UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

Emerging insights on canine hip dysplasia diagnosis: clues from radiology and genetics

Dissertação de Mestrado em Medicina Veterinária

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Orientador: Professor Doutor Mário Manuel Dinis Ginja
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I hereby declare the authenticity of the present work in order to obtain the Integrated Master degree in Veterinary Medicine at the Universidade de Trás-os-Montes e Alto Douro in Vila Real, Portugal.

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Abstract

Canine hip dysplasia (CHD) is characterized by an abnormal hip and coxofemoral joint development with both hips usually affected, and is a common disorder that affects mainly large dog breeds. Hip joint laxity is the major risk factor leading to subluxation and poor congruence between the femoral head and acetabulum. Over time, degeneration of the joint occurs. Clinical sings include pain, decreased activity and lameness. Radiograph has been the gold standard to assess and quantify the joint changes associated but, with the development of genomic techniques, there is now the possibility to try to understand the genetic basis for CHD.

One of the most popular and ancient Portuguese native breeds of shepherd dogs is the "Cão da Serra da Estrela" (CSE) (Estrela Mountain Dog). This is a large, mastiff-type molossoid dog, with high prevalence of CHD. A large database of radiographic information for CHD in CSE animals already existed, thus further justifying the development of this genetic study to try to understand which regions of the genome are associated with this condition.

A review was published to describe and discuss the different diagnostic approaches concerning this condition, with special attention given to radiographic and molecular methods.

A retrospective radiographic study on 437 dogs of several breeds (33 of CSE breed) was completed to determine the ability of the Norberg angle at ≥105° to predict a non-dysplastic hip based on a distraction index cut-off of ≤0.3 or a dorsolateral subluxation score cut-off of ≥55%. It was possible to establish that at the commonly used cutpoint of 105°, the Norberg angle is not an accurate measurement of normal hip conformation. The specificity of a non-dysplastic diagnosis was maximized with a cut-off of 112º (when compared to the distract index) or 109º (dorsolateral subluxation score).

A study on 60 CSE animals, and for comparison purposes of an additional 10 Portuguese stray dogs and 10 Iberian wolves, was conducted to investigate within-breed genetic diversity and population structure using a whole-genome SNP approach. Y-chromosomal markers were also used to investigate phylogenetic relationships and within-breed haplotype composition. These analyses are key for the subsequent investigation of the genetic basis for specific CHD traits, e.g. hip joint laxity. CSE has high genetic diversity with negligible inbreeding. Weak genetic sub-structure was observed within CSE possibly

explained by lineage sorting associated with different breeders in each geographic location where the samples were collected, i.e. the northern, central and southern regions of Portugal. All CSE animals shared the same Y-haplotype which belongs to the most prevalent H1-haplogroup found in European dogs.

The main purpose of the diagnosis and breeding schemes is to identify the individuals carrying hip dysplasia information in order to remove them from the breeding pool. This allows to eliminate carriers, to avoid the genetic inheritance associated with this condition and reduce its prevalence in the next generations. This study sets the basis for the future development of more comprehensive genomic studies to try to understand the genetic basis for CHD traits in this breed.

Key-words: 'Cão da Serra da Estrela', canine hip dysplasia, radiographic diagnosis, whole-genome SNP analyses

Resumo

A displasia da anca no cão (DA) é caracterizada por um anormal desenvolvimento da anca e da articulação coxofemoral sendo usualmente ambos os lados afetados, e é uma condição frequente que afeta essencialmente cães de grande porte. A lassitude articular é o maior fator de risco levando a subluxação e a incongruência entre o acetábulo e a cabeça do fémur. Com o decorrer do tempo, a degeneração da articulação ocorre. Os sinais clínicos incluem dor, decréscimo de atividade e claudicação. A radiografia tem sido o método mais recorrente para avaliar a articulação e quantificar as modificações associadas mas, com o desenvolvimento de técnicas genómicas, existe agora a possibilidade de tentar descortinar a base genética para a DA.

Uma das raças nativas de cães pastores Portuguesas mais populares é o "Cão da Serra da Estrela" (CSE) onde se verifica uma elevada prevalência de DA. Uma vasta base de dados de informação radiográfica para DA nos CSE já estava disponível, justificando, assim, o desenvolvimento de estudos genéticos na tentativa de compreender que regiões do genoma podem estar associadas à DA.

Um artigo de revisão foi publicado com a descrição e discussão de diferentes abordagens de diagnóstico de DA, com especial atenção prestada aos métodos radiográficos e moleculares.

Um artigo original foi publicado que consistiu num estudo radiográfico retrospetivo em 437 cães de diversas raças (dos quais 33 CSE) com o objetivo de determinar a capacidade do ângulo de Norberg ≥105° de prever uma anca não displásica baseado no índice de distração ≤0.3 ou no índice de subluxação dorsolateral ≥55%. Foi possível estabelecer que com o cut-point recomendado de 105º, o ângulo de Norberg não foi uma medida precisa da conformação normal da anca. A especificidade do diagnóstico de uma anca não displásica foi maximizado com um cut-point de 112º (quando comparado com o índice de distração) ou de 109º (índice de subluxação dorsolateral).

Um estudo em 60 CSE e, para efeitos de comparação, em 10 cães de rua Portugueses e em 10 Lobos Ibéricos, foi conduzido com o objetivo de investigar a diversidade genética e a estrutura populacional na raça CSE usando polimorfismos de uma única base nucleotídica (SNPs). Utilizaram-se, ainda, marcadores específicos do cromossoma Y para aferir relações filogenéticas e a composição haplotípica. A raça CSE demonstrou elevados índices de variabilidade genética com consanguinidade negligenciável. Foi detetada sub-estrutura subtil

nos CSE possivelmente explicada por diferentes linhagens familiares associadas a diferentes criadores em cada uma das zonas geográficas de amostragem, isto é, Norte, Centro e Sul de Portugal. Todos os CSE partilharam um único haplotipo do cromossoma Y pertencente ao haplogrupo H1 que é o mais prevalente em cães Europeus.

Os sistemas de diagnóstico têm como principal objectivo identificar os indivíduos portadores de informação genotípica e/ou fenotípica associada a DA para os remover dos programas de reprodução. Desta forma, conseguimos eliminar portadores, evitar a transmissão desta informação genética e reduzir a prevalência da DA nas gerações seguintes. Estas análises são a chave para uma investigação subsequente, nesta raça, da base genética de traços específicos associados a DA, como a lassitude articular.

Palavras-chave: Cão da Serra da Estrela, displasia da anca canina, diagnóstico radiográfico, análises genómicas com SNPs

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Gaspar A.R, Hayes G., Ginja C., Ginja M. M., Todhunter R. J., 2016. *The Norberg Angle is not an Accurate Predictor of Canine Hip Conformation Based on the Distraction Index and the Dorsolateral Subluxation Score*. Preventive Veterinary Medicine, 135, 47-52.

Gaspar A.R., Muñoz-Mérida A., Pires A.E., Godinho R., Hayward J., Todhunter R.J., Ginja M.M., Ginja C., 2016. *Genetic analysis of the Portuguese 'Cão da Serra da Estrela' dog breed using whole-genome SNPs.* (manuscript in preparation to submit to Animal Genetics, ISNN: 1365-2052).

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Gaspar A.R., Godinho R., Muñoz-Mérida A., Hayward J., Todhunter R.J., Ginja M.M., Ginja C., 2016. *Genetic analysis of the Portuguese Serra da Estrela dog breed using whole-genome SNPs.* ConGenomics 2016 - Conference on Conservation Genomics, 3-6 May 2016, ESF – CIBIO-InBIO – Universidade do Porto, Campus de Vairão, Vairão, Portugal (poster presentation)

List of abbreviations

AE – Acetabular edge

AF – Acetabular fossa

BVA/KC – British Veterinary Association/ Kennel Club

bp – Position in base pair

CHD – Canine hip dysplasia

CaAE – Caudal acetabular ridge

CFA – *Canis familiaris* autosome

CI – Confidence interval

CrAE – Cranial acetabular edge

CSE – "Cão da Serra da Estrela" breed

DA – Displasia da anca

DAE – Dorsal acetabular edge

DI – Distraction index

DJD – Degenerative joint disease

DLS – Dorsolateral subluxation score

DNA – Deoxyribonucleic acid

EBV – Estimated breeding value

FCI – *Fédération Cynologique Internationale*

Fis – Inbreeding coefficient

Fst – Coefficient of genetic differentiation

GBV – Genomic breeding value

H – Haplotype diversity

He – Expected heterozygosity

HJL – Hip joint laxity

Ho – Observed heterozygosity

ICC – Intraclass correlation coefficient

IW – Iberian wolf

JPS – Juvenile pubic symphysiodesis

k – Partition of the dataset in the Bayesian cluster analyses

Mb – Megabase

mtDNA – Mitochondrial DNA

NA – Norberg angle

nt – Nucleotide

NPV – Negative predictive value

OA – Osteoarthritis

OFA – Orthopedic Foundation for Animals

PCA – Principal components analysis

PPV – Positive predictive value

Q – Mean genotype membership coefficient

q – Individual genotype membership coefficient

QTL – Quantitative trait *loci*

ROC – Receiver operator characteristic

SD – Stray dogs

SNPs – Single nucleotide polymorphisms

STRs – Short tandem repeats

SVDV – Standard ventrodorsal hip-extended view

UTR – Untranslated region

VD - Ventrodorsal

1. Introduction

The present study was accomplished to obtain the Integrated Master degree in Veterinary Medicine and was accomplished based on collaborative research between teams from Hospital Veterinário da Universidade de Trás-os-Montes e Alto Douro (Vila Real, Portugal), Cornell University Hospital for Animals (Ithaca NY, USA) and CIBIO/InBio - Centro de Investigação em Biodiversidade e Recursos Genéticos (Vairão, Portugal). The general aims were to investigate radiographic methods for the diagnosis of canine hip dysplasia (CHD), and to try to understand the genetic basis of traits associated to this condition in the Portuguese native dog breed 'Cão da Serra da Estrela' (CSE).

This dissertation begins with a short revision of the literature regarding CHD in the second chapter. Special attention is given to the CSE breed, which is one of the oldest populations of Portuguese native dogs with high prevalence of CHD.

The third chapter consists of a review article, entitled "Emerging insights into the genetic basis of canine hip dysplasia – review", which is coauthored by M Ginja, AR Gaspar and C Ginja and published in a peer reviewed scientific journal - Veterinary Medicine: Research and Reports 2015;6:193-202. The main purpose of this review was to present and discuss medical aspects of CHD for which knowledge is incomplete and have therefore merited major current research efforts.

The fourth chapter is an original study which was designed in straight collaboration with the team of Cornell University College of Veterinary Medicine, entitled "The Norberg Angle is not an Accurate Predictor of Canine Hip Conformation Based on the Distraction Index and the Dorsolateral Subluxation Score". The purpose of this study was to determine the ability of the Norberg angle (NA) at ≥105° to predict a non-dysplastic hip based on a Distraction Index (DI) cut-off of ≤0.3 or a Dorsolateral Subluxation Score (DLS) cut-off of ≥55%. The manuscript was coauthored by AR Gaspar, G Hayes, C Ginja, M Ginja, R Todhunter and published in the specialty journal Preventive Veterinary Medicine 2016;135:47-52.

The fifth chapter, is an original study designed in collaboration with research teams from CIBIO/InBio - Centro de Investigação em Biodiversidade e Recursos Genéticos and Cornell University College of Veterinary Medicine, entitled "Genetic analysis of the Portuguese 'Cão da Serra da Estrela' dog breed using whole-genome SNPs". In this study, we aimed to carry out a comprehensive analysis of the within-breed genetic variation and

structure of CSE using whole-genome markers (i.e. single nucleotide polymorphisms, SNPs). Namely, the levels and patterns of genetic diversity and the putative population structure caused by the different breed uses (work vs pet) were investigated. Y-chromosomal markers were also used to investigate phylogenetic relationships and within-breed haplotype composition. Individuals of the distinct Iberian wolf population and Portuguese stray dogs were included in the analyses for comparison purposes. Preliminary results of these analyses were presented as a poster in the scientific meeting "*CONGENOMICS 2016 – Conference on conservation genomics"*, in May 3-6, 2016, at CIBIO/InBIO (Vairão, Portugal). A manuscript coauthored by AR Gaspar, A Muñoz-Mérida, AE Pires, R Godinho, J Hayward, R Todhunter, M Ginja and C Ginja, is being prepared for publication in the specialty journal Animal Genetics. It is imperative to first genetically characterize the CSE population to be able to pursuit an association genetic study for CHD traits in this breed, in which this condition has high prevalence.

The last chapter of this dissertation includes a brief discussion of the overall results, final considerations, and future perspectives concerning CHD diagnosis in the CSE breed.

2. Canine Hip Dysplasia

Canine hip dysplasia (CHD) is a relatively common skeletal disorder in which occurs an abnormal development of the hip and coxofemoral joint. This defect can affect mainly the health of large to giant breed dogs but it can also be seen in small breed dogs and cats (Janutta and Distl, 2006; Lopez and Schachner, 2015). Hip joint laxity (HJL) is the major risk factor for hip dysplasia leading to subluxation and poor congruence between the femoral head and acetabulum. Over time, degeneration of the joint occurs. Usually both hips are affected (Demko and McLaughlin, 2005; Ginja et al., 2010). Numerous factors influence the development and progression of hip dysplasia: genetics is considered determinant, and others aspects of environmental nature, such as rapid weight gain in growing animals can influence the disease severity (Kealy et al., 2000; Smith et al., 2001, 2006; Lopez and Schachner, 2015).

2.1. Pathogenesis

The hip capsule and chondro-osseous conformations, with two articulating surfaces – the acetabulum and the femoral head who grow in synchrony - are both major contributors to stability of the coxofemoral joint under load. For the normal development of the acetabular concavity it is fundamental the presence and pressing of the femoral head and its integrity. When these structures are affected, the joint becomes unstable (Alexander, 1992; Fries and Remedios, 1995; Todhunter and Lust, 2003).

The primary cartilage lesion results from focal stress in that area secondary to the abnormal magnitude and direction of load. Joint inflammation, pain, articular cartilage degeneration, and bone remodeling may occur. Abnormal weight bearing continues to cause excess wear on the articular cartilage and damages the underlying bone causing microfractures and sclerosis. The hip laxity eventually decreases as the capsule fibroses and the synovial effusion resolves (Alexander, 1992; Morgan, 1992; Fries and Remedios, 1995; Todhunter and Lust, 2003).

