression of the trait differs exclusively in different breeds and is influenced by environmental effects such as nutrition (Kealy *et al*., 1997). Restricted environmental control can reduce the phenotypic incidence of CHD (Kealy *et al*., 1992). During puppy development, diets that are low in protein combine with low activity levels markedly reduce the clinical signs in dogs that are genetically predisposed to CHD. However, even dogs that are not at genetic risk of developing CHD, if exercise too strongly early in life or fed diets that are too high in

calories and protein can develop CHD. Thus, it is excellent to prevent a puppy from physically powerful exercises until at least a year old of age. Besides, affected dogs may also develop disorders in other joints especially the shoulder and stifle joints, indicating that it is a systemic disease (Lust, 1997). However, there was no statistical difference in the prevalence of CHD between purebred and mixed breed dogs (Rettenmaier *et al*., 2003).

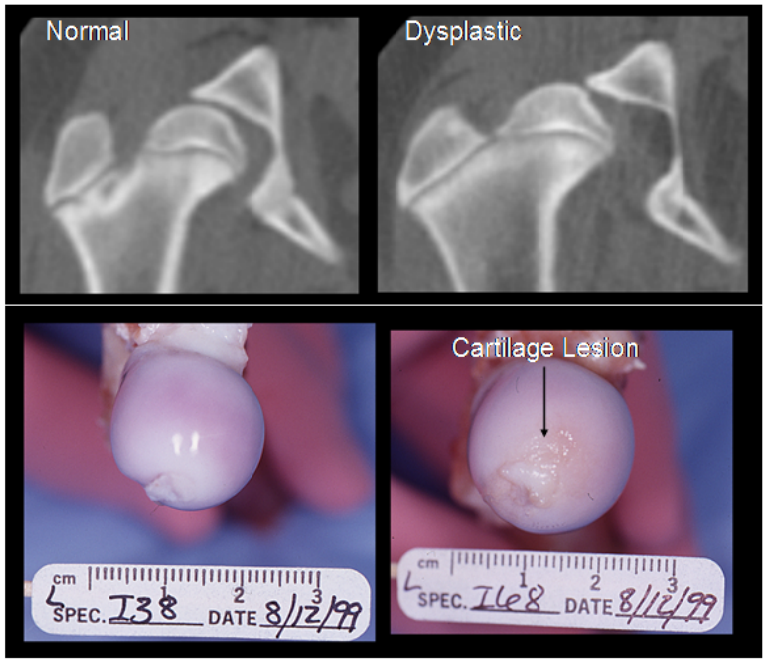


Figure 1 On the top showed computed tomography of a normal hip from 6 months Labrador Retriever. Top left panel imaged a good hip conformity of the femoral head and no lesion in the articular cartilage at necropsy (bottom left panel). In contrast, the top right and bottom right femoral heads had a lesion in the articular cartilage.

1.2 Genetic Variance and Heritability

Inheritance of polygenic traits implies that a large number of alleles, but unknown number, are segregated at many loci. Some of them might locate at the same chromosome, but others may spread throughout the genome. A major gene

could be segregated at 1 locus, with minor genes segregated at the other loci (Leighton, 1997). Moreover, variation of phenotype also has influenced from the component of genetic and environmental effects. Thus expression of CHD could differ among breeds. It supposes that alleles at these QTLs have putative positive and negative influences on the hip dysplasia traits (Todhunter and Lust, 2003). From the recent reports, heritability is widely variable among breeds ranging from 0.11-0.68 (Breur *et al*., 2002), 0.34 in Labrador Retrievers (Wood *et al*., 2002), 0.24 in Boxers and 0.15 in German Shepherds (Sturaro *et al*., 2006).

1.3 Phenotypic Measures of Hip Quality

The tentative diagnosis of CHD comes from history, clinical signs, hip palpation or radiography (Smith, 1998). In dogs, hip scores are taken at 8-24 months of age to allow dogs to be completely mature. The earliest detection can performed as early as 15 days based on necropsy evidence (Todhunter *et al*., 1997). For radiography, it can be detected as early as 4 months of age, but the dog cannot be confirmed disease-free until 2 years of age (Burton-Wurster *et al*., 1999). Physical examination may be helpful but radiography is the only means of achievement a definitive diagnosis of CHD. Even though, this method was affected by some factors such as dog age at screening time or radiologist's experience (Sturaro *et al*., 2006). Because CHD is a quantitative trait, none of these radiographic methods have 100% sensitivity and specificity (Todhunter *et al*., 2003a). Physical examination helps only to determine hip joint laxity or the degree of joint looseness. Looser hip joints tend to become dysplastic than tight ones.

Four radiographic methods including OFA score, Norberg angle (NA), Distraction index (DI) and Dorsolateral subluxation score (DLS) were used to measure different features of hip conformation.

1.3.1 OFA score

The Orthopedic Foundation for Animals (OFA) is an organization established for the purpose of standardizing the evaluation process of CHD radiographs. The OFA score consists of a broad of certified veterinary radiologists who are skilled in hip dysplasia detection. OFA score is measured from the ventrodorsal, extended-hip position (Figure 2). It is classified into 7 grades (Henry, 1992); score of 1 is excellent, score of 2 and 3 are considered unaffected, and joints with scores of 4 (borderline), 5 (mild), 6 (moderate) and 7 (severe) are considered affected with hip dysplasia (Figure 3).

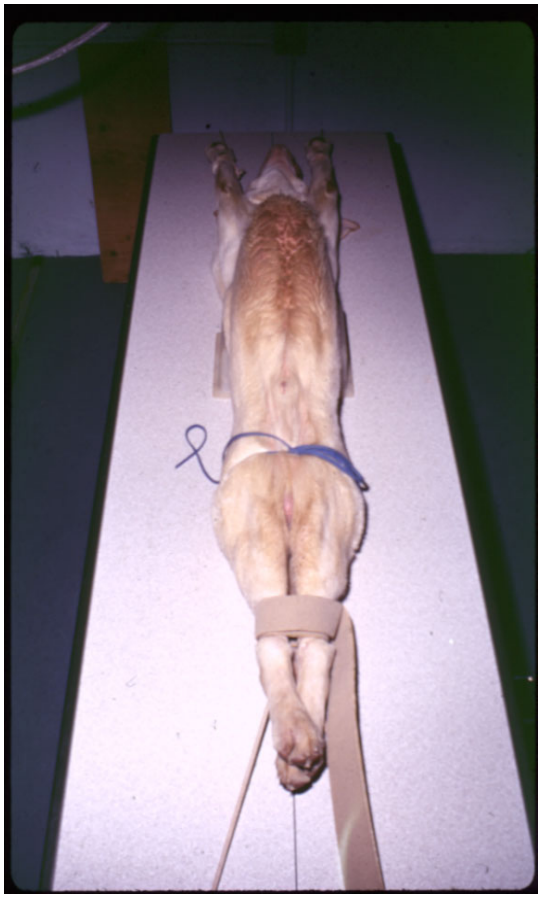


Figure 2 Illustration of a tranquilized dog in the ventrodorsal position with both high limbs extend.

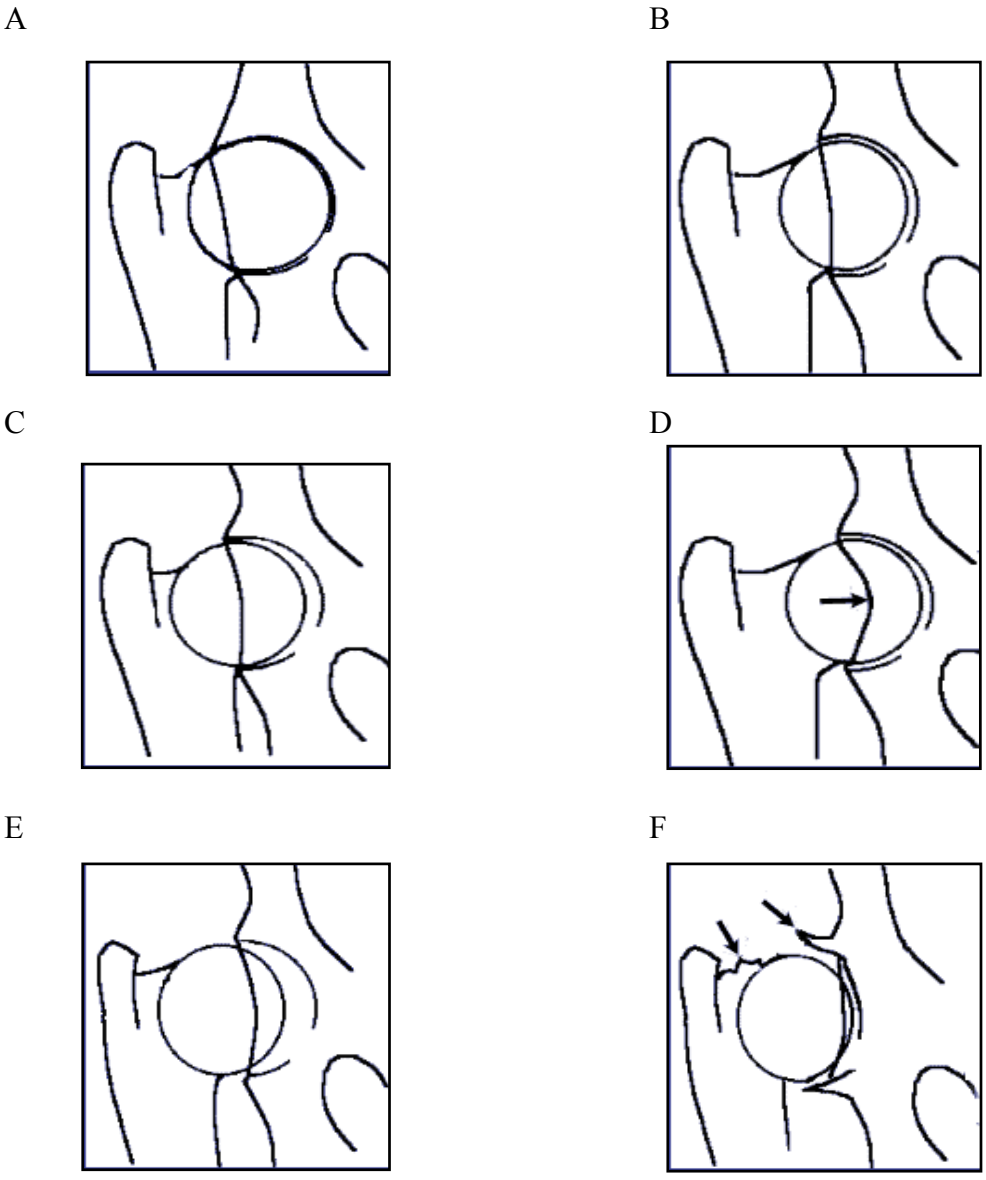


Figure 3 Illustration of hip joint conformation by OFA score method which classified hip score into 7 grades; 1 (excellent) (A), 2 (good) (B), 3 (fair) (C), 4 (borderline) (D), 5 (mild) (E), and 7 (severe) (F).

1.3.1.1 Grade 1 Excellent

Superior conformation is present with a very tight joint space and almost complete coverage of the femoral head by the socket.

1.3.1.2 Grade 2 Good

Most of the socket covers the femoral head and there is a congruent joint space.

1.3.1.3 Grade 3 Fair

Slightly incongruent (subluxated) joint space with the persistence of good femoral head coverage by the socket.

1.3.1.4 Grade 4 Borderline

There is no clear cut consensus between the radiologists to place the hip into a given category of normal or dysplastic. There is usually more incongruency present than what occurs in the minor amount found in a fair but there are no arthritic changes present that definitively diagnose the hip joint being dysplastic. There also may be a bony projection present on any of the areas of the hip anatomy. To increase the accuracy of a correct diagnosis, it is recommended to repeat the radiographs at a later date (usually 6 months). This allows the radiologist to compare the initial film with the most recent film over a given time period and assess for progressive arthritic changes that would be expected if the dog was truly dysplastic. Most dogs with this grade (over 50%) show no change in hip conformation over time and receive a normal hip rating; usually a fair hip phenotype.

1.3.1.5 Grade 5 Mild

The joint is obviously incongruent or subluxated. Usually there is a shallow socket only partially covering the femoral head.

1.3.1.6 Grade 6 Moderate

There is significant subluxation present where the ball is barely seated into a shallow socket causing joint incongruency. There are secondary arthritic bone changes usually along the femoral neck and head (termed remodeling), acetabular rim changes (termed osteophytes or bone spurs) and various degrees of trabecular bone pattern changes called sclerosis.

1.3.1.7 Grade 7 Severe

There is a shallow socket only partially covering the femoral head. There are pronounced arthritic changes at the joint (arrows).

1.3.2 Norberg angle (NA)

Norberg angle is measured from the ventrodorsal, extended-hip position (Figure 2), the same position uses in OFA score. A line is drawn between the geometric centers of each femoral head and connects to a line that contacts the craniodorsal acetarbular rims (Figure 4). The angle is range from 75 degrees (a completely subluxation hip) to 115 degrees (an unaffected hip).

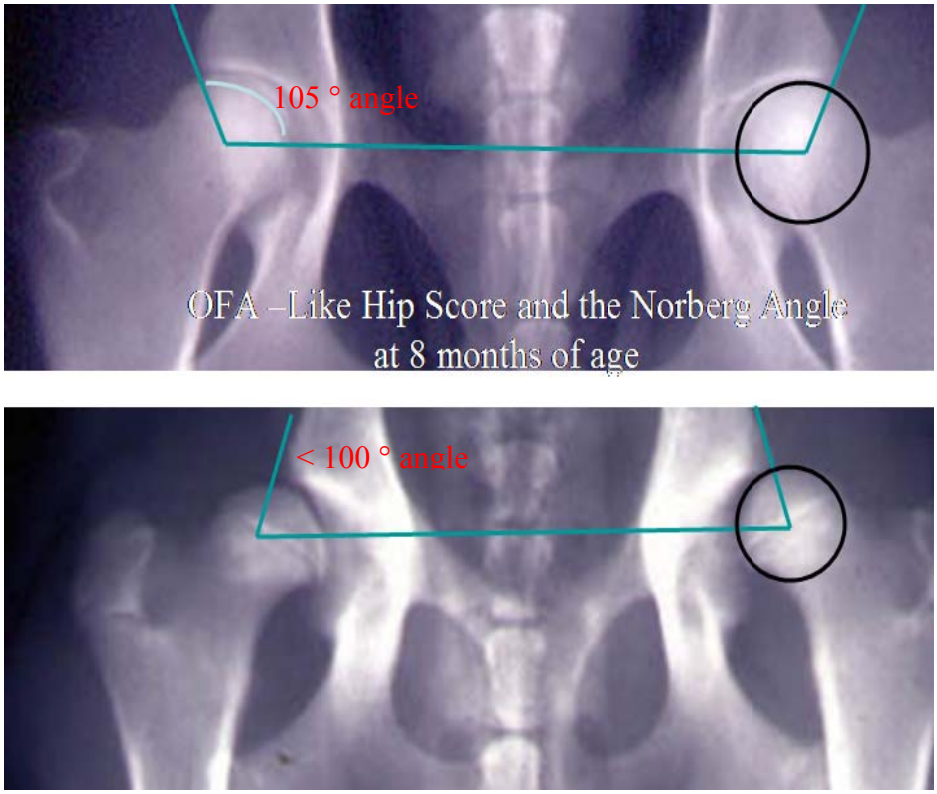


Figure 4 Norberg angle method measures the angle of femoral head and acetabular rim.

1.3.3 Distraction index (DI)

Distraction index was developed from the PennHIP (University of Pennsylvania Hip Improvement Program) program at the University of Pennsylvania and introduced in the 1980s (Smith *et al*., 1990; 1993; 1997). This technique requires different positions of the dog and flexed hip (frog like position) is requested. One advantage of this method is that it can evaluate in dogs as young as 16 weeks. From the report has shown that different breeds have different susceptibility to osteoarthritis. Therefore, this method evaluates each breed and compares to its own breed only. Only PennHIP certified veterinarians can do the DI evaluation.

Distraction index measures the degree of hip laxity. It measures as distraction index on a radiograph taken in the dorsal recumbency, distraction position

(Figure 5). This method evaluates the degree of hip laxity by measuring the displacement of femoral head from a center of acetabulum and calculated into DI value (Figure 6). It ranges from 0.1-1.0 (Smith *et al*., 1993), in which 0.0 means non-inherited condition of CHD and 1.0 means that a condition is completely under genetic control. DI less than 0.4 at eight months of age has a probability greater than 80% of not developing secondary hip osteoarthritis (OA, unaffected with hip dysplasia). Those with DI greater than 0.7 have the probability of developing hip dysplasia and subsequence hip osteoarthritis (OA).

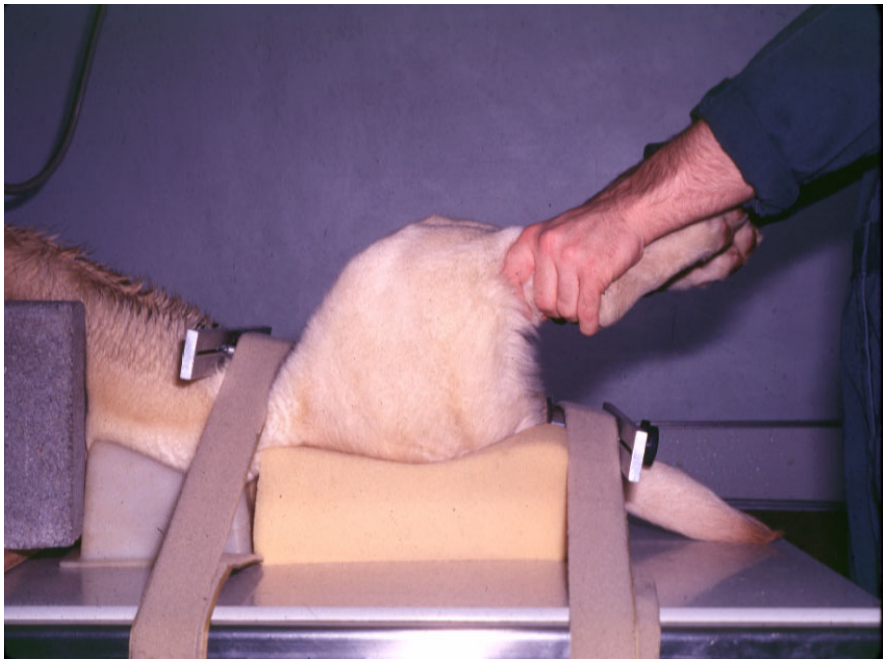


Figure 5 Illustration of a tranquilized dog in the ventrodorsal position with both high limbs flexed.

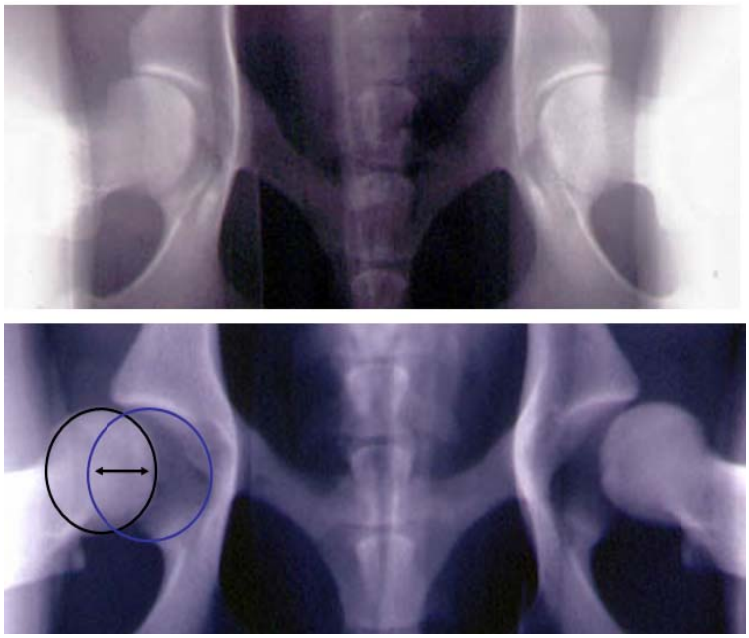


Figure 6 Distraction index method evaluates the degree of hip laxity by measuring the displacement of femoral head from a center of acetabulum and calculates into DI value.

1.3.4 Dorsolateral subluxation score (DLS)

This method was developed by researchers at Cornell University, termed the dorsolateral subluxation (DLS) test. This procedure improves upon the OFA protocol by being more accurate at a younger age than the traditional test. Studies had shown that the accuracy of the DLS test is as early as 8 months of age (Lust *et al*., 2001a). The main difference between NA and DLS is the position of the dog while being x-ray. The NA test places the hind limbs in a position that is not natural and may hide symptoms of hip dysplasia. The DLS test relies on a position much more similar to position normally found in a standing dog. Anesthetized dog was placed on a foam rubber pad and placed in a kneeling position with flexed stifles so natural load-bearing forces are transmitted to the hips by the stifles. There is a hold cut in the pad for the dog's hind legs. The stifles make contact with the x-ray table, and the dog's femurs are nearly perpendicular to the table (Figure 7). This

position similar to its natural posture allows the x-ray to show with a high level of accuracy.

Dorsolateral subluxation measures dorsal and lateral subluxation of femoral head from the acetabulum with the hips in a natural weight-bearing position. DLS is measured as the percentage of the femoral head covered by the acetabulum (Figure 8). The score ranges from 20 % to 80%. The score ranges over 55 % for tight-hipped and ~20% for dysplastic hip. This method is useful for early identification of dogs with hip dysplasia (Lust *et al.*, 2001b). Compared to DI and NA, DLS score is more sensitive and specific for prediction of early onset osteoarthritis (Lust *et al.*, 2001a).

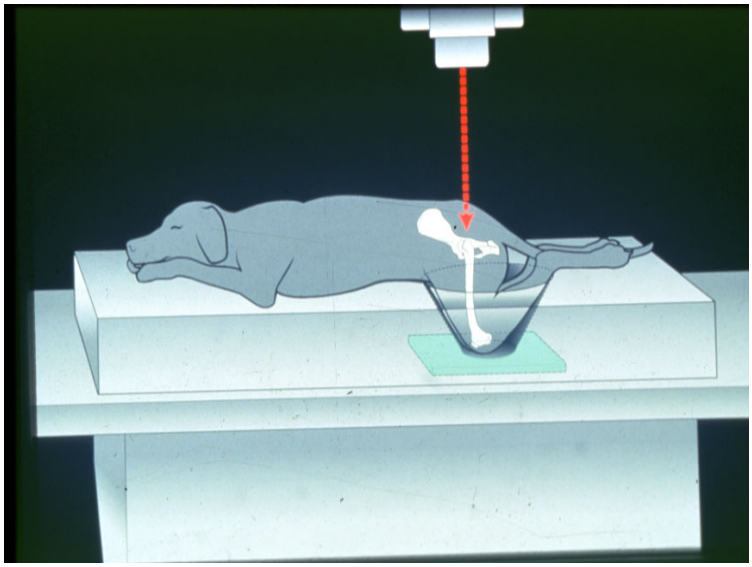


Figure 7 Illustration of a tranquilized dog in a sternal recumbency position on a foam rubber matt for the DLS test.

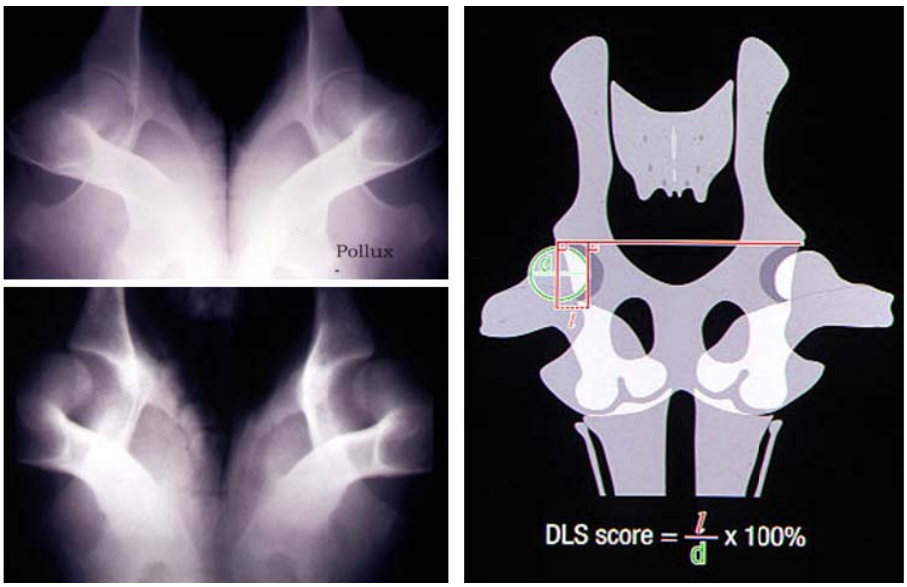


Figure 8 Illustration on the left showing DLS radiograph, on the right showing how the DLS score is calculated from a radigraph. A straight horizontal line is drawn between the acetabular lateral margins. A perpendicular line is dropped from this line at the inside edge of the femoral head and from the lateral edge of acetabulum. The distance (*l*) between these two perpendicular lines is measured in millimeters. The DLS score is expressed as percentage and it is determined by dividing " *l* " by the diameter of the femoral head (d) and multiplying by 100.

Compared sensitivity and specificity for these radiographic methods (OFA score, DI and DLS); OFA score were 38 % and 96 %; for DI 50 % and 89 %; and for DLS score 83 % and 84 %, respectively. However they had similar specificity when evaluated at 8 months of age (Todhunter and Lust, 2003).

2. Quantitative Trait Loci (QTL)

Quantitative traits such as height, weight or IQ are an interaction of genetics and environmental effects. Thus they have the model;

$$P = G + E \qquad (1)$$

where P = phenotypic value of an individual
G = genotypic value of an individual
E = environmental effect

To partition phenotypic variance into various genetic and non-genetic variance components can express as

$$V_P = V_G + V_e = V_A + V_D + V_I + V_e \quad (2)$$

where V_P = phenotypic variance
V_G = genetic variance
V_e = environmental variance
V_A = additive genetic variance
V_D = dominance genetic variance
V_I = epistatic variance

In a complex trait, phenotypic variation is usually continuous instead of discrete and condition by allelic variation at several loci, each with a relatively small effect. Moreover, the phenotype is also affected by environments. Traits that their phenotypic variations are continuous and determined by the segregation of multiple loci have often been referred to as "quantitative traits" and inheritance as polygenic. The segregation of individual alleles that affects quantitative traits usually cannot be followed from generation to generation by Mendelian analysis because each individual allele makes a small contribution to the overall phenotype. As a result, quantitative traits are studied using statistical analysis of numerical measurements of the phenotype of populations of individuals or samples taken from these populations.

In quantitative genetics studies, analysis of the phenotypic variance gives information about the differences that contribute to the overall difference in phenotypes in the population. Two main components of phenotypic variance are the genetic variance and the environmental variance. Genetic variance can be subdivided into the additive, dominance and epistasis effects that influence on the traits. The

genetic variance can be used to calculate the heritability of the trait. Heritability measures the ratio of genetic variance to total variance and used to predict the response of the trait.

3. Genetic Markers

DNA is the genetic materials of organisms hence DNA variations will be reflected the genetic difference between individuals. A wide variety of techniques can be used to measure DNA variation; direct sequencing of DNA or use PCR technique to test the difference of DNA fragment lengths. Nowadays, the advancement and discovery of DNA-based genetic markers give a new hope to investigator to find genes with complex traits. These markers have the ability to detect genetic variation at the DNA level without interfere the expression of phenotype. Therefore, not surprisingly, there has been an explosion in the use of marker-based methods in quantitative genetics. The desirable genetic markers should have these characteristics as; highly polymorphic, abundant in the genome, neutral with respect to the quantitative trait (marker has no influence), should be co-dominant markers to distinguish even heterozygous genotype and finally easily to handle. Examples of genetic or DNA-based markers that have these characteristics include Restriction Fragment Length Polymorphism (RFLP), Variable Number of Tandem Repeats (VNTR) or minisatellite, Simple Sequence Repeats (SSR) or microsatellite and Single Nucleotide Polymorphism (SNP). Randomly Amplified Polymorphic DNA (RAPD) is not included because it is a dominant marker. For QTL mapping, an average marker interval of 10-20 cM (Kruglyak, 1997) is sufficient for the whole genome-wide screen. Canine genome is 2,700 cM long, 200-300 markers should be sufficient for this mapping study.

3.1 Microsatellite markers

Animal genome has repetitive DNA sequence referred to as microsatellites or short tandem repeats that are randomly distributed throughout genomes. Number of tandem repeats found at any given microsatellite marker are

unique among individual. They are short 1-5 base pair repeats, consisting of mono-, di, tri- or tetranucleotide motifs. The differences of microsatellite alleles depend on the number of repeat at that locus. These loci do not code for proteins but could be part of gene. The mutation rates are often exceeding 10^{-2}-10^{-4} per generation and are abundant 50,000-100,000 loci in the eukaryotic genome (Weber and Wong, 1993). Therefore, they are sufficiently stable to use as genetic markers of Mendelian inheritance.

The common repeats in the canine genome are $(CA)_n$, $(GATA)_n$ or $(CAG)_n$ (Ostrander *et al*, 1992). The most frequent repeats are $(CA)_n$ or $(GT)_n$ which are distributed approximately every 43 kilobase. Repeat numbers of tri- or tetranucleotide are found every 320 kilobase and the most polymorphic tetra-nucleotide repeat is $(GAAA)_n$ (Ruvinsky and Sampson, 2001). Although tetranucleotide repeat is less frequent than dinucleotide repeat, it proves to have more polymorphism and gives less stutter bands. In dogs, microsatellite loci have heterozygosity values ranging from 36 % to 55 % within breed (Holmes *et al*., 1993; Fredholm and Wintero, 1995; Zajc *et al*., 1997 and Zajc and Sampson, 1999). Microsatellite alleles are differentiated by their size (number of repeats) which can be detected by gel electrophoresis. They show abundance, uniform distribution and a high degree of polymorphism in genomes. Therefore, they are useful tool for mapping, paternity testing, individual identification, forensic application and population studied in human beings and animals.

To generate a success linkage map, it is necessary to have a set of polymorphic markers that provide entire coverage of the whole genome. Currently the minimal screening set-1 (MSS-1) of 240 canine microsatellite markers and the minimal screening set-2 (MSS-2) of 327 canine microsatellite markers has been available to use. The combination of these screening set produce unique genotypes at 471 loci (100 loci are common between these 2 sets).

3.2 Single Nucleotide Polymorphism (SNP) markers

Among these genetic markers, microsatellite markers have been widely used because it is easy to handle, polymorphic and abundant in the genome. The recent appearance of SNP marker seems to change this trend. The SNP markers and map was developed by sequencing of 1.5X poodle and 7.6X boxer genomes. There are 2.1 million SNPs identified as part of this sequence, and 500,000 canine SNP map is available at Broad Institute of MIT and Havard. SNPs are composed of two different categories; transition and transversion SNPs. In transition SNPs, a purine is exchanged for only purine (A⇔G), while on the reverse strand a pyrimidine is exchanged for the other pyrimidine (C⇔T). Transversion SNPs consist of purine-pyrimidine and its complementary pyrimidine-purine exchanges; A⇔C (T⇔G), A⇔T (T⇔A), C⇔G (G⇔C). In the genome, occurrences of transition and transversion is not equal so make it differs in the number of these kinds of SNPs.

It is assumed that there is one SNP every 5 kb across the canine genome. In spite of the fact that SNPs are bi-allelic and less polymorphic than microsatellite markers but the quantity of highly density in the genome, low mutation rate and suitability for high-throughput genotyping make them more interesting for investigator. The mutation rate is estimated at 10^{-8} changes per nucleotide per generation (Crow, 1995; Li *et al*., 1996). However, the limitation of low heterozygosity has been overcome by genome wide association using haplotype blocks that can define multi-allelic systems for analysis (Zhang and Sun, 2005). This kind of marker is more suitable for association studies because it is almost exclusively identical by descent because of its low mutation rate. Combination of these alleles from many linked loci can be traced over several generations. In case these loci are closely linked, low possibility of crossing over so these haplotypes will be preserved for many generations.

4. Quantitative trait loci (QTL) mapping

In QTL mapping, population design is very important to refer the assessment of marker-trait association using marker genotypes. Backcross and F_2 designs are the most popular in farm animal (de Koning *et al*., 2003). Even though, inbred line approach tends to be more powerful because of the high number of informative individuals (Weller *et al*., 1990). In animal, inbred lines are not common; however crosses between lines or breeds with extreme phenotypes have been used instead as a result of long generation interval, low reproductive rate and high costs of the experiments. The QTLs information (numbers, positions and effects) in the population is unobservable so it can use only observe marker genotypes and traits for mapping QTLs. Therefore, the statistical models are very important for describing the data and abstracting the QTL information from the data. The essential data use for QTL mapping includes the map information, the trait values and the marker genotypes for each marker position of the individuals in an experimental population. The map information contains the detail of the marker positions and orders for each chromosome in an experimental organism.

The genetic variation of quantitative trait is controlled by the segregation of multiple genes. It has been assumed that the genetic variance of most quantitative trait is actually controlled by a few loci with large effects or a large number of loci with small effects. Thus, the effects of major genes should be studied via segregation analysis. However, the genes with small effects are still difficult to investigate separately. Moreover, expression of this kind of trait not only affected by many genes but also included the influence of environment effects. The power of the test of the significance of a QTL is a function of many factors including population size, QTL effect, the pedigree structure and the additive or dominance genetic effects (Davasi, 1998). Moreover, power to detect QTL would reduce if there is common allele at the same QTL in the founder populations (Todhunter *et al*., 2003b).

Population required for QTL detection depends on the purpose of the research. If it is desired to have a general picture of QTL, an F_2 is preferred. On the other hand,

if the goal to find at least some of the major QTL, a backcross is more efficient (Davasi, 1998). For genetic effect, backcross has more power than intercross in dominance QTL; however for additive QTL, an F_2 progeny is more powerful than a backcross. Major or strong QTLs can be detected with a power greater than 80% compare with weak or minor QTLs that explain only 1% or less of the trait variance. To map these minor QTLs need at least a thousand progeny to detect them with high power (Manly and Olson, 1999).

In the past decade, the application of QTL analysis was limited by the lack of polymorphic genetic markers (Lander and Botstein, 1989). However, advancement in molecular marker technology, numerous genetic markers, maps and polymerase chain reaction technique had been developed. Investigators are now able to choose these markers to investigate not only the effects of the major genes but also their locations in the genome. This is called quantitative trait loci (QTL) mapping (Lander and Botstein, 1989). The idea is to study the association between the trait of interest and random genetic makers (linkage analysis). The method is based on the analysis of recombination between the disease locus and random genetic markers with known location in the genome. This method needs the population that has mode of inheritance at the disease locus. The basic of recombination is to find the possibility of a crossover occurring between two loci that is a function of distance between them. It always indicates as θ, the small value means the two loci must be closely located in the genome. On the other hand, when two loci are far apart, they will segregate independently and θ will be equal to 50%. The recombination ranges from 0 (complete linkage or $\theta = 0$) that means no recombination ever takes place between the 2 loci and indicates that they are extremely close to one another on a chromosome. A 50% recombination fraction (independence or no linkage or $\theta = 0.5$) means that the loci are unlinked such as 2 loci on different chromosomes or loci that are on opposite ends of a chromosome. In statistical study, it should be careful to ensure that the linkage result does not occur by chance. For instance, to test the hypothesis that two loci are linked at a recombination frequency of $\theta = 0.1$ and the hypothesis that they are not linked. We calculate a likelihood ratio:

$$LR = \frac{likelihood\ of\ observing\ data\ if\ recombination\ frequency = 0.1}{likelihood\ of\ observing\ data\ if\ recombination\ frequency = 0.5} \quad (3)$$

Significant linkage at a given θ is measured by the maximum LOD-score (logarithm of the odds). LOD-scores are greater than or equal to 3 define as a significant value or are taken as evidence of linkage (Forabosco *et al*., 2005) which show the likelihood of linkage for a disease locus relative to a marker over a range of map distance . LOD scores lower than -2.0 are taken as are evidence of no linkage between two loci. In human, linkage analysis typically localizes complex disease genes to a wide region and cannot localize genes to within about 1.0 cM (around 1.0 Mb) (Lander and Schork, 1994; Weir, 1996).

The methods to improve an efficiency of gene mapping can achieve by increasing population size, improving statistical methodology or using linkage disequilibrium accumulated over generations (Lui, 1997). By using linkage analysis the identify region still has a broad range that may include hundreds of genes. However, it may not efficient enough to identify and isolate a specific gene especially genes controlling complex traits. If there are not candidate genes for that region, many fine mapping methods can be used to narrow down or estimate the location of mutant genes within the region. The variation at the DNA level is essential to trace recombination events. The more DNA sequence variation exists, the better it is to find polymorphic informative markers. Association between these loci is called linkage disequilibrium (LD). This linkage disequilibrium will provide information and power to find disease genes in the genome. Recently, LD has also been widely used for fine mapping of gene corresponding to genetic disease in human (Ardlie *et al*., 2002) and animals (Haley, 1999). The extent and distribution of LD vary among populations, in human LD extends beyond 3 kb (Kruglyak, 1999). Along with Ostrander and Kruglyak (2000), the purebred dogs in the present day are derived from a limited genetic pool, as a consequence are expected to exhibit substantial LD over their genome. In animal genetics suggest that LD is generally greater, and also extended far more distance than in human owing to frequent occurrence of the evolutionary forces causing LD (Lou *et al*., 2003). LD in dog is much more extent

than in human, suggests that dog need small number of markers for association mapping (Sutter *et al*., 2004). Sutter and coworker (2004) reported that LD in dog is varied between dog breed, it is around 400-700 kb in Golden Retriever and Labrador Retriever, 2.4 Mb in Akita and 3-3.3 Mb in Bernese Mountain Dog and Pekingese.

In this experiment, our crossbred pedigree was evaluated for the extent and distribution of LD, the result indicated an extend LD 5-10 cM throughout the canine genome in this pedigree. Owing to LD declines with the increase in distance, a low or intermediate density of markers (1 or 2 marker per 10 cM) would be sufficient for LD screening in this pedigree (Lou *et al*., 2003).

There are many statistical methods for QTL mapping compose of single marker analysis, interval mapping (IM), composite interval mapping (CIM) and association mapping.

4.1 Single marker analysis

Single marker analysis is the simplest method for QTL mapping. It is based on the idea that if there is an association between trait value and marker genotype, it is likely that a QTL locus is closed to that marker locus. By using single marker analysis, the markers close to QTLs have the highest significant level. However, the nearby markers can also have very high significant level too when the QTL effect is large or there are more than one QTL existed in a chromosome.

However this method is less power than the other methods and has many limitations when there are multiple linked QTLs situated on the chromosome. There is no way to distinguish these linked QTLs by using single marker method because the QTL positions and effects are confounded.

4.2 Interval Mapping (IM)

In single marker analysis the distribution of trait values are examined separately for each marker locus. But interval mapping has shown to provide more power particularly when markers are widely spaced and the QTL effect is large. Interval mapping uses two flanking markers to test for the existence of a quantitative locus (QTL) in the interval by performing a test statistic at many positions in the interval and to estimate the position and the effect of QTL. It is a statistical test that presents association between trait values and the genotypes of markers loci through the genome. The presence of a QTL links to the marker shows the significant association and location of putative QTL.

The use of flanking markers for interval mapping provides a more power to detect QTL location than using only a single marker (Knott and Haley, 1992). This method uses information from informative markers simultaneously to estimate QTL location and effect (Knott *et al*., 1996). Method based on least square shows similar power to the maximum-likelihood to detect QTL in single marker analysis (Haley, 1991). In 1992, Haley and Knott developed a simple regression method for mapping quantitative trait loci by using flanking markers. They showed that this method can be used the same way as maximum-likelihood method and gave very similar estimated. Moreover, it can use in data that are link or interacting QTL.

4.3 Composite Interval Mapping (CIM)

Single marker and interval mapping analysis are biased when multiple QTL are linked together. The use of all markers in the analysis can reduce the bias in the estimated position of the QTL and increase the mean maximum test statistic. Single marker and interval mapping often place QTLs in the wrong location, such as generating a ghost QTL in the position between the two real QTLs. To improve the precision and accuracy of QTL mapping, the effect of other linked QTLs on a chromosome should be separated in testing and estimation. The basic of the method is the property of multiple regression analysis (Zeng, 1993). The crucial is interval

test constructed by test statistic is unaffected by QTLs located outside a defined interval. In order to overcome interval mapping method, Zeng (1994) proposed an improved method called composite interval mapping by combining interval mapping with multiple regression analysis, in which additional markers are included as cofactors in the analysis. Composite Interval Mapping evaluates the possibility of a putative QTL at multiple analysis points across each interlocus interval. At each point, it also includes in the analysis as the effect of one or more markers that called background markers. These background markers have previously been shown to be associated with the trait and therefore possibly close to another QTL.

In composite interval mapping, it includes background markers in the model to enhance the power to detect QTL. Information from more families, more progeny per families and informative markers will help to identify the actual site of QTL (Rodriguez-Zas *et al*., 2002). Inclusion of the background markers in the analysis has a benefit, depending on whether the background markers and the target interval are linked. In case of unlinked, inclusion background markers make the analysis more sensitive to the existence of a QTL in the target interval. If they are linked, inclusion background markers may help to separate the target QTL from other linked QTLs on the far side of the background marker (Zeng, 1994).

4.4 Association mapping by linkage disequilibrium

To find the regions with genes that contribute to a disease may use the association between a marker and a disease. Association mapping uses a population-based sample instead of multigenerational families like linkage mapping. It depends strongly on the degree of linkage disequilibrium (LD). Non-random pattern of association between alleles that tend to be co-inherited is called linkage disequilibrium. The chance of alleles to be in LD is higher for loci closer together because the shorter the distance between two loci the lower the chance of recombination to disturb LD. Extensive LD need fewer markers to be genotyped than in short LD distance in the population. The test can be done by comparing the frequency of markers alleles in individual with and without the disease. When a

marker alleles in individual with disease has more frequent of this allele than in individual without disease. Therefore, this marker and its alleles are associated with the disease. This association between the marker and the disease indicates that there may be genes in that region that contribute to the disease.

LD analysis can be performed by using singular marker or combinations of alleles at multiple markers (haplotype). Haplotype occurs when a set of polymorphic loci at adjacent sites are found together more often than would be expected along a single chromosome and therefore infrequently separated from one another by recombination. There should be many haplotypes in a chromosome, but recent studies are typically finding only a few common haplotypes both in human and animal. Recombination rates do not occur at equal frequency along the whole genome. While some recombination events occur repeatedly at 'hotspots' (Jeffreys *et al*., 2001). As a result of hotspot, some areas have a higher incidence of recombination but lower in the other part of chromosomes. As a consequence, chromosomes comprise of many haplotype blocks that derived from ancestral chromosome fragments (Cargill *et al*., 1999).

5. Precision of mapping

In real QTL mapping, the QTL positions are unknown. Therefore, QTL mapping methods always use statistical value such as likelihood ratio (LR) to find the evidence of QTL. If the LR value exceeds the threshold, a QTL will be confirmed so the position and effect of QTL can be estimated. It is clear that the threshold value is very important because a high value of threshold will decrease the detection power of QTLs, in contrast, a low value of threshold will make a chance of false QTLs detection. Therefore, threshold value depends on the purpose of the QTL mapping experiment. A high value of threshold is needed if the experiment wants to find the precise position for QTL and the low value of threshold is an appropriate one if the purpose is to find as many QTLs as possible. Threshold can be affected by many experimental factors such as sample size, genome size, marker density, missing data and segregation distortion of samples. The way to obtain the accurate threshold value

comes from permutation test and to confirm the confident interval by bootstrapping method.

5.1 Permutation testing

Permutation test is used to reduce the probability of finding false marker-QTL association that is the probability of type I error. This error is determined for the entire experiment by using a random sample of permutations which is different for each experiment. Factors that affect the experiment wise error include the sample size, the genome size, the number of markers evaluated, the number of QTL that influence the trait, and the degree of the effects of the QTL's (Churchill and Doerge, 1994). One way to control the type I error is setting appropriate thresholds for the test statistic by using permutation test.

In permutation test, the data is randomly shuffled over the marker data. Analysis of the permutation data provides a test statistic that a marker is associated with the QTL. It is usually used to determine a threshold value for significance testing of the existence of a QTL effect and control the genome-wise type I error rate at a desired level. The number of permutation requirement is about 10,000 for a 1 % threshold level and recommend at least 1000 permutations for establishing a threshold for $\alpha = 0.05$ (Churchill and Doerge, 1994). The important property of this method is that it is not depend on the distribution of the data. Moreover, it can be used to obtain genome-wide threshold test statistic values at significance levels, by basically repeating the procedure across all markers.

5.2 Bootstrapping

Bootstrapping is a resampling procedure (Visscher *et al*., 1996) that uses to determine an empirical confidence interval for the QTL location, assuming that the QTL effect exists.

MATERIALS AND METHODS

1. Sample collection

1.1 Dog samples for Genome-wide screen with microsatellite markers

1.1.1 Crossbred pedigree

A canine pedigree was developed to map QTL responsible for canine hip dysplasia. Seven trait-free founding Greyhounds (2 males and 5 females) from racing stock and eight founding dysplastic Labrador Retrievers (5 males and 3 females) were intercrossed to make a crossbred population. The cross between these 2 breeds differed in the trait of CHD, offered an ideal setting for detection and mapping QTLs by using marker-trait association. Dysplastic Labrador Retrievers were bred and maintained at James A. Baker Institute for Animal Health, Cornell University, for the study of CHD since 1968 (Lust, 1997). Some of F_1's generations were backcrossed to Greyhound and Labrador Retriever founders to make a backcross progeny or intercrossed to make F_2's generations. These dogs were bred using progesterone concentration to predict ovulation timing and inseminated by artificial insemination. Puppies were weaned at 6 weeks of age and examined regularly for lameness or other clinical signs of hip dysplasia.

This group of dogs (159 dogs) in 4 generations showed a broad distribution of phenotypic range of CHD, from excellent hip joint conformation to severely dysplastic with advanced osteoarthritis (OA). This crossbred pedigree had been attributed to some of major QTL that explained around 5 % of the trait variance (Todhunter *et al*., 2003a). This crossbred pedigree had different allele's segregation among dogs with low and high susceptibility alleles to CHD (Figure 9). Simulation determined that this pedigree had sufficient power for linkage analysis following a genome-wide screen with microsatellite markers from Minimal Screening Set 1 (MSS-1) which spaced at 10 cM intervals (Todhunter *et al.*, 2003b).

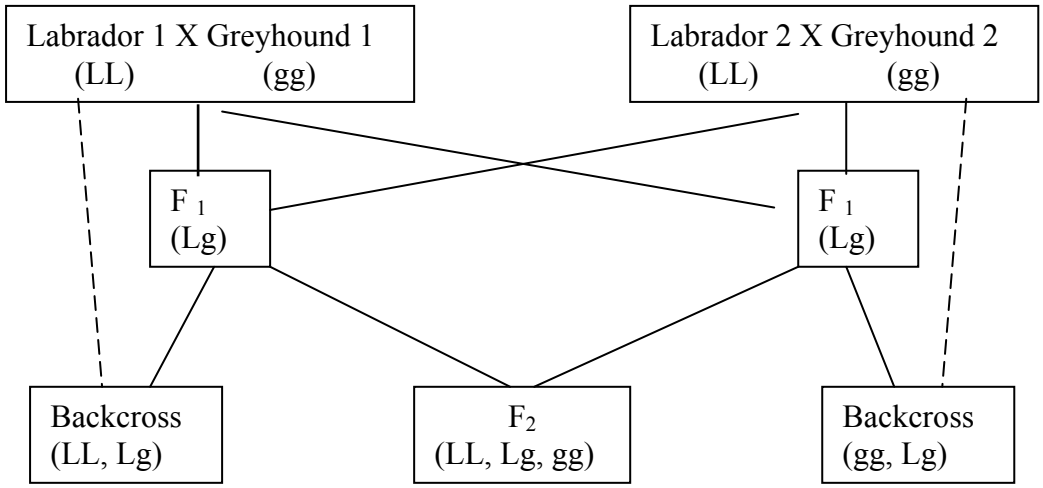


Figure 9 Illustrating the construction of crossbred pedigree. Dot lines represented backcross dogs that came from backcross F_1 to Labrador Retriever or Greyhound founders.

1.1.2 Purebred Labrador Retriever pedigree

A colony of Labrador Retrievers has been maintained at the Baker Institute, Cornell University for more than 30 years. One strain of these dogs has been bred to maximize the incidence of CHD and unrelated strain has been bred to reduce the incidence of the trait (Figure 10). These dogs had both affected and unaffected defects with a wide range of phenotypes (Figure 11). One hundred and thirty dogs from this population were genotyped at 240 Minimal Screening Set 1 (MSS-1) microsatellite markers at the Marshfield Medical Research Foundation. Additional 80 Labrador Retrievers were genotyped at 323 microsatellite loci belonging to Minimal Screening Set 2. These 2 sets had 100 microsatellite markers in common. Among these markers, there were unamplified markers resulting in an integrated genome wide screen with 284 microsatellites on the total number of 192 Labrador Retrievers in 8 generations. Some of these dogs did not have a full set of hip trait measured because some of them were maturity before doing the dorsolateral subluxation score and the distraction index. Feeding regimens were planned to attain

maximum growth rate for maximum expression of CHD both in crossbred and purebred Labrador Retrievers.

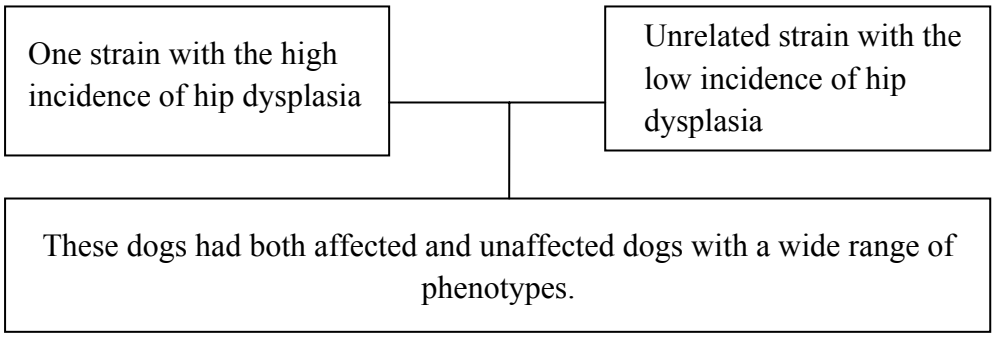


Figure 10 Illustrating the construction of purebred Labrador Retriever pedigree.

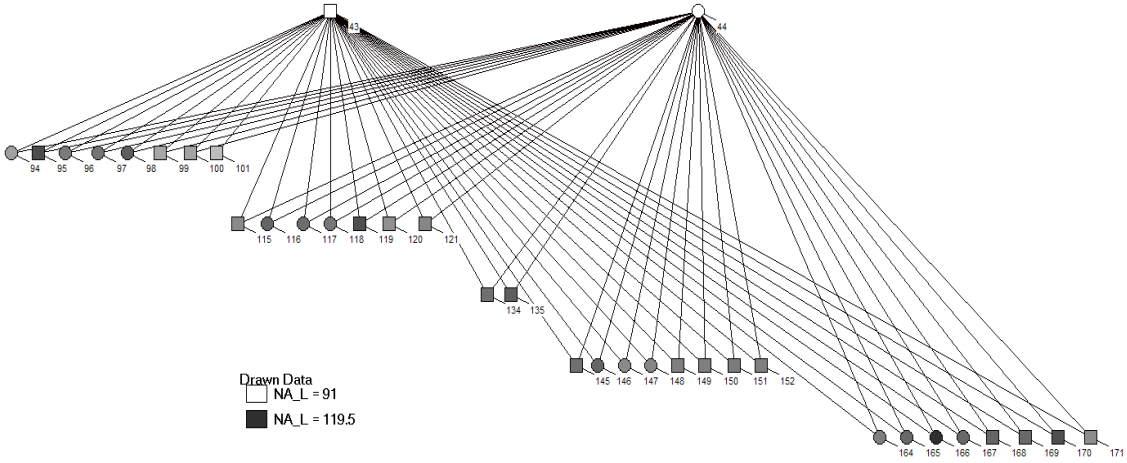


Figure 11 Diagram showed one family of 5 litters in Labrador Retriever with a wide range of phenotypes (Norberg Angle).

1.2 Dog samples for fine mapping with SNP markers

Four hundred forty nine dog samples of Cornell crossbred (158 dogs), Labrador Retrievers related to the 8 generations with 33 full sibs families and 17 loops from Cornell colony (195 dogs) and unrelated to the Cornell colony (50 dogs), Golden Retrievers (22 dogs) and German Shepherd (24 dogs) dogs were selected for fine mapping with SNP markers.

2. DNA extraction

Genomic DNA was extracted from whole blood by performing according to standard protocols (QIAGEN PureGene DNA isolation kit). Three volumes of RBC Lysis solution were added to 1 volume of blood (in heparin or EDTA as anticoagulant) in conical tube to accommodate 4 volumes in centrifuge. The tube was inverted to mix and then placed in rack on shaker (room temperature) for at least 5 minutes and up to 30 minutes. At this step, the color was changed from red to dark reddish brown. Then, tubes were balanced in rotor cups and centrifuged at 3,500 rpm (or 2000 X g) for 7 minutes. Then, tubes were carefully poured off supernatant into waste beaker. The tube was vortexed vigorously to resuspend the cells in the residual liquid and break up the pellet and added a volume of White cell lysis buffer equal to the original blood volume to resuspend cells. At this step, vortexed the tube gently without excess foaming in tube. Sample was cooled to room temperature or below by placing on ice and 4 ml protein precipitation solution (ammonium acetate) was added for every 10 ml of original blood volume. The tube was centrifuged at 3,500 rpm for 7 minutes. Supernatant was gently poured off into clean 50 ml tube. To remove all protein, a second spin was often needed. After that, supernatant sample was vortexed in 50 ml tube and sit on ice for 5-10 minutes. Tube was spinned at 3500 rpm for 7 minute and supernatant was removed to clean 50 ml tube. If there was a foam layer on top or the pellet was broken apart, transfer pipettes was used to remove foam layer rather than pouring to avoid brown clumps/flakes in supernatant. If part of pellet was entered into the supernatant, repeat this step (vortex, ice and spin) until supernatant was clean. If supernatant volume was more than 20 ml, supernatant was divided into 2 equal samples in 50 ml tubes. 2-propanol was added equally volume to supernatant volume and gently inverted to mix. The tube was kept inverting until DNA strand were visible and formed a defined white clump (invert 50 times or more as needed). The tube was centrifuged at 2,500 rpm for 5 minutes and then supernatant was poured off into waste container. At this step, did not dislodge pellet and then added 5 ml 70% ethanol to tube to cover pellet. The tube was swirled and inverted to wash pellet and tube walls. Then, the tube was centrifuged at 2,500 for 2 minutes and was carefully poured off supernatant. Pellet may dislodge at this step, so carefully

watched pellet and made sure it did not get poured into waste container. The tube was inverted on clean paper tower to dry pellet and tube walls, 5-10 minutes were usually sufficient. Pellet became too dry and therefore hard to dissolve, but residual ethanol should be avoided. TE was added to pellet, the actual volume depended on size of pellet and original blood volume. Typically, 0.75 ml was added to the pellet if the pellet was form half an original volume of 20-30 ml. Let it sit at room temperature. Heat it at 60 ºC for 1 hour and used transfer pipette to mix gently until DNA was uniform in solution. If DNA was sit at room temperature, heat at 37 ºC instead of 60 ºC. DNA sample was diluted 1:20 to check OD on biophotometer for concentration and transferred to vial for storage before use. These DNAs were kept at -70 °C at College of Veterinary Medicine, Cornell University.

3. DNA markers

3.1 Microsatellite markers

Four hundred and twenty eight and 284 unique microsatellite markers from Minimal Screening Set 1 (240 markers) and Minimal Screening Set 2 (323 markers) that 100 markers were common to both sets were used in a genome wide-screening in crossbred and purebred Labrador Retreivers respectively. Dog has 38 autosomes and one pair of sex chromosome. Average inter-marker interval was 9 cM which each multiplex set was composed of microsatellite markers located on the same chromosome. The primer sequences and inter-marker distances were available at Fred Hutchinson Cancer Research Center Institute. All the microsatellite markers were synthesized by Applied Biosystems (PE Biosystems, Foster City, CA) and forward primers were labeled with 4 fluorescent dyes; 6-FAM, VIC, NED and PET and set into multiplexed PCR. These markers were amplified using a single thermal cycling program.

3.2 SNP markers

After received the result from genome-wide screen with microsatellite markers, from crossbred and purebred Labrador Retrievers, the next step was fine mapping with SNP markers. Two chromosomes containing significant QTL with high LOD (log of the odds ratio) scores from genome-wide screen with 428 microsatellite markers on 159 crossbred dogs were chosen for the next step. SNP markers were chosen within the QTL region on 2 chromosomes that were CFA11 and CFA 29 from the database at Broad Institute of MIT and Havard. Based on the extent of linkage disequilibrium (LD) in the modern purebred dogs (average LD is 1 Mb), the LD of ancient dogs (10-25 Kb) and 800 kb of LD in Labrador Retrievers (Parker *et al*., 2004). Thus, SNP markers were selected every 25 kb for 1 Mb, skipped a Mb and repeated the selection to span the LOD score peak position of the QTL on CFA 29. For CFA11, we selected 1 SNP every 200 Kb. These SNP markers were chosen to provide more dense coverage close to the QTL LOD score peak and to be far apart in the regions flanking the peak.

These 95 and 170 SNP markers on CFA 11 and 29 were assembled into 3 and 4, 48-SNPlex pools, respectively.

4. Amplification of microsatellite and SNP loci

4.1 Amplification of microsatellite loci by PCR detection

PCR master-mixed without primers composed of 0.113 units/µl *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, PA), 1.5X Buffer B (Fisher Scientific), 4.5 mM $MgCl_2$, 1.5X MasterAmp PCR Enhancer (Epicentre Technologies, Madison, WI), 0.75 mM total dNTPs and 2.8 µl of water and adjusted to the final mastermix volume 6.65 µl. Two µl of 15 ng /µl genomic DNA was used in each reaction. Primer volumes varied up to multiplex sets of PCR.

All these microsatellite markers were amplified with a thermal cycle program: denaturation 5 minutes at 95°C followed by 5 cycles of denaturation 30 seconds at 95°C, annealing 15 seconds at 58°C, and extension 10 seconds at 72°C, and additional 30 cycles of denaturation 20 seconds at 95°C, annealing 15 seconds at 56°C, and extension 10 seconds at 72°C, with a final extension of 5 minutes at 72°C.

4.2 Amplification of SNP loci by allele-specific ligation reaction

The Applied Biosystems SNPlex genotyping used an allele-specific ligation reaction to detect SNPs in genomic DNA. Multiplexing was achieved by coupling allele-specific oligonucleotides with tag array sequences (ZipCode) and universal PCR amplification following the ligation reaction. After PCR amplification, which incorporates a biotin moiety into the amplicons, ZipCode-containing amplicons were bound to Streptavidin-coated microtiter plates and used as capture reagents. Fluorescently-labeled ZipCode molecules were captured on these plates, each containing a unique ZipCode sequence and engineer to have unique mobility/fluorescent properties. Fluorescent ZipCode were eluted from the capture plate and analyzed by capillary electrophoresis

5. Fragment analysis

5.1 Microsatellite markers

Microsatellite markers from Minimal Screening Set 1 were performed by gel based electrophoresis. Fragment separation used to size microsatellite alleles at the NHLBI Mammalian Genotyping Service, Marshfield Medical Research Foundation, Marshfield, WI, USA. The other set of Minimal Screening Set 2, PCR products from microsatellite markers were dilute 1:20 with water and mixed with an internal size standard (GeneScan 500 LIZ, PE Biosystems). Fragment analysis was done using ABI 3730 capillary-based Genetic Analyzer at the Cornell Bioresource Center (BRC).

5.2 SNP markers

The products from Fluorescent ZipCode were eluted from the capture plate and analyzed by capillary electrophoresis on an Applied Biosystems (ABI) 3730 automated DNA analyzer with fluorescent size standards included in every sample. The retention of fluorescent ZipChutes on the Streptavidin-coated plates, and subsequent detection in the electropherogram indicated the presence of a SNP in the original DNA sample. Conversely, the absence of an individual ZipChute in the electropherogram indicated the absence of a SNP (Schweitzer *et al*., 2006). Data analysis was performed using the GeneMapper^{TM} software package.

6. Genotyping

6.1 Microsatellite Genotyping

The raw data from ABI 3730 capillary-based Genetic Analyzer were analyzed for microsatellite genotyping by using Genemapper^{TM} (ABI) software package. In order to maximize the accuracy of genotypes, we developed an algorithm to check for genotype inconsistencies between parents and offspring. The program called Genoped. The program was developed in the Statistical Analysis System (SAS, Carey NC) assumed that the marker allele size in grandparents was correct, followed the flow of each allele through the pedigree, checked for inconsistencies in allele size within a narrow interval and corrected the errors. This correction program can reduce allele call error rate to 1.07 % (Mateescu *et al*., 2005).

6.2 SNP Genotyping

Based on the result of LOD score peck from the analysis of genome-wide screen with microsatellite markers, 449 dog samples from 4 generation of crossbred, Labrador Retrievers both related and unrelated to the Cornell colony, Golden Retrievers and German Shepherd dogs were genotyped. Ninety-five and 170 SNP

markers were selected on CFA11 and CFA 29, respectively from 4 pools of 48-SNPlex sets were genotyped using Genemapper^{TM} (ABI) software package.

7. Phenotypic measurement

Hip trait were measured on every dogs under general anesthesia at 8 months of age by using 4 radiographic methods; OFA score, Norberg angle (NA), Distraction index (DI) and Dorsolateral subluxation score (DLS). Because we assumed that the earlier the trait was measured, the more likely it was genetically motivated and less impacted by environmental factors. Each trait was analyzed separately on the right and the left side. Because some of these hip traits were correlated (Lust *et al*., 2001a), a principle component (PC) analysis was performed to transform the NA, DI and DLS on the right and left side, as well as on the most and least affected hip into a set of three uncorrelated variable, called principle components (factor) for each set of traits. PCs were the linear combination of traits with eigenvectors as the linear coefficients. Combined these methods always provided the best prediction of subsequent hip osteoarthritis in dogs than any single measure alone.

The main used of principle component analysis was to reduce the dimensionality of a data set while retaining as much information as possible. The first principle component (PC1) was the combination of variables that explains the greatest amount of variation. The second principle component (PC2) defined the next largest amount of variation and was independent of the first component. In this experiment, the first and second components explain approximately 70 % and 15 % of the variance in the trait data.

The traits used in this analysis composed of
DI left, right, high, low
NA left, right, high, low
DLS left, right, high, low
OFA left, right, high, low
principle component (PC) 1 or Factor 1 = principle component of NA and DI

and PC2 or Factor 2 = principle component of NA and DLS

8. Statistical analysis

8.1 The heterozygosity (Het) and polymorphism information content (PIC)

One necessity for successful QTL mapping was the sufficient number of informative markers. Marker informativeness was a function of both the number of alleles per locus and allele frequencies that represented by degree of heterozygosity (Het) and polymorphism information content (PIC) (Botstein *et al*., 1980; Lynch and Walsh, 1998). The polymorphism information content (PIC) measured the probability of differentiating the allele transmitted by a given parent to its child given the marker genotype of father, mother, and child (Botstein *et al*., 1980). Polymorphism of every locus was estimated by polymorphism information content (*PIC*). The number of alleles and allele frequencies were calculated by direct counting on the basis of Botstein (1980).

$$Het = 1 - \sum_{i=1}^{n} p_i^2 \quad (4)$$

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - 2\sum_{j=1}^{n-1} \sum_{j=i+1}^{n} p_i^2 \, p_j^2 \quad (5)$$

where p_i is the frequency of the i^{th} allele and n is the number of alleles (Botstein *et al.*, 1980).

8.2 QTL analysis

8.2.1 QTL analysis of microsatellite markers

In this study, we used genome-wide scan for the distribution of QTL as a random effect that contributed to genetic variance for canine hip dysplasia in a multi-hierarchial canine pedigree. For the linkage map, the distances between markers were estimated in centiMorgans (cM) (Breen *et al*., 2001). For the QTL

contributed to the traits, genome –wide screen were calculated on meiotic recombination observed in the pedigree. QTL mapping was performed for the left and right and the best and the worst hip and their principle components using a regression approach (Haley and Knott, 1992) implements in the web-based software QTLExpress (Seaton *et al*., 2002). The software called QTL express^{TM} was used to analyze data from crossbred pedigree by using two-step procedure for QTL mapping, first-step determined the Identity-By-Descent (IBD) probabilities between QTL alleles of individuals (probability of each QTL genotype; QQ, Qq, qQ, qq), which were derived based on observed inheritance of marker alleles from the multiple markers data at specific chromosomal locations. Then the program fits a statistical model to the observations and IBD coefficients (Seaton *et al*., 2002). Dog breed and sex were included in the model as fixed effects. The statistical model for this observation was

$$Y = \mu + \beta_1 X_1 + \beta_2 X_2 + e \qquad (6)$$

Where Y was the observed phenotype, X_1 and X_2 were the probabilities for QTL genotypes conditional on the flanking marker genotypes.

$$X_1 = P\left(\frac{QQ}{M_i M_j}\right) - P\left(\frac{qq}{M_i M_j}\right)$$

$$X_2 = P\left(\frac{Qq}{M_i M_j}\right)$$

β_1 and β_2 were the regression coefficients that measured the difference between the homozygote QTL genotypes (additive effect) and the QTL dominance effects, respectively.

The regression method assumed fixed QTL for the hip trait in each founder breed. Genome scans were performed using the forward selection approach at one centiMorgan (cM) intervals iteratively for each trait to detect multiple QTLs. Because of the breeding pressure exerted for racing performance, the Greyhound founders were assumed to be homozygous for the alleles protective against hip

dysplasia. The same as Labrador Retriever founders were assumed to be homozygous for the alleles contribute to hip dysplasia because of selection pressure to produce families with dysplastic hips. Permutation testing was used to establish the genome-wide significance threshold for the likelihood ratio at $p<0.05$ and $p<0.01$ which obtained from 1000 permutations (Doerge *et al*., 1997) and bootstrap procedure to estimate the confidence interval of a QTL location (Visscher *et al*., 1996). The position of putative QTL was judged from the F ratio statistic, the highest F ratio statistic was considered to be the best estimate for the position of the QTL. Estimate was obtained for the additive and dominance effect of the putative QTL at the location in the backcross/F_2 population.

$$\text{F-test} = \frac{\textit{Residual sum of squares (full model)}}{\textit{Residual sum of squares (reduced model)}} \quad (7)$$

In purebred Labrador Retriever, a novel module of QTL Express™ designed to analyze genotype and phenotype data from purebred populations using a variance-component approach (VCA) was used for the statistical analysis. This method uses data from marker genotypes and animal pedigree information to calculate the (co)variance matrices associated with a QTL at a particular position along the genome using a two-step approach (George *et al*., 2000). The first step uses LOKI to estimate identical-by-descent (IBD) using a Monte Carlo Markov Chain (MCHC) approach (Health, 1997). Linear mixed models using residual maximum likelihood (REML) (Gilmour *et al*., 1998) then used the identical-by-decent probabilities to model the phenotypic covariance for a putative QTL. By fitting QTL and polygenic effects simultaneously, variance component analysis generates the proportion of variance explained by the polygenic component and by the QTL. The mixed models used were

$$Y=X\beta + Z\mu + e \quad (8)$$

$$Y=X\beta + Z\mu + Z\nu + e \quad (9)$$

Where Y is a vector of phenotypic observations; β is a vector of the fixed effects of sex and litter; μ, ν, and e are vectors of additive polygenic effects, QTL effects, and random residuals respectively; and X and Z are incidence matrices relating the fixed and genetic effects, respectively.

The log likelihood ratio (log LR) was calculated to determine the likelihood of a QTL versus no QTL at the particular chromosome marker position. Twice the difference between the logarithms of the likelihood ratio of the model with and without QTL (equations 8,9) was used as a likelihood ratio test. Due to software computational limitations, confidence intervals using the bootstrap method could not be estimated, highlighting a problem with this mapping approach. LOD scores ≥ 2.0 were reported as the putative QTLs.

8.2.2 QTL analysis of SNP markers

For fine mapping with SNP markers, multipoint linkage analysis was undertaken using a Bayesian approach implemented in LOKI, version 2.4.5 (Heath, 1997). LOKI™ was a reversible Markov Chain Monte Carlo (MCMC) linkage analysis method that took whole pedigree information into account and allowed for multiple QTLs that contributed to the trait. This analysis was used to test the probability of linkage to the 4 phenotypic traits of CHD (DI, DLS, NA and OFA). The number of iterations for each Markov chain was 500,000. The number of QTL in the model was treated as a random variable and multiple QTL contributing simultaneously to the total trait variance were allowed. The Bayes factor (BF), the ratio between the posterior that a QTL signal was real or was due to the chance alone was reported along the chromosome. A BF ≥ 20 indicated strong evidence for linkage, 20 >BF ≥ 3 indicated moderate evidence for linkage and 3 > BF ≥ 1 indicated weak evidence for linkage according to BF calibration tables (Raftery, 1996).

RESULTS

To identify the location of quantitative trait loci contributed to CHD, the first step was to do genome-wide screen by using microsatellite markers on crossbred pedigree and purebred Labrador Retrievers. Minimal Screening Set 1 (MSS-1) and 2 (MSS-2) were chosen for this genome-wide screen. These sets of markers composed of highly informative markers and well spaced inter-marker interval. The analysis was undertaken on program called "QTL Express". The linkage or association between trait values and the genotypes of marker loci were presented the results in tabular and graphical format. Permutation tests were used to determine empirical significance levels and bootstrapping to estimate empirical confidence intervals of QTL locations.

1. Genome-wide screen with microsatellite markers in crossbred dogs

1.1 Crossbred pedigree

One hundred and fifty-nine crossbred dogs, including 7 Greyhound founders, 8 dysplastic Labrador Retrievers founders, 7 F_1s, 33 F_1 backcross to Greyhound (BCG), 80 F_1 backcross to Labrador Retriever (BCL), 16 F_2 (F_1 x F_1) and 7 F_3 or double backcross of Labrador Retrievers (DBCL, [(F_1 x L) x (F_1 x L)]) dogs were used for genome-wide screen with microsatellite markers. This group of dogs came from 4 generations, 19 families in which there were 1-11 offsprings (Figure 12). According to Darvasi (1998), an F_2 will give a general picture of the number of QTLs segregation and backcross should be the most efficient for detection of at least some of the major QTLs. Our crossbred pedigree had both of the F_2 and backcross so it should be the suitable pedigree for QTL analysis.

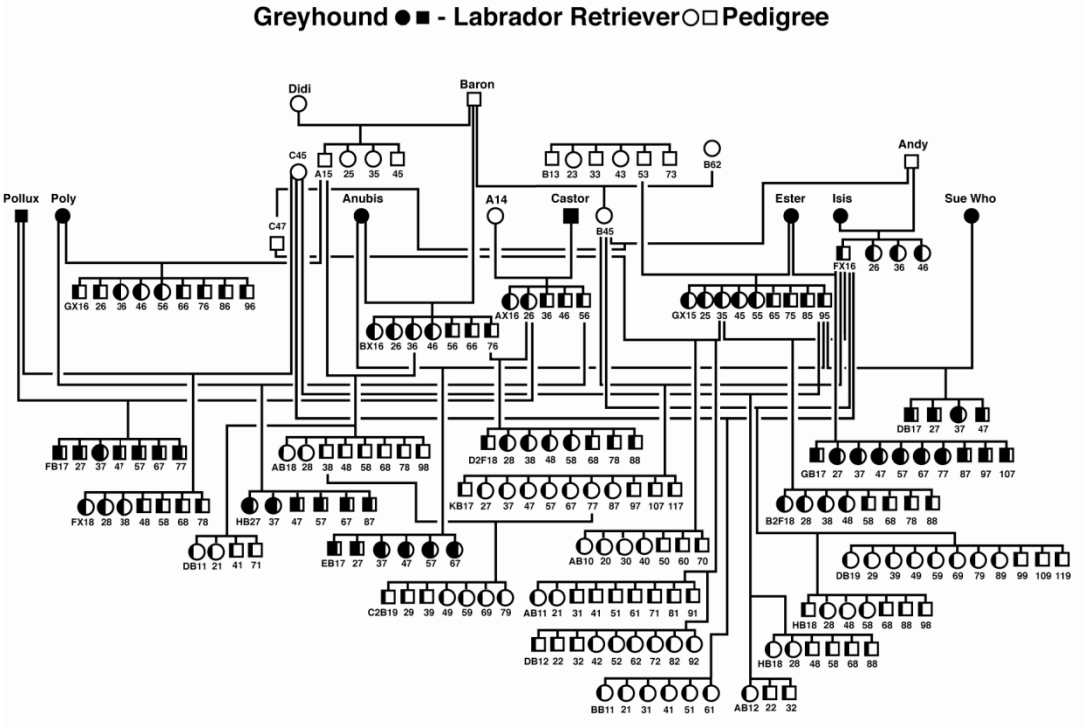


Figure 12 Diagram of a crossbred pedigree founded on trait-free Greyhounds and dysplastic Labrador Retrievers. Squares and circles represent males and females, respectively. Filled and open portions of each symbol represent the proportion of Greyhound and Labrador Retriever alleles, respectively, possessed by that dog.