2.2. Clinical signs

The main clinical signs are hip pain, resulting in decreased activity, difficulty in rising, reluctance in running or climbing stairs, lameness, atrophy of the thigh muscles, hypertrophy of the shoulder muscles, crepitus, reduced hip joint motion and HJL. However, a number of stoic animals don’t show any clinical sign and this condition goes undetected to the owners (Fry and Clark, 1992; McLaughlin, 2001; Demko and McLaughlin, 2005).

The age of clinical detection varies depending on the trait's severity and the owner's awareness. It is assumed that dysplastic dogs during growth, around 8-12 months, often develop lameness or gain abnormalities. However, sometimes, the young dog will often spontaneously improve to normal function due to fibrosis that contains and stabilizes the joint improving clinical signs. By the time it becomes adult, and osteoarthritis is already severe, clinical signs resurge and the dog is presented for veterinary care in a late stage of the disease (Todhunter and Lust, 2003; Demko and McLaughlin, 2005).

2.3. Diagnosis

2.3.1. Physical examination

Clinical examination of an animal with a CHD suspicious should include a thorough retrieve of phenotypic information as well of the relevant history. A detailed orthopedic and neurologic examination is recommended to rule out other causes of pelvic limb lameness. Palpation and manipulation of the hip joint is an important step, both with the animal conscious and under anesthesia (McLaughlin, 2001; Ginja et al., 2010). Synovitis, joint capsule thickening, and articular injury are uniformly present (McLaughlin, 2001; Todhunter and Lust, 2003).

The Ortolani (Ortolani, 1976), Barlow (Barlow, 1962), and Bardens (Bardens and Hardwick, 1968) tests are a subjective evaluation of HJL. The first two were originally designed for diagnosis of human congenital hip dysplasia and then adapted for veterinary medicine. In most instances, general anesthesia is necessary to demonstrate these tests adequately (Ortolani, 1976; Fry and Clark, 1992). The Ortolani test will be clarified in detailed ahead in the review article (third chapter).

For the Barlow's test, while in lateral recumbency, the application of an axial pressure down the femoral shaft towards the coxofemoral joint produces subluxation. If the femoral head slips forward into the acetabulum the hip has been dislocated. Barlow's test is essentially a modification of the Ortolani test to be used in younger puppies, since, occasionally, when the hips are abducted the dislocated femoral head slides so smoothly over the low rim of the acetabulum that it does not make a click, and therefore appears to be normal (Barlow, 1962; Fry and Clark, 1992).

For the Bardens test, the dog is similarly positioned in lateral recumbency and the proximal femur is elevated laterally from the body. Simultaneously, a thumb or forefinger on one hand is placed over the greater trochanter of the femur while the other hand firmly grasps

the thigh, lifting it laterally without abduction. This displacement is measured and a positive sign is considered when the distance is greater than 2 mm (Bardens and Hardwick, 1968; Fry and Clark, 1992). These palpation techniques should be considered with other clinical data when attempting to diagnose CHD.

2.3.2. Radiographic examination

Radiograph has been the gold standard to assess and quantify joint changes associated with CHD and is the diagnostic method evaluated in the present study. It is possible to assess canine radiographic coxofemoral joint conformation and degenerative changes in adult animals or to estimate HJL in young dogs. Presently, for the first scenario, worldwide, there are 3 popular standardized scoring systems: *Fédération Cynologique Internationale* (FCI), Bristish Veterinary Association/ Kennel Club (BVA/KC) and Orthopedic Foundation for Animals (OFA). These systems are similar to each other and it is possible to establish correlations between them (detailed ahead in the review article, third chapter). To quantify HJL 2 techniques are commonly used: the PennHIP method and the dorsolateral subluxation (DLS) test.

The FCI grading scheme is majorly used in Europe, South America, Africa and Asia. Limb radiographs in standard ventrodorsal hip-extended view (SVDV) (Figure 1), performed at 1 year of age (18 months for large breed dogs), are scored by a radiologist approved by breed-specific kennel clubs using FCI scoring system. The Norberg angle (NA), the degree of subluxation, acetabular shape and depth and signs of secondary joint disease are taken in consideration. The hip joint is scored from A (normal) to E (severe dysplasia), being the final grade based on the worst hip (Table 1). The first two categories are considered non-dysplastic while the last three are dysplastic (Flückiger, 2007; Ginja et al., 2010; Verhoeven et al., 2012; Lopez and Schachner, 2015; Fédération Cynologique Internationale website, 2016a).

The BVA/KC scoring scheme is popular in Britain, Ireland, Australia and New Zealand. For scoring, the dogs must be at least 1 year of age and the criteria used are the NA, subluxation, acetabular shape and depth and femoral head and neck's shape and with eventual signs of degenerative joint disease (DJD) (Table 2). In a SVDV (Figure 1) both hips are individually classified with a score, 0-53 (the sum being 0-106), with higher values implicating worse hip status (Gibbs, 1997; Flückiger, 2007; Ginja et al., 2010; Dennis, 2012; Verhoeven et al., 2012; Lopez and Schachner, 2015; Kennel Club website, 2016).

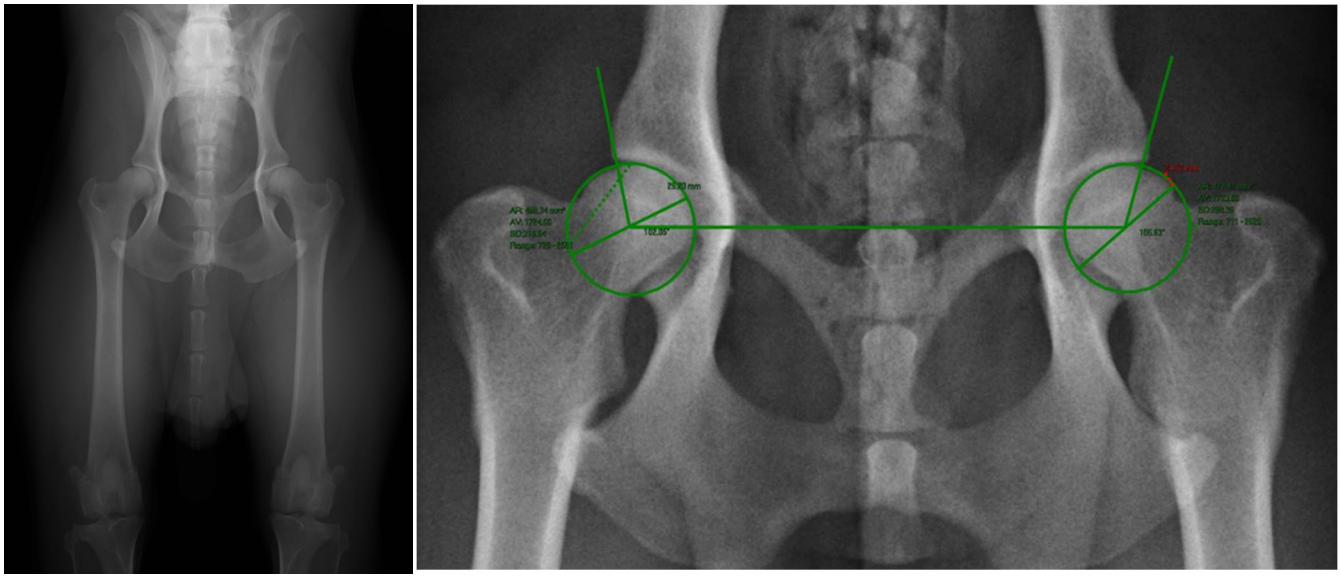

Figure 1: Standard ventrodorsal hip extended view (left) and Norberg angle calculation. Hospital Veterinário da Universidade de Trás-os-Montes e Alto Douro.

Table 1: *Fédération Cynologique Internationale* grading scheme for canine hip dysplasia (Flückiger, 2007).

A **No signs of CHD**	The femoral head and the acetabulum are congruent. The craniolateral acetabular rim appears sharp and slightly rounded. The joint space is narrow and even. The NA ≈ 105°. In excellent hip joints the craniolateral rim encircles the femoral head somewhat more in caudolateral direction.
B **Near normal hip joints**	The femoral head and the acetabulum are slightly incongruent and the NA ≈ 105° or the femoral head and the acetabulum are congruent and the NA<105°.
C **Mild CHD**	The femoral head and the acetabulum are incongruent, the NA ≈ 100° and/or there is slight flattening of the craniolateral acetabular rim. No more than slight signs of osteoarthrosis on the cranial, caudal, or dorsal acetabular edge or on the femoral head and neck may be present.
D **Moderate CHD**	There is obvious incongruity between the femoral head and the acetabulum with subluxation. The NA>90° (only as a reference). Flattening of the craniolateral rim and/or osteoarthritic signs are present.
E **Severe CHD**	Marked dysplastic changes of the hip joints, such as luxation or distinct subluxation are present. The NA<90°. Obvious flattenting of the cranial acetabular edge, deformation of the femoral head (mushroom shaped, flattening) or other signs of osteoarthrosis are noted.

CHD, canine hip dysplasia; NA, Norberg angle.

The OFA grading system is mainly represented in the United States of America and Canada. Standard ventrodorsal hip-extended views (Figure 1) performed at 2 years of age are assessed by three independent board-certified radiologists in excellent, good, fair, borderline, mild, moderate, or severe (Table 3). The first three categories are considered non-dysplastic while the last three are dysplastic. Hip conformation and evidence of DJD are considered (Flückiger, 2007; Ginja et al., 2010; Verhoeven et al., 2012; Lopez and Schachner, 2015).

The PennHIP method measures HJL which reveals the susceptibility for a dog to develop DJD at 16 weeks of age. Three different radiographic views are taken: a SVDV for evidence of DJD, a compression view for congruity between the femoral head and the acetabulum, and a distraction view to measure HJL (Figures 2 and 3). Joint laxity is estimated measuring the distraction index (DI), based on quantification of the relative displacement of the femoral head center from the acetabular center and it ranges from 0-1. This way passive HJL is measured in addition to subjection radiographic information. Dogs with looser hips ($DI \geq 0.7$) are more likely to develop hip dysplasia than dogs with tighter hips ($DI < 0.3$) (Smith et al., 1990, 1993; Smith, 1997).

The DLS test reveals HJL in a weight-bearing position at 4-8 months of age (Figures 4 and 5). With the hip joints in a normal standing orientation, the DLS measures the femoral head coverage by the lateral aspect of the cranial acetabular rim. It is assumed that DLS scores equal to or above 55% are related to low susceptibility to develop osteoarthritis while joints with less than 45% coverage of the femoral head have a higher probability of developing osteoarthritis (Farese et al., 1998; Todhunter et al., 2003a).

Table 2: British Veterinary Association/ Kennel Club scoring scheme for canine hip dysplasia (Flückiger, 2007; Dennis, 2012; Verhoeven, 2012).

Score	Norberg angle*	Subluxation	Cranial acetabular edge	Dorsal acetabular edge	Cranial effective acetabular rim	Acetabular fossa	Caudal acetabular edge	Femoral head and neck exostoses	Femoral head recountouring
0	+15 and over	Femoral head is well centred in the acetabulum	Even curve, parallel to the femoral head throughout	Slight curve	Sharp, clean cut junction of the DAE and CrAE	Fine bone line curves medial and caudal from the caudal end of the CrAE	Clean line	Smooth, rounded profile	Nil
1	+10 to +14	Femoral head centre lies medial to the DAE. The lateral or medial joint space is increased slightly	Lateral or medial quarter of the edge is flat and the lateral or medial joint spaces diverge slightly	Loss of S curve only in the presence of other dysplastic change	Indistinct junction of the DAE and CrAE	Slight increase in medial bone density. The 'fine line' is hazy or lost	Small exostosis at the lateral edge	Slight exostosis in 'ring form' and/or dense vertical line adjacent to the trochanteric fossa ('Morgan line')	Femoral head does not fit in a circle due to exostosis or bone loss
2	+5 to +9	Femoral head centre is superimposed on the DAE. There is an obvious increase in the medial joint space	Flat throughout most of its length	Very small exostosis cranially	Very small exostosis or very small facet	'Fine line' is lost and the ventral AE is hazy due to new bone. The notch at the CaAE is clear	Small exostosis at the lateral and medial edge	Slight exostosis visible on the skyline and/or density on the medial femoral head	Some bone loss and/or femoral head/neck ring of exostosis
3	0 to +4	Femoral head centre is just lateral to the DAE. Half of the femoral head is within the	Slight bilabiation	Obvious exostosis, especially cranially, and/or minor 'loss	Facet and/or small exostosis and/or slight bilabiation	Incomplete remodelling of the acetabulum, with the medial face lateral to	Large exostosis and narrow notch	Distinct exostosis in 'ring form'	Obvious bone loss and distinct exostosis giving a slight conical appearance

Score	Norberg angle*	Subluxation	Cranial acetabular edge	Dorsal acetabular edge	Cranial effective acetabular rim	Acetabular fossa	Caudal acetabular edge	Femoral head and neck exostoses	Femoral head recountouring
		acetabulum		of edge'		the AF. The ventral AE is lost, the AF is hazy and the notch is irregular			
4	-1 to -5	Femoral head centre is clearly lateral to the DAE. A quarter of the femoral head is within the acetabulum	Moderate bilabiation	Exostosis well lateral to the edge and/or moderate 'loss of edge'	Obvious facet and/or obvious exostosis and/or moderate bilabiation	Marked remodelling. The medial face of the acetabulum is clearly lateral to the AF. The ventral AE is lost and the notch is partly closed	Marked exostosis and 'hooking' of the lateral end	Obvious complete collar of exostosis	Gross remodelling. There is obvious bone loss and exostosis gives a mushroom-like appearance
5	-6 to -10	Femoral head centre is well lateral to, and just touches, the DAE	Gross bilabiation	Marked exostosis all along the edge and/or gross 'loss of edge'	Gross exostosis and/or facet and/or gross bilabiation	Gross remodelling, with dense new bone throughout the acetabulum. The CaAE notch is lost and the AF is obscured	Gross distortion due to mass of new bone in the acetabulum. The notch is lost completely	Massive exostosis giving a mushroom-like appearance	Very gross remodelling with marked bone loss and much new bone
6	-11 and over	Complete pathological	Entire edge slopes	Massive exostosis from	Complete remodelling.	Complete remodelling and	Void	Massive exostosis and	Femoral head is improperly shaped

Score	Norberg angle*	Subluxation	Cranial acetabular edge	Dorsal acetabular edge	Cranial effective acetabular rim	Acetabular fossa	Caudal acetabular edge	Femoral head and neck exostoses	Femoral head recountouring
		dislocation	cranially	the cranial to caudal edge	Massive exostosis and/or gross facet	new articular surface, well lateral to the AF. The notch is lost		infill of the trochanteric fossa and below the femoral head	due to maldevelopment of the femoral head centre

DAE, dorsal acetabular edge; CrAE, cranial acetabular edge; CaAE, caudal acetabular edge; AE, acetabular edge; AF, acetabular fossa; *relative to 90º.

Table 3: Orthopedic Foundation for animals grading system for canine hip dysplasia (CHD) (Flückiger, 2007; Verhoeven, 2012).

Excellent	Superior hip conformation in comparison to other animals of the same age and breed. There is a deeply seated femoral head which fits tightly into a well-formed acetabulum with minimal joint space. There is almost complete coverage of the acetabulum over the femoral head.
Good	Slightly less than superior but well-formed congruent hip joint. The femoral head fits well into the acetabulum and good coverage is present.
Fair	Minor irregularities are present. The hip joint is wider than a good hip phenotype due to slight subluxation causing a minor degree of joint incongruence. There may also be slight receding of the weight-bearing surface of the dorsal acetabular rim causing the acetabulum to appear slightly shallow
Mild CHD	The femoral head is partially subluxated causing an incongruent and widened joint space. The acetabulum is usually shallow only partially covering the femoral head. There are usually no arthritic changes present and if the dog is young (24 to 30 months of age), a second radiograph may be submitted for re-evaluation when the dog is older. Most dogs will remain dysplastic showing progressive degenerative joint disease.
Moderate CHD	There is significant subluxation present with the femoral head barely seated into a shallow acetabulum. There are secondary arthritic changes usually along the femoral neck and head (remodelling), acetabular osteophytes and various degrees of trabecular bone pattern changes (sclerosis).
Severe CHD	Radiographic evidence of marked CHD. There is significant subluxation with the femoral head partly or completely out of a shallow acetabulum. There are massive secondary arthritic bone changes along the femoral neck and head, acetabular rim changes and large amounts of abnormal bone pattern changes.

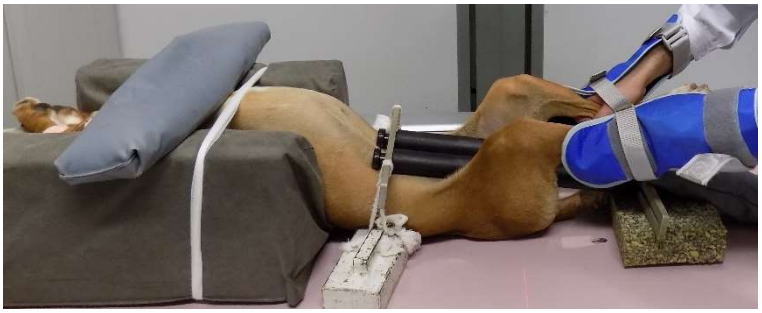

Figure 2: Animal placement for the PennHIP distraction view. Hospital Veterinário da Universidade de Trás-os-Montes e Alto Douro.

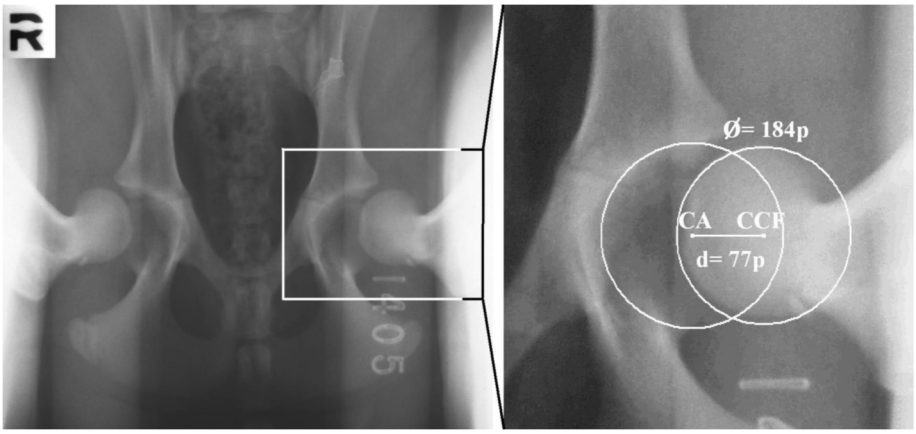

Figure 3: PennHIP distraction view and distraction index calculation. Hospital Veterinário da Universidade de Trás-os-Montes e Alto Douro.

Figure 4: Animal placement for the dorsolateral subluxation test radiograph. Cornell University Hospital for Animals.

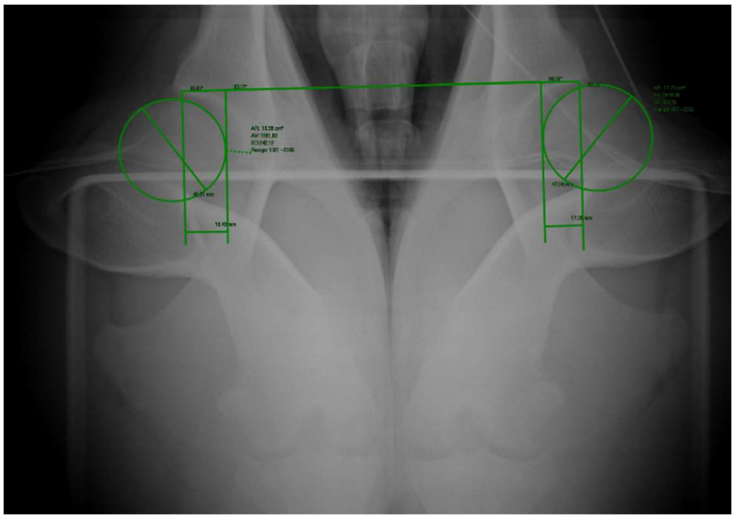

Figure 5: Dorsolateral subluxation score ilustration. Cornell University Hospital for Animals.

2.3.3. Other diagnostic techniques

There are other tests available for CHD diagnosis such as computed tomography imaging. This technique is becoming more popular, it has been used in several studies and it is more suitable to evaluate bone structure (Fujiki et al., 2007; Ginja et al., 2009; Andronescu et al., 2015). Magnetic resonance imaging can also be used, nevertheless its cost is more prohibitive and it is more appropriate to evaluate the structure of articular soft tissues (Ginja et al., 2009). The use of ultrasonography in dogs is not supported due to the non-congenital nature of CHD and early femoral head ossification preventing the correct visualization of the acetabulum (Greshake and Ackerman, 1993; Adams et al., 2000; Ginja et al., 2009). Examination of arterial blood supply to the hip joint with Doppler sonography was also investigated but not associated to HJL or hip dysplasia (Rademacher et al., 2005).

Molecular studies have been developed for CHD diagnosis searching genetic markers linked to *loci* responsible for different CHD phenotypes (Todhunter et al., 1999; Chase et al., 2004, 2005; Todhunter et al., 2005; Marschall and Distl, 2007; Phavaphutanon et al., 2009; Sánchez-Molano et al., 2014a; Fels and Distl, 2014; Fels et al., 2014). Genome-wide association studies, considering the joint effect of multiple SNPs, have been published by several researchers (Zhou et al., 2010; Pfahler and Distl, 2012; Lavrijsen et al., 2014; Fels et al., 2014; Hayward et al., 2016). Presently, there is a commercial DNA-based test for the Labrador Retriever breed that predicts the risk for the dog to develop CHD (Sanchez et al.,

2013). However, the applicability of such test to diagnose CHD in dogs from other breeds remains limited.

The cost of genomic techniques is becoming much more accessible. The availability of complete genomes from several breeds of dogs will increase. Also, the inexpensive genotyping by sequencing of genomic regions of interest will allow to characterize genetic variability in a wide range of breeds and to investigate more efficiently the regions of the genome that influence CHD traits in dogs.

The main purpose of the diagnosis and breeding schemes is to identify the individuals carrying CHD information in order to remove them from the breeding pool. This allows to eliminate carriers, to avoid the genetic inheritance associated with this disease and reduce its prevalence in future generations.

2.4. The Portuguese native dog breed 'Cão da Serra da Estrela'

One of the most popular Portuguese native dog breeds is "Cão da Serra da Estrela" (Estrela Mountain Dog), a large, mastiff-type molossoid dog recognized by FCI - number 173 (Fédération Cynologique Internationale website, 2016b) with approximately 500 to 600 registrations annually in the Portuguese Kennel Club (Clube Português de Canicultura website, 2016a).

Considered one of the oldest breeds in Portugal, this dog breed has been protecting flocks of sheep for many centuries. Shepherds depend on their ability to identify and scare off wolves and other predators. This breed has been developed over a period of hundreds of years. Shepherds would have chosen to breed the dogs that had the necessary characteristics to survive in their mountain environment and to do their job: large size, strength, endurance, agility, a deep chest, ability to tolerate a marginal diet, a powerful set of the legs and mouth, a warm coat, and a watchful, mistrustful, yet loyal temperament. The first CSE dog entered a show ring in 1908, and the official breed standard was established by 1933. In 1972, the United States of America became the first foreign country to receive CSE individuals outside of Portugal. Animals of this breed can now be found in several countries around the world. We can distinguish two different varieties – long-haired (Figure 6) mainly related to show and pet dogs and short-haired (Figure 7) being used mostly as work dogs (Pye, 2002).

In the CSE breed, which is characterized by large sized dogs, CHD is a frequent problem breeders have to face. A comprehensive radiology study of the CSE breed reported 65% of the animals affected (Ginja et al., 2009). For this reason, and for being one of the most

important Portuguese native breeds of shepherd dogs, it was decided to carry out this study to investigate the genetic basis for CHD traits.

Figure 6: 'Cão da Serra da Estrela' individuals - long-haired variety.

Figure 7: 'Cão da Serra da Estrela' individual - short-haired variety.

3. Emerging insights into the genetic basis of canine hip dysplasia

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3.1. Abstract

Canine hip dysplasia (CHD) is the most common inherited polygenic orthopedic trait in dogs with the phenotype influenced also by environmental factors. This trait was described in the dog in 1935 and leads to a debilitating secondary hip osteoarthritis. The diagnosis is confirmed radiographically by evaluating signs of degenerative joint disease, incongruence, and/or passive hip joint laxity. There is no ideal medical or surgical treatment so prevention based on controlled breeding is the optimal approach. The definitive CHD diagnosis based on radiographic examination involves the exposure to ionizing radiation under general anesthesia or heavy sedation but the image does not reveal the underlying genetic quality of the dog. Phenotypic expression of CHD is modified by environmental factors and dogs with a normal phenotype can be carriers of some mutations and transmit these genes to their offspring. Programs based on selection of dogs with better individual phenotypes for breeding are effective when strictly applied but remain inferior to the selection of dogs based on estimation of breeding values. Molecular studies for dissecting the genetic basis of CHD are ongoing, but progress has been slow. In the future, the recommended method to improve hip quality in controlled breeding schemes, which will allow higher selection pressure, would be based on the estimation of the genomic breeding value. Since 2012, a commercial DNA (deoxyribonucleic acid) test has been available for Labrador Retrievers using a blood sample and provides a probability for development of CHD but we await evidence that this test reduces the incidence or severity of CHD.

Keywords – canine hip dysplasia, phenotype, breeding stock, genome-wide association studies, screening

3.2. Introduction

Canine hip dysplasia (CHD) is the most common inherited polygenic orthopedic trait with the phenotype influenced by environmental factors (Ginja et al., 2010). This trait was described in the dog in 1935, in the USA, and leads to a debilitating secondary hip osteoarthritis (Ginja et al., 2009). Heritability estimates for CHD vary from 0.1 to 0.83 (Ginja et al., 2008; Zhu et al., 2009), due to different pedigrees, methods used to calculate the heritability, and the hip phenotypes analyzed (Silvestre et al., 2007). Canine hip dysplasia is more prevalent in large and giant breeds of dogs often resulting in mild or no clinical signs (Barr et al., 1987; Ginja et al., 2010). However, for some dogs, clinical signs can be severe and resistant to medical management needing aggressive and expensive surgical treatments (Manley et al., 2007; Ginja et al., 2010). The definitive diagnosis of CHD is made if characteristic radiographic signs are evident on a standard or stressed ventrodorsal view of the pelvis, occurring along a gradual scale from nearly normal to severely affected (Ginja et al., 2010). This is a crucial aspect of CHD as the radiographic diagnosis has been essential for the selection of breeding stock (Ginja et al., 2010). Studies attempting to find genetic markers for CHD diagnosis are now frequent (Chase et al., 2004; Todhunter et al., 2005; Fels and Distl, 2014; Sánchez-Molano et al., 2014a). The sequencing and annotation of the canine genome has resulted in renewed interest in research of the genetic underpinnings of canine orthopedic disorders, particularly those of a multifactorial etiology, such as CHD (Breur et al., 2012). Recently, the first commercial CHD diagnostic genetic test for Labrador Retrievers appeared (Sanchez et al., 2013), but, the imaging diagnosis continues to be of major importance for disease screening and treatment. Humans are also affected by hip dysplasia and both conditions have phenotypic similarities of joint subluxation and the development of osteoarthritis (Zhou et al., 2010). However, the main medical approach in humans is different, being mainly based on the preventive management and with good results (Wenger and Bomar, 2003; Ginja et al., 2010). Currently, molecular CHD studies are considered useful for the understanding of the genetic basis of analogous conditions in humans, mainly because the heterogeneity in human populations and the complexity of this disorder makes the genetic dissection of human hip osteoarthritis more difficult (Breur et al., 2012). The main purpose of this review is to present and discuss medical aspects of CHD for which knowledge is incomplete and have therefore merited major current research efforts.