1.2 Phenotypic Characteristics in Crossbred pedigree

1.2.1 Phenotypic evaluation within crossbred pedigree

Four radiographic methods; Distraction index (DI), Dorsolateral subluxation score (DLS), Norberg angle (NA) and OFA score (Orthopedic Foundation for Animal) were used to evaluate phenotypic characteristics in CHD of crossbred pedigree. This crossbred pedigree was founded by crossbreeding between Greyhound and Labrador Retriever founders composed of F_1, BCL, BCG, F_2 and DBCL that expressed normal and affected phenotypic characteristics. The DI method was used to measure maximum lateral passive laxity of the hip. The DLS test was

used to assess the amount of passive dorsolateral subluxation of the hip in a weight-bearing position. The standard, hip-extended; NA and OFA score methods were used to estimate hip subluxation and the presence of osteoarthritis.

Summary statistics from SPSS v11.5 program, including number of dogs, mean, median, mode, standard deviation (SD), minimal value, maximal value, variance, range, the 25^{th} quartile and the 75^{th} quartile of phenotypic distribution in the crossbred pedigree were described in Table 1. Among 4 radiographic methods, DLS scores were not available on all founders at 8 months of age because the method was introduced after some of the founders were older. The mean phenotypic scores for the left and the right of Labrador Retriever founders were 0.52, 0.58 for DI, 51.40, 44.13 for DLS, 103.83, 103.33 for NA and 3.89, 3.89 for OFA score indicating bad hip quality. On the contrary, the mean phenotypic scores for the left and the right of Greyhound founders were 0.15, 0.13 for DI, 74, 74 for DLS, 105, 110.36 for NA and 2, 2 for OFA score indicating good hip quality.

The analysis results showed that mean DI for the left and the right hip of the F_1 generation (0.45, 0.48), BCL (0.5, 0.55) and F_3 (0.49, 0.63) were similar to the mean DI of the Labrador Retriever founders (0.52, 0.58) than Greyhound founders (0.15, 0.13) (Table 1). This result showed that our founders were in the group of tight hips (DI< 0.3-0.4) in Greyhound founders and susceptible to CHD group in Labrador Retriever founders (DI> 0.4) (Lust *et al*., 1993; Smith 1997; Smith *et al*., 2001). The distribution of the distraction index for the BCG and F_2 were between the two founders although in the F_1 and F_3 generation was skewed to the Labrador Retriever founders (Figure13).

Only some of the data for DLS was available for the founders and F_1s dog. The mean DLS scores for the left and the right of the Labrador Retriever and Greyhound founders were 51.40, 44.13 and 74, 74 respectively. The mean DLS for the left and the right hip of F_1 (63.80, 67.80) , BCG (66.70, 66.11) and F_2 (67.41, 66.23) were closed to Greyhound founders however BCL (50.96, 50.72) and F_3 (44.49, 41.09) were similar to Labrador Retriever founders (Table 1).

The evaluation of NA measurement in Labrador Retriever and Greyhound founders expressed affected and normal hip respectively. BCL, BLG and F_2 represented that most of these progenies had tendency to normal hip. The phenotypic distribution of NA for these dogs was skewed to normal hip (NA>105). The founding Greyhounds had the mean of good OFA score (OFA=2) and the founding Labrador Retrievers had dysplastic hips with OFA scores of fair to borderline hip dysplasia (OFA=3.89) while the others (F_1, BCL, BCG, F_2 and F_3) had hip score inclined to normal hip (Table 1). Among these radiographic methods, DI showed the best phenotypic distribution (Figure 13).

Table 1 Summary statistics included mean, median, mode, standard deviation (SD), minimal value, maximal value, variance, range, the 25th quartile and the 75th quartile of phenotypic distribution according to pedigree in the crossbred dogs.

	Breed	L	G	F_1	BCL	BCG	F_2	F_3
DI_L	**N**	7	7	7	79	33	16	7
	mean	0.52	0.15	0.45	0.50	0.31	0.39	0.49
	median	0.54	0.14	0.42	0.50	0.28	0.39	0.54
	mode	0.32	0.14	0.30	0.30	0.08	0.25	0.16
	SD	0.21	0.07	0.15	0.18	0.15	0.10	0.21
	min	0.32	0.03	0.30	0.13	0.03	0.25	0.16
	max	0.92	0.21	0.74	0.91	0.62	0.57	0.73
	variance	0.05	0.01	0.02	0.03	0.02	0.01	0.05
	range	0.60	0.18	0.44	0.91	0.59	0.32	0.57
	P25	0.32	0.12	0.33	0.33	0.19	0.30	0.32
	P75	0.62	0.21	0.52	0.64	0.42	0.47	0.69
DI_R	**N**	7	6	7	8	32	16	7
	mean	0.58	0.13	0.48	0.55	0.33	0.36	0.63
	median	0.61	0.11	0.46	0.54	0.30	0.35	0.64
	mode	0.33	0.11	0.46	0.48	0.29	0.39	0.50
	SD	0.18	0.07	0.12	0.19	0.15	0.10	.076
	min	0.33	0.04	0.33	0.08	0.07	0.20	0.50
	max	0.87	0.21	0.64	1.00	0.62	0.54	0.73
	variance	0.03	0.01	0.01	0.03	0.02	0.01	0.01
	range	0.54	0.17	0.31	0.92	0.55	0.34	0.23
	P25	0.42	0.09	0.34	0.43	0.25	0.28	0.58
	P75	0.70	0.21	0.60	0.66	0.43	0.47	0.68
DLS_L	**N**	3	1	1	80	33	16	7
	mean	51.40	74.00	63.80	50.96	66.70	67.41	44.49
	median	49.00	74.00	63.80	51.70	66.60	66.60	42.00
	mode	35.20	74.00	63.80	62.00	66.00	66.60	35.70
	SD	17.52	na	na	13.83	5.41	4.80	9.62
	min	35.20	74.00	63.80	21.40	55.10	60.00	35.70
	max	70.00	74.00	63.80	85.30	76.60	76.00	61.50
	variance	307.08	na	na	191.34	29.21	23.06	92.50
	range	34.80	0.00	0.00	63.90	21.50	16.00	25.80
	P25	35.20	74.00	63.80	40.40	62.80	64.35	36.00
	P75	70.00	74.00	63.80	62.00	70.20	70.83	53.50

Table 1 (Continued)

	Breed	L	G	F_1	BCL	BCG	F_2	F_3
DLS_R	**N**	3	1	1	80	33	16	7
	mean	44.13	74.00	67.80	50.72	66.11	66.23	41.09
	median	48.00	74.00	67.80	51.80	66.10	66.60	39.20
	mode	34.40	74.00	67.80	66.60	64.00	66.60	39.20
	SD	8.49	na	na	12.66	4.21	4.91	7.56
	min	34.40	74.00	67.80	25.00	57.10	56.20	32.10
	max	50.00	74.00	67.80	74.00	73.30	74.00	55.70
	variance	72.05	na	na	160.22	17.68	24.14	57.18
	range	15.60	0.00	0.00	49.00	16.20	17.80	23.60
	P25	34.40	74.00	67.80	40.33	63.70	61.83	35.40
	P75	50.00	74.00	67.80	62.38	69.05	69.80	44.00
NA_L	**N**	8	7	7	80	33	15	7
	mean	103.83	105.00	104.29	107.23	108.95	110.00	104.76
	median	104.00	103.50	104.50	108.50	109.00	109.00	105.00
	mode	103.50	103.00	97.50	108.00	107.00	108.00	105.00
	SD	5.13	3.69	4.08	6.11	2.97	3.52	1.12
	min	94.00	100.00	97.50	85.00	103.50	103.50	103.00
	max	113.00	111.00	110.00	116.50	118.00	115.00	106.00
	variance	26.31	13.58	16.65	37.29	8.82	12.39	1.25
	range	19.00	11.00	12.50	31.50	14.50	11.50	3.00
	P25	101.75	103.00	102.00	104.13	107.00	107.50	103.50
	P75	106.25	107.50	108.00	111.50	111.00	113.00	105.80
NA_R	**N**	8	7	7	80	33	15	7
	mean	103.33	110.36	108.93	107.74	110.54	110.07	105.57
	median	102.00	109.00	107.00	108.25	110.50	109.50	105.00
	mode	98.00	107.00	107.00	111.00	109.00	109.00	101.50
	SD	5.67	4.54	4.16	5.64	3.77	3.91	2.73
	min	96.00	107.00	105.50	90.50	103.00	104.00	101.50
	max	112.00	119.50	117.50	117.00	117.80	115.50	110.00
	variance	32.188	20.64	17.29	31.81	14.19	15.32	7.45
	range	16.00	12.50	12.00	26.50	14.80	11.50	8.50
	P25	98.00	107.00	106.00	105.00	108.50	106.00	104.00
	P75	108.25	113.00	110.50	111.88	113.50	114.00	107.50

Table 1 (Continued)

	Breed	L	G	F_1	BCL	BCG	F_2	F_3
OFA_L	**N**	8	7	7	80	33	15	6
	mean	3.89	2.00	2.14	2.65	2.03	1.73	2.50
	median	3.00	2.00	2.00	2.00	2.00	2.00	2.00
	mode	3.00	2.00	2.00	2.00	2.00	2.00	2.00
	SD	1.83	0.00	0.69	1.51	0.47	0.46	1.38
	min	2.00	2.00	1.00	1.00	1.00	1.00	1.00
	max	7.00	2.00	3.00	6.00	3.00	2.00	5.00
	variance	3.36	0.00	0.48	2.28	0.22	0.21	1.90
	range	5.00	0.00	2.00	5.00	2.00	1.00	4.00
	P25	3.00	2.00	2.00	2.00	2.00	1.00	1.75
	P75	5.50	2.00	3.00	3.00	2.00	2.00	3.50
OFA_R	**N**	8	7	7	80	33	15	6
	mean	3.89	2.00	2.14	2.66	2.00	1.73	2.50
	median	3.00	2.00	2.00	2.00	2.00	2.00	2.00
	mode	3.00	2.00	2.00	2.00	2.00	2.00	2.00
	SD	1.83	0.00	0.69	1.54	0.43	0.46	1.38
	min	2.00	2.00	1.00	1.00	1.00	1.00	1.00
	max	7.00	2.00	3.00	6.00	3.00	2.00	5.00
	variance	3.36	0.00	0.48	2.38	0.19	0.21	1.90
	range	5.00	0.00	2.00	5.00	2.00	1.00	4.00
	P25	3.00	2.00	2.00	2.00	2.00	1.00	1.75
	P75	5.50	2.00	3.00	4.50	2.00	2.00	3.50

Note: L = Labrador Retriever, G = Greyhound, F_1 = L x G, BCL = F_1 backcross to Labrador Retriever, BCG = F_1 backcross to Greyhound, F_2 = F_1 x F_1, F_3 = (F_1 x L) x (F_1 x L), na = not applicable, DI=distraction index, DLS = dorsolateral subluxation score, NA = Norberg angle, OFA = OFA score, L = left hip, R = right hip, N = Number of observations on which calculations were based, mean = arithmetic mean, median = middle value (50^{th} percentile), mode = most frequent value (if not unique, the smallest mode), SD = standard deviation, min = smallest (minimum) value, max = largest (maximum) value, range = difference between the maximum and minimum values, P25 = lower quartile (25^{th} percentile), P75 = upper quartile (75^{th} percentile)**.**

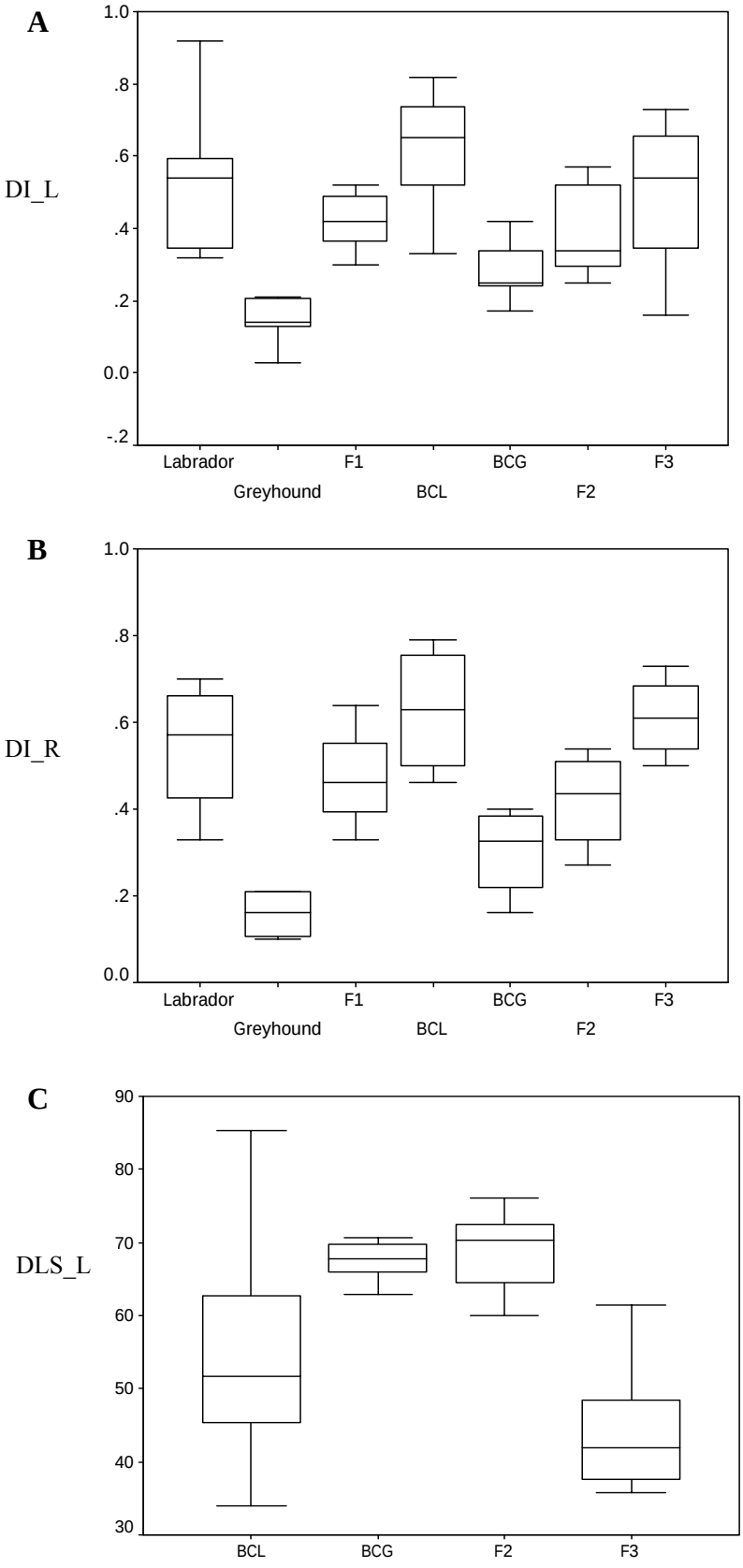


Figure 13 (Continued)

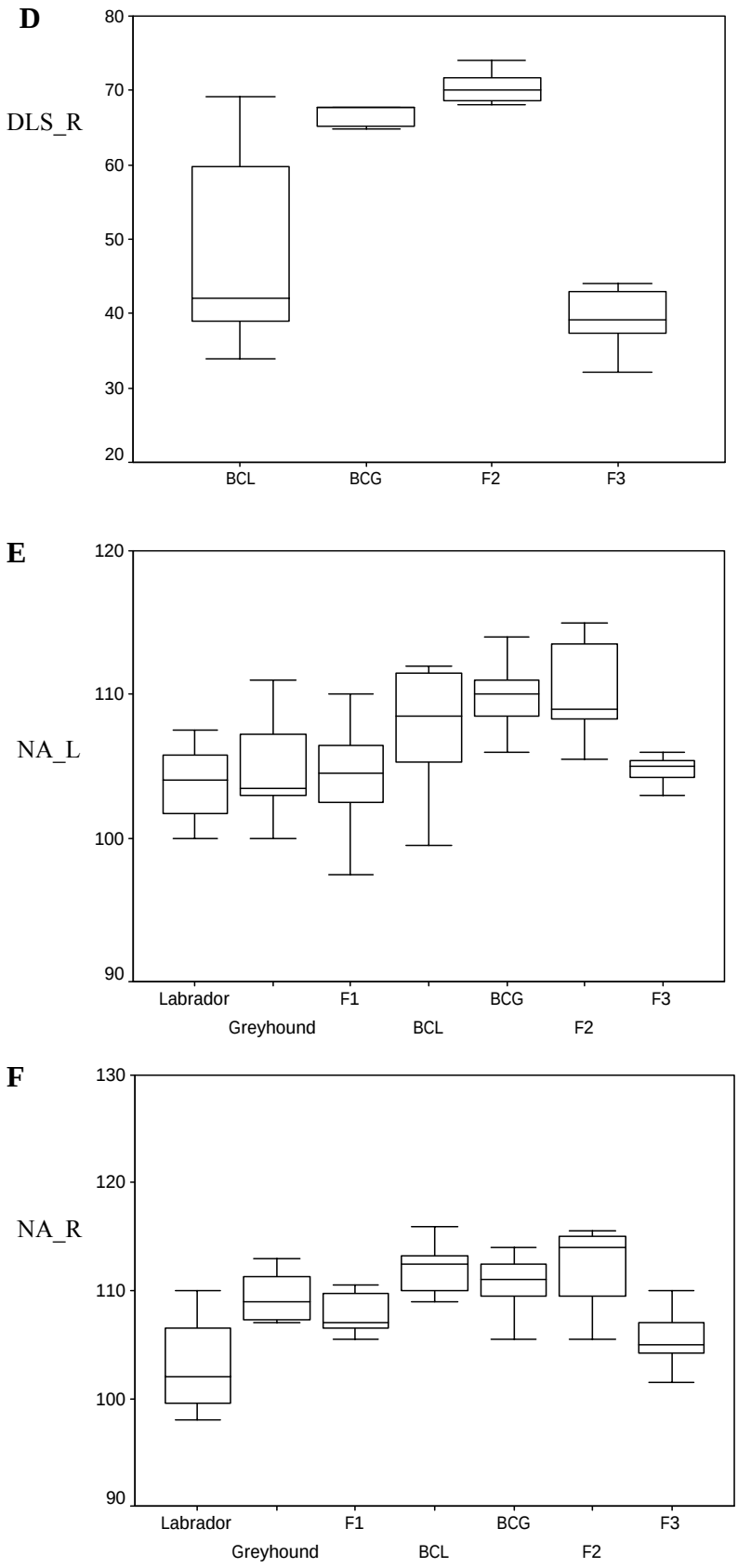


Figure 13 (Continued)

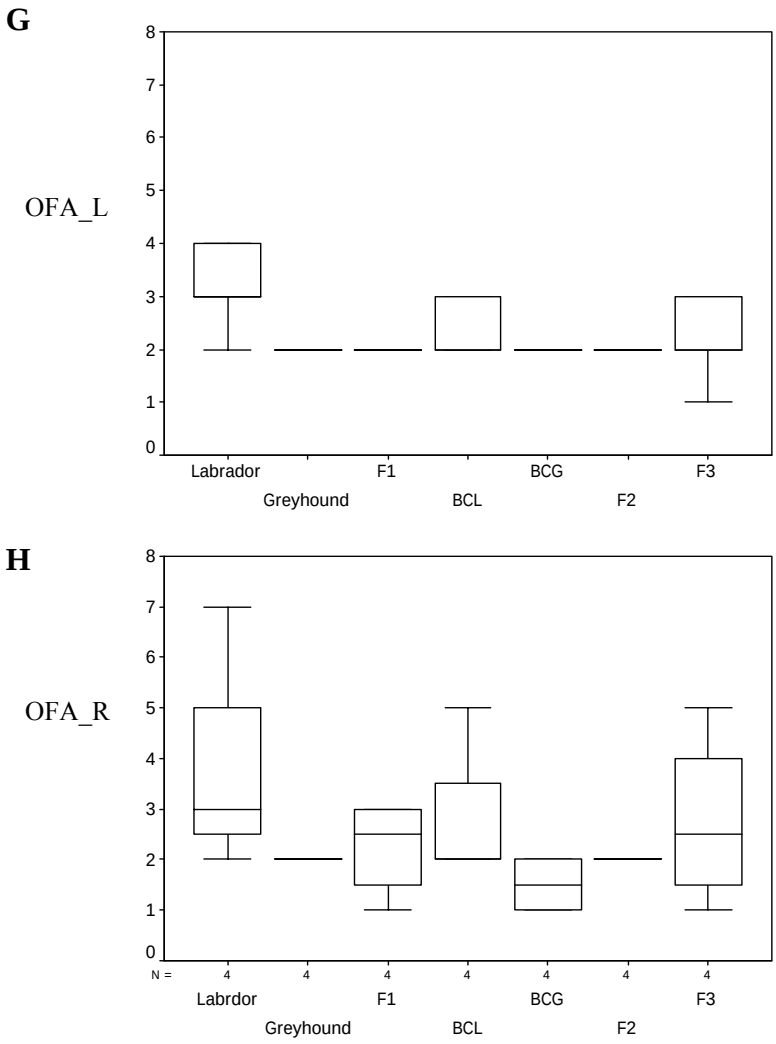


Figure 13 Box plots comparing the distribution of the DI_L (A), DI_R (B), DLS_L (C), DLS_R (D), NA_L (E), NA_R (F), OFA_L (G) and OFA_R (H) between Labrador Retriever founders, Greyhound founders, F_1, BCL, BCG, F_2 and F_3. The plots showed the mean, the 25^{th}, and the 75^{th} quartile, and the lowest and the highest phenotypic measurement.

Note: There were too few valid cases to create a chart in DLS and OFA score. DI = Distraction index, DLS = Dorsolateral subluxation score, NA = Norberg angle, OFA = OFA score, L = left hip, R = right hip, BCL = backcross to Labrador Retriever founder, BCG = backcross to Greyhound founder, $F_2 = F_1 x\ F_1$ and $F_3 = [(F_1 \times L) \times (F_1 \times L)]$.

1.2.2 Phenotypic evaluation in group of crossbred pedigree

To compare the phenotypic distribution for distraction index (DI), dorsolateral subluxation score (DLS), Norberg angle (NA) and OFA score of these crossbred pedigree with their parents, data from every parents were analyzed separately (Table 2). The mean phenotypic scores for the left and the right hips of these parents were 0.38, 0.39 for DI, 51.26, 48.01 for DLS, 103.64, 106.62 for NA and 2.96, 2.96 for OFA score. The mean of these 4 traits were closed to borderline and showed normal distribution of the traits (Figure 14).

In order to observe the phenotypic distribution in group of crossbred pedigree, data from every dog were evaluated together. Data for DI, DLS, NA and OFA score were available for 153, 141, 158 and 157 dogs, respectively (Table 3). Among these phenotypic measurements in group of dogs, DI was the best methods that showed normal distribution of the phenotype both in parents and in group of dogs. The distribution of DLS and NA methods were skewed to the right (normal hip) and OFA score was skewed to the left (normal hip) (Figure 15). When observed the data in group, the mean of DLS, NA and OFA scores in crossbred dogs were greater than 55%, 105 and 2 respectively (normal hip). While dysplastic-susceptible dogs had DLS, NA and OFA scores less than 55%, 105 and 2. It may be assumed that the Greyhound effect on the DLS, NA and OFA was dominant.

Table 2 Summary statistics included mean, median, mode, standard deviation, minimal value, maximal value, variance, range, the 25th quartile and the 75th quartile of hip trait measured in crossbred pedigree parents.

	DI		DLS		NA		OFA	
	L	**R**	**L**	**R**	**L**	**R**	**L**	**R**
N	25	25	7	7	25	25	25	25
mean	0.38	0.39	51.26	48.01	103.64	106.62	2.96	2.96
median	0.33	0.42	49.00	48.00	103.70	106.92	2.58	2.58
mode	0.00	0.00	24.00	28.00	103.00	107.00	2.00	2.00
SD	0.26	0.29	18.72	17.59	4.83	5.82	1.54	1.54
min	0.00	0.00	24.00	28.00	92.00	96.00	1.00	1.00
max	0.92	1.00	74.00	74.00	113.00	119.50	7.00	7.00
variance	0.07	0.08	350.54	309.30	23.37	33.90	2.37	2.37
range	0.92	1.00	50.00	46.00	21.00	23.50	6.00	6.00
P25	0.19	0.12	37.10	34.03	101.67	102.75	1.88	1.88
P75	0.55	0.61	68.45	63.35	107.08	109.63	3.56	3.56

Note: DI=distraction index, DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score,

L=left hip, R=right hip,

N = Number of observations on which calculations were based,

mean =arithmetic mean,

median =middle value (50th percentile),

mode = most frequent value (if not unique, the smallest mode),

SD = standard deviation,

min = smallest (minimum) value,

max = largest (maximum) value,

range = difference between the maximum and minimum values,

P25 = lower quartile (25th percentile),

P75 = upper quartile (75th percentile).

Table 3 Summary statistics included mean, median, mode, standard deviation, minimal value, maximal value, variance, range, the 25th quartile and the 75th quartile of hip trait measured in group of crossbred pedigree.

	DI		DLS		NA		OFA	
	L	**R**	**L**	**R**	**L**	**R**	**L**	**R**
N	156	153	141	141	158	158	157	157
mean	0.431	0.472	56.450	55.750	107.32	108.37	2.446	2.446
median	0.420	0.480	60.400	59.200	108.00	109.00	2.000	2.000
mode	0.300	0.500	66.600	66.600	111.00	111.00	2.000	2.000
SD	0.192	0.201	13.839	13.032	5.206	5.206	1.308	1.327
min	0.030	0.040	21.400	25.000	85.000	90.500	1.000	1.000
max	0.920	1.000	85.300	74.000	118.00	119.50	7.000	7.000
variance	0.037	0.040	191.51	169.84	27.103	27.097	1.710	1.761
range	0.890	0.960	63.900	49.000	33.000	29.000	6.000	6.000
P25	0.293	0.330	45.250	44.300	104.50	105.50	2.000	2.000
P75	0.570	0.600	66.600	66.600	111.00	112.00	3.000	3.000

Note: DI=distraction index, DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score,
L=left hip, R=right hip,
N = Number of observations on which calculations were based,
mean =arithmetic mean,
median =middle value (50th percentile),
mode = most frequent value (if not unique, the smallest mode),
SD = standard deviation,
min = smallest (minimum) value,
max = largest (maximum) value,
range = difference between the maximum and minimum values,
P25 = lower quartile (25th percentile),
P75 = upper quartile (75th percentile).

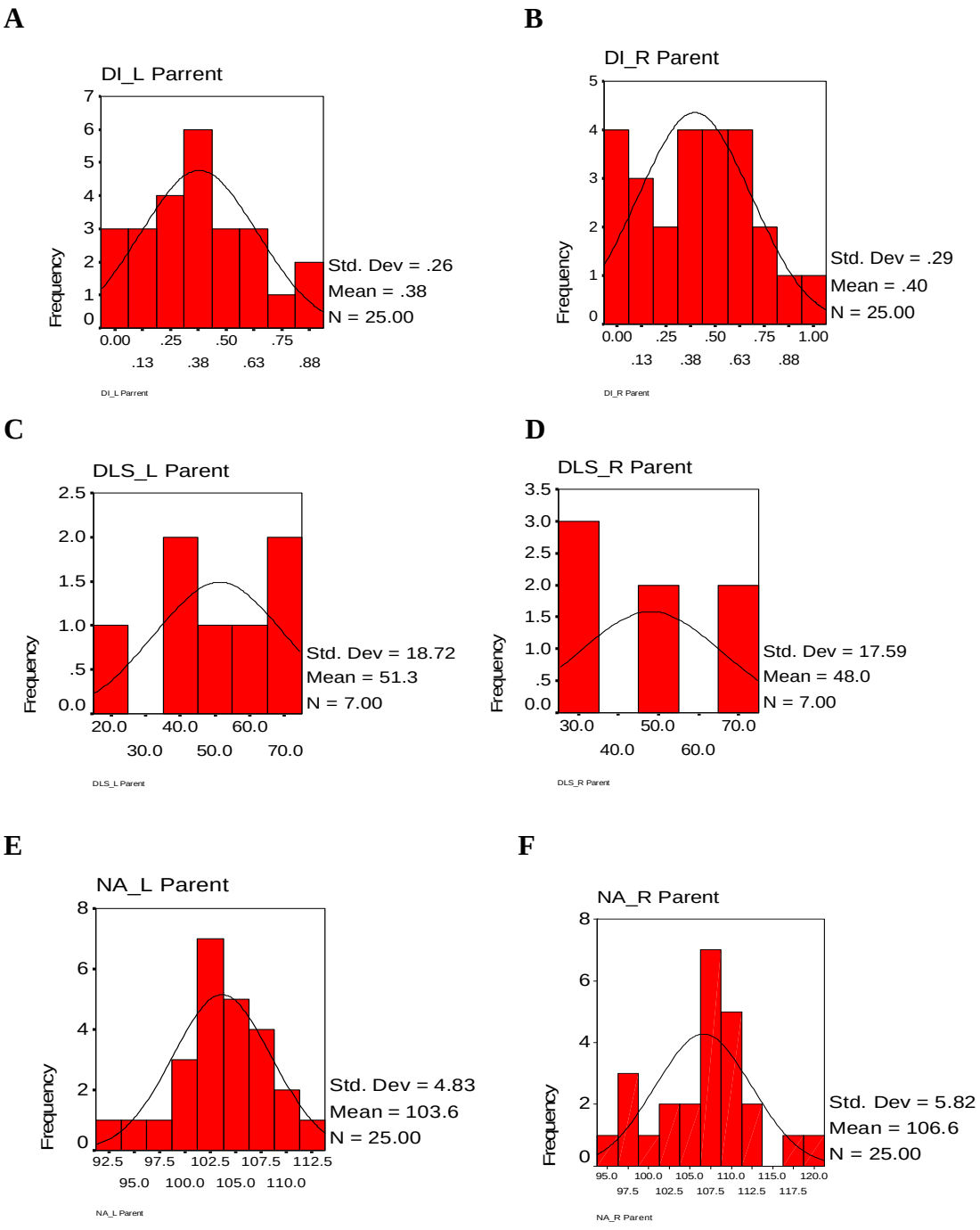
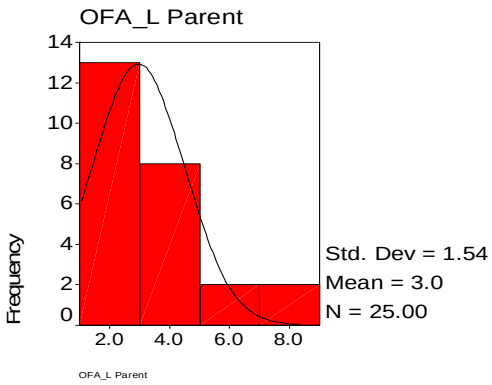


Figure 14 (Continuted)

G



H

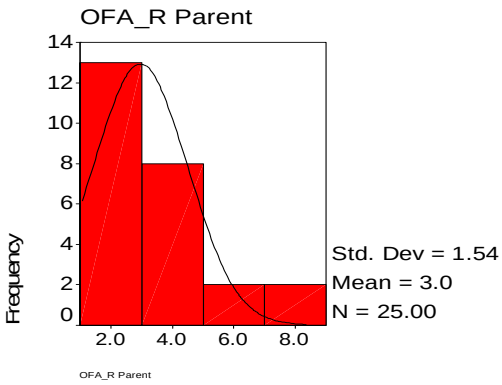


Figure 14 These graphs were shown frequency distribution of canine hip dysplasia traits in crossbred pedigree parents where the height of each bar gave the number of individuals with the trait value on the X-axis. They showed phenotypic distribution for the left DI (A), right DI (B), left DLS (C), right DLS (D), left NA (E), right NA (F), left OFA (G) and right OFA (H).

Note: DI=distraction index, DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score, L=left hip, R=right hip.

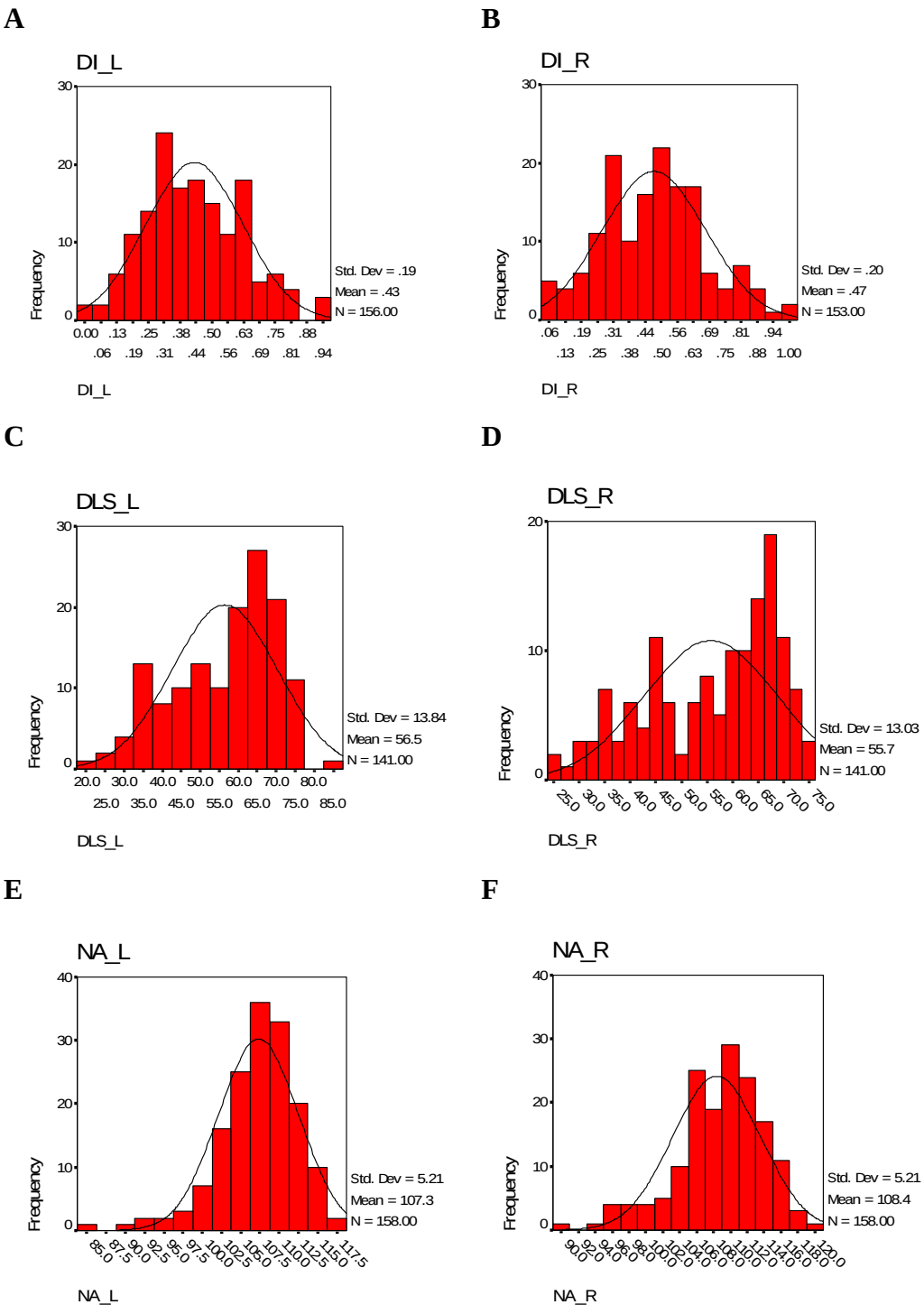


Figure 15 (Continuted)

G **H**

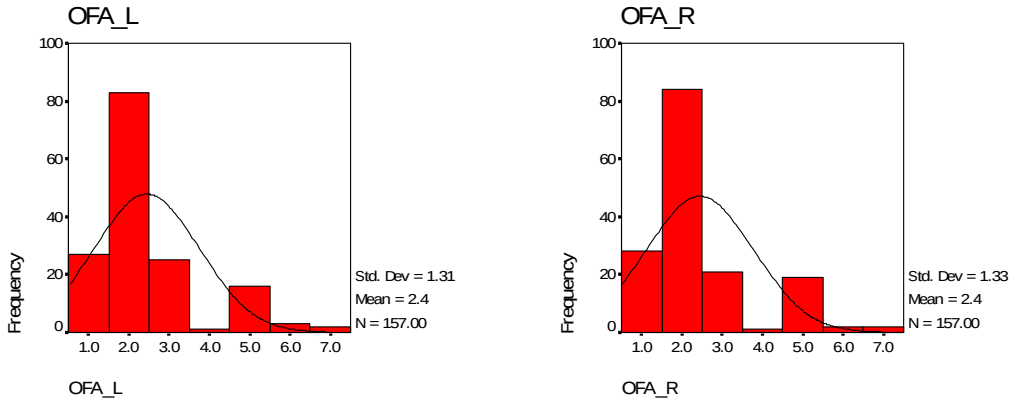


Figure 15 These graphs were shown frequency distribution of canine hip dysplasia traits in group of crossbred pedigree where the height of each bar gave the number of individuals with the trait value on the X-axis. They showed phenotypic distribution for the left DI (A), right DI (B), left DLS (C), right DLS (D), left NA (E), right NA (F), left OFA (G) and right OFA (H).

Note: DI=distraction index, DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score, L=left hip, R=right hip.

1.3 Microsatellite informativeness

1.3.1 Marker Allele Fidelity

Software called “Genoped” was used to verify marker allele fidelity. It checked Mendelian inheritance errors. Parents were always genotypes with their offspring for each litter providing an internal control. Markers with error were corrected assuming that the marker allele size in the grandparent was correct.

1.3.2 Microsatellite informativeness in crossbred pedigree

The analysis was undertaken on 428 microsatellite markers in crossbred pedigree. These markers spanned across 38 autosomes and X chromosome. For the analysis reported here we used the Guyon *et al*., (2003) map marker order. The orders and numbers of the genome-wide screen with these markers had showed in Figure 16. The average inter-marker interval among 38 autosomes and X chromosme was 6.10 cM that ranged from 4.42-8.76 cM. The widest interval was found on CFA 28 and 38 (8.76 cM), the narrowest interval was on CFA05 (4.42 cM). Marker coverage within each chromosome ranged from 44.49-100 % and average across the genome was 91.10 %. There were 4 chromosomes (CFA10, 22, 24 and 37) having 100 % marker coverage. In these marker spanning, only CFA16 and X had marker coverage less than 80 % (Table 4).

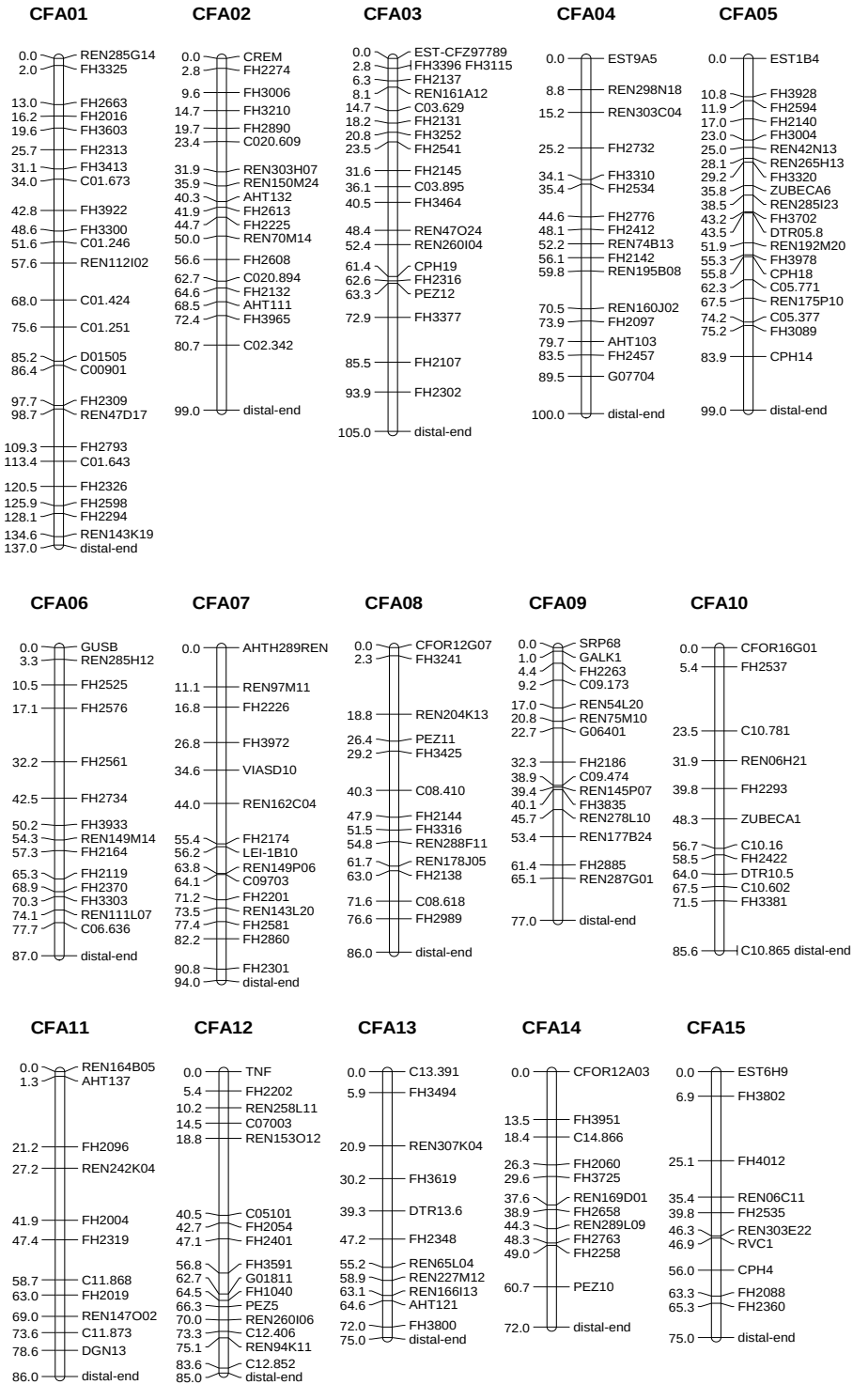


Figure 16 (Continued**)**

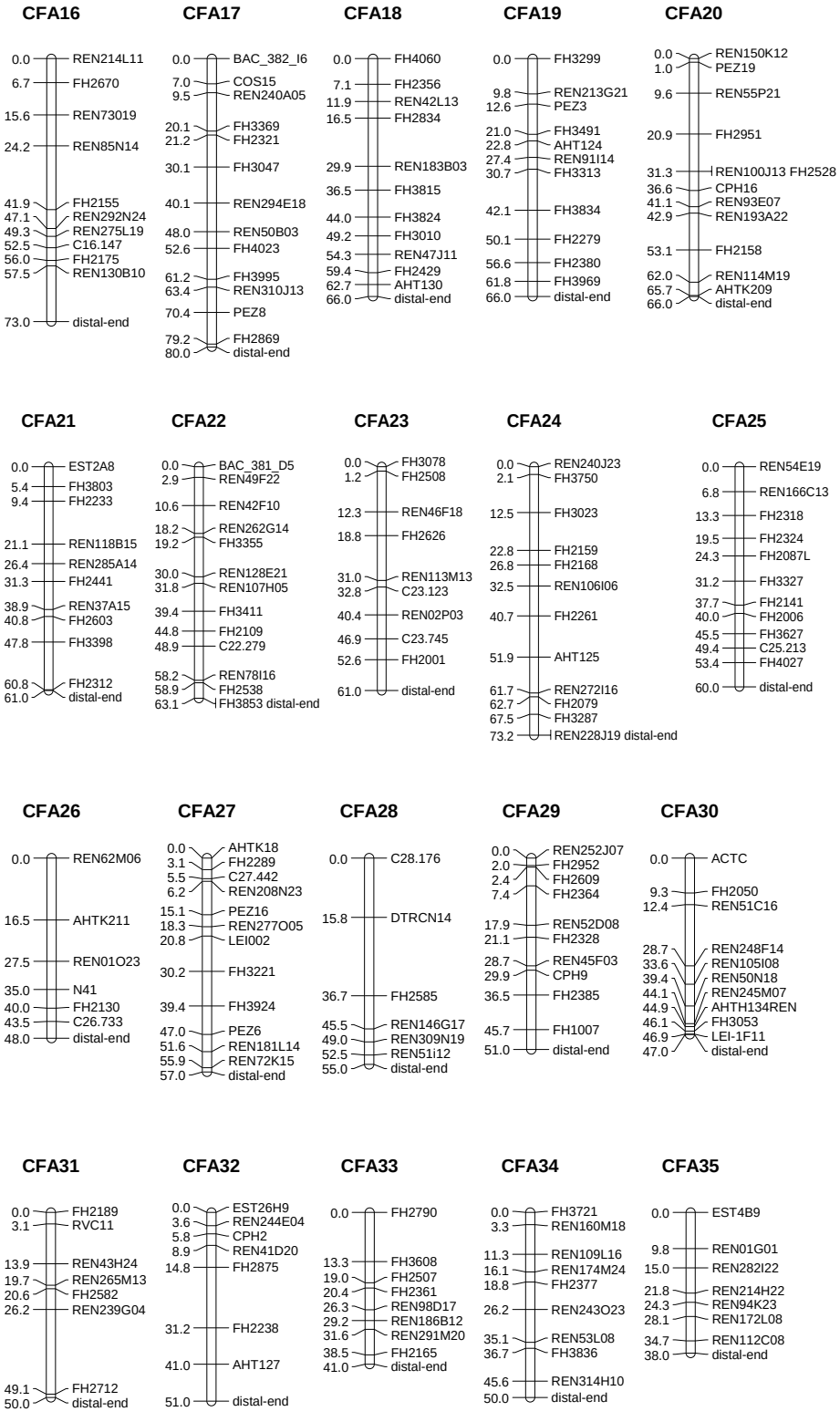


Figure 16 (Continued**)**

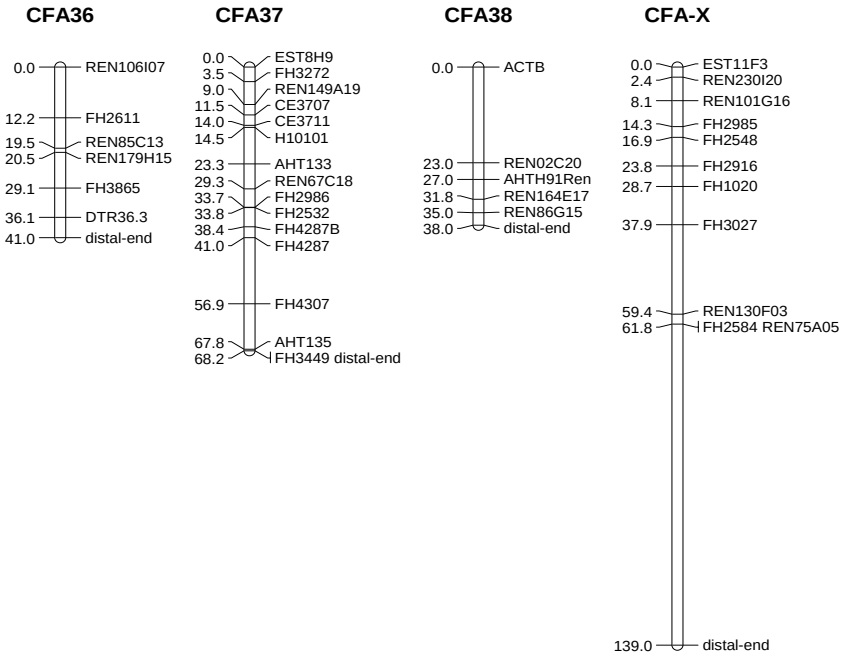


Figure 16 This pictures showed microsatellite order and number using for genome-wide screen in crossbred pedigree. The total number of markers was 428 microsatellite markers with average inter-marker interval was 6.10 cM. There were not any chromosomes that have inter-marker interval more than 10 cM.

Table 4 Average inter-marker interval and marker coverage of the 428 microsatellite markers set in crossbred pedigree.

CFA	number of markers	average inter-marker interval (cM)	marker coverage (%)
1	23	5.85	97.81
2	17	4.74	81.47
3	19	4.94	89.42
4	15	5.97	89.53
5	19	4.42	84.78
6	13	5.98	89.29
7	14	6.49	96.65
8	12	6.38	89.04
9	14	4.65	84.59
10	11	7.78	100.00
11	10	7.86	91.36
12	15	5.57	98.35
13	11	6.54	95.96
14	10	6.07	84.24
15	9	7.25	87.01
16	10	5.75	78.73
17	12	6.60	99.05
18	11	5.70	95.00
19	11	5.62	93.59
20	11	5.98	99.61
21	9	6.76	99.72
22	12	5.26	100.00
23	9	5.85	86.30
24	11	6.65	100.00
25	11	4.85	89.00
26	5	8.71	90.69
27	11	5.08	98.04
28	6	8.76	95.51
29	9	5.08	89.59
30	9	5.21	99.74
31	7	7.02	98.24
32	6	6.84	80.46
33	8	4.82	93.96
34	9	5.07	91.22
35	6	5.79	91.37
36	5	6.02	88.07
37	14	4.98	100.00
38	4	8.76	91.18
X	10	6.18	44.49
Total	**428**	**6.10**	**91.10**

The number of alleles range from 1-17 alleles. Among these 428 markers, there were 8 markers having only one allele (monomorphic marker). The remaining (420 markers) revealed polymorphic of the markers that had allele more than one allele. The most frequent alleles were 5 alleles (74 markers) (Figure 17).

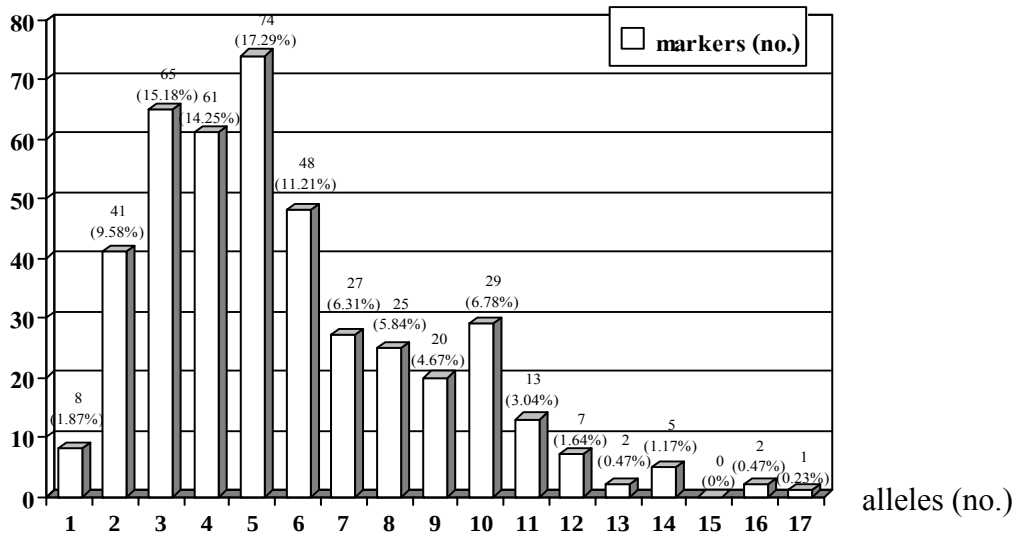


Figure 17 The number of alleles of 428 microsatellite markers screened on 159 crossbred dogs.

Marker informativeness was a function of both the number of alleles per locus and allele frequencies. These values were represented in term of polymorphic information content (PIC) and degree of heterozygosity (Het) (Botstein *et al*., 1980; Lynch and Walsh 1998). PIC and heterozygosity at each marker locus was calculated for each breed or crossbred as a measure of the marker informativeness in the pedigree. Because this crossbred pedigree was derived from various unrelated founders, multiple alleles of each marker should be segregated in the progeny samples.

Based on the criteria of Botstein, most of the markers were moderately to highly informative. Thirty-four of the markers (8 %) were uninformative (PIC and heterozygosity < 0.3), 154 (36 %) and 104 markers (24 %)

were moderately informative (PIC and heterozygosity between 0.3 and 0.59), and the remainder 240 (56 %) and 290 markers (68%) were highly informative ((heterozygosity > 0.59) (Table 5).

Table 5 The polymorphic information content (PIC) and heterozygosity of 428 microsatellite markers screened on 159 crossbred dogs.

	Marker informativeness		
	Uninformative	**Moderately informative**	**Highly informative**
PIC	**< 0.3**	**0.3 – 0.59**	**> 0.59**
No. of markers	34 (8%)	154 (36%)	240 (56%)
Heterozygosity	**< 0.3**	**0.3 – 0.59**	**> 0.59**
No. of markers	34 (8%)	104 (24%)	290 (68%)

1.4 QTL mapping results

1.4.1 Mapping result in crossbred pedigree

Canine genome is 2,700 cM long, from the assumption of QTL mapping 200-300 markers should be enough for genome wide-screen with a marker interval at an average distance of 10-20 cM (Kruglyak, 1997). In this study, we used 428 microsatellite markers spanning across 38 autosomes and X chromosome with average inter-marker interval was 6.10 cM. Genome-wide screen showed 11 chromosomes with log of the odds ratio (LOD) scores over 2.0 were harboring putative QTL affecting CHD on CFA02, 03, 04, 05, 06, 09, 10, 11, 16, 29 and 37 ($p<0.05$, chromosome-wide) and on CFA 02, 06 and 11 ($p<0.01$, chromosome-wide). Chromosome-wide significance was reached by QTL on CFA02 at 16-21 cM, on CFA03 at 4-8 cM, on CFA04 at 33-36 cM, on CFA05 at 0 cM, on CFA06 at 63-66 cM, on CFA09 at 50 cM, on CFA10 at 52-53 cM, on CFA11 at 0-7 cM, on CFA16 at 52 cM, on 29 at 12-19 cM and on CFA37 at 7 cM (Table 6).

The significant QTL on CFA 11 was identified around 0-7 cM between marker AHT137 and FH2096. This QTL had the highest F ratio (6.12) and was chromosome-wide significant at P<0.01 (Table 6). Flanking marker intervals and position of the putative QTLs were displayed in Table 6. The analysis from different traits were identified putative QTLs on the same chromosome in the same position area, the flanking marker interval between QTL position ranged from 1.05 cM on CFA05 to 19.86 cM on CFA 11 (Table 6). Moreover, most of the flanking markers on each QTL were mapped on the same position on each chromosome. The combined traits showed the most power for QTL mapping especially the combination between NA and DLS (Factor2). However, DI and NA revealed the most power for single trait analysis.

Analysis of this QTL model showed 3 significant putative QTLs on CFA02, 06 and 11, with a LOD score 2.259, 2.214 and 2.528 at p<0.01 chromosome-wide. The same QTL on these 11 chromosomes appeared to affect multiple traits because they each mapped to the same chromosome location and contributed similar additive effects on the traits in both magnitude and direction (Table 7).

Table 6 Parameter estimated for the QTL in crossbred pedigree with F tests significant at p<0.05 (chromosome-wide) and p<0.01 for several hip dysplasia traits following a genome-wide screen of QTL for the left, right, low and high distraction indices (DI), dorsolateral subluxation (DLS) hip scores (%), Norberg angle (NA)(degrees) and the factor1 and 2 of these traits. Chromosome (CFA for *Canis familiaris*), bracketing markers, position on the chromosome (in cM), F statistic, log of the odds (LOD) ratio score and threshold at the locus were shown.

Trait	CFA	Bracketing markers (cM)	Pos.	F	LOD	Threshold	Sig. level
Factor2 worst	2	AHT111- FH2132 (3.97)	16	5.43	2.258	4.554	*
Factor2 best	2	C02.894 – FH2608 (6.07)	19	5.44	2.259	5.320	**
Factor2 left	2	C02.894 – FH2608 (6.07)	19	5.35	2.225	4.425	*
Factor2 right	2	C02.894 – FH2608 (6.07)	21	4.84	2.02	4.720	*
Factor1 best	3	FH2137 – REN161A12 (1.85)	4	5.75	2.381	5.386	*
Factor1 worst	3	FH2137 – REN161A12 (1.85)	4	4.82	2.011	4.870	*
Factor1 left	3	FH2137 – REN161A12 (1.85)	4	5.52	2.293	5.182	*
Factor2 left	3	REN161A12 – C03.629 (6.55)	6	6.4	2.639	5.144	*
Factor2 worst	3	REN161A12 – C03.629 (6.55)	7	6.57	2.705	5.364	*
Factor2 best	3	REN161A12 – C03.629 (6.55)	8	5.09	2.122	5.414	*
Factor2 right	3	REN161A12 – C03.629 (6.55)	8	5.12	2.131	5.246	*
DI left	4	FH2534 – FH2776 (9.22)	33	4.84	2.023	4.962	*

Table 6 (Continued)

Trait	CFA	Bracketing markers (cM)	Pos.	F	LOD	Threshold	Sig. level
Facotr1 best	4	FH2776-FH2412 (3.48)	36	5.65	2.344	4.788	*
Factor2 best	5	FH3928 – FH2594 (1.05)	0	5.32	2.212	5.178	*
Factor2 worst	5	FH3928 – FH2594 (1.05)	0	5.41	2.248	5.276	*
Factor2 left	5	FH3928 – FH2594 (1.05)	0	5.47	2.27	4.904	*
Factor2 right	5	FH3928 – FH2594 (1.05)	0	4.82	2.012	4.904	*
Factor2 worst	6	FH2576 – FH2525 (6.57)	63	5.2	2.165	4.526	*
Factor2 right	6	FH2576 – FH2525 (6.57)	64	4.87	2.032	4.742	*
Factor2 left	6	FH2576 – FH2525 (6.57)	65	5.2	2.165	4.660	*
Factor2 best	6	FH2576 – FH2525 (6.57)	66	5.33	2.214	5.339	**
Factor2 left	9	REN278L10 – REN177B24 (7.63)	50	5.07	2.11	4.606	*
NA left	10	C10.16 – FH2422 (1.75)	52	4.8	2.006	5.168	*
NA high	10	C10.16 – FH2422 (1.75)	53	7.08	2.906	5.221	*
Factor2 best	10	C10.16 – FH2422 (1.75)	53	6.03	2.493	5.088	*
DI low	11	AHT137 – FH2096 (19.86)	0	5.82	2.411	4.524	*
DI left	11	AHT137 – FH2096 (19.86)	7	6.12	2.528	6.109	**
Factor2 worst	16	REN275L19 – C16.147 (3.15)	52	5.03	2.103	4.202	*
NA low	29	FH2364 – REN52D08 (10.53)	12	7.25	2.97	4.763	*
NA left	29	REN52D08 – FH2328 (3.21)	19	6.46	2.665	4.815	*
Factor2 right	37	REN149A19 – CE3707 (2.50)	7	4.9	2.044	4.683	*

Note: Factor1 best = principle component of high NA and low DI (the best hip combination).

Factor1 worst = principle component of low NA and high DI (the worst hip combination).

Factor1 left = principle component of the left NA and left DI.

Factor1 right = principle component of the right NA and right DI.

Factor2 best = principle component of high NA and high DLS (the best hip combination).

Factor2 worst = principle component of low NA and low DLS (the worst hip combination).

Factor2 left = principle component of the left NA and left DLS.

Factor2 right = principle component of the right NA and right DLS.

* Chromosome-wide p value <0.05, significant at the 5% chromosome-wide level

** Chromosome-wide p value <0.01, significant at the 1% chromosome -wide level

The general pictures of QTL position on each chromosome were illustrated on Figure 18. The analysis result represented the likelihood ratio test statistic that plotted at every 1 cM along the chromosome. Each color line represented each phenotypic trait; most traits were mapped on the same chromosomal location.

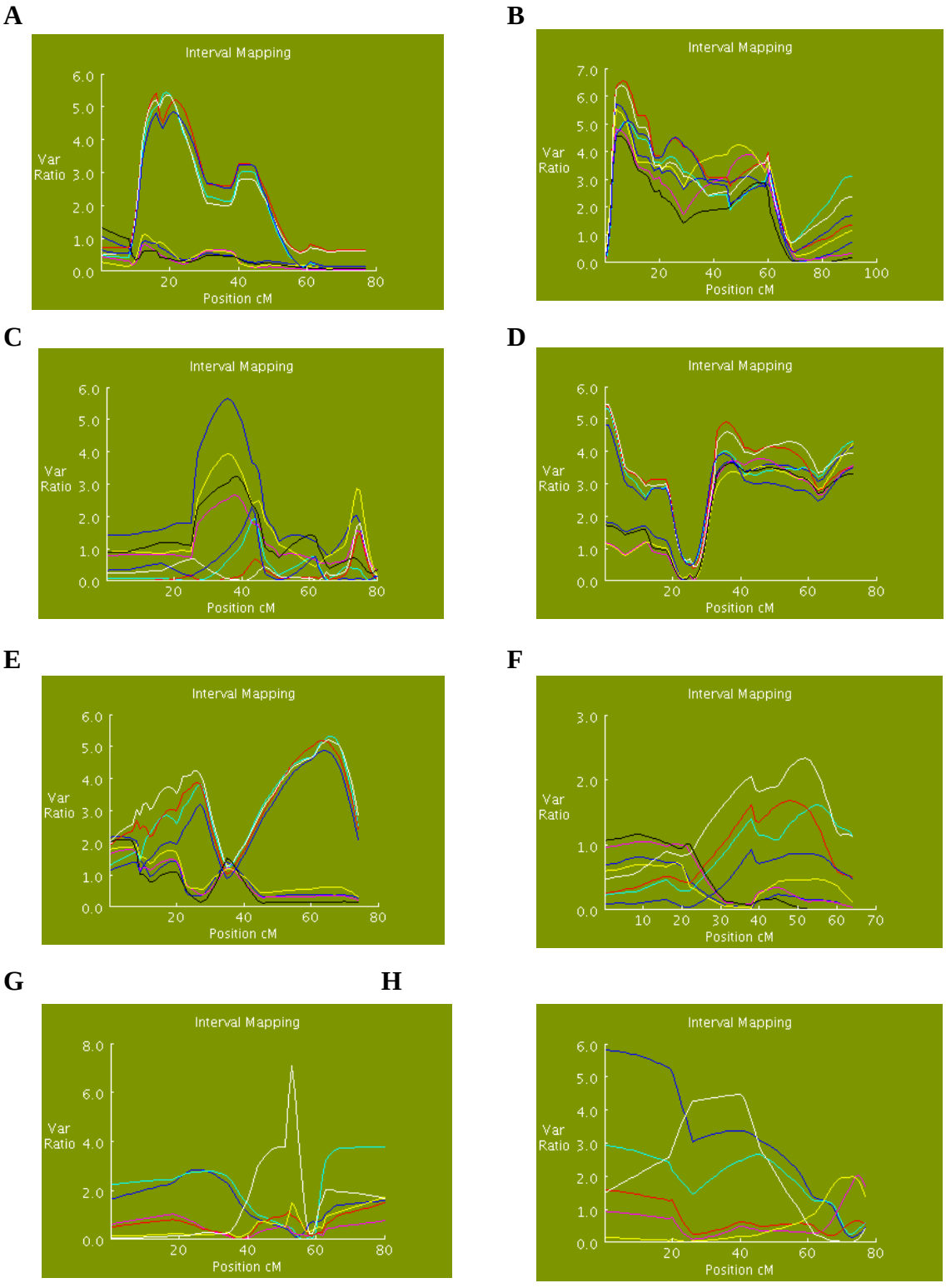


Figure 18 (Continued)

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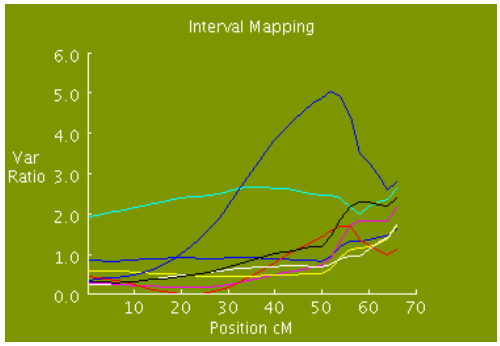

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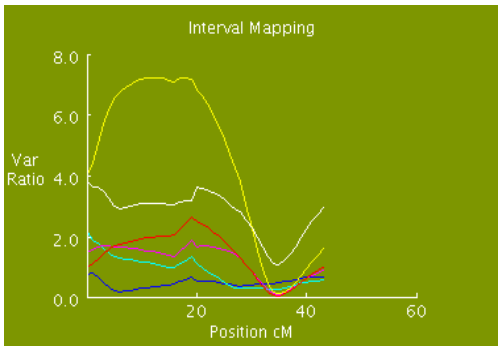

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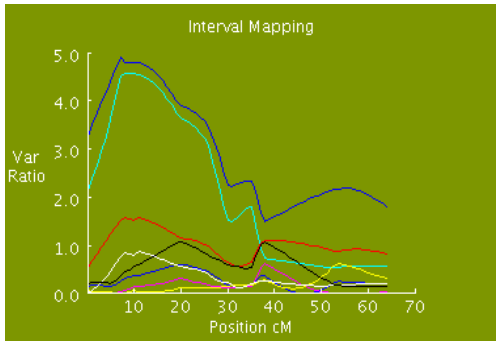

Figure 18 In this graph the likelihood ratio test statistic was plotted at regular (e.g. 1 cM) intervals along the chromosome, with the peak value representing the most likely position of the QTL on CFA02 (A), CFA03 (B), CFA04 (C), CFA05 (D), CFA06 (E), CFA09 (F), CFA10 (G), CFA11 (H), CFA16 (I), CFA29 (J), CFA37 (K). The peaks represent the most likely position of the QTL in cM on the X axis across the chromosome. Each color line represented each trait. The likelihood ratio test statistic revealed putative QTL with LOD scores > 2 at chromosome-wide p value <0.05 (CFA02, 03, 04, 05, 06, 09, 10, 11, 16, 29 and 37) and <0.01 (CFA02, 06 and 11) respectively.

Table 7 This table revealed additive (*a*) and dominance (*d*) QTL effects and trait mean at the locus correspond to genotype value of +*a*, *d*, and –*a* for dogs having inherited two Greyhound QTL alleles, heterozygote and dogs with two Labrador Retrievers alleles respectively.

			QTL effect estimates (SE)		
Trait	**CFA**	**Position**	***a***	***d***	**Mean**
Factor2 best	2	19	0.42 (0.16)	-0.22 (0.17)	0.63 (0.38)
Factor2 worst	2	16	0.44 (0.16)	-0.16 (0.17)	0.71 (0.38)
Factor2 left	2	19	0.39 (0.16)	-0.24 (0.17)	0.71 (0.37)
Factor2 right	2	21	0.44 (0.17)	-0.19 (0.18)	0.65 (0.39)
Factor1 best	3	4	0.56 (0.19)	0.56 (0.19)	0.53 (0.39)
Factor2 best	3	8	-0.50 (0.19)	-0.54 (0.19)	0.53 (0.38)
Factor1 worst	3	4	0.52 (0.19)	0.52 (0.20)	0.68 (0.40)
Factor2 worst	3	7	-0.58 (0.19)	-0.59 (0.19)	0.66 (0.38)
Factor1 left	3	4	0.53 (0.20)	0.59 (0.20)	0.53 (0.40)
Factor2 left	3	6	-0.50 (0.18)	-0.62 (0.19)	0.66 (0.37)
Factor2 right	3	8	-0.57 (0.19)	-0.49 (0.20)	0.52 (0.39)
DI left	4	33	0.04 (0.03)	-0.08 (0.04)	0.33 (0.07)
Factor1 best	4	36	-0.30 (0.17)	0.35 (0.18)	0.59 (0.33)
Factor2 best	5	0	-0.55 (0.17)	-0.30 (0.19)	0.65 (0.41)
Factor2 worst	5	0	-0.56 (0.17)	-0.29 (0.19)	0.74 (0.41)
Factor2 left	5	0	-0.55 (0.17)	-0.27 (0.18)	0.69 (0.40)
Factor2 right	5	0	-0.54 (0.17)	-0.31 (0.19)	0.69 (0.42)
Factor2 best	6	66	0.40 (0.17)	-0.26 (0.18)	0.72 (0.39)
Factor2 worst	6	63	0.42 (0.17)	-0.19 (0.18)	0.84 (0.39)
Factor2 left	6	65	0.38 (0.17)	-0.26 (0.18)	0.77 (0.38)
Factor2 right	6	64	0.43 (0.18)	-0.18 (0.19)	0.77 (0.40)
Factor2 left	9	50	0.39 (0.18)	-0.25 (0.19)	0.19 (0.36)
NA left	10	52	0.89 (1.01)	-2.77 (1.06)	111.82 (1.97)

Table 7 (Continued)

			QTL effect estimates (SE)		
Trait	**CFA**	**Position**	***a***	***d***	**Mean**
NA high	10	53	1.33 (0.81)	-2.21 (0.83)	113.69 (1.59)
Factor2 best	10	53	0.31 (0.18)	-0.42 (0.18)	0.21 (0.35)
DI left	11	7	-0.01 (0.03)	0.10 (0.03)	0.22 (0.06)
DI low	11	0	-0.03 (0.03)	0.07 (0.03)	0.18 (0.06)
Factor2 worst	16	52	0.32 (0.17)	-0.32 (0.18)	0.02 (0.35)
NA left	29	19	-2.13 (0.94)	1.64 (0.95)	108.01 (1.91)
NA low	29	12	-2.71 (1.01)	1.47 (1.00)	106.81 (1.97)
Factor2 right	37	7	na	na	na

Note: na = not applicable

Factor1 best = principle component of high NA and low DI (the best hip combination).

Factor1 worst = principle component of low NA and high DI (the worst hip combination).

Factor1 left = principle component of the left NA and left DI.

Factor1 right = principle component of the right NA and right DI.

Factor2 best = principle component of high NA and high DLS (the best hip combination).

Factor2 worst = principle component of low NA and low DLS (the worst hip combination).

Factor2 left = principle component of the left NA and left DLS.

Factor2 right = principle component of the right NA and right DLS.

* Chromosome-wide p value <0.05, significant at the 5% chromosome-wide level

** Chromosome-wide p value <0.01, significant at the 1% chromosome –wide level

Additive and dominance genetic effects on a particular trait were important components of statistical analysis. The power of detecting the significance of a QTL was a function of many factors such as pedigree structure, marker informativeness, genetic effect etc. An examination of the additive (i.e., half the difference between homozygotes) and dominance effects (i.e., the difference between heterozygotes and the mean of the homozygotes) revealed that additive effect was always in the direction opposite to the dominance effects except on CFA03 and CFA05 that both effects were in the same direction. QTL for each trait on the same chromosome had similar additive or dominance genetic contribution in both magnitude and direction of effect (Table 7). This may be suggested that they should be the same QTL affecting multiple traits. There were not differed between the QTL for the left and right hip. To compare the QTL effects between single and combined traits such as NA and DI or NA and DLS in principle component 1 or 2, combined traits tended to have the high estimation of QTL effects as compared to the single trait.

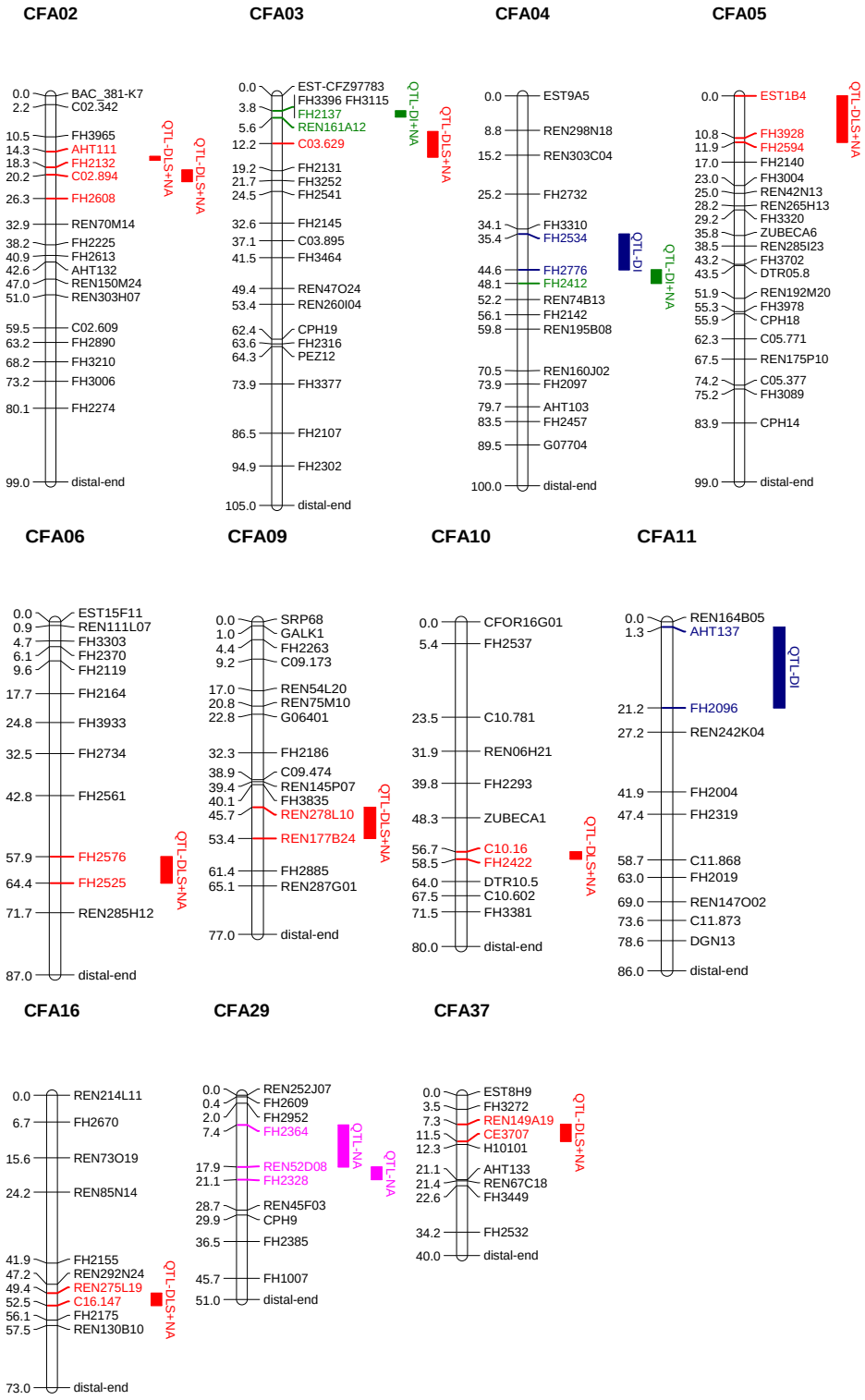

Figure 19 This picture showed QTL position on CFA02, 03, 04, 05, 06, 09, 10, 11, 16, 29 and 37. Color blocks represent flanking marker interval and trait for the QTL.