3.3. Epidemiology, physical signs, and outcomes

Canine hip dysplasia continues to be a common trait mainly in large and giant breeds, in pet and working dogs, with prevalence higher than 50% in some breeds (Ginja et al., 2009). The clinical presentation of the disease is not correlated with the radiographic changes (Barr et al., 1987; Ginja et al., 2009). Clinical signs of CHD are more evident in dogs younger than 1 year of age due to hip instability, or in adult dogs with chronic pain from osteoarthritis (Riser, 1975; Manley et al., 2007). Chronic hip alterations, such as fibrosis and thickening of the joint capsule, result in joint stability and improvement of limb function, masking the clinical signs and functional limitations in middle-aged animals (Barr et al., 1987; Ginja et al., 2009). Clinical signs warrant medical and/or surgical treatment (Farrell et al., 2007; Vezzoni et al., 2008). Preventive conservative or surgical management could be indicated in puppies at risk of developing CHD (Lust et al., 1992; Vezzoni et al., 2008), but early intervention is hampered because there are no pathognomonic clinical signs for CHD. Common clinical signs are: slight to moderate lameness; gait and running abnormalities, such as shortened stride length and bunny hopping; difficulty in rising and reluctance to climb stairs (Fry and Clark, 1992).

3.4. Diagnosis – physical examination and imaging

Information about the conformation of the hip joint can be obtained using clinical or diagnostic imaging tests (Fry and Clark, 1992; Vezzoni et al., 2005). These medical tests are usually performed on sedated or anesthetized animals and are separated into two main categories: to evaluate hip joint laxity (HJL), mainly used on young animals; to detect clinical or radiographic signs of osteoarthritis, as crepitation and reduced range of motion on joint palpation or degenerative joint disease (DJD) signs on radiographs (Fry and Clark, 1992; Ginja et al., 2010). However, it would be very helpful to develop medical CHD screening techniques for fully conscious young animals, similar to hip dysplasia screening examination of human neonates (Ginja et al., 2010). The imaging diagnosis of CHD has been the main area of research of CHD in the last 50 years, for the purposes of reproductive control. Clearly, in terms of human medicine the main focus has been different, trying to refine diagnostic accuracy and preventive management (Wenger and Bomar, 2003; Ginja et al., 2010).

3.4.1. Physical examination

The Ortolani test is the most common and popular physical maneuver that is used in veterinary medicine to diagnose HJL in young dogs (4–12 months of age) (Chalman and Butler, 1985; Ginja et al., 2008). Other clinical tests are described, like the Barlow's and Barden tests for puppies younger than 4 months of age but their clinical accuracy is more questionable (Ginja et al., 2009; Ginja et al., 2010). The Ortolani test is performed with the dog awake, sedated or anesthetized with the patient in lateral or dorsal recumbency (Chalman and Butler, 1985). The test has two steps, first apply proximal force to the stifle joint on the non-dependent limb, with the hip at a normal weight-bearing angle, and while still applying this force, slowly abduct the joint. In hips with abnormal laxity, the dysplastic femoral head, may be displaced dorsally beyond the dorsal acetabular rim (in the first step, Figure 8A) and then the limb abduction promotes its reduction back into the acetabulum (in the second step, Figure 8B) which elicits a typical palpable and/or audible clunk, of variable magnitude, commonly called a positive Ortolani sign (Chalman and Butler, 1985; Ginja et al., 2010). Advanced stages of CHD with destruction of the acetabular rim or in dogs younger than 4 months of age with an inadequate acetabular ossification can result in false negative cases based on the Ortolani maneuver even though HJL may be present (Puerto et al., 1999; Ginja et al., 2008; Ginja et al., 2009). The Ortolani test showed an excellent sensitivity in prediction of CHD when used in dogs younger than 1 year of age that later developed moderate or severe CHD (Ginja et al., 2008).

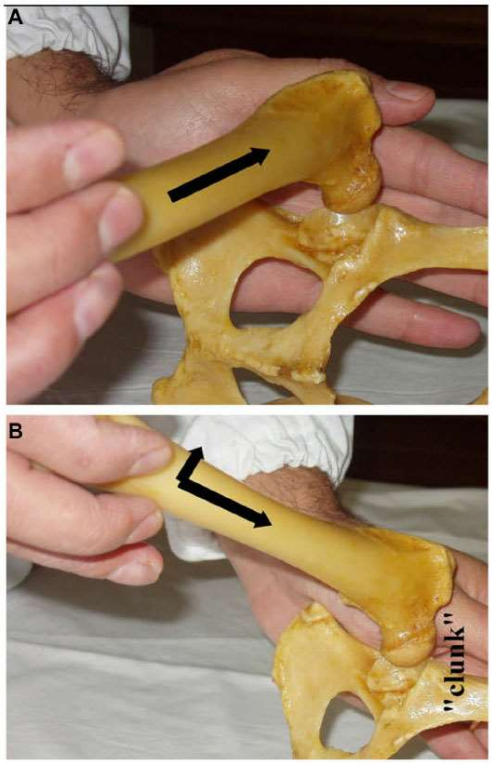

Figure 8: Ortolani test performed with the dog in lateral recumbency. Reprinted from Ginja et al. 2010. Copyright with permission from Elsevier.

3.4.2. Diagnostic imaging

Radiography is the reference technique for the definitive diagnosis of CHD from its first description in 1935. This imaging technique uses different radiographic views of the hip joint for genetic screening purposes or for diagnosis and treatment of dogs with clinical CHD. All these radiographic techniques should be performed under anesthesia or heavy sedation, which facilitates accurate positioning and elicitation of passive HJL (Manley et al., 2007; Ginja et al., 2008; Vezzoni et al., 2008; Ginja et al., 2010). Given the complexity of the topic and the objectives of this review, we will cover particularly the radiographic studies used for genetic screening of CHD, which are used to detect HJL, the major risk factor for CHD, or signs of DJD.

The radiographic information on HJL is obtained using radiographic techniques such as PennHIP (Smith et al., 1990), dorsolateral subluxation (DLS) (Farese et al., 1998), Flückiger (Flückiger et al., 1999) and half-axial position methods (Vezzoni et al., 2005). Signs of DJD are evaluated using the standard ventrodorsal hip-extended view (SVDV) (Corley, 1992; Gibbs, 1997; Ginja et al., 2009).

3.4.2.1. Radiographic estimation of hip joint laxity

In this group of methods, the PennHIP was the pioneer and is the most popular. It was developed at the University of Pennsylvania in the 1980s with the main purpose of CHD breeding control (Smith et al., 1990). One of the main advantages of this procedure is its precocity, being performed with accuracy on dogs at 16 weeks of age, compared with 1 or 2 years of age for previous screening systems (Smith et al., 1990; Corley, 1992; Gibbs, 1997). The PennHIP method requires certified members and is performed with three hip radiographic views: hip-extended, compression, and distraction (Smith et al., 1990). The distraction view is used to measure HJL. It is performed with the dog in dorsal recumbency, with the hips at a neutral position and the PennHIP distractor between hind limbs acting as a fulcrum lateralizing the femoral heads under examiner force (Smith et al., 1990). The radiographs are sent to the PennHIP Analysis Center at University of Pennsylvania for an official report, and the dogs are placed in rank-order with the other dogs of the breed in the database. The HJL is evaluated in the distraction view calculating the distraction index (DI), which measures the relative degree of femoral head displacement from the acetabulum (Smith et al., 1990; Ginja et al., 2008). The DI ranges from 0 to >1, with 0 representing a tight hip and 1 a loose hip (Smith et al., 1990).

The DLS is also a passive stress radiographic or tomographic imaging technique; the HJL is measured as the DLS score (Farese et al., 1998). The hip stress is caused by weight bearing. The DLS score has a strong correlation with DI. This method was reported in dogs at 4 and 8 months of age to evaluate the chondro-osseous acetabular and femoral head structure as an indicator of functional joint stability (Farese et al., 1998, 1999).

In the Flückiger method, the HJL is estimated with the subluxation index in a similar manner to the DI (Flückiger et al., 1999). The stress in hip joints is caused by the dorsocranial force exerted by the examiner, with the dog in dorsal recumbency (Flückiger et al., 1999). No follow-up studies were published and the research was performed in adult animals together with the SVDV to better assess the quality of the hips.

The half-axial position method was used by positioning the dog and performing the hip stress similar to the PennHIP, using a trapezoidal-shaped distractor (Vezzoni et al., 2005). The HJL measures using this method are performed mainly with the purpose of early CHD diagnosis and treatment, using the juvenile pubic symphysiodesis (JPS).

3.4.2.2. Radiographic evaluation of DJD

The DJD is evaluated using the SVDV, a universal radiographic view used in dogs older than 1 (*Fédération Cynologique Internationale*'s [FCI] system) or 2 years (Orthopaedic Foundation for Animals [OFA] system) of age (Corley, 1992; Ginja et al., 2010). This view has been used since the 1960s (Whittington, 1961). The dog is placed in dorsal recumbency on the X-ray table, with hind limbs extended parallel to each other with the stifles internally rotated (Whittington, 1961; Ginja et al., 2010). Many international systems are used to evaluate DJD, including the FCI (Ginja et al., 2010), OFA (Corley, 1992), British Veterinary Association/Kennel Club (BVA/KC) (Gibbs, 1997), and the Flückiger (Flückiger, 1994) method with more influence in continental European countries, USA, UK and Australia, and Switzerland, respectively. As the main guideline of all of these scoring methods is based on the degree of subluxation, joint congruence and remodeling of the femoral head and acetabulum, these scoring systems may be equal (Table 4). However, these direct comparisons between grades and schemes are considered speculative, due to their subjective nature (Soo and Worth, 2015). These scoring systems have some particularities: FCI requires a minimum age of 12 months in medium breeds and the OFA method 24 months; FCI scrutinizers are not certified; FCI, OFA, and BVA/KC are voluntary screening schemes. The SVDV is not strongly evaluative of HJL. It is underestimated. The parallel hip-extended positioning and the internal rotation of stifles twist the hip soft tissues, tightening the tensile elements of the joint capsule and may reduce some degree of luxation (Smith et al., 1990).

Table 4: Comparison of four canine hip dysplasia scoring systems: *Fédération Cynologique Internationale* (FCI), Orthopaedic Foundation for Animals (OFA), British Veterinary Association/Kennel Club (BVA/KC) and Flückiger method.

FCI	OFA	BVA/KC	Switzerland (Flückiger method)
	Excellent	0-4 (no >3/hip)	
A	Good	5-10 (no >6/hip)	0-2
	Fair	11-18	
B	Borderline	19-25	3-6
C	Mild	26-35	7-12
D	Moderate	36-50	13-18
E	Severe	51-106	>18

3.4.2.3. Other imaging techniques

Ultrasonography in human neonates is the reference technique for the definitive diagnosis of developmental hip dysplasia (Gerscovich, 1997). However, the use of ultrasound in puppies for the confirmation of CHD is not recommended, as the acetabulum cannot be evaluated after 8 weeks of age because femoral head ossification and acetabular chondro-osseous alterations are only evident after this age (Ginja et al., 2009). Increased synovial fluid volumes in hip joints detected by magnetic resonance imaging in 8-week-old puppies were correlated with later HJL and CHD (Ginja et al., 2009). Dynamic ultrasonography was used in puppies at 8–16 weeks of age, to quantify HJL (O´Brien et al., 1997). Hip joint laxity and osseous acetabular structure can be evaluated confidently using computed tomography (Farese et al., 1998).

3.5. Prevention and treatment

Some preventive conservative and surgical treatments have been proposed for young dogs with clinical predisposition for CHD (Manley et al., 2007; Vezzoni et al., 2008). The main conservative management recommendations are based on limiting food consumption and controlled weight-bearing activity to prevent obesity and develop muscular tissues (Kealy et al., 2000; Ginja et al., 2010). Disease-modifying osteoarthritis drugs given by injection are recommended, as they retard breakdown and may promote the synthesis of cartilage matrix and reduce pain and inflammation (Lust et al., 1992). Analgesic or anti-inflammatory medications are effective to manage pain and lameness but should only be used for the short term due to their undesirable side effects. However, the long-term effectiveness of this conservative treatment is questionable, since their ability to prevent the development and progression of osteoarthritis is at best limited (Manley et al., 2007; Vezzoni et al., 2008).

Juvenile pubic symphysiodesis is a surgical treatment used on puppies at 14–20 weeks of age and at risk of developing CHD (Patricelli et al., 2002; Vezzoni et al., 2008), with greater improvements achieved when surgery is performed at 15 weeks of age (Patricelli et al., 2002). Juvenile pubic symphysiodesis is a minimally invasive procedure based on induction of thermal necrosis in chondrocytes of the growth plate of the pubis (Patricelli et al., 2002; Vezzoni et al., 2008). The pubic growth plate undergoes premature closure resulting in an underdeveloped ventral pelvis and normal dorsal development (Manley et al., 2007). This modified pelvic growth results in an increase in acetabular coverage of the femoral head and reduction of subluxation forces (Patricelli et al., 2002). This technique has been recommended

in puppies with slight to moderate signs of CHD but not in animals with severe signs of CHD. In the JPS, acetabular ventroversion occurs slowly and in severe cases of CHD the femoral head continues to slide laterally, the dorsal acetabular edge becomes round and femoral head stability is never obtained (Vezzoni et al., 2008).

Triple pelvic osteotomy is a reasonable surgical treatment option for CHD being used in animals between 5 and 12 months old, without radiographic signs of DJD and with minimal or clinical signs of CHD (Manley et al., 2007). However, triple pelvic osteotomy is more effective in preventing the development of DJD when used in dogs younger than 7 months of age (Manley et al., 2007). The pelvis is cut in the pubis, ischium, and ilium, rotated and the ilium fixed with a surgical plate. This surgical procedure results in ventrolateral rotation of the acetabulum and provides immediate increased femoral head stability. However, the hips of dogs with extant osteoarthritic changes or with a high HJL continue to deteriorate and have a less favorable outcome (Manley et al., 2007). When osteoarthritis is already at an advanced stage, treatment should be performed to alleviate pain and maintain joint function (Johnson et al., 1998; Farrell et al., 2007).

Femoral head and neck excision reduces the pain produced by abnormal bone to bone hip joint contact, but it does not effectively maintain the full range of hip motion and limb function. The total hip replacement is the best treatment to preserve long-term limb functionality.

3.6. Genetic and environmental factors in the pathogenesis of canine hip dysplasia

Canine hip dysplasia is a complex polygenic disease due to the small additive effect of many genes (Chase et al., 2004; Zhu et al., 2009). Environmental factors such as sex, age, and body weight can influence the expression and severity of the disease (Kealy et al., 2000). It appears that CHD is not a congenital disease, hips are normal at birth with adequate femoral head and acetabular congruence. The first 60 days of a puppy's life is thought to be the most critical period in terms of development of the hip joint (Riser, 1975). In this period, the depth of the acetabular cavity and the proximal femoral head and neck conformation are susceptible to modeling according to the stress loading (Wenger and Bomar, 2003).

In a normal congruent hip joint, the normal weight bearing force is transmitted between femoral head and acetabulum across the surface of the articular cartilage. The joint incongruence favors the reduction of contact between the cartilaginous surfaces, the early

destruction of chondrocytes by increasing pressure and the cyclic cascade of osteoarthritis. Small synovial joint volume and low intracapsular pressure, high pelvic muscle mass, and a reduced level of the hormones that promote soft tissue relaxation maintain stability and prevent the development of CHD signs (Smith et al., 1990). The HJL is the primary risk factor, well-evaluated in a radiographic study that is associated with CHD development (Smith et al., 2001). Acetabular and proximal femoral head and neck conformation are directly associated with the magnitude of transmitted hip forces (Weigel and Wasserman, 1992). So, genetic factors associated with CHD can be related to hip conformation, cartilage susceptibility to pressure forces, joint soft tissues or even to hormonal factors.

Published heritabilities of CHD traits are variable and commonly range between 0.1 to 0.60 (Breur et al., 2012; Hou et al., 2013). Differences of heritability estimates depend on the trait used, calculation method, selection, the population and sample used for estimation (Silvestre et al., 2007; Hou et al., 2013). For example, heritability reached as high as 0.83 for passive hip laxity in the Estrela Mountain dog breed from Portugal (Ginja et al., 2008). The genetic improvement used in selection of traits with higher heritabilities and similar selection pressures will be bigger per generation (Ginja et al., 2010).

3.7. Selection of breeding stock with low hip scores – progress toward reducing incidences of hip dysplasia

Because there is no ideal medical or surgical treatment, veterinarians in practice are at the forefront, and their main focus is on prevention of CHD through reproductive control (Sánchez-Molano et al., 2014b). Selection of breeding stock has clearly been a priority intervention area of veterinary medicine. Control CHD programs based on radiographic phenotype quality of hips were used in some countries as early as the 1960s (Leighton, 1997; Swenson et al., 1997; Leppänen et al., 2000). These programs were based on radiographic screening of CHD using the SVDV and scores of hip quality based on DJD signs and hip congruence. Selection of breeding stock was based on an individual dog's hip phenotype and subjective pedigree evaluation performed by the breeder. The results of phenotype-based genetic screening on CHD prevalence and severity are somewhat disparate in different countries and breeds. When the CHD control schemes are voluntary, as the OFA, FCI, and BVA/KC, only the better hip phenotypes are scrutinized and the studies performed using these databases are biased (Ginja et al., 2009; Hou et al., 2013). Using the OFA scoring scheme and the best linear unbiased prediction method for the estimation of breeding values

and its application in the selection for hip joint conformation, for Labrador Retrievers the total genetic improvement in four decades (1970–2007) corresponded to only ~17% of the total phenotypic standard deviation (Breur et al., 2012). However, radiographs of dogs with normal-appearing hips are several times more likely to be submitted for evaluation in the OFA system than radiographs of dogs that are severely dysplastic (Paster et al., 2005). For example, in a closed breeding colony of German Shepherd dogs and Labrador Retrievers, CHD prevalence over five generations of selection decreased from 55% to 24%, and from 30% to 10%, respectively (Leighton, 1997). Selective breeding against CHD was less effective in decreasing its prevalence and severity in Sweden (Swenson et al., 1997). In other countries, such as Finland, the general CHD control program was even considered ineffective in reducing the prevalence of CHD in various dog breeds (Leppänen et al., 2000). For the PennHIP method, there are no reports of its effectiveness in reducing the prevalence of CHD in different dog populations. Theoretically, it is a promising method since studies have shown that HJL has a higher heritability than the CHD scores based on DJD. The lack of desired success of CHD control programs that rely totally on individual phenotype has been due partly to its sensitivity to environmental factors. Animals with a normal individual radiographic phenotype can still be carriers of CHD genes, which will be transmitted to their offspring and maintained in the population (Mäki et al., 2000). Estimated breeding values (EBV) are commonly used in farm animal selection for complex polygenic traits (phenotype expression is influenced by environmental factors), such as milk yield or growth rate (Flückiger et al., 1999; Hou et al., 2010). So, the phenotypic expressions of these traits are very similar to CHD, determined by heredity and environment. The EBV for CHD is a genetic parameter derived from the hip quality of relatives, and is thus more representative of the dog's genetic quality (Swenson et al., 1997; Breur et al., 2012), and allows monitoring of the genetic trends in dog populations (Wilson et al., 2011), being recommended for CHD selection purposes (Malm et al., 2013; Wilson et al., 2013).

With the rapid development of high-throughput sequencing technology and emergence of high-density genome-wide single nucleotide polymorphisms (SNPs) canine arrays, associations between genetic markers in linkage disequilibrium and CHD genes have been discovered (Zhou et al., 2010; Hou et al., 2013). The molecular genetic information can be applied for CHD selection purposes, particularly if the most informative SNPs are used to estimate the genomic breeding value (GBV) of an individual. Such genomic selection was successfully applied in livestock animal breeding programs and can be used for selection

against prevalence of undesired traits with greater genetic improvement (Guo et al., 2011; Hou et al., 2013; Sánchez-Molano et al., 2014a; Sánchez-Molano et al., 2014b). In the near future, GBV might become the recommended method to improve hip quality in CHD control schemes (Guo et al., 2011; Breur et al., 2012; Sánchez-Molano et al., 2014b). In a particular dog breed, pedigree and phenotypic data can be used to obtain EBV and combined with genomic data to derive a predictive formula for the GBV (Breur et al., 2012). Then, the genotyping of a puppy for a set of informative SNPs can be combined with radiographic information, and used to determine the susceptibility to CHD and make decisions regarding breed management.

3.8. Genome-wide association studies and genetic analyses in the identification of susceptibility alleles

The genetic etiology of CHD has been proven and accepted by the scientific community (Leighton et al., 1977). The first molecular studies for CHD diagnosis were developed by Todhunter et al. in the 1990s at Cornell University, who began by searching for molecular genetic markers that were linked to quantitative trait *loci* (QTL) responsible for different CHD phenotypes (Todhunter et al., 1999, 2005). To optimize the linkage of genes to CHD traits an outcross between breeds with high and low susceptibility to develop CHD, Labrador Retrievers and Greyhounds, respectively, was implemented. Twelve chromosomes were identified to harbor putative QTL for different CHD traits (Todhunter et al., 2005). Quantative trait *loci* were also associated to the Norberg angle (Chase et al., 2004) and acetabular osteophyte formation (Chase et al., 2005). Recently, more QTL were associated with other CHD traits (Marschall and Distl, 2007; Sánchez-Molano et al., 2014a).

Pedigree and CHD phenotype analysis showed some evidence of a major QTL (contributing about 20% of variance) associated to CHD in several studies (Leighton, 1997; Todhunter et al., 2003; Mäki et al., 2004; Janutta et al., 2006).

However, the QTL region may contain hundreds of genes and the identification of genes remains problematic (Zhu et al., 2009). The strategy that is followed by some researchers is to refine the QTL interval using SNPs and across-breed-mapping thus reducing the linkage disequilibrium interval (Zhu et al., 2009). Unrelated affected animals with CHD probably share more common disease alleles than an unaffected dog population (Zhu et al., 2009). The associated SNPs might be physically next to the responsible gene (Zhou et al., 2010). Genome-wide association studies consider the joint effect of multiple SNPs, being

much more effective than individual SNP analysis, in the identification of common genetic variants for complex diseases (Zhou et al., 2010). In eight different dog breeds using genome-wide association studies, four SNPs were significantly associated with CHD on CFA3, 11, and 30, and two with osteoarthritis on CFA17 and 37 (Zhou et al., 2010). In German Shepherd dogs, 13 SNPs were also associated with CHD on chromosome CFA14 and 37 (Pfahler and Distl, 2012), CFA19, 24, 26, and 34 (Fels and Distl, 2014), and CFA3, 9, 26, 33, and 34 (Fels et al., 2014). In other recent studies on Labrador Retrievers, four SNPs were associated with CHD on chromosome CFA1 and 21,10 and 31 SNPs on CFA1, 5, 8, 15, 20, 25, and 32 positioned within or in the vicinity of 24 different genes (Table 5) (Lavrijsen et al., 2014). Candidate genes involved in hypertrophic differentiation of chondrocytes and extracellular matrix integrity of basement membrane and cartilage were located in significantly associated regions on CFA1, 8, 20, and 25 (Lavrijsen et al., 2014). These results confirm the complex genetic architecture of CHD, based on many genes with small individual effect, which encourages circumspection about a marker-assisted, accurate CHD diagnostic test in the near future. The immediate importance of CHD molecular diagnosis will probably be their use in genomic (many markers assessed for their combined contribution) selection.

One mutation in the FBN2 gene on CFA11 chromosome was significantly associated with CHD in Labrador Retrievers and other dog breeds (Friedenberg et al., 2011). However, other genes must be involved in CHD because the FBN2 locus only explains a small part of the genetic trait variation in CHD (Friedenberg et al., 2011). Studies on QTL and/or SNPs associated with other phenotypes of CHD, such as the passive hip laxity, could provide additional information on the genetic basis of this condition. Passive hip laxity is the highest risk factor for CHD and is the trait associated with the highest heritability (Smith et al., 2001; Ginja et al., 2008).

Genetic studies regarding the developmental hip dysplasia in humans were unable to make much progress, so knowledge on the *loci*-linked hip dysplasia in humans is still limited (Breur et al., 2012). Despite recent developments in whole-genome analysis in humans, with the finding of a number of genetic variants associated with this condition in affected patients (Feldman et al., 2010, 2014), understanding the genetics of hip dysplasia in humans can benefit from similar studies in the dog.

Table 5: Summary of the single nucleotide polymorphism (SNP) and nearby candidate genes identified in six genome-wide association studies of canine hip dysplasia (CHD) and other related traits in reference breeds (Zhou et al., 2010; Pfahler and Distl, 2012; Fels et al., 2014; Fels and Distl, 2014; Sánchez-Molano et al., 2014a; Lavrijsen et al., 2014).

SNP/trait	CFA	Position (bp)	Allele	Distance (Mb)	Gene(s)	Reference/breed
BICF2S2459425/ CHD	3	74720873	G	0.900	EVC, EVC2	Zhou et al., 2010
BICF2P550340/CHD	11	32935770	T	0.220	PTPRD	eight different breeds
BICF2S23432143/CHD	11	57517597	G	0.394	COL15A1	
BICF2P799261/CHD	30	13883057	C	0.109	MAGP1	
BICF2G63020552/OA	17	48092910	T	0.060	REG3A	
BICF2P1242205/OA	37	17299306	C	0.070	PARD3B	
BICF2P1089246/CHD	14	23811133	C	0.183	PON2	Pfahler and Distl, 2012
BICF2P1282232/CHD	14	59537633	T	-	-	Bernese Mountain dog
BICF2S23052396/CHD	37	25095511	A	0.361	FN1	
G:37139132G>A /CHD	3	77186	A	-	PGM2	Fels et al., 2014
BICF2S22937555/CHD	9	54312	G	-	-	Germans shepherd dog
BICF2P844355/CHD	26	32016	A	-	-	
G:973418T>G/CHD	33	3975	T	-	EPHA3, EPHA6, and	
G:4052195T>C/CHD	33	7022	T	-	PCNP	
G:7967386T>C/CHD	33	10983	C	-		
G:414871G[A/CHD	34	3422	A	-	TRIO, SEMA5A,	
G:4846215A>G/CHD	34	7851	G	-	SLC6A3 and FGF12	
G:11239992A>G/CHD	34	14246	A	-		
G:11547417T>G/CHD	34	27034	G	-		
TIGRP2P265674/ CHD	19	35.533	C	-	-	Fels and Distl, 2014
BICF2S2367279/ CHD	24	28.944	G	-	SRC	Germans shepherd dog
BICF2P281364/CHD	26	17.181	G	-	KSR2	
BICF2P1086886/CHD	34	4.239	T	0.7	TRIO	
BICF2P355865/CHD	34	39.346	A	-	-	
BICF2P219706/Left CrAE	1	100106009	G	Genes in region 99Mb-110Mb	SHC3, SEMA4D, OMD, OGN, PHF2,	Sánchez-Molano et al., 2014a Labrador retriever
BICF2S2443186/Left CrAE	1	100138261	A		BARX1, ZNF677	
BICF2P1285984/Left CrAE	1	107719908	G			

SNP/trait	CFA	Position (bp)	Allele	Distance (Mb)	Gene(s)	Reference/breed
BICF2P429643/Right NA	21	43337454	G	1.5	OTOG, SOX6, SAA, MYOD1, SERGEF	
-/CHD	1	70938018	T/A	Intron	LAMA2	Lavrijsen et al., 2014
-/CHD	1	70997779	A/T	Exon, synonymous	LAMA2	Labrador retriever
-/CHD	5	59194609	G/–	3′-UTR	KLHL17	
-/CHD	5	62929475	C/T	5′-UTR	NPHP4	
-/CHD	8	29247021	C/T	3′-UTR	LRR1	
-/CHD	8	31496895	C/T	Downstream	PTGDR	
-/CHD	8	31496910	C/T			
-/CHD	20	45260332	A/G	Exon, non-synonymous	LTF	
-/CHD	20	46671813	C/G	Intron	PBX4	
-/CHD	20	46706734	A/G	3′-UTR	CILP2	
-/CHD	20	47636184	G/A	3′-UTR	GDF15	
-/CHD	20	47714388	G/A	5′-UTR	LSM4	
-/CHD	20	48071192	G/A	Upstream	INSL3	
-/CHD	20	48243399	G/–	3′-UTR	NA	
-/CHD	20	48804130	G/A	Exon, synonymous	NWD1	
-/CHD	20	49019261	T/C	Intron	CHERP	
-/CHD	20	49318725	C/T	Exon, synonymous	CIB3	
-/CHD	25	47629272	C/T	Upstream	INPP5D	
-/CHD	25	47666842	G/A	Exon, synonymous	INPP5D	
-/CHD	25	47685188	C/T	Intron	INPP5D	
-/CHD	25	48075297	C/G	Exon, non-synonymous	LOC100688622	
-/CHD	25	48076278	G/A	Downstream	LOC100688622	
-/CHD	25	48396330	C/T	Intron	SPP2	
-/CHD	25	50050076	G/A	Downstream	ASB18	
-/CHD	25	50228063	C/G	Intron	IQCA1	
-/CHD	25	51029326	G/A	Intron	COL6A3	
-/CHD	25	51031100	A/G	Exon,	COL6A3	

SNP/trait	CFA	Position (bp)	Allele	Distance (Mb)	Gene(s)	Reference/breed
-/CHD	25	51040259	A/G	synonymous		
-/CHD	25	51046607	G/A			
-/CHD	25	51736576	T/C	Upstream	HES6	
-/CHD	32	11265116	–/T	Downstream	LOC487839	

OA, osteoarthritis; CrAE, cranial acetabular edge; CFA, Canis familiaris autosome; UTR, untranslated region

3.9. Prospects for the development of commercially available DNA tests for screening and diagnosis

A DNA-based test for CHD is a desirable tool for early identification of dogs susceptible or resistant to the disease. In 2012, such a test was registered by Bioiberia. Called Dysgen, it was the first commercial marker-based DNA test for susceptibility to CHD in the Labrador Retriever breed. This test analyzes blood samples using a DNA kit containing seven SNPs (Sanchez et al., 2013). The Dysgen diagnosis is reported as a prediction, classifying the dog into a risk group for developing CHD – minimal, low, moderate, and high. However, the performance of the Dysgen diagnosis test was not independently tested and there are no published studies reporting its success in the control for CHD at the population level. Breeding of dogs with minimal or low risk of developing CHD is recommended by the manufacturer. This is a first step in the molecular diagnosis of CHD, but until all the genes involved in the disease are detected, CHD control programs continue to require the combination of an accurate phenotype screening, EBV, and the information of available genetic tests. Particularly, if the heritability of the trait is low, effectiveness of selection will benefit from combining information on major gene genotypes and EBV. Moreover, if we consider that CHD affects a rather large group of distinct dog breeds, from the Alaskan Malamute to the Portuguese Water Dog and which are raised in different environments, we require a deep understanding of the genetics underlying the incidence of this condition at the population level.