The result of genome-wide screen with a combined set of MSS-1&2 of 428 microsatellite markers revealed putative QTLs for CHD ($p < 0.05$ chromosome-wide) on 11 chromosomes with significant LOD score over 2.0 (CFA02, 03, 04, 05, 06, 09, 10, 11, 16, 29 and 37). The highest LOD score was on CFA29, 10, 03 and 11 respectively. Figure 19 showed 11 QTLs on chromosome 2, 3, 4, 5, 6, 9, 10, 11, 16, 29 and 37. The color blocks represented flanking marker interval for that QTL. On CFA02, 03, 04 and 29, there were 2 color blocks on each chromosome. These blocks represented the position of flanking marker interval on the chromosome that may be one or two QTLs in this area. From the analysis results having in this study, we still cannot make the absolute conclusion of these QTLs.

2. Genome-wide screen with microsatellite markers in purebred Labrador Retriver

2.1 Labrador Retriever pedigree

The total number of 192 Labrador Retrievers in 7 generations, 33 full-sib families, 17 loops, 25 families in which there were 1-8 offspring was used for the analysis (Figure 20).

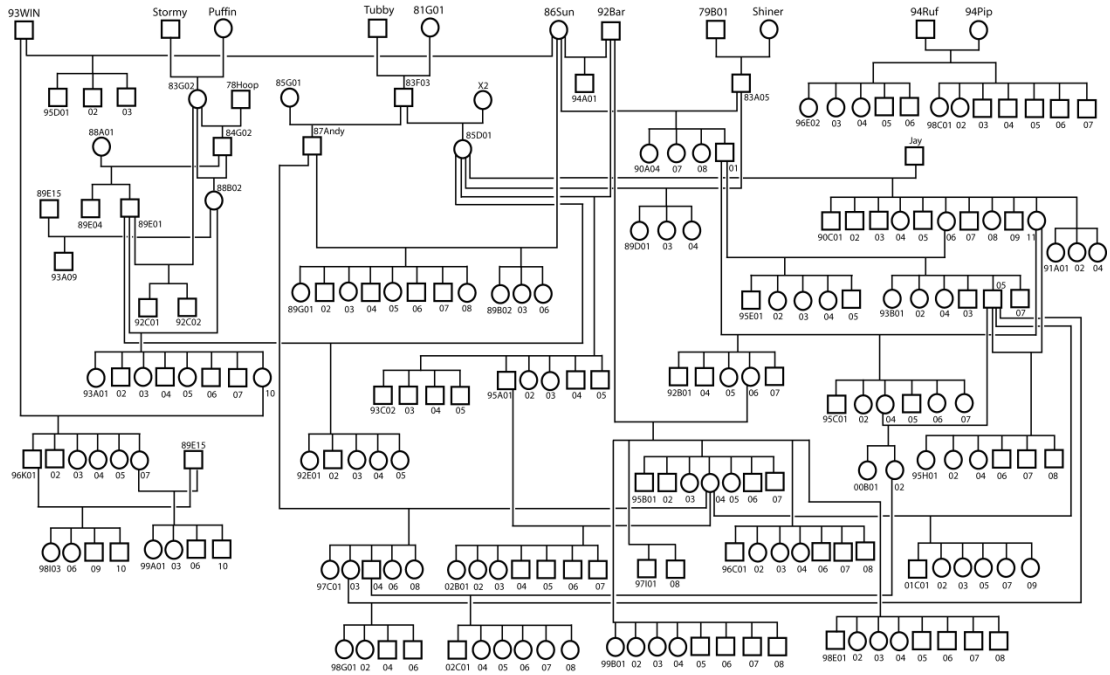

Figure 20 Structure of purebred Labrador Retriever pedigree used for genetic mapping study of canine hip dysplasia. Circles and boxes represent females and males respectively.

2.2 Phenotypic distribution in purebred Labrador Retriever pedigree

The phenotypic data from parents of the offspring were analyzed separately to observe the phenotypic values (Table 8) and distribution (Figure 21). The mean phenotypic distribution of 192 Labrador Retriever dogs were 0.53, 0.53 on DI, 49.92, 52.09 on DLS, 105.3, 106.64 on NA and 2.68, 2.66 on OFA score for the left and right hip respectively (Table 9). The mean values of these traits in parents and group of 192 dogs were in the range of borderline to identify for CHD. On DI, DLS and NA the distributions closed to normal distribution. Although, OFA score revealed the skewed to the left (normal hip) (Figure 22).

Table 8 Summary statistics included mean, median, mode, standard deviation, minimal value, maximal value, variance, range, the 25th quartile and the 75th quartile of hip trait measured in Labrador Retriever parents.

	DI		DLS		NA		OFA	
	L	**R**	**L**	**R**	**L**	**R**	**L**	**R**
N	22	22	9	9	21	21	6	6
mean	0.47	0.51	53.19	55.50	103.81	105.21	2.67	2. 67
median	0.45	0.53	47.00	53.00	104.67	106.60	2.75	2.75
mode	0.19	0.56	30.30	34.40	104.00	106.50	3.00	3.00
SD	0.21	0.21	17.09	15.57	6.99	8.72	1.03	1.03
min	0.18	0.00	30.30	34.40	85.50	86.00	1.00	1.00
max	0.92	0.87	76.00	78.20	116.00	123.00	4.00	4.00
variance	0.04	0.05	292.15	242.42	48.89	76.01	1.07	1.07
range	0.74	0.87	45.70	43.80	30.50	37.00	3.00	3.00
P25	0.31	0.42	40.90	41.93	103.00	100.44	2.000 0	2.00 00
P75	0.61	0.633	69.30	71.10	107.81	110.63	3.50	3.50

Note: DI = distraction index, DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score, L=left hip, R=right hip,
N = Number of observations on which calculations were based,
mean =arithmetic mean,
median =middle value (50th percentile),
mode = most frequent value (if not unique, the smallest mode),
SD = standard deviation,
min = smallest (minimum) value,
max = largest (maximum) value,
range = difference between the maximum and minimum values,
P25 = lower quartile (25th percentile),
P75 = upper quartile (75th percentile).

Table 9 Summary statistics included mean, median, mode, standard diviation, minimal value, maximal value, variance, range, the 25th quartile and the 75th quartile of hip trait measured in Labrador Retrievers.

	DI		DLS		NA		OFA	
	L	**R**	**L**	**R**	**L**	**R**	**L**	**R**
N	162	162	80	80	164	164	79	79
mean	0.53	0.53	49.92	52.09	105.30	106.64	2.68	2.66
median	0.52	0.52	48.05	50.95	107.00	108.00	2.00	2.00
mode	0.50	0.50	44.00	50.00	107.00	110.00	2.00	2.00
SD	0.17	0.17	13.02	11.82	8.12	7.82	1.50	1.48
min	0.18	0.18	22.20	30.90	70.00	68.00	1.00	1.00
max	1.00	1.00	76.00	78.20	119.50	123.00	7.00	6.00
variance	0.03	0.03	169.38	139.79	65.87	61.07	2.25	2.18
range	0.82	0.82	53.80	47.30	49.50	55.00	6.00	5.00
P25	0.41	0.41	40.08	42.985	103.00	104.00	2.00	2.00
P75	0.62	0.62	59.90	62.50	110.00	112.00	3.00	3.00

Note: DI = distraction index, DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score, L=left hip, R=right hip,
N = Number of observations on which calculations were based,
mean =arithmetic mean,
median =middle value (50th percentile),
mode = most frequent value (if not unique, the smallest mode),
SD = standard deviation,
min = smallest (minimum) value,
max = largest (maximum) value,
range = difference between the maximum and minimum values,
P25 = lower quartile (25th percentile),
P75 = upper quartile (75th percentile).

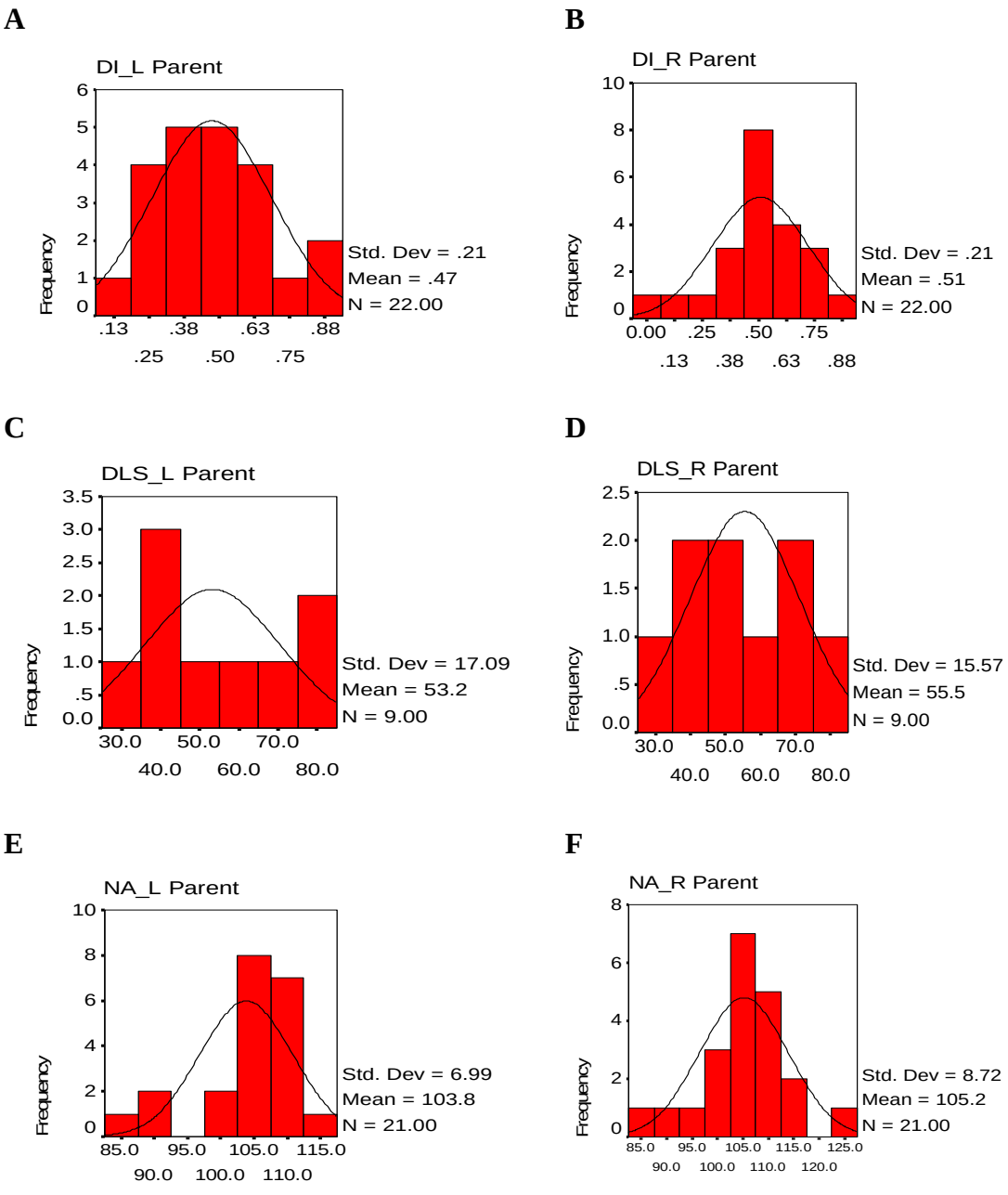

Figure 21 (Continued)

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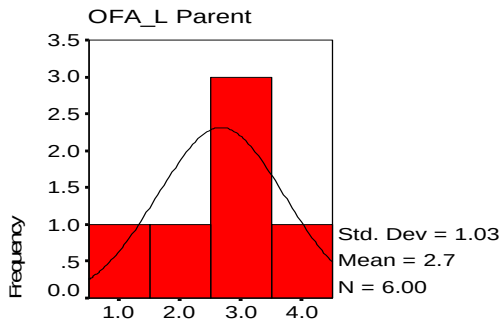

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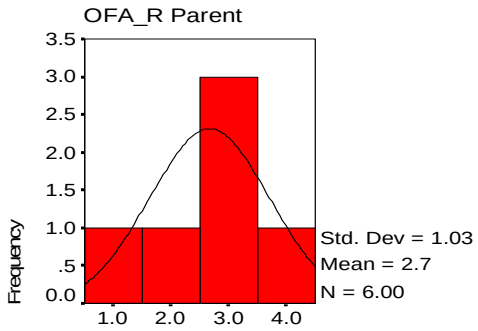

Figure 21 The phenotypic distribution for the left DI (A), right DI (B), left DLS (C), right DLS (D), left NA (E), right NA (F), left OFA (G) and right OFA (H) in purebred Labrador Retriever parents were shown.

Note: DI=distraction index; DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score, L=left hip, R=right hip.

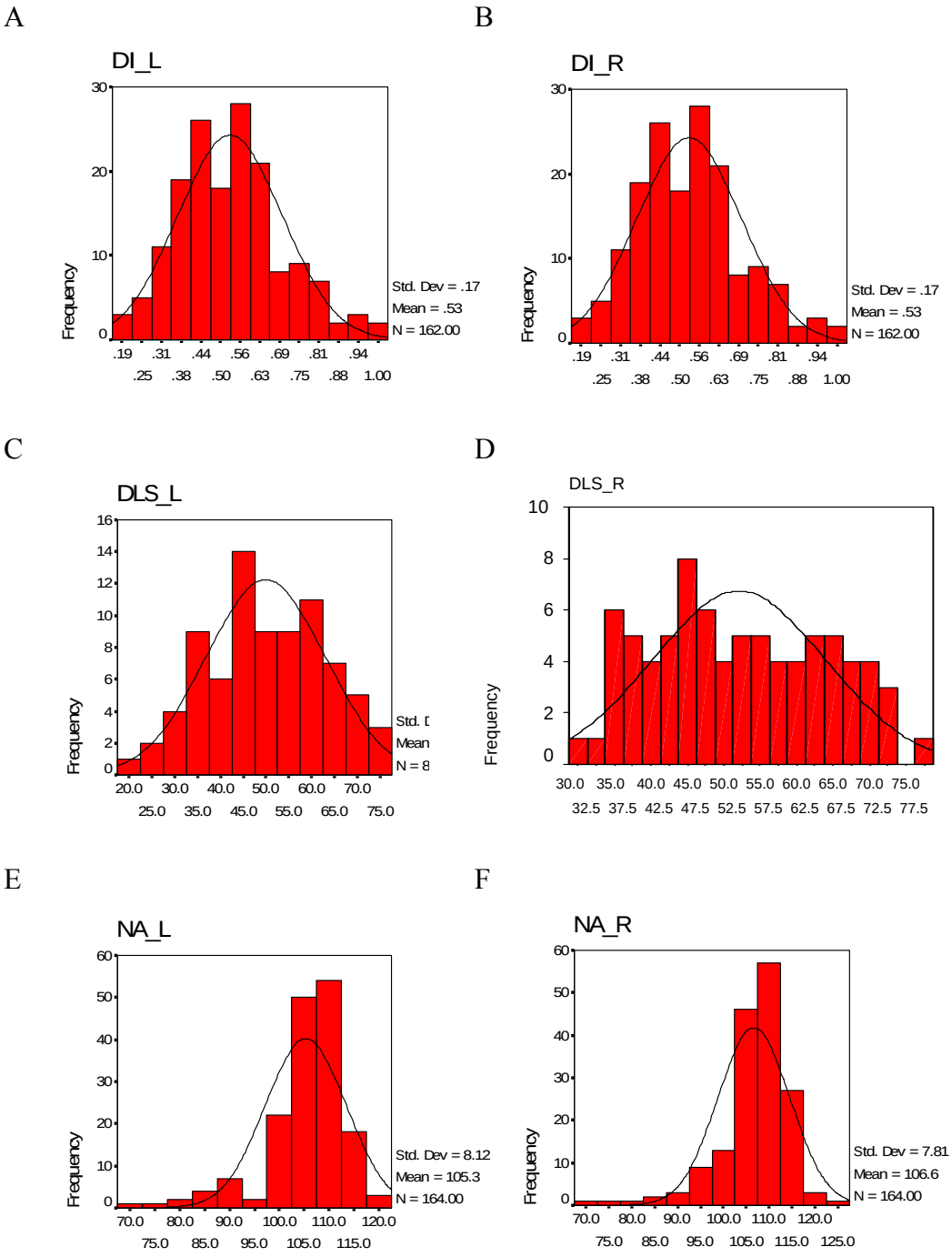

Figure 22 (Continued)

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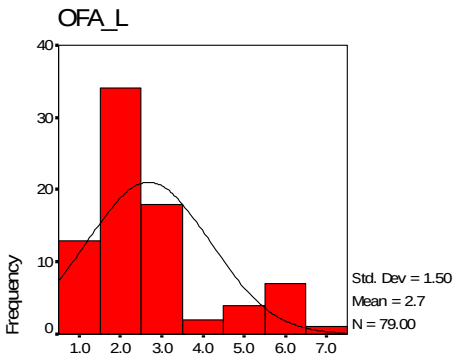

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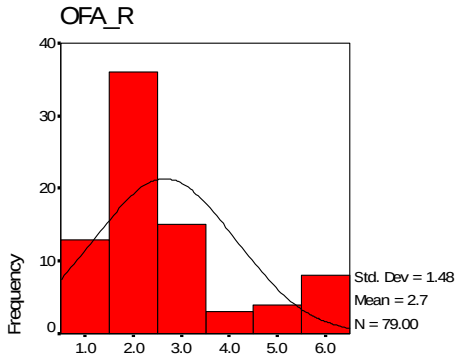


Figure 22 The phenotypic distribution for the left DI (A), right DI (B), left DLS (C), right DLS (D), left NA (E), right NA (F), left OFA (G) and right OFA (H) in purebred Labrador Retriever were shown.

Note: DI=distraction index; DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score, L=left hip, R=right hip.

2.3 Microsatellite markers informativeness in Labrador Retriever dogs

In Labrador Retrievers, analysis was undertaken on 284 microsatellite markers that spanned the entire genome (Figure 23) with an average inter-marker interval of 8.92 cM. There were 10 chromosomes that had an inter-marker interval more than 10 cM (CFA07, 10, 12, 13, 14, 15, 18, 26, 30 and 38). The widest interval was found on CFA 30 (15.38 cM) and the narrowest interval was on CFA 34 (4.56 cM). Marker coverage within each chromosome ranged from 44.50-100% and average was 88.31%. There were 3 chromosomes (CFA 23, 36 and X) having marker coverage less than 80 % (Table 10). The order and position of markers were showed in Figure 23.

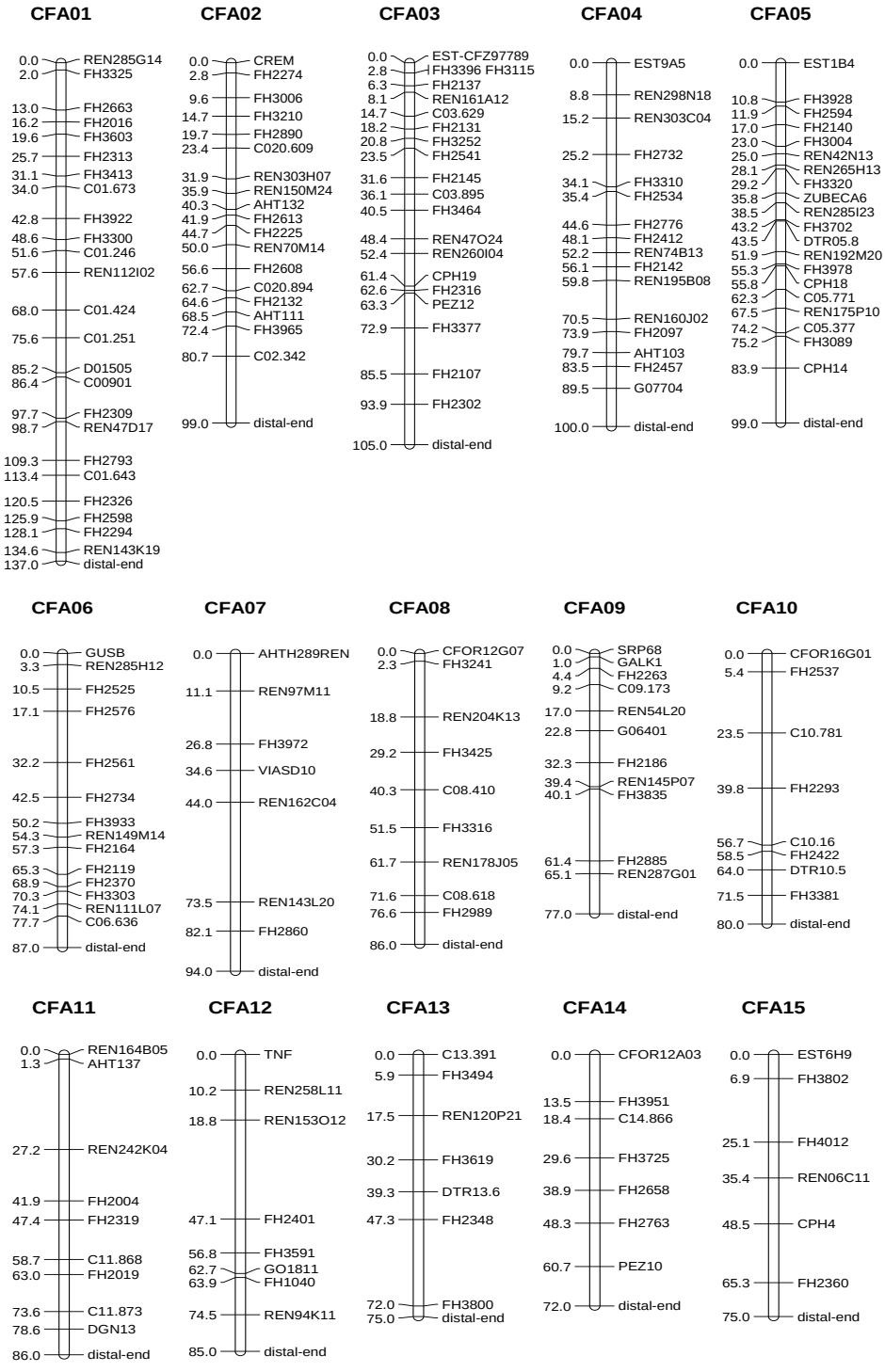
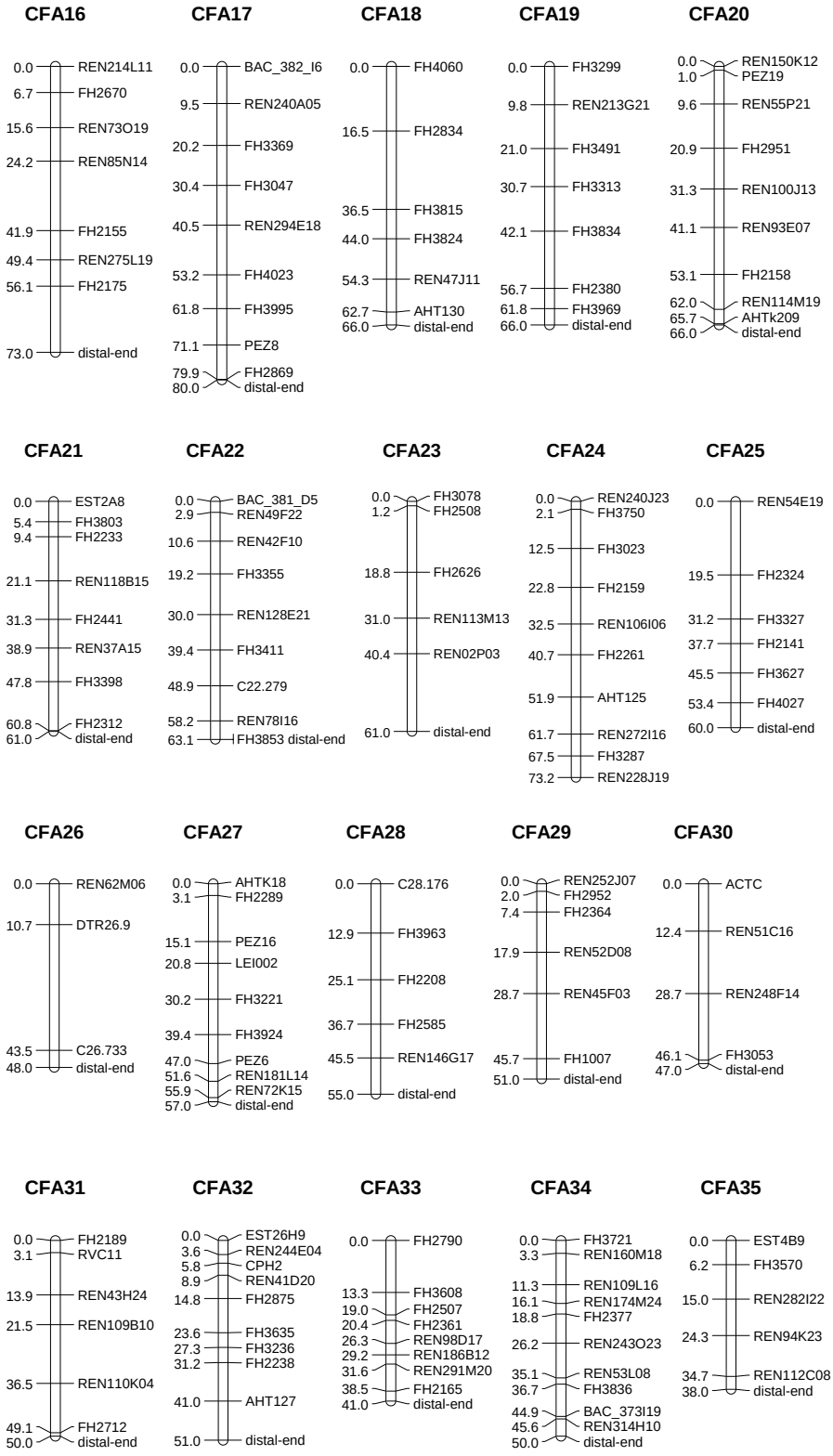


Figure 23 (Continued)



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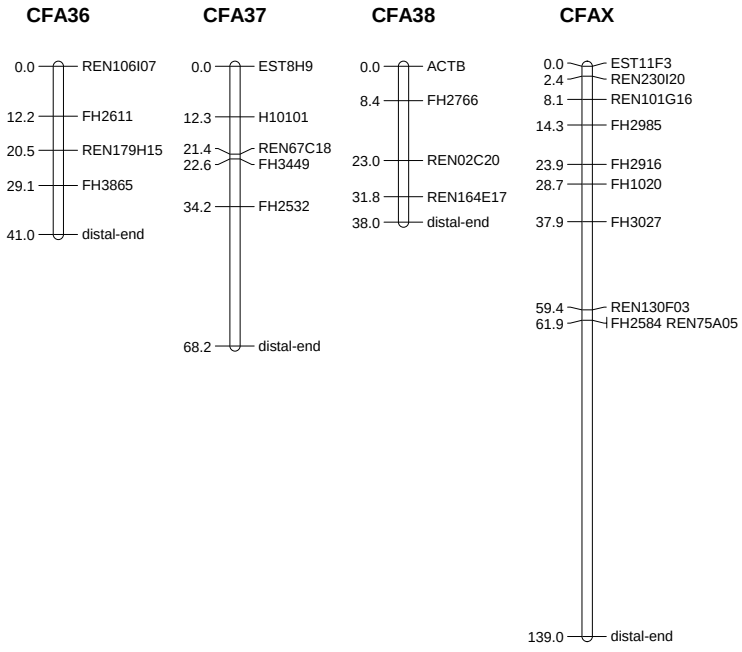


Figure 23 This picture showed microsatellite order and number using for genome-wide screen in purebred Labrador Retriever pedigree. The total number of markers was 284 microsatellite markers with average inter-marker interval was 8.92 cM. There were 10 chromosomes that had inter-marker interval more than 10 cM (CFA07, 10, 12, 13, 14, 15, 18, 26, 30 and 38).

Table 10 Average inter-marker interval and marker coverage of the 284 microsatellite markers set in Labrador Retrievers.

CFA	No. of markers	average intermarker interval (cM)	Marker coverage (%)
1	15	8.97	98.22
2	11	7.33	81.46
3	10	9.39	89.40
4	10	8.95	89.53
5	12	6.99	84.77
6	10	7.41	85.22
7	6	13.69	87.38
8	8	9.57	89.05
9	10	6.51	84.60
10	7	10.21	89.31
11	8	9.82	91.37
12	7	10.64	87.64
13	7	10.28	95.96
14	6	10.11	84.24
15	6	10.88	87.01
16	7	8.01	76.78
17	8	9.99	99.88
18	6	10.45	95.00
19	7	8.82	93.62
20	8	8.22	99.60
21	7	8.69	99.72
22	8	7.89	100.00
23	5	8.07	66.18
24	9	8.13	100.00
25	6	8.90	89.00
26	3	14.51	90.69
27	8	6.99	98.04
28	5	9.11	82.78
29	5	9.14	89.59
30	3	15.38	98.16
31	6	8.19	98.24
32	8	5.13	80.46
33	8	4.82	93.96
34	10	4.56	91.22
35	4	8.68	91.36
36	4	7.28	71.05
37	4	8.55	85.50
38	3	10.59	83.62
X	9	6.87	44.50
	284	**8.92**	**88.31**

Among these markers, there were 8 monomorphic markers represented in the 192 dogs genotyped (all 192 dogs were homozygous for one allele). Two hundred and seventy-six markers were polymorphic with the number of alleles ranging from 2 to 19 alleles (Figure 24).

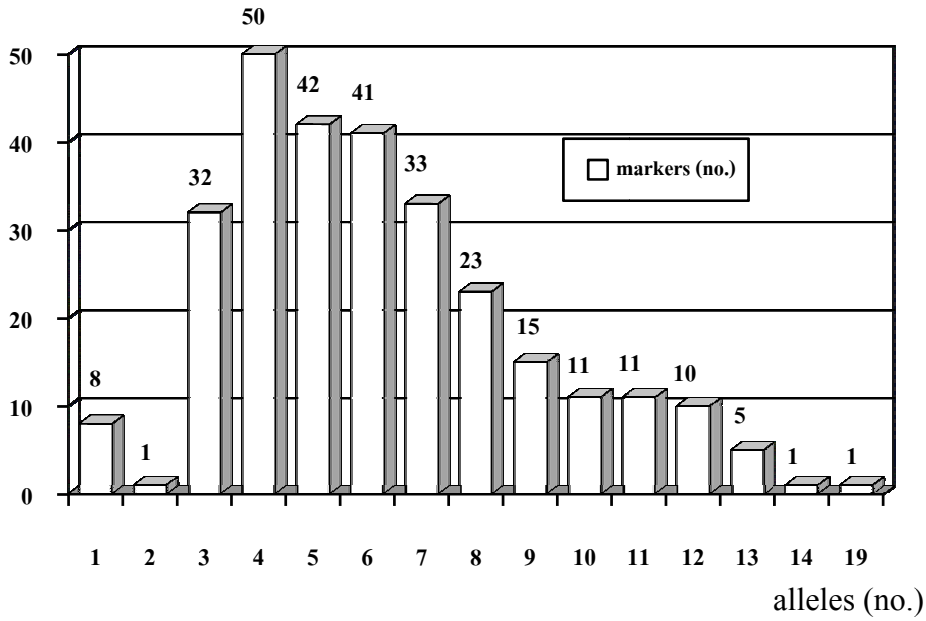


Figure 24 The number of alleles of 284 microsatellite markers screened on 192 purebred Labrador Retrievers.

Heterozygosity ranged from 0-0.93. The polymorphism information content (PIC) measures the probability of differentiating the allele transmitted by a given parent to its child given the marker genotype of father, mother, and child (Botstein *et al*. 1980). The PIC values ranged from 0-0.90. PIC and heterozygosity of these 284 microsatellite markers were informative markers. More than 80 % of the markers were moderately to highly informative and only 12 % were uninformative (Table 11).

Table 11 The polymorphic information content (PIC), heterozygosity of 284 microsatellite markers screened on 192 purebred Labrador Retriever dogs.

	Marker informativeness		
	Uninformative	**Moderately informative**	**Highly informative**
PIC	**< 0.3**	**0.3 – 0.59**	**> 0.59**
No. of markers	25 (8.8%)	105 (37%)	154 (54.2%)
Heterozygosity	**< 0.3**	**0.3 – 0.59**	**> 0.59**
No. of markers	33 (11.6%)	85 (29.9%)	166 (58.5%)

2.4 Quantitative Trait Locus Analysis

Quantitative trait loci were detected for each trait or for the principle components of each trait. A novel module of QTL Express™ designed to analyze genotype and phenotype data from purebred populations using a variance-component approach was used for the statistical analysis. QTLExpress analyzed all the data and calculated likelihood ration (LR) to reveal the peak and position of QTLs (Figure 25).

Six chromosomes harbored putative QTLs for one or more traits at LOD score > 2.0 (CFA02, 10, 22 and 32) and LOD score >3.0 (CFA01 and 20) (Table 12). There were 3 chromosomes (CFA02, 22 and 32) that mapped the putative QTLs for only one trait. However, QTL on chromosomes 10 and 20 were mapped for several traits and the QTL for several traits mapped to the same chromosomal location except one trait on CFA20 mapped to the different position. Mean estimates of QTL location ranged from 3 to 10 cM (based on the flanking marker interval) (Table 12). The narrowest interval was found on CFA32 (3.07 cM) and the widest was on CFA20 (10.43 cM). The direction and position of these QTLs strengthening the assumption that the QTL were not detected by chance. The most significant QTL (LOD = 3.32) was found for the second principle component of the right hip DI and NA on CFA20. Putative QTL on CFA 10 was mapped to the same chromosomal region in the Labrador Retriever pedigree as in the greyhound/Labrador Retriever cross breed pedigree.