3.10. Conclusion

Despite phenotypic screening and breeding programs, CHD continues to be one of the most common orthopedic hereditary diseases in dogs. There is no ideal diagnosis or treatment for CHD and reproductive control schemes have been, in the last 50 years, a priority area of veterinary medicine to deal with the disease.

The genetic architecture of CHD is complex, as the many associated genes have small individual effect. This fact makes the development of a marker-assisted accurate CHD diagnosis test difficult, despite intensive research worldwide.

The molecular diagnosis of CHD will be based on genomic selection until all contributing and critical mutations are identified, and may have a significant impact for a better understanding of the genetic basis of similar conditions in humans.

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4. The Norberg Angle is not an Accurate Predictor of Canine Hip Conformation Based on the Distraction Index and the Dorsolateral Subluxation Score

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4.1. Abstract

Canine hip dysplasia is a common complex trait characterized by abnormal hip joint development. Hip joint laxity, an early characteristic of hip dysplasia, results in degeneration of the joint due to mechanical trauma, which is a clinical problem mostly in medium to large breed dogs. Clinical sings include pain, decreased activity and lameness. Imaging has been the gold standard to assess and quantify the joint changes associated. A retrospective multi-center cross-sectional study was performed to determine if a Norberg angle of ≥105° accurately predicts a non-dysplastic hip based on a distraction index cut-off of ≤ 0.3 or a dorsolateral subluxation score cut-off of≥55%. The Norberg angle, distraction index and/or dorsolateral subluxation score were measured on pelvic radiographs from 437 dogs with canine hip dysplasia screening radiographs. The predictive capacity of the Norberg angle against a distraction index ≤ 0.3 or a dorsolateral subluxation score≥55% was assessed using area under the receiver operator characteristic curve analysis. The area under the curve of Norberg angle for the prediction of a distraction index ≤ 0.3 was 0.59, 95% CI=0.50-0.69 and for the prediction of dorsolateral subluxation score≥55% was 0.69, 95% CI=0.63-0.75. Optimizing the specificity of the Norberg angle to≥80% for prediction of a distraction index ≤ 0.3 and a dorsolateral subluxation score≥55% gave a cutpoint for the Norberg angle of≥112º and 108.7º, respectively. The Norberg angle, the distraction index and the dorsolateral subluxation score measure different aspects of canine hip dysplasia. In conclusion, at the commonly used cutpoint of 105°, the Norberg angle is not an accurate measurement of normal hip conformation. The specificity of a non-dysplastic diagnosis was maximized with a

Norberg angle cut-off of 112º (distraction index) or 109º (dorsolateral subluxation score). Clinically, application of screening methods for CHD based on hip laxity, such as the distraction index or the dorsolateral subluxation score, would help to remove dysplastic susceptible dogs from the breeding pool.

Keywords – canine hip dysplasia, Norberg angle, distraction index, dorsolateral subluxation

4.2. Introduction

Canine hip dysplasia (CHD) is an inherited, developmental trait with both genetic and environmental factors such as sex, age, growth rate, and body condition affecting its expression (Zhang et al., 2009; Powers et al., 2010; Wilson et al., 2011). First described in 1935 (Schnelle, 1935), it has been the subject of intensive research and methods for reproductive control because it is one of the most common, and clinically-important, orthopedic traits of the dog. Canine hip dysplasia continues to be prevalent in large and giant dog breeds reaching 48.1% in St. Bernard, 47.6% in Boykin Spaniel (Corley, 1992), 59.7% in Cane Corso (Genevois et al., 2008), 65.8% in Serra da Estrela Dogs (Ginja et al., 2009b), 57% in German Shepherd (Grosu et al., 2013), and 29.6% in Molossoid breeds (Lavrijsen et al., 2014).

The diagnosis of CHD is worldwide based on characteristic radiographic signs observed on the standard ventrodorsal (VD) hip-extended projection in dogs older than 1 or 2 years of age (Ginja et al., 2010). However, a predisposition to CHD can be identified at earlier ages using hip joint laxity determination (Smith et al., 1990; Farese et al., 1998). The Norberg Angle (NA) is determined on the VD view, being a measure of the relationship between the femoral head and the acetabulum (Morgan and Stephens, 1985). The NA is used in some national and at least in two important international CHD scoring systems: the *Fédération Cynologique Internationale* (FCI) and the British Veterinary Association/ Kennel Club (BVA/KC) (Gibbs, 1997; Ginja et al., 2010). The FCI scores CHD in five categories using as reference the most dysplastic hip and the NA ≥105º is the main criteria to score normal or borderline hips (Douglas, 1970; Morgan and Stephens, 1985). Other FCI categories are reserved for dysplastic hips (mild, moderate and severe CHD), which have a NA <105º and evidence of radiographic signs of degenerative joint disease (DJD) (Flückiger, 2007; Ginja et al., 2010; Verhoeven et al., 2012). The BVA/KC grading system classifies each hip individually with a score ranging from 0-53 and the sum over both hips from 0-106. The

BVA/KC higher scores indicate worse hip status and the NA of each hip is scored from 0 (NA≥105º) to 6 (NA<80º) (Gibbs, 1997; Dennis, 2012). Another international CHD scoring system is applied through the Orthopedic Foundation for Animals (OFA) in the USA and Canada. This organization scores CHD into seven categories (three normal, one borderline and three dysplastic), does not use the NA, and scoring is focused on hip conformation associated with joint congruity, subluxation and signs of DJD (Corley, 1992).

Hip laxity is considered the main risk factor to predict the development of DJD in dysplastic hips (Smith et al., 2001; Todhunter et al., 2003b). The presence of DJD signs on a radiograph is considered the clinical gold standard for diagnosis of CHD. Radiographic methods used to estimate hip joint laxity are the PennHIP and the dorsolateral subluxation (DLS) test (Smith et al., 1990; Farese et al., 1998). The PennHIP method is used in dogs older than 4 months of age and is used to calculate the distraction index (DI) (Smith et al., 1990). The DI measures the relative displacement of the geometric center of the femoral head from the center of the acetabulum when lateral stress is applied to the proximal femur by a PennHIP distractor (Smith et al., 1990). The DLS test reveals hip joint subluxation by positioning dogs in a weight-bearing position at 4 to 8 months of age (Farese et al., 1998). The DI and DLS are negatively correlated, dogs with tight hips (DI ≤0.3 or DLS score ≥55%) are significantly less likely to develop CHD than dogs with loose hips (DI >0.7 or DLS score <45%) (Smith et al., 1990; Farese et al., 1998; Todhunter et al., 2003a).

The main purpose of this study was to determine the ability of the NA ≥105° to predict a non-dysplastic hip based on a DI ≤0.3 cut-off or a DLS ≥55% cut-off.

4.3. Materials and methods

4.3.1. Animals

This was a retrospective multi-center cross sectional study conducted on 437 dogs from both clinical and research settings that underwent screening for CHD using VD radiographic projections, distraction method projections or DLS projections, between 1998 and 2015. The dogs were radiographed at Cornell University Hospital for Animals (USA), at the Veterinary Teaching Hospital of University of Trás-os-Montes and Alto Douro (Portugal) or at the Baker Institute for Animal Health at Cornell University College of Veterinary Medicine (USA). Recorded data included animal sex, age at the time of the radiographs, breed, bodyweight, body score condition and geographic location of evaluation.

The inclusion criteria were availability of pelvic radiographs that allowed assessment of either the NA and DI, the NA and DLS score, or all three projections on the same dog, at concurrent time points. Due to the observational nature of the study, the client consent was waived.

4.3.2. Sedation

For the radiographic study, different sedation protocols were used: at Cornell University Hospital for Animals, a combination of medetomidine (0.02 mg/kg IV) and butorphanol (0.1 mg/kg IV) was used prior to 2007 and then was replaced with dexmedetomidine (0.005 mg/kg IV) and butorphanol (0.1 mg/kg IV); at the Veterinary Teaching Hospital of University of Trás-os-Montes and Alto Douro medetomidine (0.02 mg/kg IV), butorphanol (0.1 mg/kg IV) and atropine sulphate (0.020 mg/kg IV) was used; while acepromazine (0.02 mg/kg IV) and glycopyrrolate (0.010 mg/kg SQ) and then anesthesia was induced with propofol (6 mg/kg IV), and maintained with inhalant halothane at the Baker Institute for Animal Health.

4.3.3. Radiographic projections and measurement

For the VD projection, dogs were placed in dorsal recumbency on the X-ray table, with the rear limbs extended parallel to each other and to the table top, and the stifles internally rotated (Corley, 1992; Ginja et al., 2010). The NA measurements were performed by RJT or MMG, on digital images visualized in imaging softwares (CARESTREAM Vue PACS. Carestream Health, Inc. Rochester, NY and OSIRIS Version 3.1. University Hospitals of Geneva. Geneva, Switzerland) or on hard copy radiographs. The NA was measured in degrees between a line drawn connecting the center of both femoral heads and a line connecting the center of the femoral head and the craniolateral aspect of the ipsilateral acetabular rim (Henricson et al., 1966; Comhaire and Schoonjans, 2011).

For the distraction projection, dogs were placed in dorsal recumbency on the X-ray table, with hips at a neutral position and the flexed stifles pointing vertical to the table top. The examiner pushed both rear limbs medially at the level of the tibia against the PennHIP distractor which was placed between them, thus forcing the femoral heads away from the acetabulum (Smith et al., 1990). This view was performed by RJT or MMG. The DI was calculated at the PennHIP Analysis Center (PennHip. Synbiotics Inc. Malvern, PA).

For the DLS projection, dogs were radiographed in sternal recumbency in a kneeling position with the stifles in an opening in a foam pad or in an acrylic positioning device (Ogsen et al., 2012), with the femora approximately perpendicular to the table, the stifles flexed and the hock joints extended (Farese et al., 1998; Lust et al., 2001). The DLS measurements were performed by RJT on digital images using imaging software (CARESTREAM Vue PACS. Carestream Health, Inc. Rochester, NY) or on hard copy radiographs. A best fitting circle was placed over each femoral head to calculate their diameter. For the determination of the DLS score, a line was drawn connecting both craniolateral edges of the acetabular rims. Then, two perpendicular lines were dropped from this line at the most medial edge of the femoral head and at the craniolateral edge of the acetabulum, respectively. The displacement distance between these perpendicular lines was measured. The DLS score was determined by dividing this distance by the ipsilateral femoral head diameter (Farese et al., 1998).

4.3.4. Statistical Analysis

One hip from each dog was randomly selected for the statistical analysis. Descriptive statistics were computed for all variables. Normality was assessed using graphical methods and the Shapiro-Wilk test. Continuous data are presented as mean ± standard deviation where normal or median and interquartile range for non-normal distribution and categorical data as number (%) unless otherwise indicated. All comparisons were two-tailed and $p<0.05$ was considered to be statistically significant. T-tests were conducted where data was normal and Mann-Whitney tests where data was not normal. We performed univariable logistic regression analyses against binary outcomes as the dependent factors consisting of DI ≤0.3 and DLS score ≥55% for non-dysplastic and DI >0.3 and DLS <55% for dysplastic hips. The NA was considered as the independent variable. The Hosmer Lemeshow goodness-of-fit test was performed and odds ratios [±95% confidence interval (CI)] were computed. The predictive capacity of the NA against a DI ≤0.3 or a DLS score ≥55% was assessed using area under the receiver operator characteristic (ROC) curve analysis. The area under ROC curves and 95% CI were reported. Two-by-two classification tables were constructed, and overall sensitivity and specificity of the NA for each outcome at the selected cut-point was calculated, together with positive and negative predictive values. All analyses were performed with statistical software (Stata Statistical Software: Release 13. StataCorp. College Station, TX, USA).

4.4. Results

Radiographs were available on 437 dogs, 218 right hips and 219 left hips were randomly selected as the measurement for that dog. The most prevalent breeds were Labrador Retriever (33%, 146/437), mixed breed (30%, 135/437), and Serra da Estrela dogs (8%, 33/437). Forty-nine percent (212/437) were intact female, 5% (23/437) were spayed females, 41% (177/437) were intact male and 5% (24/437) were neutered males. Thirty-five percent (152/437) were client-owned dogs evaluated in a clinical setting, 52% (227/437) were laboratory dogs evaluated in a research setting, and 13% (58/437) had radiographs from referring veterinarians. Bodyweight was available on 124 dogs, mean 31.4±11.5 kg. Median age of 425 dogs was 8 months (interquartile range ±2 months).

Following radiographic review, DI and NA and DLS score could be measured on 160 dogs, DI and NA on 106 dogs, and DLS score and NA on 171 dogs. The median NA (437 hips) was 105.5±10°, the mean DI (266 hips) was 0.51±0.19, the mean DLS score (331 hips) was 57.8±22.6%. The prevalence of dogs with DI ≤0.3 was 13.9% (37/266) and with DLS score ≥55% was 56% (185/331). Differences in NA between groups of dogs with DI ≤0.3 and DI>0.3 did not reach statistical significance (p=0.07) with a mean NA of 108.4±7.0° and 106.5±8.5°, respectively. The NA was significantly different for dogs with a DLS score ≥55% and DLS score <55% (p<0.001) with a median NA of 107±7.9° and 103±16.3°, respectively (Table 6 and Figure 9).

Table 6: Norberg angles scores for distraction indices (DI) and dorsolateral subluxation scores (DLS) groups.

Variable/ Group	Mean ± SD	N	Norberg angle					
			Minimum	Maximum	P_{25}	P_{50}	P_{75}	Mean ± SD / Median ± IQR*
DI≤0.3	0.23±0.06	37	100º	116.5º	105º	108.4º	112º	108.4º±7.0^{oa}
DI>0.3	0.56±0.16	229	70º	120º	102.5º	106.5º	111º	106.5º±8.5^{oa}
DLS≥55%	66±6.10%	185	88.6º	118º	103.1º	107º	111º	107º±7.9^{oa}
DLS<55%	41±8.92%	146	54.8º	119.5º	91.3º	103º	107.5º	103º±16.3^{ob}

*Norberg angle means with different superscripts, for the same variable, are statistically different (p<0.05).

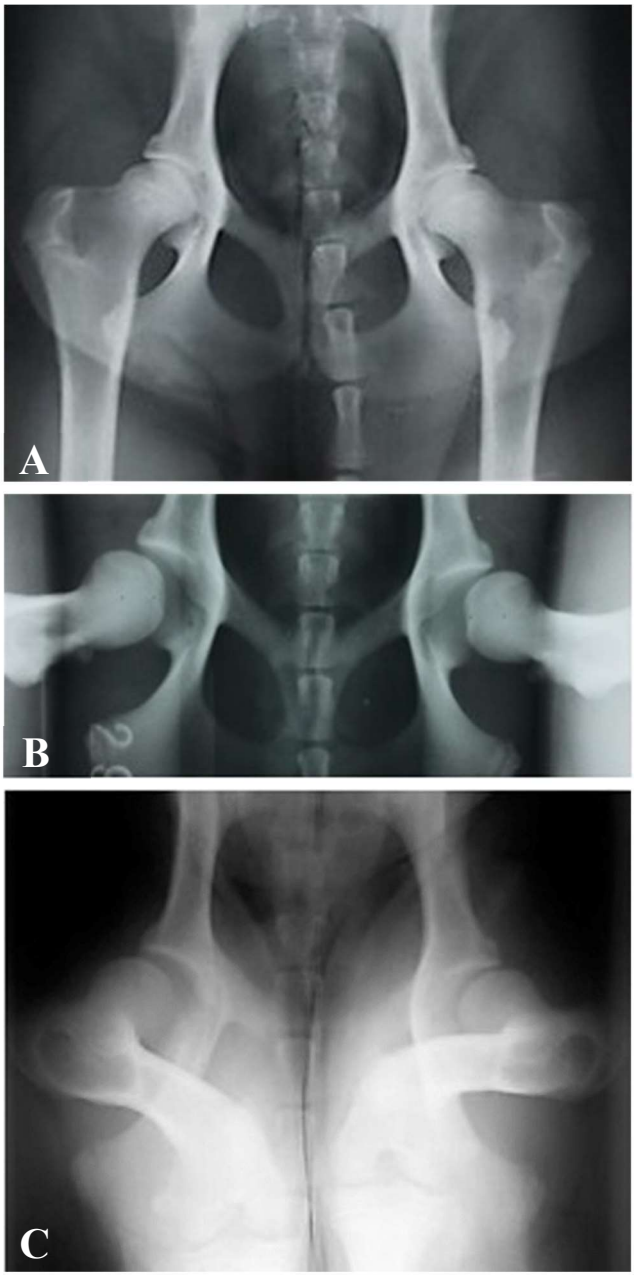
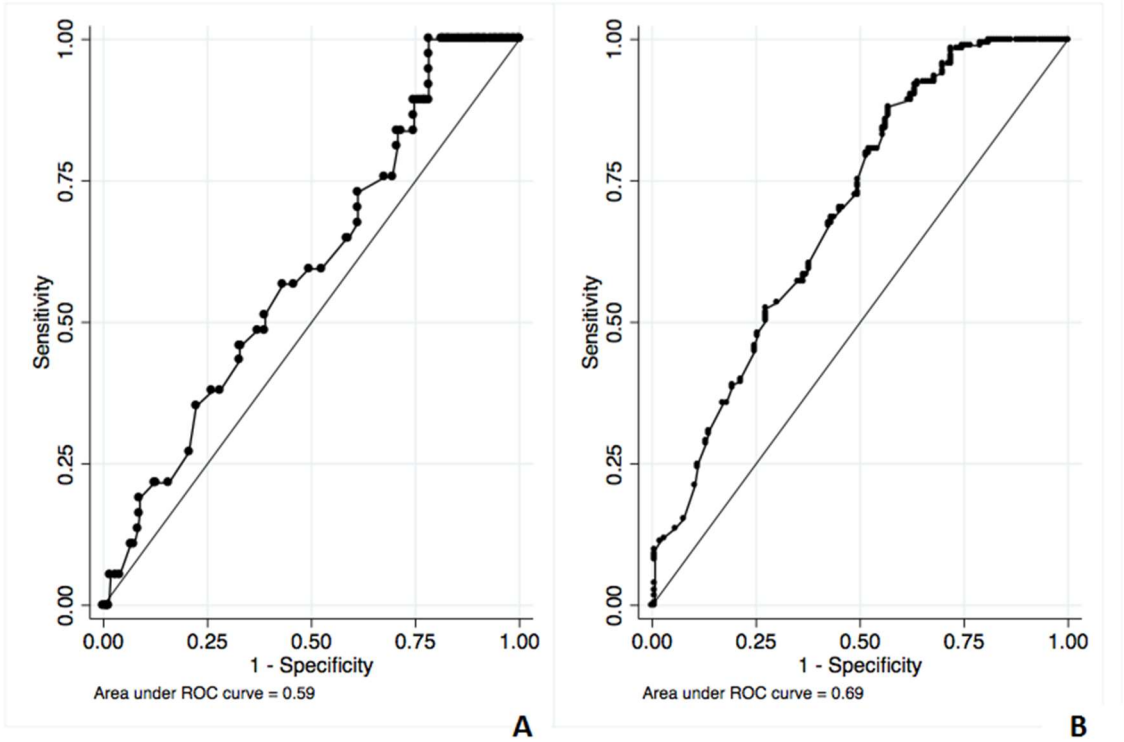


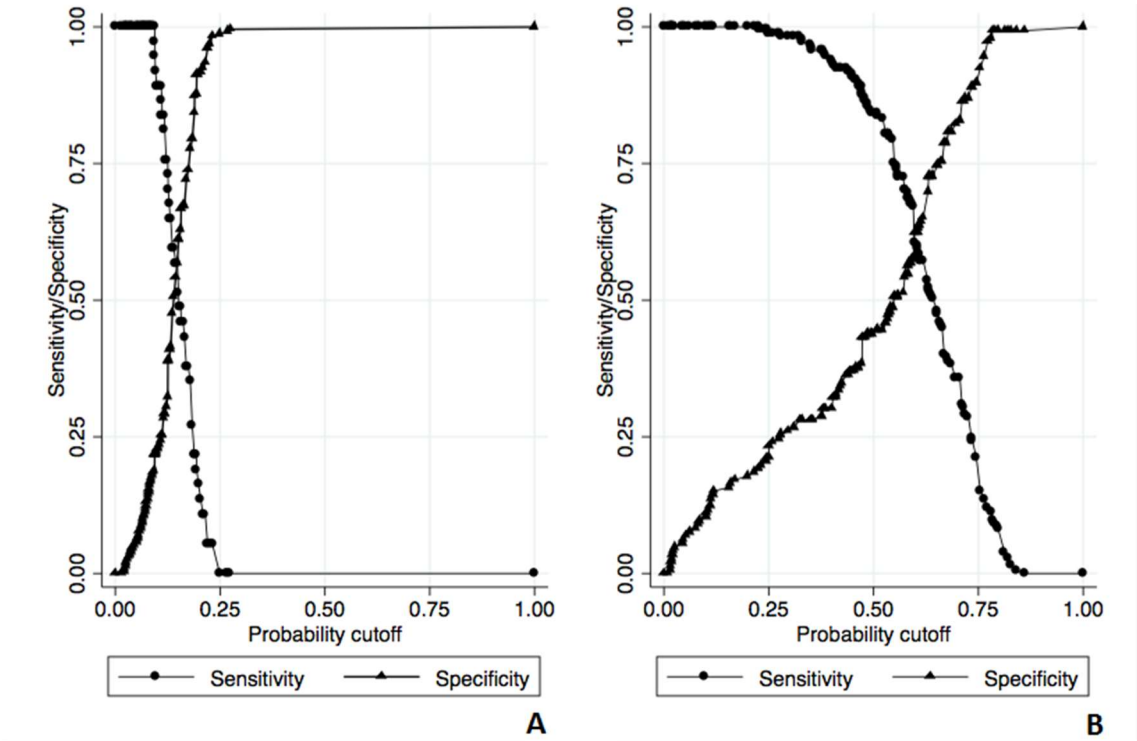
Figure 9: Hip views of an 8 months old dog. (A) Ventrodorsal hip extended projection with a Norberg angle >105°. (B) PennHIP distraction projection showing a distraction index >0.30. (C) Dorsolateral subluxation projection showing a femoral head coverage score <55%.

4.4.1. Prediction analysis

The NA was significantly associated with a DI ≤0.3. Each degree increase in NA was associated with increased odds of DI ≤0.3 (odds ratio=1.07, p=0.04 and 95% CI=1.01-1.13, n=266). The area under the ROC curve of the NA for the prediction of DI ≤0.3 was 0.59, 95% CI=0.50-0.69 (Graphic 1A). At a specificity of >80% for a DI ≤0.3 (equivalent to a false positive fraction of <20%), the cut point for the NA would be ≥ 112º. At this cut point, sensitivity fell to 27%, PPV (positive predictive value) was 17.5% and NPV (negative predictive value) was 87.1%, with 72.2% overall correctly classified (Graphic 2A). The NA was significantly associated with a DLS score ≥55%. Each degree increased in the NA increased the odds of a DLS score ≥55% (odds ratio=1.11 p<0.001, 95% CI 1.06-1.14, n=331). The area under the ROC curve of NA for the prediction of DLS score ≥55% was 0.69, 95% CI=0.63-0.75 (Graphic 1B). At a specificity of >80% for a DLS score ≥55% (equivalent to a false positive fraction of <20%), the cut-point for the NA would be ≥108.7º. At this cut-point, sensitivity fell to 38.4%, PPV was 71.72%, and NPV was 50.9%, with 57.1% overall correctly classified (Graphic 2B).



Graphic 1: Area under receiver operating characteristic (ROC) curves. For Norberg angle≥105º as a predictor of distraction index≤0.3 and dorsolateral subluxation score≥55% the ROCs were 0.59 (A) and 0.69 (B), respectively.



Graphic 2: Sensitivity and specificity curves. At a specificity of >80%, for a distraction index ≤0.3 and for a dorsolateral subluxation score ≥55%, the cut-point for the Norberg angle would be ≥112º (A) and ≥108.7º (B), respectively.

4.5. Discussion

Our study showed that the NA measured from a VD, extended hip radiographic projection was not an accurate predictor of normal hip conformation at the common cut-off of NA ≥105º. Our results hinge on the knowledge that hip laxity has been shown to be the main risk factor for the development of secondary DJD in dogs with CHD (Smith et al., 1993; 2001; Todhunter et al., 2003b). Hip laxity can be measured with the DI and the DLS score with dogs placed in weight-bearing position (Smith et al., 1990; Farese et al., 1998). We used as the criterion of normality, a DI ≤0.3 or a DLS score ≥55%, because hips with no laxity are highly unlikely to develop DJD and, therefore, were never dysplastic (Smith et al., 1993; 2001; Todhunter et al., 2003b).

The NA is highly correlated with the OFA hip score (Zhang et al., 2009). In 2 year old dogs, when the OFA usually certifies hip conformation, hips with a NA ≥105° will be scored as good or excellent (normal) (Verhoeven et al., 2012). The FCI and BVA/KC scoring systems use a NA □105º threshold in adult animals to distinguish between dysplastic or non-dysplastic hips, together with other radiographic signs of DJD (Gibbs, 1997; Ginja et al., 2009b). However, a subset of dogs with normal hips in the VD view (NA ≥105º), have palpable laxity (positive Ortolani sign) or loose hips according to the DI or DLS score (Smith et al., 1990; Farese et al., 1998; Ginja et al., 2008a; 2009a) as it is shown in Figure 9 a dog with disparate readings in the three methods accessed. Other studies, report that radiographs scored as normal by the OFA had NAs of ~100º (Tomlinson and Johnson, 2000) or high hip laxity (Belkoff et al., 1989). Therefore, we should reconsider the conclusion that preliminary hip evaluations using the VD view and the OFA scoring system in young dogs (less than 2 years of age) were considered reliable to predict CHD at later ages (Corley et al., 1997). The NA is not adequate to measure hip laxity on the VD hip view, as the extension of the hind legs tightens the joint capsule, the ligament of the femoral head and associated muscles fostering joint congruence (Smith et al., 1990). Thus, the DLS score in weight bearing position also should not be compared with similar measures of femoral-acetabular coverage described on other studies (Belkoff et al., 1989; Janssens et al., 2014), when is used the VD hip extended projection.