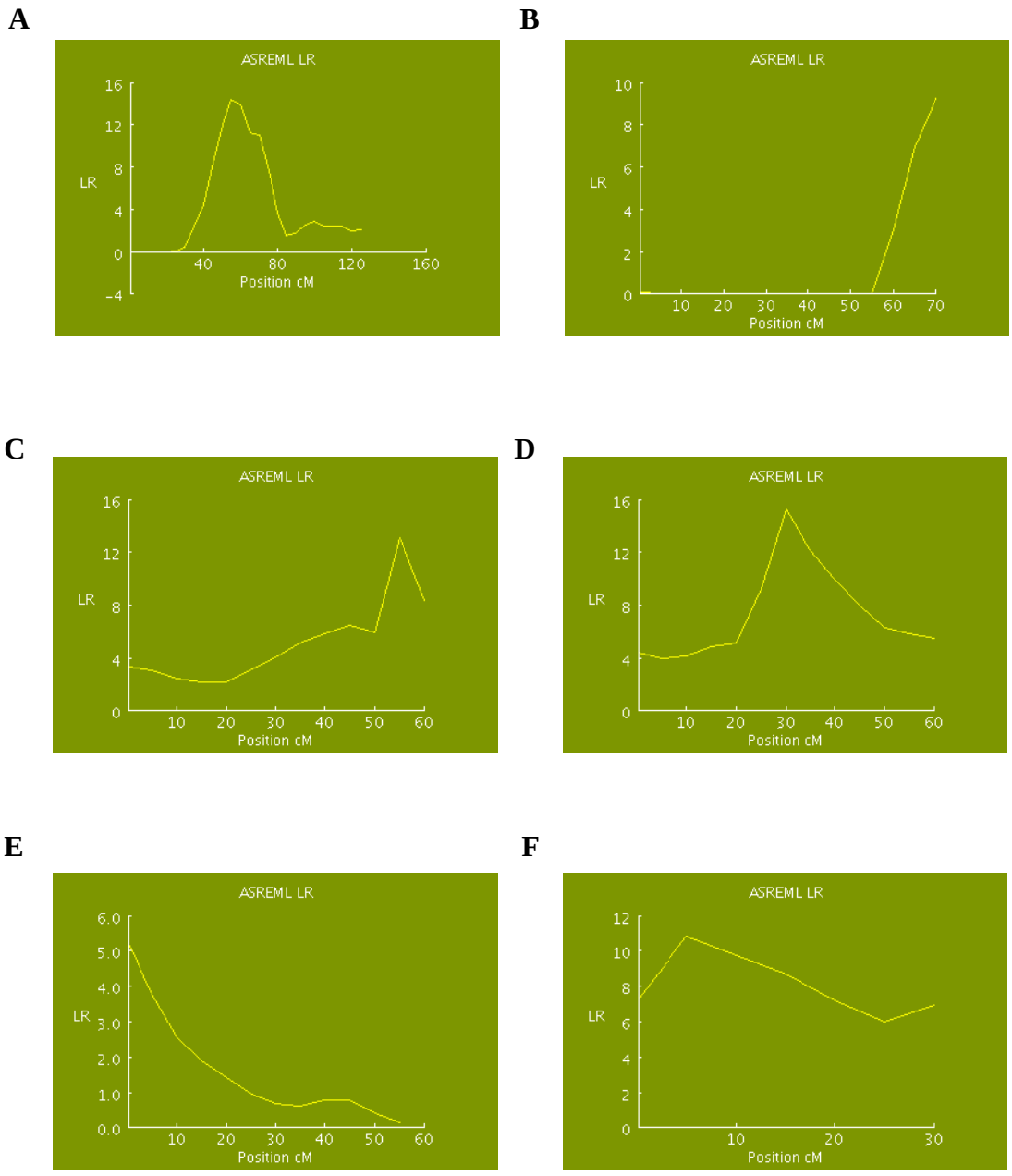


Figure 25 Likelihood ratio plots for detection of the QTL on CFA01 (A), CFA02 (B), CFA10 (C), CFA20 (D), CFA22 (E) and CFA32 (F) with the QTL heritability 0.31, 0.51, 0.27, 0.47, 0.34 and 0.36 respectively. The peaks represent the most likely position of the QTL in cM on the X axis across the chromosome. The likelihood ratio test statistic revealed putative QTL at LOD scores > 2 on CFA02, 10, 22, 32 and QTL at LOD score > 3 on CFA01 and 20.

Table 12 Quantitative trait loci for the hip trait, the chromosome, the flanking marker interval, the estimated QTL position, and the LOD score mapped in a Labrador Retriever pedigree following a microsatellite- based genome-wide screen. A variance component method was used for QTL mapping.

Trait	CFA	Flanking markers (cM)	Position	LOD
PC2LeftDN	1	FH3300-REN112I02 (9.03)	55	3.13
PC2RightDN	1	C01.424-C01.251 (7.66)	70	2.32
PC1Right	2	FH3965-C02.342 (8.24)	70	2.01
NA, R	10	FH2422-DTR10.5 (5.46)	55	2.66
NA, Hi	10	FH2422-DTR10.5 (5.46)	55	2.85
PC2RightDN	10	FH2422-DTR10.5 (5.46)	55	2.33
PC2RightDN	20	FH2951-REN100J13 (10.43)	30	3.32
DLS, L	20	FH2158-REN114M19 (8.83)	60	2.01
DLS, Lo	20	FH2158-REN114M19 (8.83)	60	2.27
PC1Right	20	FH2158-REN114M19 (8.832)	60	2.44
DLS, Lo	22	REN49F22- REN42F10 (7.74)	0	2.10
PC2RightDN	32	CPH2- REN41D20 (3.07)	5	2.35

Note: L = left, R = right, Lo = low, Hi = high, DLS = dorsolateral subluxation, NA = Norberg angle, PC1 = first principle component, PC2 = second principle component, CFA = chromosome for *Canis familiaris*, LOD = logarithm of the odds ratio, PC1 Right = first principal component of the right DLS, the right DI and the right NA, PC2 LeftDN = second principal component of left DI and left NA, PC2 RightDN = second principal component of right DI and right NA.

Quantitative genetic variation results from the combined effects of genetic and environmental factors. In this study, QTL heritability ranged from $2.51x10^{-8}$ to 0.54 (data not shown). The highest QTL heritability was found on principle component 1 on CFA02 and 20 (Table 13). For QTL with a small effect (QTL heritability = 0.05) (Martinez *et al*., 1997) there was a lower power to estimate QTL location than one with a large effect, as we found in this experiment. No QTL effect with a QTL heritability less than 0.25 was mapped or given a LOD score over 2. Most of the traits had low polygenic heritability. As the same result with QTL heritability, no polygenic heritability less than $7.63\ E^{-7}$ were given a LOD score over 2.

Table 13 Quantitative trait loci for the hip trait, the chromosome and its heritability mapped in a Labrador retriever pedigree following a microsatellite- based genome-wide screen. A variance component method was used for QTL mapping.

Trait	CFA	polygenic heritability	QTL heritability	SE
PC2LeftDN	1	0.08	0.31	0.09
PC2RightDN	1	0.08	0.28	0.13
PC1Right	2	3.73 E^{-7}	0.51	0.10
NA, R	10	4.31 E^{-7}	0.26	0.10
NA, Hi	10	0.12	0.27	0.11
PC2RightDN	10	0.05	0.26	0.12
DLS, L	20	3.11 E^{-7}	0.38	0.14
DLS, Lo	20	6.76 E^{-7}	0.39	0.14
PC2RightDN	20	3.73 E^{-7}	0.47	0.06
PC1Right	20	3.44 E^{-7}	0.54	0.06
DLS, Lo	22	7.63 E^{-7}	0.34	0.14
PC2RightDN	32	1.17 E^{-6}	0.36	0.11

Note: L = left, R = right, Lo = low, Hi = high, DLS = dorsolateral subluxation, NA = Norberg angle, CFA = chromosome for *Canis familiaris*, LOD = logarithm of the odds ratio, PC1 = first principle component, and PC2 = second principle component, PC1 Right = first principal component of the right DLS, the right DI and the right NA, PC2 LeftDN = second principal component of left DI and left NA, PC2 RightDN = second principal component of right DI and right NA.

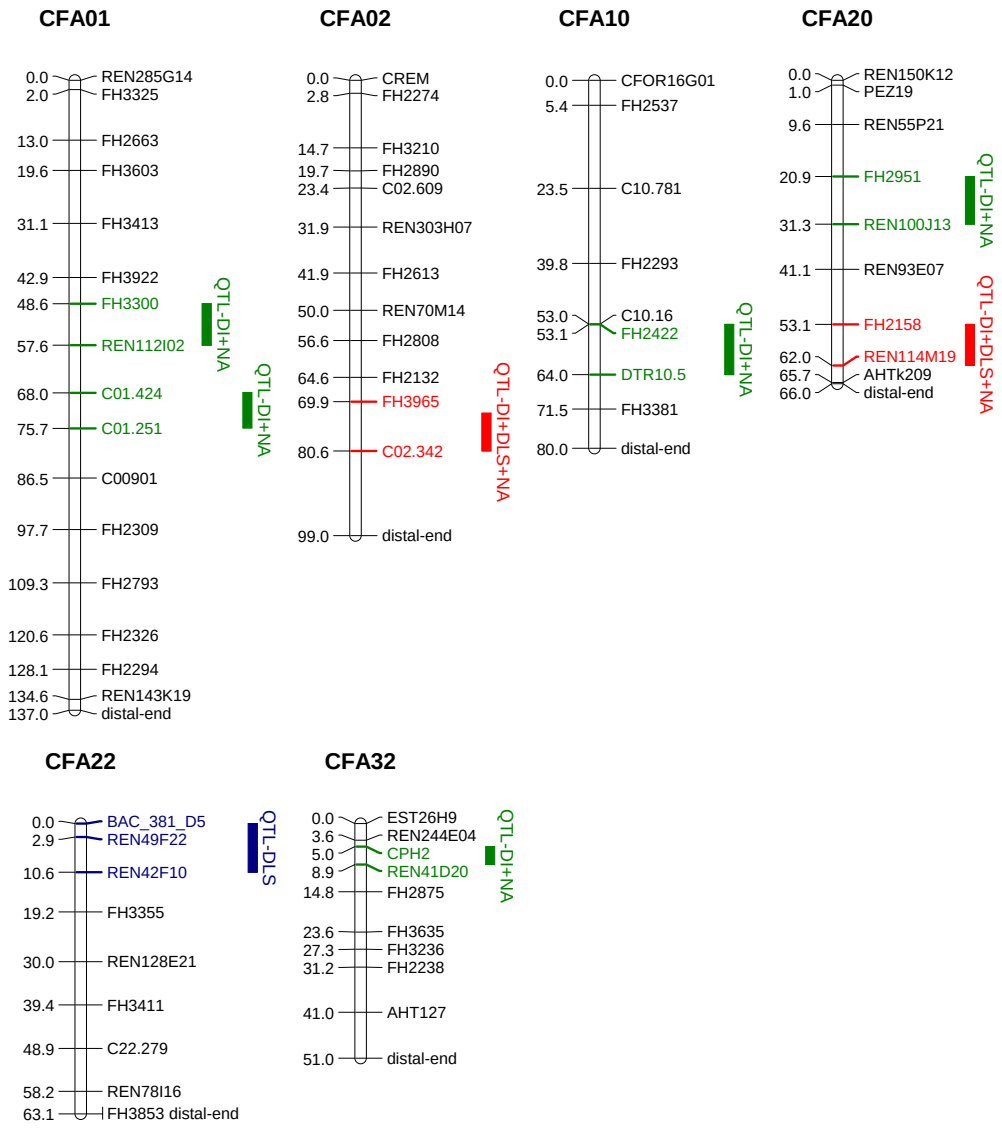


Figure 26 Each vertical bar illustrated the QTL position on CFA01, 02, 10, 20, 22 and 32. Color blocks represented flanking marker interval and trait for the QTL.

The locations of putative QTLs on 6 chromosomes were appeared on Figure 26. On CFA01 and 20, there were 2 small color blocks indicated the different location of QTL. From this study we cannot proved that they were the same or different QTL.

3. Fine mapping with SNP markers

The QTL mapping results from genome-wide screen with 428 microsatellite markers on 159 crossbred dogs revealed 11 chromosomes harbored putative QTL (LOD > 2.0) contributing to CHD. Among these, CFA11 and CFA29 showed significant evidence of linkage (chromosome-wide significant at P< 0.01 and 0.05 respectively). These 2 chromosomes were selected as a model to narrow down the regions containing the putative QTL. The selection of these chromosomes depended on the results from crossbred dogs only because we decided to do fine mapping before we got the results from genome-wide screen with microsatellite markers in purebred Labrador Retrievers. Fine mapping with SNPs markers was undertaken by using multipoint linkage analysis.

3.1 Dog samples

In order to increase the power for QTL mapping analysis , 449 dogs composed of 191 Labrador Retrievers from Baker Institute, crossbred founders (8 Labrador Retrievers and 7 Greyhounds), F_1 dogs (n = 7), F_2 dogs (n = 16), F_1 dogs backcross to founder Labrador Retrievers (n = 80), F_1 dogs backcross to Greyhounds founder (n = 33), F_2 dogs (n = 16), German Shepherds (n = 24), Golden Retrievers (n = 22) and unrelated Labrador Retrievers (n = 54) were genotyped with SNP markers on CFA 11 and CFA 29. These dogs had both DNA samples and phenotypic traits. We used many dog breeds to do this fine mapping because we wanted them to be the representative of dog affecting CHD. Labrador Retrievers, Golden Retrievers and German Shepherds were the breed that had high incident of CHD. In the continuing study, we plan to select more dog breeds to use for the analysis.

3.2 Canine hip dysplasia phenotypic distribution

Phenotypic measurement from 4 radiographic methods (DI, DLS, NA and OFA score) were undertaken on 449 dogs of 167 crossbred dogs, 236 Labrador Retrievers, 22 Golden Retrievers and 24 German Shepherds. The evaluation was

obtained from each trait or principal components on the left and the right hips separately.

The mean of parents on the left and on the right for DI was 0.42 and 0.45, for DLS was 53.49 and 52.22, for NA was 104.84 and 107.12 and for OFA was 3.00 and 2.93 respectively (Table 14). The mean of 449 dogs on the left and right for DI was 0.47 and 0.50, for DLS was 54.14 and 54.37, for NA was 105.72 and 106.45, and for OFA score was 2.72 and 2.73 respectively (Table 15). Phenotypic distribution on each trait of parents and 449 dogs were illustrated on the left and the right hip (Figure 27, 28). DI showed the most normal phenotypic distribution. The others (DLS, NA and OFA score) methods were skewed the distribution to the right (DLS and NA) and left (OFA score) which was the distribution of normal hip.

Table 14 Summary statistics included mean, median, mode, standard deviation, minimal value, maximal value, variance, range, the 25th quartile and the 75th quartile of hip trait measures in parent of 449 dogs using for fine mapping analysis.

	DI		DLS		NA		OFA	
	L	**R**	**L**	**R**	**L**	**NA**	**L**	**R**
N	41	40	16	16	45	45	46	46
mean	0.42	0.45	53.49	52.22	104.84	107.12	3.00	2.93
median	0.41	0.47	62.50	51.60	104.90	107.11	2.49	2.45
mode	0.21	0.33	42.80	3.50	107.50	106.50	2.00	2.00
SD	0.23	0.24	21.84	21.04	6.12	6.62	1.81	1.74
min	0.03	0.00	3.00	3.50	85.50	86.00	1.00	1.00
max	0.92	1.00	76.00	78.20	118.00	123.00	7.00	7.00
variance	0.051	0.06	476.96	442.85	37.41	43.79	3.29	3.04
range	0.89	1.00	73.00	74.70	32.50	37.00	6.00	6.00
P25	0.23	0.27	37.73	36.85	102.88	105.25	1.65	1.63
P75	0.55	0.61	70.70	70.95	108.25	110.42	3.80	3.78

Note: DI=distraction index, DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score, L=left hip, R=right hip.

N = Number of observations on which calculations were based

mean =arithmetic mean

median =middle value (50th percentile)

mode = most frequent value (if not unique, the smallest mode)

SD = standard deviation

min = smallest (minimum) value

max = largest (maximum) value

range = difference between the maximum and minimum values

P25 = lower quartile (25th percentile)

P75 = upper quartile (75th percentile)

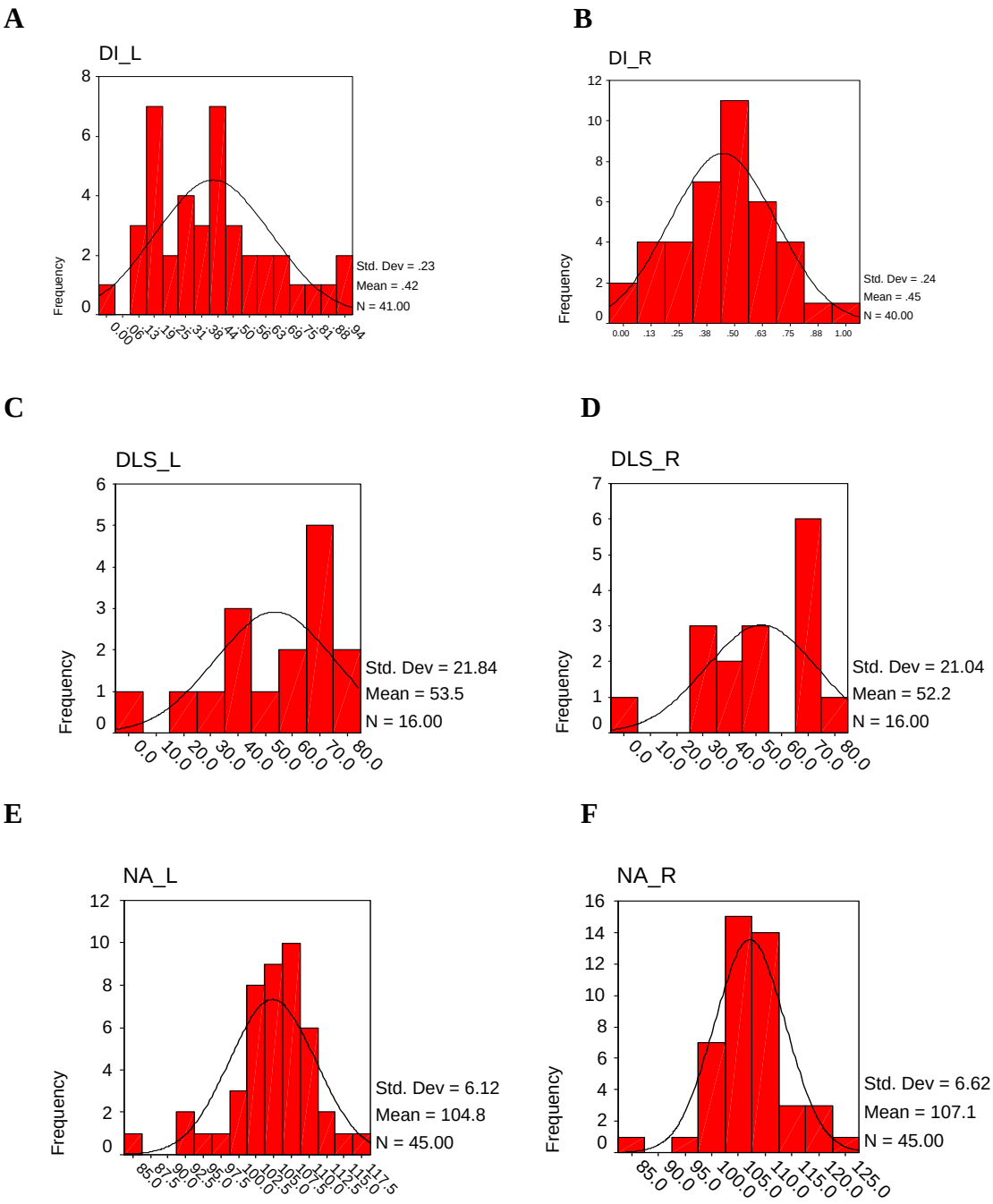
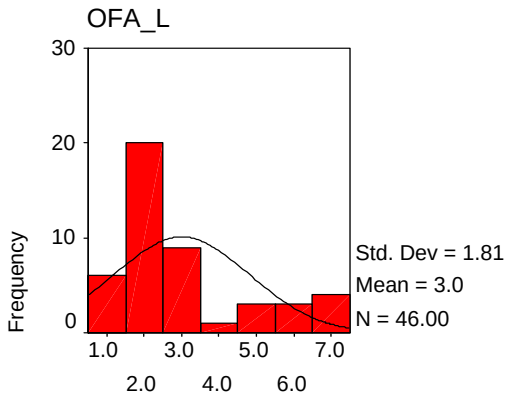


Figure 27 (Continued)

G



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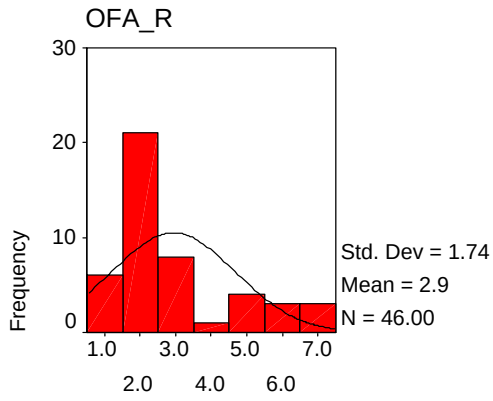

Figure 27 The phenotypic distribution of the parent of 449 dogs using for fine mapping analysis for the left DI (A), right DI (B), left DLS (C), right DLS (D), left NA (E), right NA (F), left OFA (G), right OFA (H) were shown.

Note: DI=distraction index, DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score, L=left hip, R=right hip.

Table 15 Summary statistics included mean, median, mode, standard deviation, minimal value, maximal value, variance, range, the 25th quartile and the 75th quartile of hip trait measures in 449 dogs.

	DI		DLS		NA		OFA	
	L	**R**	**L**	**R**	**L**	**NA**	**L**	**R**
N	329	330	229	229	423	423	359	359
mean	0.47	0.50	54.14	54.37	105.72	106.45	2.72	2.73
median	0.46	0.50	57.10	56.20	107.50	108.00	2.00	2.00
mode	0.50	0.50	66.00	64.00	110	110	2.00	2.00
SD	0.19	0.20	14.55	13.53	8.60	8.28	1.68	1.69
min	0.03	0.04	3.00	3.50	50.00	56.00	1.00	1.00
max	1.00	1.05	85.30	80.00	119.50	123.00	7.00	7.00
variance	0.05	0.04	211.56	183.14	74.03	68.50	2.84	2.86
range	1.00	1.05	82.30	76.50	69.50	67.00	6.00	6.00
P25	0.34	0.37	42.80	44.00	103.50	104.00	2.00	2.00
P75	0.60	0.62	66.00	66.60	111.00	111.50	3.00	3.00

Note: DI=distraction index, DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score, L=left hip, R=right hip.

N = Number of observations on which calculations were based

mean =arithmetic mean

median =middle value (50th percentile)

mode = most frequent value (if not unique, the smallest mode)

SD = standard deviation

min = smallest (minimum) value

max = largest (maximum) value

range = difference between the maximum and minimum values

P25 = lower quartile (25th percentile)

P75 = upper quartile (75th percentile)

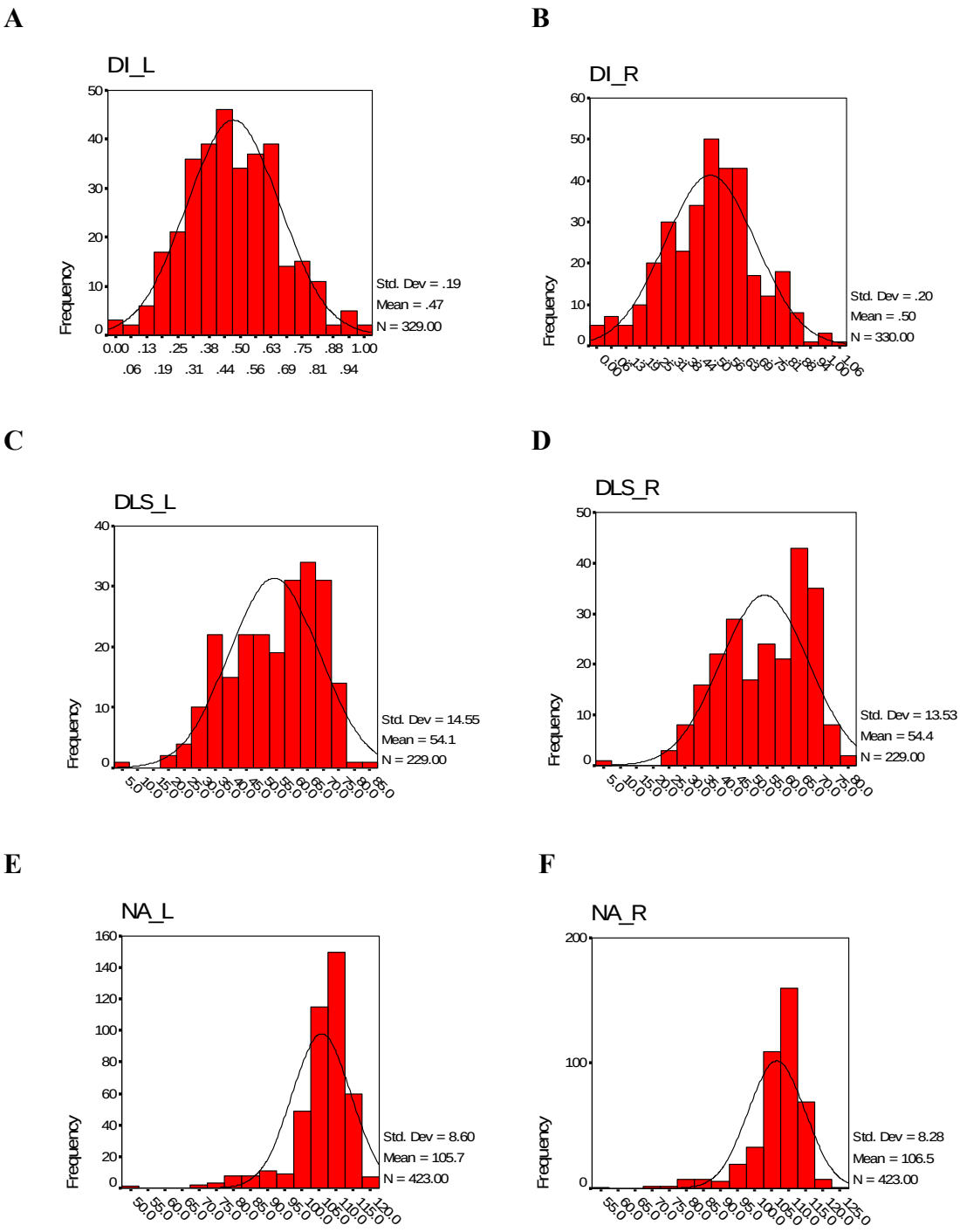

Figure 28 (Continued)

G **H**

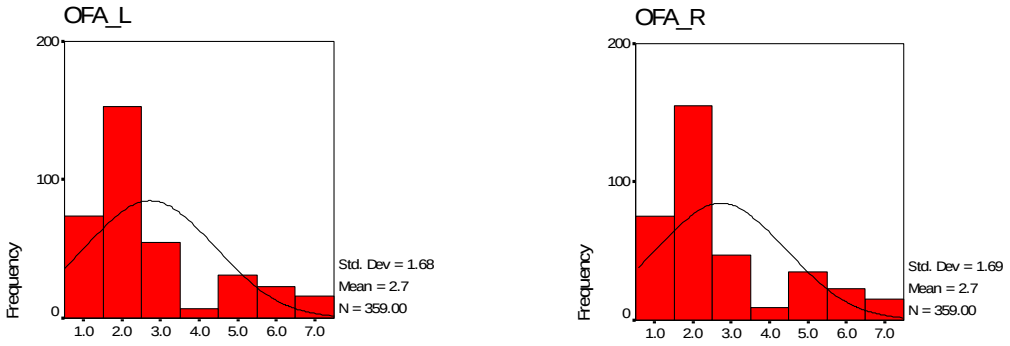

Figure 28 The phenotypic distribution for the left DI (A), right DI (B), left DLS (C), right DLS (D), left NA (E), right NA (F), left OFA (G), right OFA (H) were shown.

Note: DI=distraction index, DLS = dorsolateral subluxation score, NA=Norberg angle, OFA= OFA score, L=left hip, R=right hip.

3.3 SNP marker informativeness

Four hundred and forty-nine dog samples were used for fine mapping with 95 and 170 SNP markers on CFA11 and 29 respectively. Out of 95 SNP markers set on CFA11, there were 6 unamplified markers. Across all breed, more than 80% of the markers were polymorphic with 2 alleles. Among the purebred dogs, Labrador Retriever had the highest marker alleles. There was no number of alleles different between Greyhound, Golden Retriever and German Shepherd dogs (Table 16).

Table 16 The number of alleles of 89 SNP markers on CFA11 screened on 449 dogs.

Breed	no. of dogs	SNP with 2 alleles	SNP with 1 allele
All breeds	499	77 (86.52)	12 (13.48)
Lab	245	73 (82.02)	16 (17.98)
Grey	7	52 (58.43)	37 (41.57)
F_1	7	50 (56.18)	38 (42.70)
BCL	80	66 (74.16)	23 (25.84)
BCG	33	64 (71.91)	25 (28.09)
F_2	16	59 (66.29)	30 (33.71)
$(LGL)_2$	15	47 (52.81)	42 (47.19)
GSD	24	58 (65.17)	30 (33.71)
GD	22	54 (60.67)	32 (35.96)

Note: percentage represent in blanket

Lab = Labrador Retriever dog

Grey = Greyhound dog

F_1 = Labrador Retriever x Greyhound

BCL = backcross to Labrador Retriever founder

BCG = backcross to Greyhound founder

$F_2 = (F_1 x\ F_1)$

$(LGL)_2 = [(F_1 \text{ x } L) \text{ x } (F_1 \text{ x } L)]$

GSD = German Shepherd dog

GD = Golden Retriever dog

Table 17 The number of alleles of 170 SNP markers on CFA29 screened on 449 dogs.

Breed	no. of dogs	SNP with 2 alleles	SNP with 1 allele
All breeds	449	162 (95.29)	8 (4.71)
Lab	245	146 (85.88)	24 (14.12)
Grey	7	116 (68.24)	54 (31.76)
F_1	7	135 (79.41)	35 (20.59)
BCL	80	133 (78.24)	37 (21.76)
BCG	33	144 (84.71)	26 (15.29)
F_2	16	131 (77.06)	39 (22.94)
$(LGL)_2$	15	97 (57.06)	73 (42.94)
GSD	24	131 (77.06)	39 (22.94)
GD	22	135 (79.41)	35 (20.59)

Note: percentage represent in blanket. Lab = Labrador Retriever dog, Grey = Greyhound dog, F_1 = Labrador Retriever x Greyhound, BCL = backcross to Labrador Retriever founder, BCG = backcross to Greyhound founder, F_2 = (F_1x F_1), $(LGL)_2$ = [(F_1 x L) x (F_1 x L)], GSD = German Shepherd dog, GD = Golden Retriever dog

In these 170 SNP markers on CFA29, only 2 loci did not work (nothing was detected, maybe due to an error in the design process). Across all breeds, only 8 SNPs had only one allele represented in the 449 dogs genotyped (all 449 dogs were homozygous for one allele). By breed, Labrador Retriever had the higher rate of 2 SNP alleles (85.88%) (Table17). German Shepherd and Golden Retriever dogs had the similar percentage of 2 allele markers (77.06 % and 79.41% respectively). The order and location of SNP markers on CFA11 and 29 were represented in Figure 29. The result from genome-wide screen with microsatellite markers identified the putative QTL at 0-7 cM on CFA11 and 12-19 cM on CFA29. Therefore, these markers were selected to span across the whole chromosome with additional dense markers around the QTL area (Figure 29).

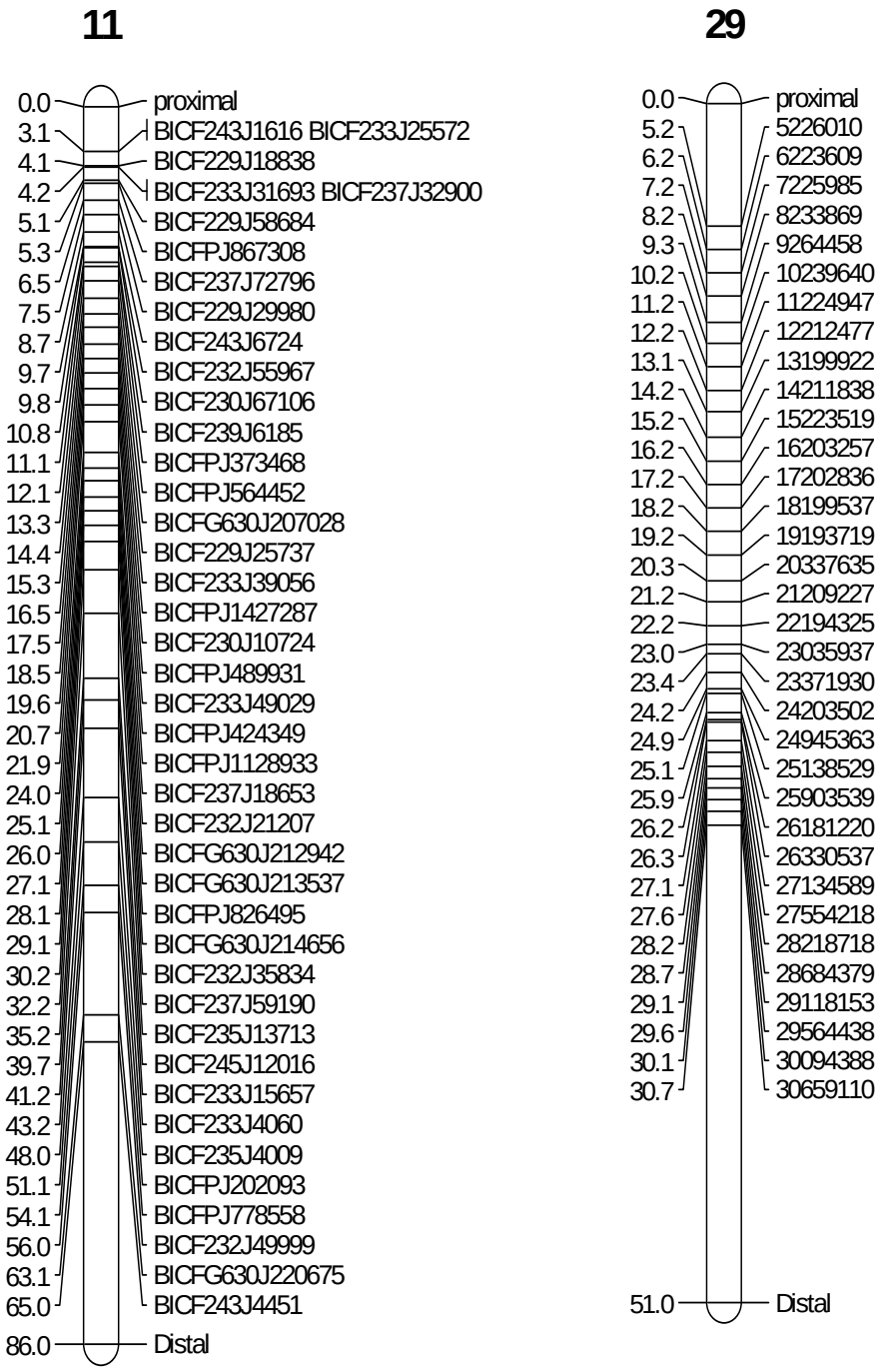

Figure 29 The order and position of SNP markers used for fine mapping on CFA 11 and 29.

Table 18 Polymorphic information content (PIC) of 89 SNP markers on CFA 11 represented across different dog breeds.

Breed	PIC		
	≤ 0.15	**0.16 - 0.3**	**0.31 - 0.375**
All breeds	30 (33.71)	24 (26.97)	35 (39.33)
Lab	39 (43.82)	25 (28.09)	25 (28.09)
Grey	44 (49.44)	21 (23.60)	24 (26.97)
F_1	39(43.82)	24 (26.97)	26 (29.21)
BCL	33 (37.08)	26 (29.21)	30 (33.71)
BCG	33 (37.08)	21 (23.60)	35 (39.33)
F_2	36 (40.45)	16 (17.98)	37 (41.57)
$(LGL)_2$	44 (49.44)	11 (12.36)	34 (38.20)
GSD	41 (46.07)	17 (19.10)	31 (34.83)
GD	45 (50.56)	18 (20.22)	26 (29.21)

Note: percentage represent in blanket.

no = number, Lab = Labrador Retriever dog

Grey = Greyhound dog

F_1 = Labrador Retriever x Greyhound

BCL = backcross to Labrador Retriever founder

BCG = backcross to Greyhound founder

$F_2 = (F_1 x F_1)$

$(LGL)_2 = [(F_1 x L) x (F_1 x L)]$

GSD = German Shepherd dog

GD = Golden Retriever dog

Table 19 Polymorphic information content (PIC) of 170 SNP markers on CFA 29 represented across different dog breeds.

Breed	PIC		
	≤0.15	**0.16 – 0.3**	**0.31 - 0.375**
All breeds	40 (23.53)	51 (30.00)	79 (46.47)
Lab	33 (19.41)	36 (21.18)	77 (45.29)
Grey	12 (7.06)	38 (22.35)	66 (38.82)
F_1	12 (7.06)	37 (21.76)	86 (50.59)
BCL	20 (11.76)	38 (22.35)	75 (44.12)
BCG	21 (12.35)	54 (31.76)	69 (40.59)
F_2	0 (0.00)	36 (21.18)	95 (55.88)
$(LGL)_2$	1 (0.59)	19 (11.18)	77(45.29)
GSD	28 (16.47)	28(16.47)	75 (44.12)
GD	25 (14.71)	43 (25.29)	67 (39.41)

Note: percentage represent in blanket. no = number, Lab = Labrador Retriever dog, Grey = Greyhound dog, F_1 = Labrador Retriever x Greyhound, BCL = backcross to Labrador Retriever founder, BCG = backcross to Greyhound founder, F_2 = (F_1x F_1), $(LGL)_2$ = [(F_1 x L) x (F_1 x L)], GSD = German Shepherd dog, GD = Golden Retriever dog

PIC values in the crossbred pedigrees on CFA11 and 29 revealed higher value in F_1 and F_2 than in the parental lines (Greyhounds and Labrador Retrievers founders) (Table 18, 19). The variation of PIC values was shown in Figure 30 and 31. The PIC values across different dog breeds on CFA11 and 29 were not different. Every breed had a high polymorphic information content (PIC > 0.15) especially on CFA29 (Figure 32 and 33). The maximum possible PIC value for a SNP was 0.375 for 2 alleles.

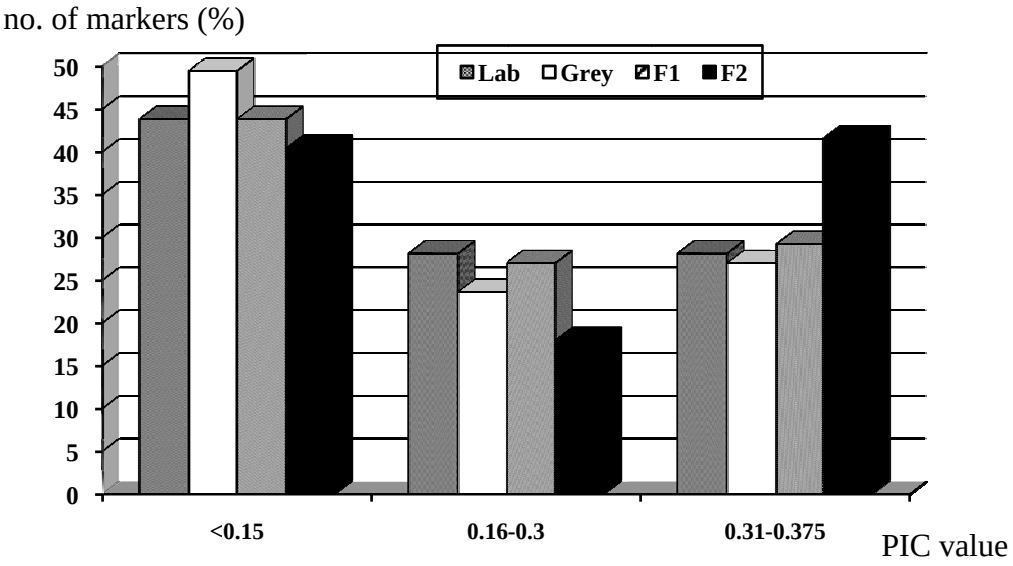

Figure 30 PIC values of 89 SNP markers on CFA11 illustrated in the crossbred pedigrees. (Lab = Labrador Retriever, Grey = Greyhound, F_1 = Lab x Grey, $F_2 = F_1 \times F_1$).

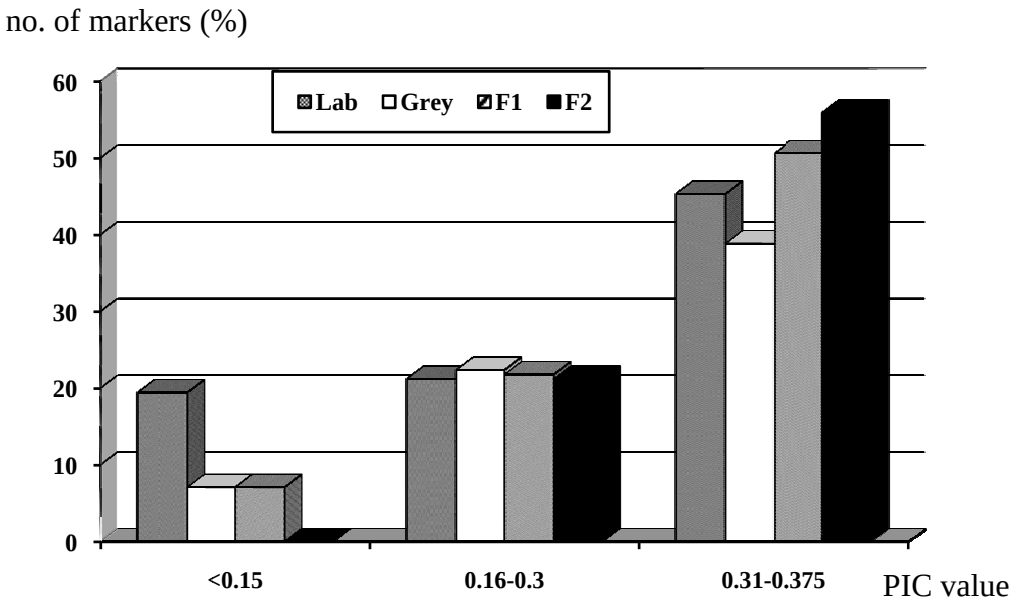

Figure 31 PIC values of 170 SNP markers on CFA29 illustrated in the crossbred pedigrees. (Lab = Labrador Retriever, Grey = Greyhound, F_1 = Lab x Grey, $F_2 = F_1 \times F_1$).

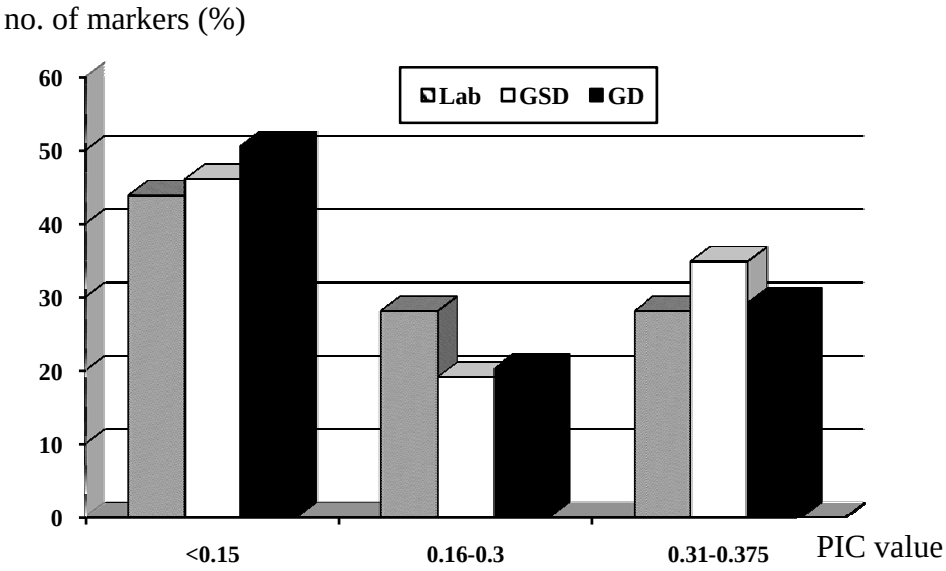

Figure 32 PIC values of 89 SNP markers on CFA11 illustrated across different breeds. (Labs = Labrador Retriever, GSD = German Shepherd dog, GD = Golden Retriever).

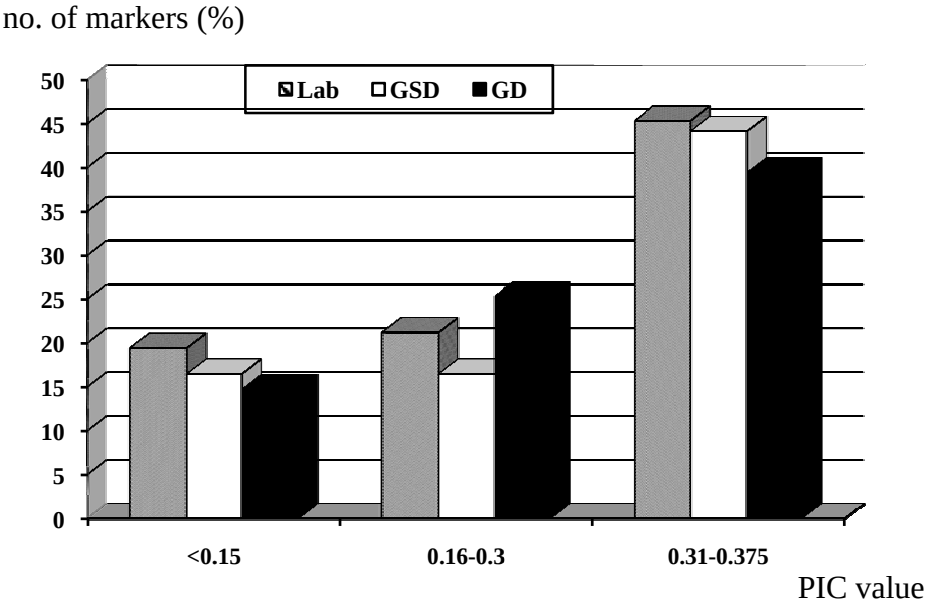

Figure 33 PIC values of 170 SNP markers on CFA29 illustrated across different breeds. (Labs = Labrador Retriever, GSD = German Shepherd dog, GD = Golden Retriever).

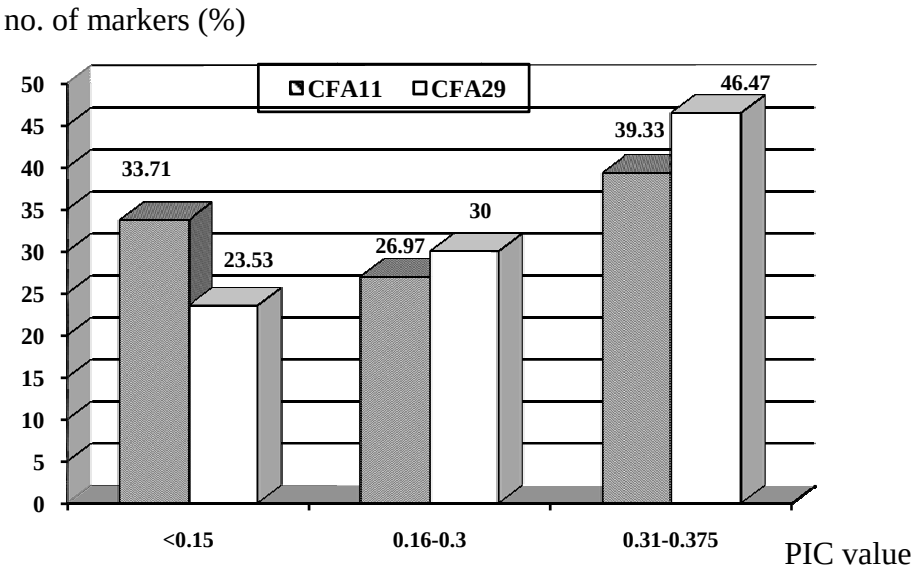

Figure 34 PIC values of SNP markers on CFA11 and 29 ac5ross every breeds.

When analyzing SNP genotypes from every breed together, most of the markers revealed high PIC value. CFA29 had higher polymorphic markers than on CFA11 (Figure 34).

The heterozygosity, sometimes called the observed heterozygosity, was simply the proportion of heterozygous individuals in the data set (maximum is 1 if all individuals were heterozygous for that particular marker). Heterozygosity across different dog breeds on CFA11 and 29 were shown in Table 20 and 21 respectively. On CFA11, percentage of markers having heterozygosity more than 0.25 were 41.57% in Labrador Retrievers, 29.22 % in Greyhounds, 33.71% in German Shepherds and 28.09 % in Golden Retrievers. On CFA29, percentage of markers having heterozygosity more than 0.25 were 61.18% in Labrador Retrievers, 55.89 % in Greyhounds, 55.29% in German Shepherds and 50 % in Golden Retrievers. Among crossbred dogs, 49.44% in F_1, 47.2 % in BCL, 52.81 % in BCG, 47.19 % in F_2 and 42.69 % in $(LGL)_2$ had markers having heterozygosity > 0.25. The result was the same on CFA29 but had the higher percentage of markers; 62.94 % in F_1, 62.35 % in BCL, 58.23 % in BCG, 64.11 % in F_2 and 42.95 % in $(LGL)_2$ (Figure 35 and 36). Across every dog breeds, heterozygosity ranged from 0-0.53 on CFA11 and 0-0.8 on CFA29 with 43.82 and 63.53 % over 0.25 respectively (Figure 37).

Table 20 Heterozygosity of 89 SNP markers on CFA 11 illustrated across different dog breeds.

	Heterozygosity			
	<0.25	**0.26-0.5**	**0.51-0.75**	**0.76-1.0**
All breeds	50 (56.18)	36 (40.45)	3 (3.37)	0 (0.00)
Lab	52 (58.43)	34 (38.20)	3 (3.37)	0 (0.00)
Grey	63 (70.79)	21 (23.60)	5 (5.62)	0 (0.00)
F_1	44 (49.44)	28 (31.46)	7 (7.87)	9 (10.11)
BCL	47 (52.81)	32 (35.96)	10 (11.24)	0 (0.00)
BCG	42 (47.19)	28 (31.46)	19 (21.35)	0 (0.00)
F_2	47 (52.81)	24 (26.97)	14 (15.73)	4 (4.49)
$(LGL)_2$	51 (57.30)	15 (16.85)	15 (16.85)	8 (8.99)
GSD	58 (65.17)	20 (22.47)	10 (11.24)	0 (0.00)
GD	61 (68.54)	21 (23.60)	4 (4.49)	0 (0.00)

Note: percentage represent in blanket.

no = number

Lab = Labrador Retriever dog,

Grey = Greyhound dog

F_1 = Labrador Retriever x Greyhound

BCL = backcross to Labrador Retriever founder

BCG = backcross to Greyhound founder

$F_2 = (F_1 x\ F_1)$

$(LGL)_2 = [(F_1 \text{ x } L) \text{ x } (F_1 \text{ x } L)]$

GSD = German Shepherd dog

GD = Golden Retriever dog

Table 21 Heterozygosity of 170 SNP markers on CFA 29 illustrated across different dog breeds.

	Heterozygosity			
	<0.25	**0.26-0.5**	**0.51-0.75**	**0.76-1.0**
All Breeds	62 (36.47)	98 (57.65)	10 (5.88)	0 (0.00)
Lab	48 (28.24)	75 (44.12)	25 (14.71)	4 (2.35)
Grey	20 (11.76)	59 (34.71)	30 (17.65)	6 (3.53)
F_1	25 (14.71)	45 (26.47)	53 (31.18)	9 (5.29)
BCL	27 (15.88)	67 (39.41)	36 (21.18)	3 (1.76)
BCG	44 (25.88)	72 (42.35)	26 (15.29)	1 (0.59)
F_2	15 (8.82)	55 (32.35)	50 (29.41)	4 (2.35)
$(LGL)_2$	24 (14.12)	41 (24.12)	31 (18.24)	1 (0.59)
GSD	36 (21.18)	61 (35.88)	33 (19.41)	0 (0.00)
GD	49 (28.82)	62 (36.47)	23 (13.53)	0 (0.00)

Note: percentage represent in blanket.

no = number

Lab = Labrador Retriever dog

Grey = Greyhound dog

F_1 = Labrador Retriever x Greyhound

BCL = backcross to Labrador Retriever founder

BCG = backcross to Greyhound founder

$F_2 = (F_1 x\ F_1)$

$(LGL)_2 = [(F_1 \text{ x } L) \text{ x } (F_1 \text{ x } L)]$

GSD = German Shepherd dog

GD = Golden Retriever dog

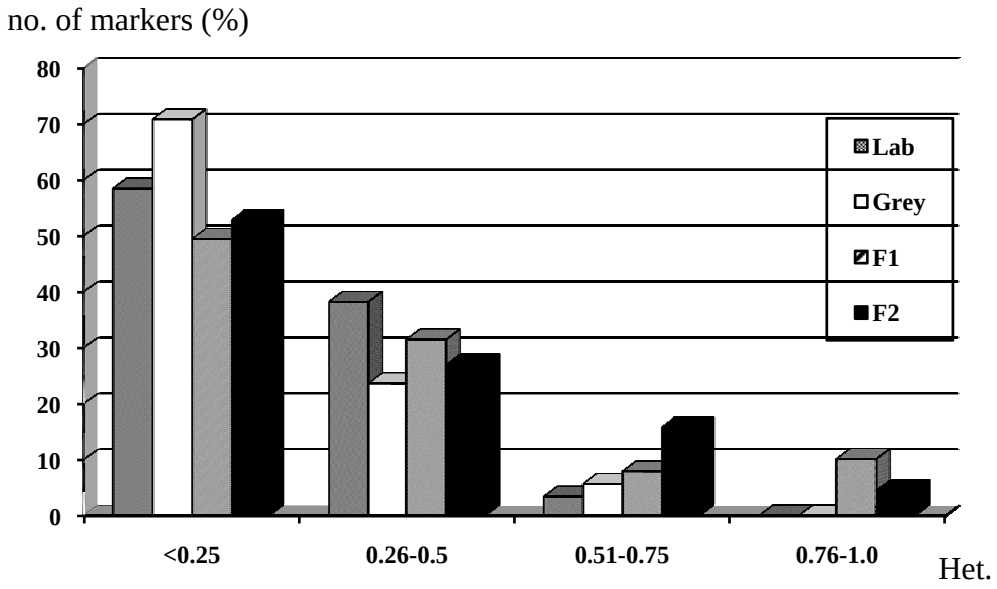

Figure 35 Heterozygosity values of 89 SNP markers on CFA11 represented in the crossbred pedigrees. (Lab = Labrador Retriever, Grey = Greyhound, F_1 = Lab x Grey, F_2 = F_1 x F_1, Het. = Heterozygosity).

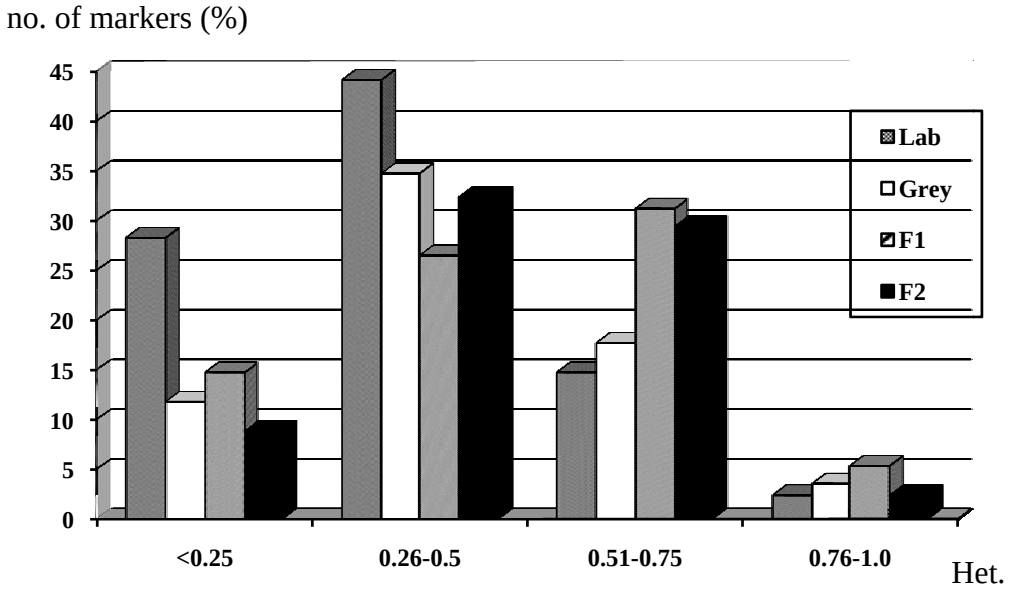

Figure 36 Heterozygosity values of 170 SNP markers on CFA29 represented in the crossbred pedigrees. (Lab = Labrador Retriever, Grey = Greyhound, F_1 = Lab x Grey, F_2 = F_1 x F_1, Het. = Heterozygosity).

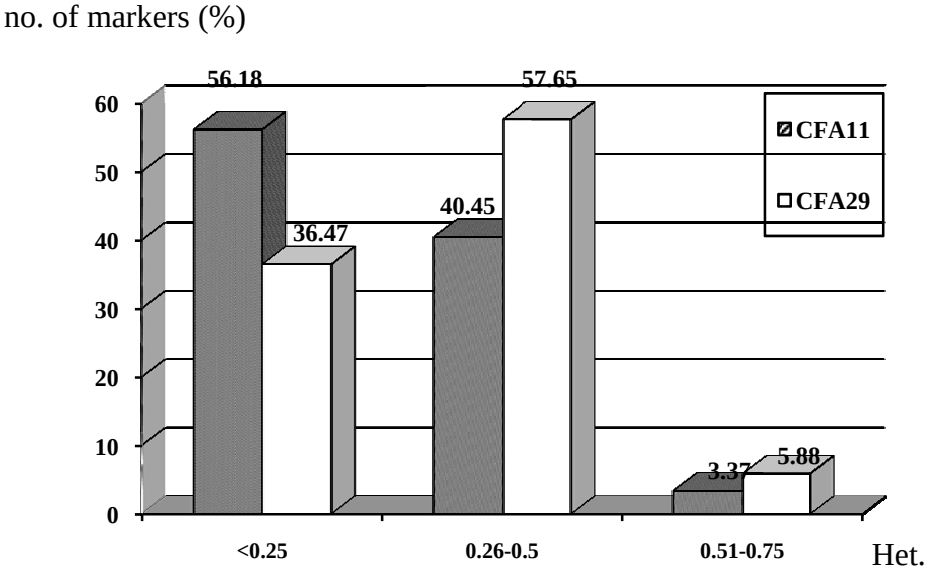

Figure 37 Heterozygosity of SNP markers on CFA11 and 29 represented across every dog breeds. (Lab = Labrador Retriever, Grey = Greyhound, F_1 = Lab x Grey, F_2 = F_1 x F_1, Het. = Heterozygosity).

3.4 QTL mapping with SNP markers

The analysis was undertaken using a Bayesian approach implemented in LOKI™ (Health, 1997). This software has enabled to localize the QTL interval for the hip traits on CFA 11 and 29 to a far narrower interval than analysis using microsatellite-based linkage model software (QTL Express™). In this study, DI, DLS, NA and OFA were chosen as the phenotypic traits for the first step of fine mapping with SNP markers.

The analysis result from multipoint linkage analysis using LOKI, version 2.4.5 (Health, 1997) was used to test the probability of linkage to DI, DLS and NA traits on CFA11 (Figure 38) and CFA29 (Figure 39). On CFA11, DI, DLS and NA traits revealed strong to moderate evidence for linkage to CHD ($20 > BF \geq 3$) especially for DI trait. The result was the same on CFA29 except for DLS trait that had $BF < 3$. Among these traits, DI showed the strong evidence for QTL on these 2 chromosomes. The OFA score did not show the evidence of QTL on these 2 chromosomes.

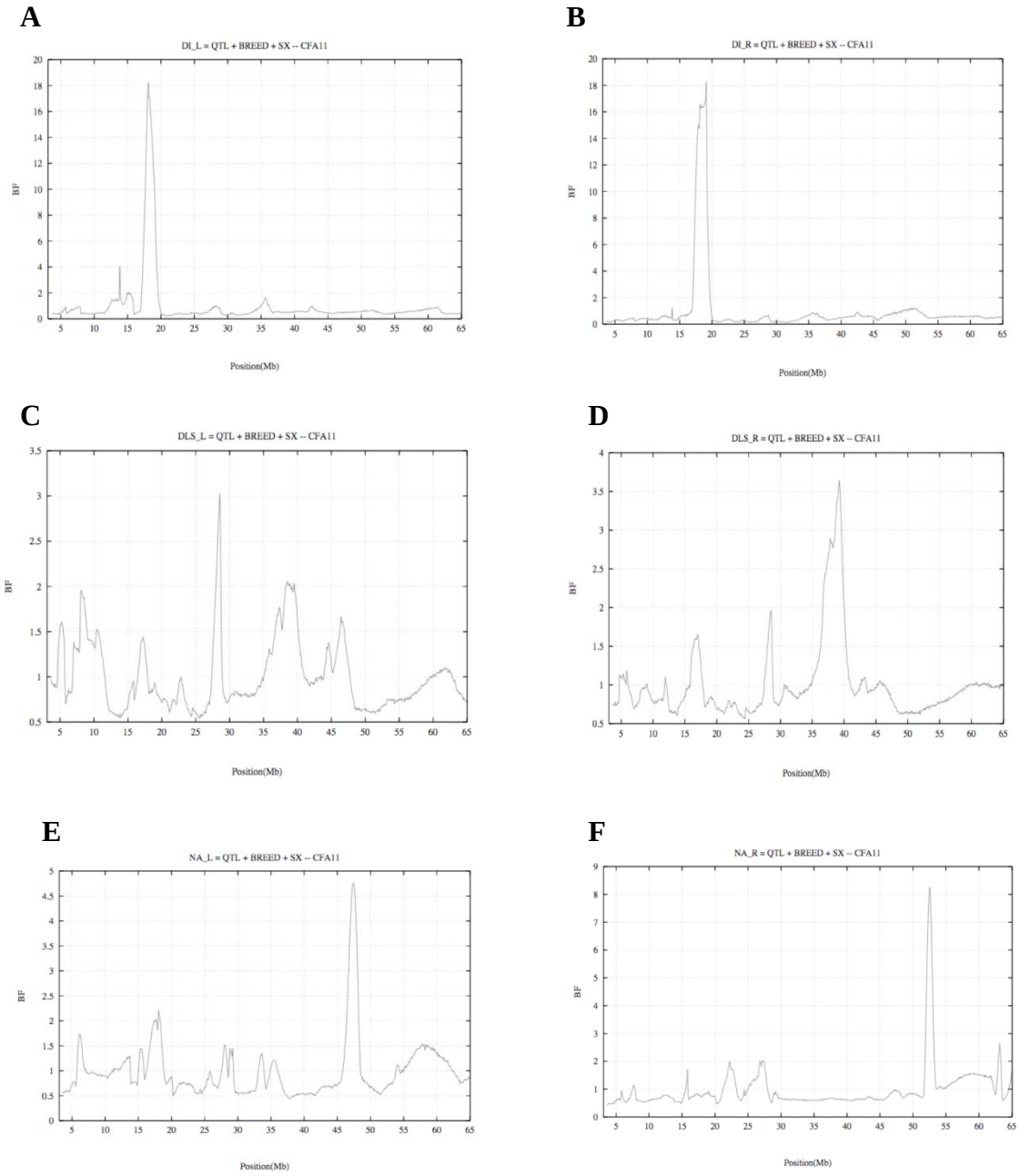

Figure 38 Multiple linkage analysis from SNP markers for fine mapping QTL on CFA11 for DI_L (A), DI_R (B), DLS_L (C), DLS_R (D), NA_L (E) and NA_R (F).

Note: DI_L = distraction index on the left hip, DI_R = distraction index on the right hip, DLS_L = dorsolateral subluxation on the left hip, DLS_R = dorsolateral subluxation on the right hip, NA_L = Norberg angle on the left hip, NA_R = Norberg angle on the right hip.

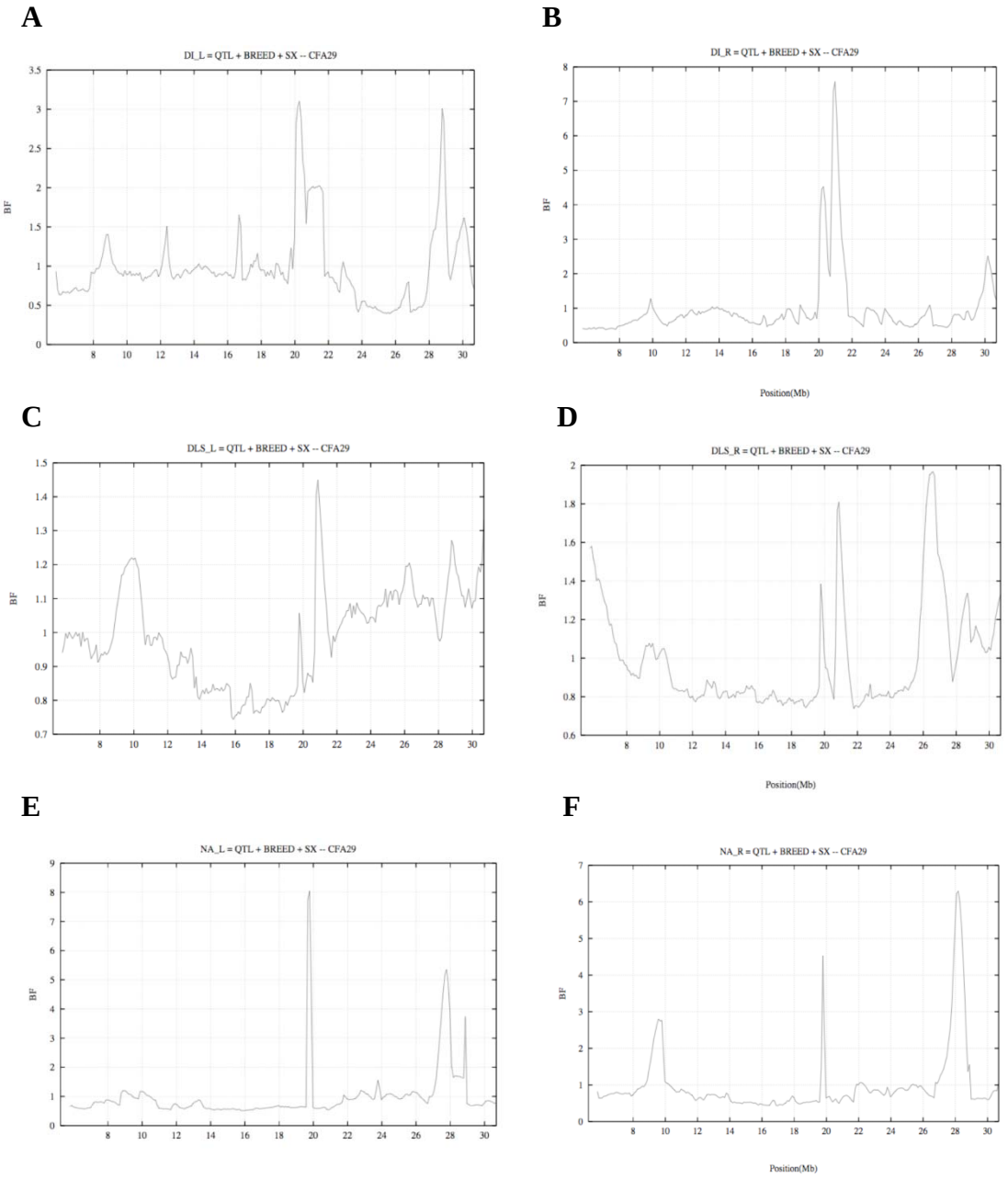

Figure 39 Multiple linkage analysis from SNP markers for fine mapping QTL on CFA29 for DI_L (A), DI_R (B), DLS_L (C), DLS_R (D), NA_L (E) and NA_R (F).

Note: DI_L = distraction index on the left hip, DI_R = distraction index on the right hip, DLS_L = dorsolateral subluxation on the left hip, DLS_R = dorsolateral subluxation on the right hip, NA_L = Norberg angle on the left hip, NA_R = Norberg angle on the right hip.

The results from Bayesian approach implemented in LOKI^{TM} analyzed on DI, DLS and NA revealed the strongest evidence of QTL on DI. When we observed at the variance of this trait, it was approximately normally distributed (Table 15). The proportion of affected dogs (DI > 0.7) was 11.7 % for the left DI (DIL) and 13.2 % for the right DI (DIR). The unaffected dogs proportion (DI < 0.4) was 37.7 % for DIL and 30.4 % for DIR. The percentage of total variance in DI due to QTL on CFA11 was higher than on CFA29. About 14-18 % of total variation (72.9-75.3 % of the total genetic variance) was explained by the QTL on CFA 11. About 11-14 % of total variation (55.4-72.3 % of the total genetic variance) in DI was due to QTL on CFA 29. There was no more different between the total genetic variance of singular or combined chromosomes (Table 22).

Table 22 The percentage of total variance in distraction index due to QTL on CFA 11 and 29.

Chromosome	DIL	DIR
CFA11	14.7 (75.3)	18.3 (72.9)
CFA29	11.3 (72.3)	13.6 (55.4)
CFA11 + CFA29	15.3 (79.8)	17.6 (72.4)

Note: The percentage of genetic variance due to QTL was in parentheses.
DIL = Distraction index on the left hip, DIR = Distraction index on the right hip.

On CFA11 at 19.7 cM (BF = 22.3) and 19.6 cM (BF = 21.6) for DIL and DIR was found strong evidence of one QTL (Figure 40). The 95% and 99% posterior probability intervals for DI on CFA11 were 4.8 cM (16.2-21 cM) and 1 cM (19.1-20.1 cM) respectively (Table 23). On CFA29 at 20.3 cM also found moderate evidence (BF = 3.1) of one QTL for DIL and 2 QTL for DIR at 20.3 (BF = 4.5) and at 21 cM (BF = 7.6) (Figure 40). The 95% and 99% posterior probability intervals for DI on CFA29 were 1.5 cM (20-21.5 cM) and 1 cM (20.1-21.1 cM) respectively (Table 23). Compared the results between these 2 chromosomes, CFA11 had a higher evidence of QTL than in CFA29. This evidence correlated with the small effect of the QTL on CFA29.

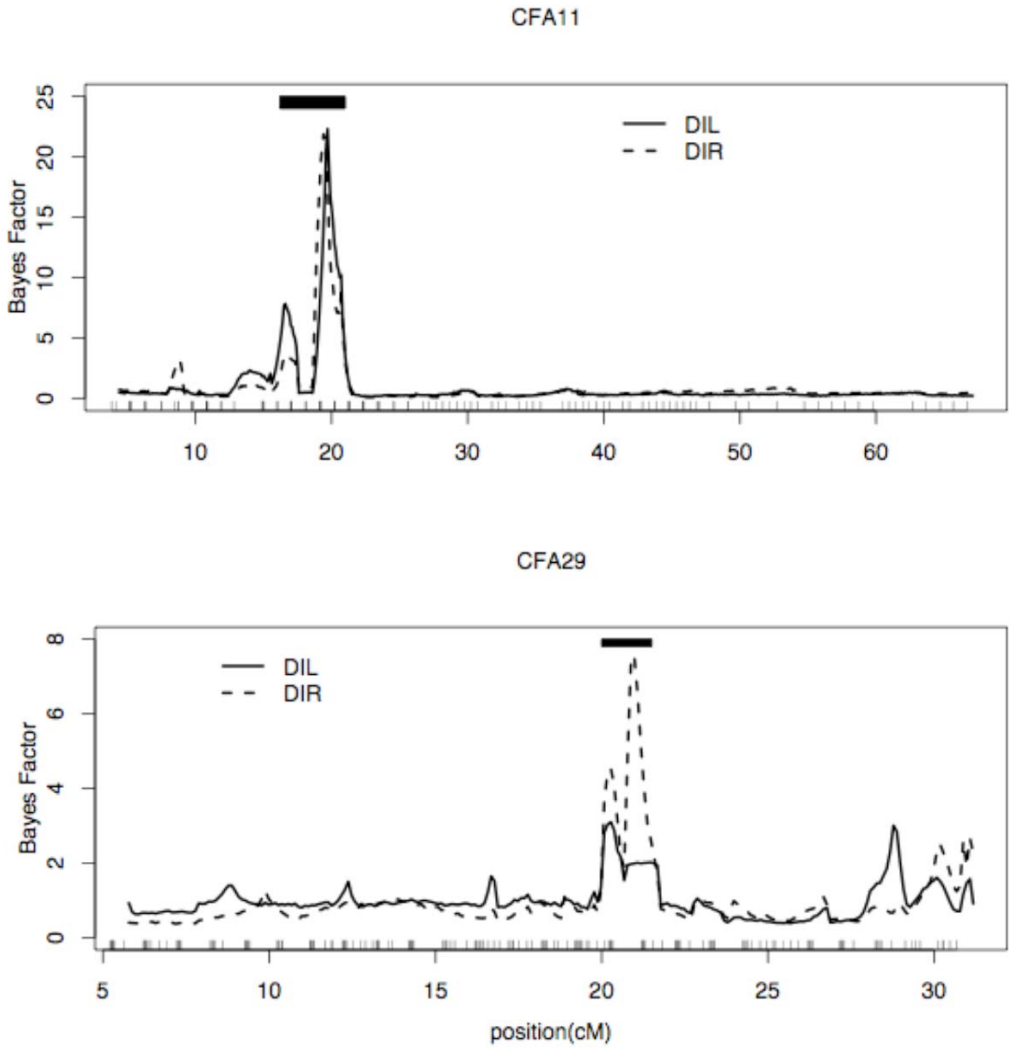

Figure 40 QTL mapping results by using Bayes Factor for linkage (y-axis) as a function of position (cM) along CFA11 and CFA29 (x-axis) for the distraction index on the left (DIL) and the right (DIR) hips from SNP markers. Black horizontal bars above the peaks indicate the 95% posterior probability intervals. Black upright tick on the x-axis represents SNP marker positions (cM) along the chromosomes.

Table 23 Estimate of the QTL peak for the Distraction Index (DI) on the left (DIL) and on the right (DIR), the associated Bayes Factor (BF) at the peak location in centiMorgans (cM), and the 95% and 99% posterior probability interval (PPI) by fitting of each chromosome separately and jointly.

	CFA	**Trait**	**Peak (cM)**	**BF**	**95% PPI**	**99% PPI**
Single chromosome	CFA11	DIL	19.7	22.3	16.2-21.0	19.5-20.1
		DIR	19.6	21.6	16.5-21.0	19.1-19.7
	CFA29	DIL	20.3	3.1	20.0-21.5	20.1-20.3
		DIR	20.3	4.5	20.0-21.4	20.8-21.1
			21.0	7.6	20.0-21.4	20.8-21.1
Two chromosomes jointly	CFA11	DIL	19.6	24.5	16.3-21.0	19.4-20.0
		DIR	19.4	18.2	19.8-21.0	19.1-19.7
	CFA29	DIL	na	<1	na	na
		DIR	20.3	2.9	20.0-20.9	20.1-20.4

Note: na = not applicable

The percentage of total variation of QTLs on both CFA11 and CFA29 was not simply the addition of the two chromosome percentage (Table 22). To identify the possible interaction of these 2 chromosomes, we jointly analyzed CFA11 and CFA29 using LOKI as shown in table 23. CFA11 revealed strong evidence of linkage between QTL and SNP markers while signals on CFA29 were decreased. The possible reason could be the relatively small effect of the QTL on CFA29 in the presence of QTL on CFA11 or its effect may be interacted with genes on CFA11.

DISCUSSION

Its polygenic nature of CHD has contributed to the difficulty of the eradiation. The disease characteristic is marked by hip joint laxity and subluxation. Distraction index (DI), Dosolateral Subluxation Score (DLS), Norberg angle (NA) and OFA score were the radiographic methods that used for CHD diagnosis for many years. A single radiograph with the standard diagnostic methods, hip extended radiograph has proved difficult to succeed the carrier dog eradication. Currently, accepted radiographic methods for the early diagnosis of this disease were not completely accurate predictor. From the previous study found that even in the progeny of normal parent, 19-36% of puppies were hip dysplasia (Willis, 1989). However, strict breeding screening with radiograph is the only way to reduce the incidence and to improve hip quality. In the advent of PCR technique and molecular genetic markers, the causative genes affecting many heritable genetic diseases were mapped and identified. Canine hip dysplasia was first reported since 1983 therefore morphology and histopathology were studied for many years. After that different radiographic methods were created to find the most appropriate method to identify the disease. However, it was not satisfied for disease eradication. Molecular genetic marker and linkage QTL mapping for CHD would be a good choice for detecting susceptible dogs at very young of age which is an alternative choice for owners prior to purchase a new puppy.

In this study, we used crossbred and purebred Labrador Retriever pedigrees for QTL mapping of CHD traits. According to many studies in the past, the data suggested that the QTLs that control CHD may be expressed differently in different purebred and crossbred dogs (Gustafsson *et al*., 1972; Lust *et al*., 1973 and Cardinet *et al*., 1983). In this study, crossbred pedigree between Greyhound and Labrador Retriever were chosen for the first QTL mapping. Greyhound dog was proved to be the breed having excellent hip conformation and Labrador Retriever using in this study was selected to susceptible to CHD. The objective of creating this crossbred pedigree was having the pedigree with the wide range of phenotypic traits.

The phenotypic methods such as DI, DLS, NA and OFA score were reliable as a good predictor of affected and unaffected CHD when used more than one method to make the diagnosis. The reason was that single radiographic method alone such as DI was not sufficience to make the definitive diagnosis for dogs with DI between the borderline (0.4-0.7). In this study, we measured the traits at young age which was the age for medium to large breed dogs to reach skeletal maturity. An advantage of mapping a complex phenotype in young dogs was that it was less to be influenced by non-genetic factors and more of the phenotypic variance should come from their genetic factors.

In crossbred and purebred Labrador Retriever pedigrees, the main differences between these 2 pedigrees were the phenotypic distribution of DLS. In crossbred, the phenotypic distribution was skewed to normal hip but in Labrador Retriever dogs the value was normal distribution. It may be possible that DLS in Greyhound dogs have the protective gene for CHD. An analysis of genetic effect of this crossbred pedigree explained that DI and DLS were significant at an additive genetic effect. Furthermore, DLS was also significant at dominance genetic effect (Bliss, *et al*., 2002). In our study, most of the principal components having DLS measurement were mapped in crossbred pedigree more than in purebred Labrador Retriever pedigree. It may come from the pedigree structure. For additive QTL, an F_2 intercross pedigree was more powerful than a backcross. However, for dominance QTL, an informative backcross can be twice as powerful as an intercross (Davasi, 1998). Our crossbred pedigree had both of these populations so it displayed more power for QTL analysis. This result suggested that our crossbred pedigree may be the most efficient pedigree structure for detection of QTL underlying the DLS score.

High microsatellite marker informativeness was observed in both crossbred and purebred Labrador Retriever pedigrees. These markers were spanned across the entire genome with inter-marker interval less than 10 cM (6.10 cM in crossbred and 8.92 cM in Labrador Retrievers). Among these effective factors such as genetic markers, phenotypes and dog pedigrees, many putative QTLs were identified. The result from genome-wide screen with microsatellite markers identified 11 (CFA02,

03, 04, 05, 06, 09, 10, 11, 16, 29 and 37) and 6 chromosomes (CFA01, 02, 10, 20, 22 and 32) containing the significant putative QTLs in crossbred and purebred Labrador Retriever pedigrees.

QTL affecting CHD have now been identified on several chromosomes such as 2 QTLs for hip dysplasia in the right and the left hip joints by using a genome-wide screen with 500 microsatellite markers of 286 Portuguese Water Dogs (PWD). These QTLs explained 14-16 % of the variation in Norberg angle. They located on each end of CFA01 (Chase *et al*., 2004) and on CFA03 that was significantly related to acetabular osteophyte formation of the hip joints (Chase *et al*., 2005). In German Shepherd dogs, QTLs were mapped at 23.5 and 82.3-115.1 on CFA01 and 44.6-49.9 and 79.4-91.7 on CFA03 (Table 24) (Marschall and Distl, 2007). Our study used crossbred and purebred pedigree. These chromosomes in our purebred Labrador Retrievers (CFA01) and crossbred dogs (CFA03) also harbored the QTLs. On CFA01 in Labrador Retrievers, QTL was associated with the second principle component of Norberg Angle and distraction index. On CFA03 in crossbred dogs, this QTL was associated with the first and second principle components of Norberg Angle, distraction index and dorsolateral subluxation score.

QTLs detected in crossbred and Labrador Retrievers, in German Shepherds and in Portuguese Water Dogs (PWD) on CFA01 had one QTL with the similar chromosomal location (distal end of CFA01). Marschall and Distl (2007) used multipoint test statistics for the whole genome scan with 261 microsatellite markers and identified QTLs on 18 chromosomes (CFA01, 03, 04, 05, 08, 09, 10, 16, 18, 19, 21, 22, 26, 29, 32, 33, 34 and 35) for the FCI grade in German Shepherd dogs (Table 24). Additional, our study, Marschall and Distl and Chase identified QTL for CHD on CFA03 in crossbred, German Shepherds and PWD.

Table 24 Comparison the results for the whole genome-wide screen with microsatellite markers for canine hip dysplasia on crossbred and Labrador Retriever in the present study with studies by Marschall, Y. and O. Distl (2007) in German Shepherd and by Chase *et al*. (2004, 2005) in Portuguese Water Dogs.

	QTL position (cM)			
	Present study		**Marschall, Y. and O. Distl (2007)**	**Chase *et al.* (2004, 2005)**
CFA	**Crossbred**	**Labrador Retriever**	**German Shepherd**	**Portuguese Water dog**
1	-	-	23.5 (FCI grade)	26.8 (NA)
	-	55.0 (DI and NA)	-	-
	-	70.0 (DI and NA)	82.3-115.1 (FCI grade)	111.3 (NA)
2	16.0-21.0 (NA and DLS)	-	-	-
	-	70.0 (DI, DLS and NA)	-	-
3	4.0-8.0 (DI, DLS and NA)	-	-	-
	-	-	44.6-49.9 (FCI grade)	44.8 (NA)
	-	-	79.4-91.7 (FCI grade)	-
4	-	-	4.7-12.7 (FCI grade)	-
	33.0-36.0 (DI and NA)	-	-	-
5	0.0 (DLS and NA)	-	9.3-14.8 (FCI grade)	-

Table 24 (Continued)

	QTL position (cM)			
	Present study		Marschall, Y. and O. Distl (2007)	Chase *et al.* (2004, 2005)
CFA	**Crossbred**	**Labrador Retriever**	**German Shepherd**	**Portuguese Water dog**
6	63.0-66.0 (DLS and NA)	-	-	-
8	-	-	29.0-34.0 (FCI grade)	-
9	-	-	14.1-28.0 (FCI grade)	-
	50.0 (DLS and NA)	-	37.4-54.6 (FCI grade)	-
10	52.0-53.0 (DLS and NA)	55 (DI and NA)	69.0 (FCI grade)	-
11	0.0-7.0 (DI)	-	-	-
16	-	-	8.5-21.1 (FCI grade)	-
	-	-	30.9-35.8 (FCI grade)	-
	52.0 (DLS and NA)	-	-	-
18	-	-	8.1 (FCI grade)	-
	-	-	54.3-61.2 (FCI grade)	-
19	-	-	8.0-11.2 (FCI grade)	-
	-	-	36.3-50.5 (FCI grade)	-

Table 24 (Continued)

QTL position (cM)				
	Present study		Marschall, Y. and O. Distl (2007)	Chase *et al.* (2004, 2005)
CFA	**Crossbred**	**Labrador Retriever**	**German Shepherd**	**Portuguese Water dog**
20	-	30 (DI and NA)	-	-
	-	60 (DI, DLS and NA)	-	-
21	-	-	38.0 (FCI grade)	-
22	-	0.0 (DLS)	3.9-5.5 (FCI grade)	-
	-	-	23.4-37.9 (FCI grade)	-
26	-	-	23.4-37.9 (FCI grade)	-
29	12.0-19.0 (NA)	-	27.8 (FCI grade)	-
32	-	5.0 (DI and NA)	-	-
	-	-	16.0-20.0 (FCI grade)	-
33	-	-	3.2-12.7 (FCI grade)	-
	-	-	32.6 (FCI grade)	-
34	-	-	4.5 (FCI grade)	-
	-	-	34.9 (FCI grade)	-

Table 24 (Continued)

QTL position (cM)				
	Present study		Marschall, Y. and O. Distl (2007)	Chase *et al.* (2004, 2005)
CFA	**Crossbred**	**Labrador Retriever**	**German Shepherd**	**Portuguese Water dog**
35	-	-	22.5-23.4 (FCI grade)	-
37	7.0 (DLS and NA)	-	-	-

Note: CFA = chromosome for *Canis familiaris*, cM = centiMorgans
DI = Distraction index, DLS = Dorsolateral subluxation score, NA = Norberg angle, FCI grade = FCI grade is scored hip joints into A, B, C, D and E grade; A for normal hips to E for severely dysplastic hip joints.

The identification of QTL in independent studies in different dog breeds provided convincing evidence that there was at least one QTL on CFA01, 03 and 10 that truly informs hip conformation in more than one breed of dog. Moreover, QTLs that control CHD may be expressed differently in different breed as convinced from the mapping results from the different studies (Table 24). Twenty-three out of 38 chromosomes revealed putative QTLs, QTL position, dog breed and mapping trait were showed in Figure 41. Among these chromosomes, CFA01 and 03 were found putative QTLs on every study; it may be possible to be the major QTL on these dogs or every dog breeds. However, it still needs more experimental data from another breed to support this observation before reaching the final conclusion. These 3 research teams used different radiographic methods and statistical analysis to do QTL mapping, Chase et al (2004) used NA, Marschall and Distl (2007) used FCI grade and our team used DI, DLS, NA and OFA score. This may be one of the factors that affected the QTL mapping results

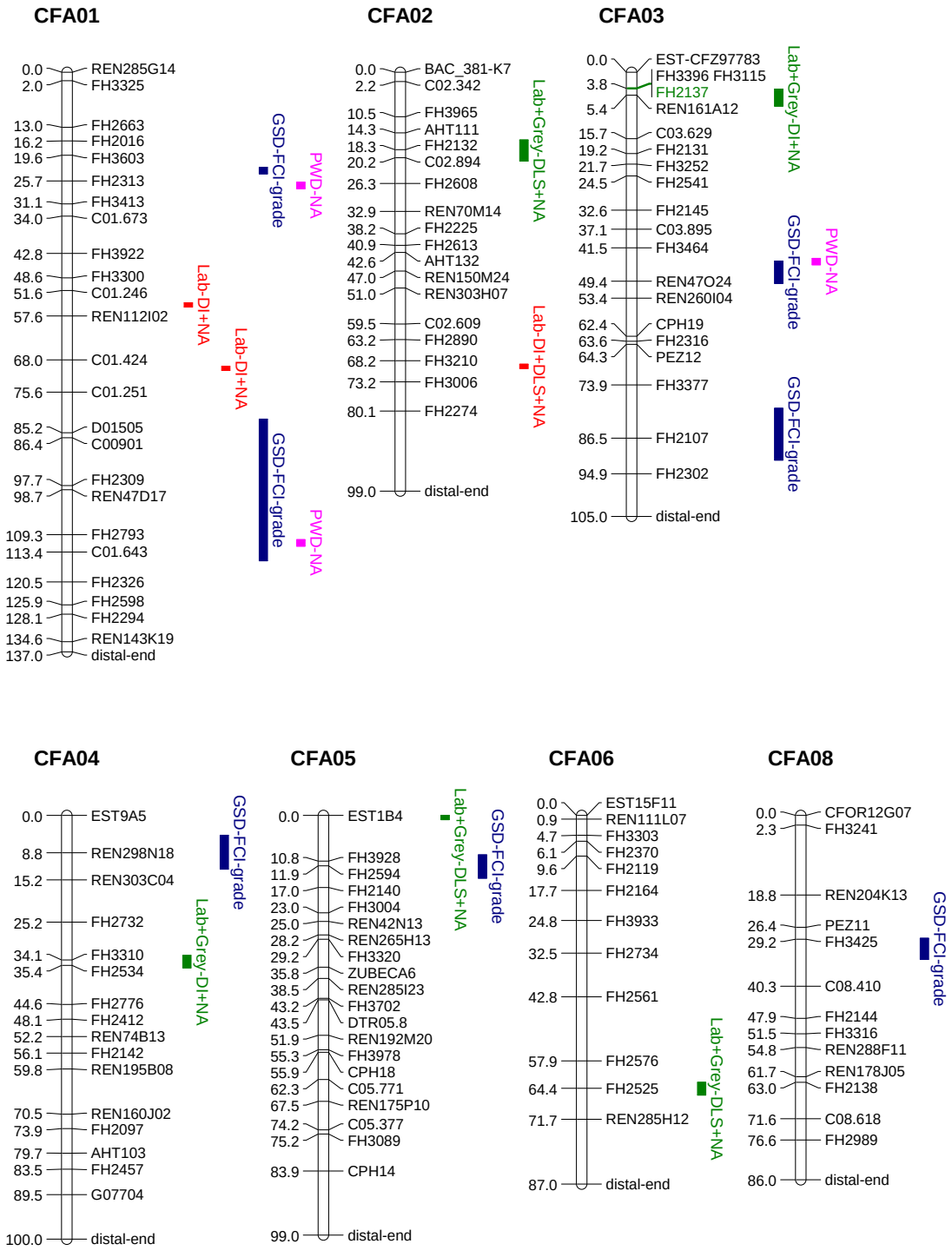

Figure 41 (Continued)

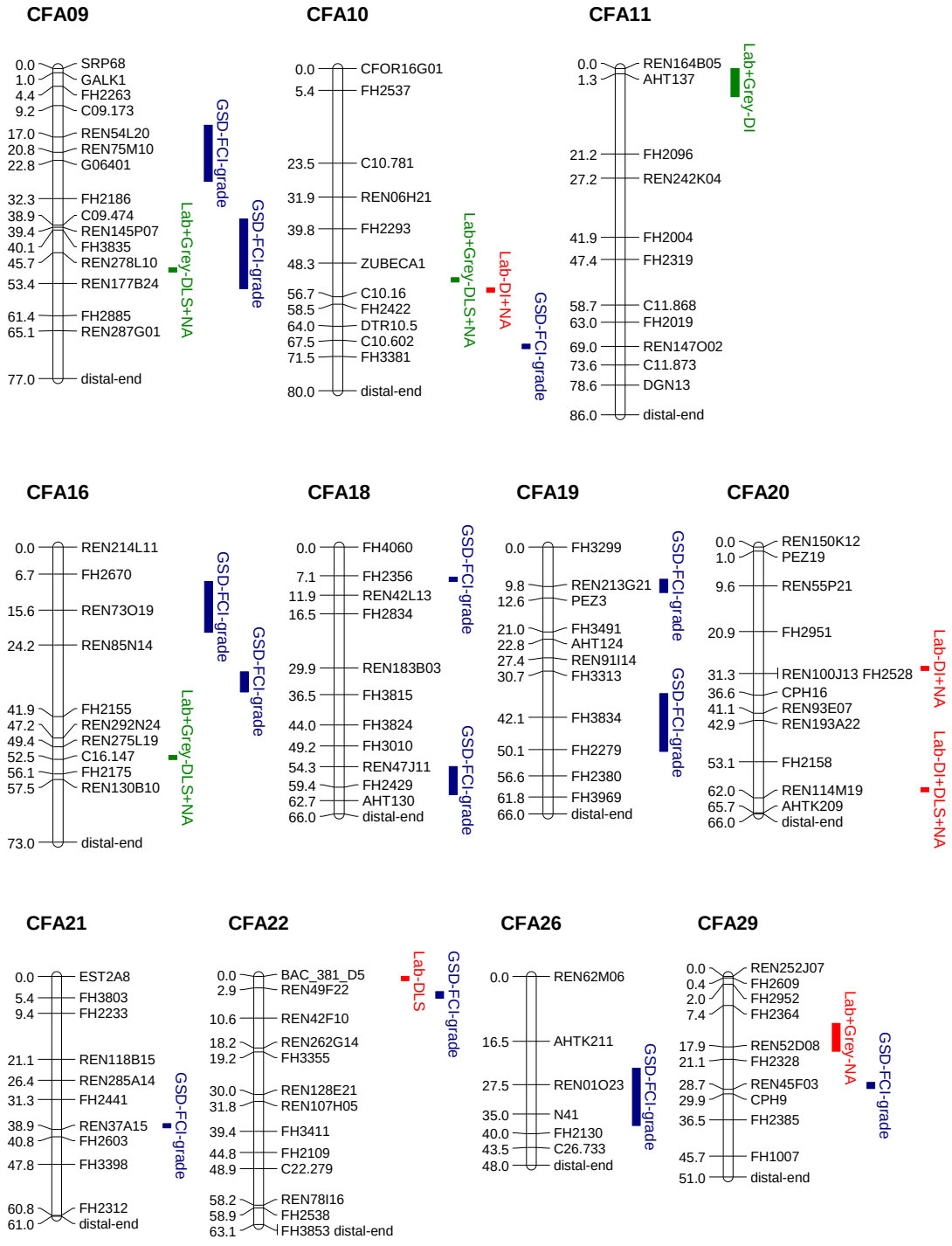

Figure 41 (Continued)

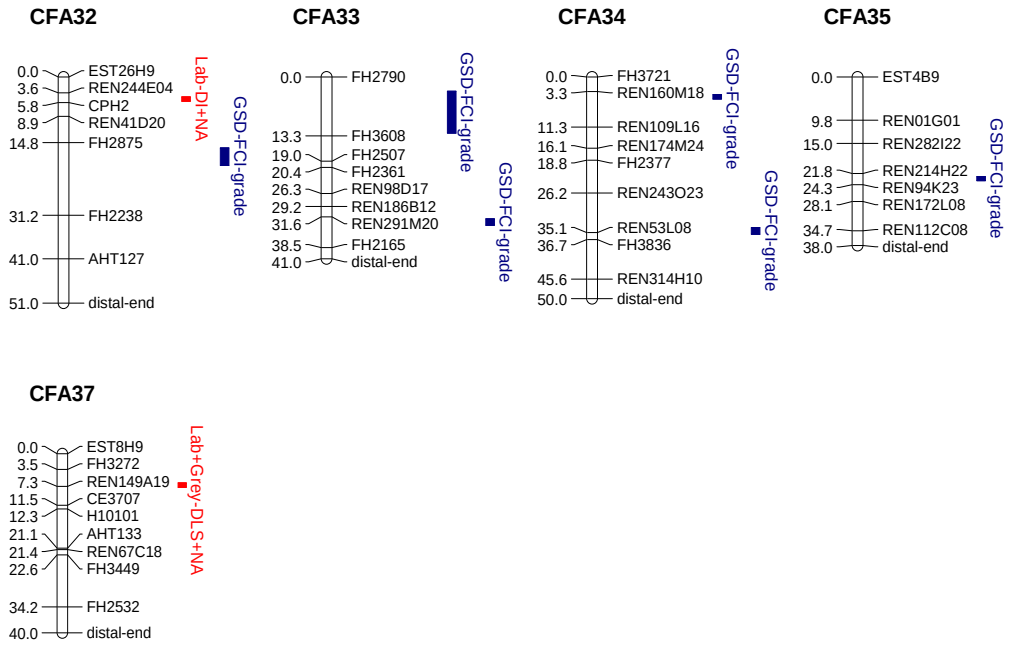

Figure 41 This picture showed the compared results for the whole genome-wide screen with microsatellite markers for canine hip dysplasia on crossbred and Labrador Retriever in the present study with studies by Marschall, Y. and O. Distl (2007) in German Shepherd and by Chase *et al*. (2004, 2005) in Portuguese Water Dogs. Color blocks represented QTL position, dog breed and trait for the QTL.

Note: Lab = Labrador Retriever dog, Grey = Greyhound dog, GSD = German Shepherd dog, DI = Distraction index, DLS = Dorsolateral subluxation score, NA = Norberg angle, FCI grade = FCI grade is scored hip joints into A, B, C, D and E grade; A for normal hips to E for severely dysplastic hip joints.

As a complex trait, the multiple genes that contributed to CHD may not be essentially located on a single chromosome. Our crossbred and purebred Labrador Retriever pedigrees were mapped to the different chromosomes that agreed with the previous reports (Chase *et al*., 2004; Chase *et al*., 2005; Marschall and Distl, 2007). In addition, these colonies were maintained in a controlled environment that should reduce the non-genetic factors that affected the expression of hip conformation. The results from our study and the previous reports may be concluded that it is possible to have more than one gene controlled the expression of CHD and the affect of genes may diverse among dog breeds.

Canine hip dysplasia is usually expressed bilaterally but can be unilateral. The trait measures on each hip in our study were always not the same values as has been indicated in some report (Chase, *et al*., 2004). In human, the left hip was more predisposed to hip dysplasia than the right (Smith, *et al*., 1963). In this study, there were no statistically different between the traits for the right or left hip that converse the concept of a directed asymmetry for QTL on CFA01 for the Norberg angle in Portuguese Water dogs (Chase, *et al*., 2004).

The combination of the traits provided a more power of assessment putative QTLs than using one trait alone as showed on QTL mapping result in crossbred and Labrador Retriever (Table 6, Table 12). Moreover, the data showed that these different traits were often linked to QTL in the same or similar marker interval. These QTLs could contain one or more than one genes contributing to each trait. On the other hand, they may be possible to have more than one QTL in some of these intervals. However, it appeared that each of these QTLs informed the different traits should be correlated although some traits had greater power than others for QTL detection.

Although discovery of the genes that underlie CHD is our major goal, understanding the factors that affect linkage mapping power and resolution will influence how confidently one should proceed with fine mapping and candidate gene screening. The QTL mapping results vary with the statistical analytical method used

and according to the structure of the mapping population. Power to detect the QTL was limited by many factors. In situation, an offspring has the same marker genotype as both of its parents or known only one of the parent genotypes. As a consequence, the inheritance of this canine pedigree was unknown. Therefore, the use of less informative markers and a lot of missing marker genotypes also affects the power of QTL detection (George *et al*., 2000). There were limitations of current results with regression based, interval mapping such as some markers were uninformative. Moreover, mapping in a modest-sized pedigree may exaggerate parameter estimates or limit the number of recombination by the number of animals.

In complex trait such as CHD, many genes were involved for the expression of the phenotypes. These genes may be linked to each other so in this case; QTL mapping was limited in its ability to locate these linked QTLs. From the other study, QTL detection was more power when the relative QTL variance was equal or greater than 0.5 and marker interval decreases (Mayer *et al*., 2004). The use of multiple markers to predict QTL genotype can increase the test statistic. Moreover, information content of markers also increases the test statistic (Haley *et al*., 1994).

There were limits to our ability to locate and to estimate the position of individual and linked QTLs. Multiple linked QTL usually bias the estimation of QTL location and effects. This bias has a more serious effect on small effect QTL than on larger effect QTL. Moreover, QTL mapping was also complicated by factors such as QTL by environmental interaction or QTL epistasis. Our methods do not specifically test for QTL interaction but there were methods for doing so in large populations (Wang, *et al*., 1999). The Labrador Retrievers in our mapping population were reared in the same environment and were fed for maximal growth. Therefore, this pedigree was not designed to study QTL by environmental interactions. Further, the Labrador Retriever pedigree we used for mapping in this experiment was not structured specifically for genetic mapping studies. It was constructed to study the pathological change of secondary hip osteoarthritis. As such, the mapping power of this pedigree was not optimized. Yet, our analysis showed that there were likely several QTL segregating in the Labrador Retriever pedigree and that their effects varied from locus

to locus. Moreover, QTL heritability varied from trait to trait which affected the power to detect QTL. The higher the heritability, the greater the opportunity to detect and to locate the QTL. High heritability for CHD was found in Flat Coated Retrievers (0.74±0.25), Newfoundlands (0.49±0.08), Golden Retrievers (0.34±0.09 and 0.47±0.08 for male and female, respectively) and Labrador Retrievers (0.54±0.21 and 0.60±0.13 for male and female, respectively) (Swenson, *et al*., 1997; Wood, *et al*., 2000a). But the heritability of CHD was low in Gordon Setters (0.20±0.10) (Wood, *et al*., 2000b). The ability to detect QTL in these Labrador Retrievers was probably enhanced by the high heritability of CHD in this breed.

Small effect QTL can be detected with higher power if the family is large enough (Martinez *et al*., 1997) which may explain why we did not detect some QTL for CHD that were discovered in other populations like the German shepherd (Marschall and Distl, 2007). Further, mapping QTL in a pure breed of dog may investigate different major and minor QTL compared to those within a crossbreed population based on one of the same pure breeds. Some QTL alleles may be fixed in one population, will not be segregating, and therefore will not be detectable. The polygenic background of a particular breed will also affect the ability to detect QTL and influence or modify QTL expression.

In our purebred Labrador Retriever pedigree, we did not find significant QTL on CFA11 and 29 that reached the statistical threshold at the 1% level as we found in crossbred dogs. There were some markers missing, especially on CFA29, in our experiment in this pedigree. These chromosomes had a high QTL heritability (0.31-0.46) associated with the NA but the QTL did not reach an arbitrary LOD score above 2. In this study, some phenotypes were mapped to different chromosomes. It is possible that loci that control CHD trait such as NA, DI or DLS may come from different loci. The result from genome wide-screen with microsatellite makers in crossbred pedigree revealed highly significant QTLs on CFA11 and 29. Therefore, we decided to do fine mapping with SNP markers on these 2 chromosomes. Even though, these chromosomes had never been reported in other studies.

However, the result from QTL mapping with SNP markers was consistent with the microsatellite makers that we used in this study. It suggested that there was a major locus for the DI involving in the CHD expression. Compared to microsatellite markers analysis, multipoint linkage analysis in SNP markers reduced the QTL intervals from 20-30 cM to 1-5 cM, and effective number of QTL was about 1 or 2 for this trait on these two chromosomes. This should provide a reasonable starting point for candidate gene selection. There was not different power with the probability interval of QTL mapping on CFA11 and CFA29. Even though on CFA 29, we selected SNP markers about 1 SNP/25 kb but on CFA11, the SNP markers span every 200 kb. This result provided a strategy for markers selection. It will benefit for the marker selection on the next chromosome for fine mapping with SNP markers. There were no differences between the analysis of QTL position on the left and the right hips independently, indicating symmetric effects of both hip laxity.

Candidate genes in the 99% posterior probability interval for the QTL on CFA11 between 19.1 and 20.1 cM included membrane-associated RING-CH protein III, MEGF10 (multiple EGF domains 10) and MGC12103. The 95% posterior probability interval for the QTL on CFA11 contains at least 10 genes. Candidate genes in the 99% and 95% posterior probability interval for the QTL on CFA29 between 20.1 and 21.1 cM and 20 and 21.5 cM included DEP domain containing 2 isoform a and proteasome subunit alpha type 1. These genes will be used as candidate genes for the future study. The benefit of identification these genes that contribute to dysplastic hip development in dogs may point to candidate genes and related biochemical pathways for comparative studies in dogs and human on this or similar complex traits.

Recently, in human hip dysplasia was mapped to an 11-cM region on human chromosome HSA 4q35 (Roby *et al.* 1999). In Italy, the incidence of human hip dysplasia was high, ranging between 10 and 18.5 per 1000 live births and was higher in the Adriatic region (Baronciani *et al.* 1997). CFA03 in dog is the syntenic region to human 4q35q and 15q24-26 (Roby *et al.* 1999) where a putative QTL for hip dysplasia in dogs in our experiment. Canine hip dysplasia is one of the hereditary

diseases that have remarkable clinical signs similarity to human hip dysplasia. The genes we uncovered in the dogs may well be the same genes that contributed to hip dysplasia in human or at least encoding the same proteins in biochemical pathway common to both canine and human. Therefore, CHD should be the potential model to identify causative genes in canine and human study. Moreover, the genes and related markers can apply to improve the diagnostic tools to identify CHD in the future.

CONCLUSION

Canine hip dysplasia is a heritable genetic disease that marked by hip laxity and subluxation. It is one of the most common orthopedic diseases in any breed of dogs that affects health problem and quality of life. It is estimated that more than 50 percent of some breeds are affected. Control breeding by using radiographic screening can reduce the incident but cannot completely eradicate the disease. Dysplastic parents tend to have dysplastic offspring. However, there are some dysplastic dogs that have the normal parents. The inheritance of the traits implies that more than one gene should affect the expression of the disease and heritability is widely variable among dog breeds. To identify genes influencing complex trait such as CHD, molecular genetics technology and advance statistic analysis are very important.

In this study, we used crossbred, purebred Labrador Retrievers, Golden Retrievers and German Shepherds as a population model for mapping the QTL affecting for CHD. Phenotypic measurement from 4 radiographic methods; Distraction index (DI), Dorsolateral Subluxation Score (DLS), Norberg angle (NA) and OFA score were measured on every dog samples. To identify some of the major genes contribute to CHD and reduction the size of significant interval as much as possible, many analytical methods such as genome-wide screen with microsatellite markers and QTL fine mapping with SNP markers were undertaken.

Under the effective markers, phenotypes and dog pedigrees, many putative QTLs were identified. The result from the genome-wide screen with microsatellite markers identified 11 (CFA02, 03, 04, 05, 06, 09, 10, 11, 16, 29 and 37) and 6 chromosomes (CFA01, 02, 10, 20, 22 and 32) contained the significant putative QTLs in crossbred and purebred Labrador Retrievers. Two chromosomes (CFA11 and 29) were chosen for fine mapping with SNP markers to narrow down the QTL position. Compared to microsatellite markers analysis, multipoint linkage analysis in SNP markers on CFA11 and 29 reduced the QTL intervals from 3-20 cM to 1-5 cM, and effective number of QTL was about 1 or 2 for this trait on these two chromosomes.

In conclusion, the result from this study and the others implied that in CHD a large number of alleles, but unknown numbers are segregated at many loci. Some of them might locate at the same chromosome, but others may spread throughout the genome. Moreover, variation of the gene affecting the expression of the trait may be different among breeds. The aim of QTL mapping is to apply genetic testing and marker-assisted selection that may improve susceptibility of hip trait screening at a very young age. Because some dogs show clear signs of hip dysplasia at a very young age, before the severe clinical signs such as lameness, crippling or osteoarthritis has developed. Genetic testing should assist in preventing carriers with mutant alleles from entering the genetic pool before breeding time and thus decrease the incidence of the disease. This study is only some parts of the main project of tracing gene for CHD. Rather than using each strategy in isolation, the combination of several analysis such as QTL mapping, SNP haplotype analysis, microarray-based transcriptome analysis, comparative mapping and Northern analysis or RT-PCR have been used to identify strong candidate genes. Mutation screening in affected and unaffected dogs will be undertaken in the final step. Finding QTLs from this study could be used in subsequent candidate gene mapping and applied to marker-assisted selection to early identify of susceptible loci for CHD.

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APPENDICES

Appendix A

Glossary

Allele

Specific string of DNA at the locus.

Association mapping

Gene localization by linkage disequilibrium without cloning.

Association methods

Methods concerned with testing whether single-locus allele or genotype frequencies (or, more generally, multilocus haplotype frequencies) are different between 2 groups (typically designated cases and controls).

Association probability

Probability that a random haplotype at 2 specified diallelic loci is descended without crossing-over from an ancestral haplotypes at maximal disequilibrium.

Haplotype

Set of closely linked alleles present on 1 chromosome, which tend to be inherited together.

Gametic disequilibrium

Linkage disequilibrium.

Genomics

The functions and interactions of all genes in the genome.

Genotype

The pair of alleles at a locus.

Hardy-Weinberg equilibrium (HWE)

The situation in which the genotype frequencies for a locus are determined by the allele frequencies.

Heterozygote

An individual who has 1 copy of 2 different alleles at a locus.

Homozygote

An individual who has 2 copies of the same allele at a locus.

Independence

The situation in which the probability of one event occurring does not depend on another event; for example, 2 alleles from 2 different loci on the same chromosome are independent if the probability of observing 1 allele does not depend upon the presence of the other allele.

Linkage disequilibrium (LD)

A relationship between 2 alleles that arises more often than can be accounted for by chance, since those alleles are physically close on a chromosome and infrequently separated from one another by recombination.

Linkage methods

Methods involving estimation of the recombination fraction between 2 loci, 1 that is observed and 1 that is typically unobserved (the disease locus).

Locus (plural, loci)

Any polymorphic stretch of DNA in the genome.

Marker

Short DNA sequence that is polymorphic and useful for mapping by linkage association.

Mendelian diseases

Disease that follow either a dominance or recessive pattern and for which genotype relative risks are on the order of 1,000 or more.

Penetrance

The probability that an individual is affected with a specific disease if the individual has a certain number of copies of a disease locus. Mathematically, it is written as f_i = Pr (affected / i copies of disease allele).

Power

The probability that the test statistic indicates (usually when the statistic has a large value) that the observed data are near an (unobserved) disease locus.

Recombination

The event in which an exchange of genetic material between homologous chromosomes takes place.

Recombination fraction

This word refers to the probability that a recombination will take place between 2 loci.

Single nucleotide polymorphism (SNP)

A Single Nucleotide Polymorphism, or SNP (pronounced "snip"), is a small genetic change, or variation, that can occur within a person's DNA sequence. SNP variation occurs when a single nucleotide, such as an A, replaces one of the other three nucleotide letters—C, G, or T.

Type I error rate

The probability that the test statistic indicates that the observed marker loci are near a disease locus when in the fact there is no disease locus nearby; this rate is determined by the researcher.

Appendix B

DNA Isolation Solution

1. Working Solution:

1.1 Red Blood Cell Lysis Solution (500 ml)

1 ml 0.5 M EDTA
10 ml 0.5 M Sodium Bicarbonate
155 ml 0.5 M Ammonium Chloride
334 ml dH_2O
pH ~ 7.3 no adjustment required.
Autoclave before used

1.2. White Blood Cell Lysis Solution (500 ml)

Combine and mix the following
469 ml autoclaved milli-Q H_2O
5 ml 1 M Tris Base
1 ml 0.5 M EDTA
Add SDS gently to avoid foaming
25 ml 10% SDS
Filter sterilize

1.3 10 M Ammonium Acetate (1 liter)

Combine and mix the following:
300 ml autoclaved milli-Q H_2O
770.8 g Ammonium Acetate (77.08 MV)
Adjust to final volume
Note that an overnight stir may be necessary before all the ammonium acetate is in solution.

1.4 TE Solution (10 mM Tris base, 0.2 mM EDTA) (1 liter)

Combine and mix the following
989.8 ml autoclaved milli-Q H_2O
400 µl 0.5 M Tris base
Final solution does not need to be autoclaved.

2. Stock Solution

2.1 1 M Tris Base (500 ml, pH 8.0)

400 ml autoclaved milli-Q H_2O
60.57 g Tris (base, MW 121.14)
Adjust to final volume
Filter sterilize

2.2 0.5 M EDTA (100 ml, pH 8.0)

80 ml autoclaved milli-Q H_2O
18.61 g EDTA (disodium, MW 372.24)
~ 5 ml 10 M NaOH required for EDTA to go into solution
Initial pH ~ 8.6
Adjust to final volume
Filter sterilize

2.3 10% SDS (500 ml)

400 ml autoclaved milli-Q H_2O
50.0 g SDS (MW 288.4)
Adjust to final volume

2.4 0.5 M Sodium Bicarbonate (1L, pH 8.0)

900 ml d H_2O
42.0 g Sodium Bicarbonate (MW 84.01)
Initial pH ~ 8
Adjust to final volume
Filter sterilize

2.5 0.5 M Ammonium Chloride (1L)

900 ml d H_2O
26.75 g Ammonium Chloride (MW 53.49)
pH ~ 5 no adjustment required
Adjust to final volume

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