Most of the dogs in our study were about 2 years of age reducing the confounding of ageing because the NA may change throughout a dog's life, being directly influenced by capsular fibrosis, the degree of acetabular ossification and degenerative remodeling, and joint geometry (Ginja et al., 2008a; 2009a; Pinna et al., 2013). The articular cartilage lesions and

synovitis, which occur in the early stages of the DJD that accompanies CHD, are not detectable on any hip radiograph projection and DJD signs take years to develop in mild CHD cases. One study showed that 55% of dogs which were categorized as unaffected with CHD at 2 years of age had radiographic evidence of DJD at 12 years of age (Smith et al., 2012). Our results suggest that additional diagnostic information concerning hip laxity in dogs with fair, good or even excellent VD hip conformation might be beneficial to remove more potentially dysplastic, dogs from the breeding pool.

The NA reflects the relationship between the femoral head and acetabulum and is correlated with the percentage of the femoral head covered by the acetabulum in the VD projection (Janssens et al., 2014). If the geometry of the pelvis is altered, it can influence the NA and the proportion of the femoral head covered by the acetabulum, both were increased after intertrochanteric varus osteotomy in dysplastic dogs (Tomlinson and Johnson, 2000; Pinna et al., 2013). However, the NA was only moderately associated with both the DI and DLS score in this investigation, as observed in previous studies (Todhunter et al., 2003b; Culp et al., 2006; Ginja et al., 2008a; Comhaire and Schoonjans, 2011). In dogs treated with triple pelvic osteotomy, the DLS score increased while the DI was unaffected (Farese et al., 1999). This suggests that the DLS score is assessing more than direct hip laxity and is affected by the osseous relationships on the hip joint.

According to our prediction analysis, if using the VD extended hip projection as the only criterion for CHD diagnosis, excluding dogs that would later develop hip DJD would be achieved more rapidly using a NA cut-off of 112º (DI ≤0.3) or 109º (DLS score ≥55%). However, some breeds with a high CHD prevalence contain dogs with NA >105°, but those dogs lie in the tails of the distribution (Ginja et al., 2009b). Application of such a rigorous threshold to the selection of breeding pairs may reduce genetic diversity. Application of methods like inbreeding coefficients can guard against loss of genetic diversity (Hou et al., 2013).

All CHD scoring systems based on VD view, NA measurement, and/or DJD signs are not accurate to identify normal hips especially in young dogs (Ginja et. al., 2015). In Europe, the effectiveness of these schemes in reducing CHD prevalence in canine populations has been not effective, even with very controlled breeding programs (Leppänen et al., 2000), or have met with limited success (Swenson et al., 1997). Incremental improvement in genetic quality of hip conformation has been achieved over 45 years through the OFA scoring scheme and its registry (Hou et al., 2013). These studies indicate that breeders might apply additional

selection pressure in breeding decisions. The DI showed excellent within- and between-examiner repeatability, reproducibility and high heritability (Smith et al., 1997; Ginja et al., 2006; 2008b; Powers et al., 2010). The DLS score showed a strong correlation with the DI and high repeatability (Farese et al., 1998). As the NA, the DI and the DLS score measure different aspects of CHD, the DI or DLS score inclusion in hip evaluation could improve the accuracy of CHD scoring systems which at present are based exclusively on VD projection analysis. At a minimum, the result of an Ortolani test could be included on the application form that is submitted by veterinarians to the VD hip extended scoring. Rigorous application of the DI to selection of breeding pairs has markedly reduced the incidence and severity of CHD in closed breeding populations (Leighton, 1997). When the OFA score, the DI, the DLS score, and the NA were used on radiographs of 8 months old dogs to predict DJD (articular cartilage lesions) at 12 months of age, the most accurate single predictor of DJD was the DLS score but the combination of the DLS score and the NA was the most accurate predictor of hip conformation (Todhunter et al., 2003b). At a minimum, selection of dogs for breeding or purchase using more than a single VD hip extended projection is recommended, until estimated breeding values for hip conformation and molecular genetic tests are widely accepted (Ginja et al., 2015).

Limitations of the present study include its retrospective multi-center cross sectional nature and inherent intra- and inter-examiner variability in radiographic examinations and measurements as well the use of different anesthetic protocols. Therefore, we cannot assume that there were no differences between measurements made on digital and on printed radiographs (Baroni et al., 2003). The inclusion of a limited number of pure dog breeds and mixed breeds in the present study may limit the applicability of these findings to other dog breeds with different CHD prevalence and severity. A large proportion of the dogs in our study were of laboratory origin rather than client owned animals, which may limit the transferability of our results. Finally, not all assessments were performed on all dogs, which may have resulted in selection bias.

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4.6. Conclusion

In conclusion, at the commonly used cut-point of NA ≥105°, the NA is not an accurate measurement of normal hip conformation. The specificity of a non-dysplastic diagnosis was maximized with cut-off at NA ~112º (DI) or ~109º (DLS) but it would be difficult to find

enough animals to breed, especially in breeds with high CHD prevalence. Careful application of screening methods for CHD based on hip laxity, like the DI or the DLS score, would help to remove dysplastic-susceptible dogs from the breeding pool.

4.7. Acknowledgments

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5. Genetic diversity and structure of the Portuguese 'Cão da Serra da Estrela' dog breed using whole-genome SNPs

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5.1. Abstract

'Cão da Serra da Estrela'(CSE) is a mastiff-type molossoid dog, one of the most ancient and diverse Portuguese native breeds. A total of 60 CSE animals were genotyped with the Illumina CanineHD Beadchip. For comparison purposes, 10 Iberian wolves and 10 Portuguese stray dogs were also included. After filtering, two subsets of markers were selected, including 29,439 and 16,793 SNPs for the three canid populations and within-breed CSE analyses, respectively. We used a whole-genome SNP approach to investigate the genetic diversity and population structure of these canid populations. Y-chromosomal markers were used to infer haplotype composition and investigate phylogenetic relationships. The average within-breed heterozygosity was 0.26 for CSE with no significant inbreeding found. Results of the PCA, genetic distances and Bayesian clustering were mostly consistent, with the three canid populations well-differentiated. Genetic structure related to breed uses, i.e. work versus pet dogs, was not detected. But for k=2, we observed weak differentiation between CSE dogs sampled in the north (mean Q=0.893±0.251) and south (mean Q=0.476±0.342) of Portugal, whereas dogs from the center split between these two clusters, possibly reflecting lineage sorting associated to specific breeders.

Y-chromosome diversity was low with a single haplotype (H1) of the major H1 lineage detected in 32 CSE males.

In our study, a pedigree dog population native to the Iberian Peninsula was analyzed for the first time with whole-genome SNPs. These results indicate that CSE breed retains high genetic diversity and is well differentiated, supporting the development of management and conservation programs to preserve key domestic animal genetic resources.

Keywords – 'Cão da Serra da Estrela', whole-genome SNPs, genetic diversity, population structure, Y chromosome

5.2. Introduction

There are 10 Portuguese canine breeds registered in the Portuguese Kennel Club (Clube Português de Canicultura website, 2016b), of which 8 are recognized by the *Fédération Cynologique Internationale* (FCI) (Fédération Cynologique Internationale website, 2016d). 'Cão da Serra da Estrela'(CSE) is a mastiff-type molossoid dog, the most popular Portuguese native breed (FCI number 173) (Fédération Cynologique Internationale website, 2016b), with approximately 250 females registered annually in the Portuguese Kennel Club (Clube Português de Canicultura website, 2016a). The breed comprises two coat varieties: the long and short-haired individuals. Long-haired CSE dogs are mostly used as pets, due to their alertness and loyalty, while the latter are mostly working dogs, being athletic, fearless and protective. The breed is one of the livestock guarding dogs used to promote the reduction of wolf damage on livestock in Iberian wolf (IW) conservation programs (Life COEX website, 2016). The official register of CSEs was started in 1932 with both coat varieties recorded (Pye, 2002).

The genetic diversity of CSE breed has been characterized using traditional genetic markers such as autosomal short tandem repeat polymorphisms (STRs, i.e. microsatellites), mitochondrial DNA (mtDNA) sequences, as well as Y-chromosome specific single nucleotide polymorphisms (SNPs) and STRs. 'Cão da Serra da Estrela' breed is one of the most diverse Portuguese canid breeds with high expected heterozygosity as inferred from STRs (He=0.76±0.10, mean overall Portuguese native breeds=0.74±0.05) (Pires et al., 2009), and high haplotype diversity for mtDNA sequence data (H=0.90±0.05, mean overall Portuguese native breeds H=0.58±0.33) (Pires et al., 2006).

In Portugal, breeds of shepherd dogs are at risk due to abandonment of traditional husbandry practices, as well as the important decline of grey wolf (*Canis lupus*) over the past

century (Petrucci-Fonseca 1990). Additionally, dog shows are becoming more popular, likely resulting in within-breed sub-structure and genetic differentiation between work and pet dogs. This type of sub-structure was already detected in a Portuguese native dog breed "Cão Fila de São Miguel" from the Azores (Pires et al., 2009). A recent study also reported diversification of work and show dogs within Labrador Retrievers and Border Collies (Fadel et al., 2016).

The level of genetic differentiation observed among Portuguese shepherd dog breeds is generally low, namely between CSE and Alentejo Shepherd Dogs for STR loci (*Fst*=0.02) (Pires et al., 2009) and for mtDNA sequences (*Fst*=0.055) (Pires et al., 2006). This is also supported by results of the Bayesian clustering analysis with STRs, showing a common cluster for CSE and Alentejo Shepherd Dogs, which were well-differentiated from other populations of native dogs (Pires et al., 2009).

DNA markers located on the non-recombining region of the Y chromosome have been used to investigate the origin of several canid populations (Bannasch et al., 2005; Natanaelsson et al., 2006; Brown et al., 2011; Ding et al., 2012; Sacks et al., 2013). For Iberian dog breeds, Y-STR diversity was relatively high (*H*=0.52±0.11) with a total of 19 haplotypes detected. Among Portuguese shepherd dogs, CSE had the highest Y-STR diversity with a total of 8 haplotypes found (Pires et al., 2016). The level of genetic differentiation among Iberian dog patrilines was low with all animals belonging to a single major haplogroup (H1/HG1 (Ding et al., 2012)) defined by Y-SNPs and STR markers (Pires et al., 2016).

In studies of domestic dogs, it is often relevant to investigate their genetic composition in comparison to that of their wild ancestor, the gray wolf (*Canis lupus*). In the Iberian Peninsula an isolated population of gray wolves - the Iberian wolf subspecies (IW; *Canis lupus signatus*) - persists (Godinho et al., 2011; Pilot et al., 2014). The IW population represents a genetic diversity reservoir for the species with particular genetic variants described. Recently, the total number of maternal lineages reported in IW increased to 24 haplotypes (*H*=0.32±0.080), and 11 Y-STRs haplotypes were disclosed (*H*=0.41±0.04) (Pires et al., 2016). Wolves and dogs are very close genetic entities (Fan et al., 2016), and can well be inferred from their sharing of several maternal lineages (i.e. haplogroups A-E) (Savolainen et al., 2002). This is also valid for paternal markers, which have shown that dogs originated from at least 13-24 different wolf Y-chromosome haplotypes (Ding et al., 2012). Despite, hybridization between IW and dogs was described, it appears to be sporadic, and IW and Portuguese native dogs are significantly differentiated (Godinho et al., 2011; Pires et al., 2016).

Recently, the use of commercial SNP-arrays for the fast and reliable screening of nuclear genetic variation has become generalized. Also, STRs are time consuming and need standardization between different laboratories, and mtDNA is genealogically restrictive in the inferences that can be produced (vonHoldt et al., 2013). High density SNP-arrays can disclose relevant information for our understanding of the evolution, domestication and breed relationships of canids (vonHoldt et al., 2011; Frantz et al., 2016). They also allow, among other things, to investigate in detail population structure in purebred and stray/village dogs (Parker et al., 2004; Lindblad-Toh et al., 2005; Boyko et al., 2009; Shannon et al., 2015). A thorough analysis, i.e. whole-genome, of the within-breed genetic diversity and population structure of CSE has not been carried out thus far. In sub-divided populations, such as those of domestic animal breeds, the global genetic diversity of the species is maintained at the cost of a loss in the genetic variability of sub-populations (Cañón et al., 2011; Ginja et al., 2013). Characterizing the within-breed genetic structure can help to manage threatened animal resources, to minimize inbreeding and maintain genetic diversity.

In this study, we aim to refine our understanding of CSE breed using a whole-genome SNP approach to investigate levels and patterns of genetic diversity, and the putative population structure caused by the different breed uses (work vs pet). Y-chromosomal markers will also be used to investigate phylogenetic relationships and within-breed haplotype composition. Individuals of the IW population were included for comparison purposes. As in other studies of whole-genome SNP data (Ollivier et al., 2013; Hayward et al., 2016), we also aim to set the basis for subsequent analyses of specific traits, namely canine hip dysplasia, a prevalent condition in these dogs (Ginja et al., 2015).

5.3. Materials and Methods

5.3.1. Sampling and SNP genotyping

A total of 60 dog samples belonging to the CSE breed (53 pet and 7 work dogs; 23 males and 37 females) were collected. For comparison purposes, 10 Portuguese IW (5 males and 5 females) and 10 Portuguese stray dogs (5 males and 5 females) were also sampled. Details on the animals sampled are described in supplementary Table S1, namely gender, geographic location of the sampling, CSE phenotypes for canine hip dysplasia and breed use (work vs pet). High quality genomic DNA was extracted from whole blood using innuPREP

Blood DNA Midi Kit (Analytik Jena, Germany) and from tissue samples using the DNeasy Blood and Tissue kit (Qiagen).

Genotyping of the Illumina CanineHD Beadchip containing 173,662 (Illumina, 2010) SNPs was done through service acquisition (AROS Applied Biotechnology, Denmark and Cornell University, U.S.A.). All SNP positions are mapped to the reference dog genome canFam2.0 (Broad Institute of MIT and Harvard, 2016). Filtering was done using PLINK v1.07 software (Purcell, 2007), to discard SNPs with a genotyping rate below 95% and SNPs in linkage disequilibrium. We confirmed that phenotypic and genotypic gender matched. Two subsets of markers were selected for subsequent analyses, 29,439 and 16,793 SNPs for the three canine populations and within-breed CSE, respectively.

5.3.2. Genetic diversity and population structure

Genetic diversity parameters were estimated with R statistical program (R Development Core Team, 2011) using the Diversity tool (Keenan et al., 2013; Keenan, 2015), namely observed (*Ho*) and unbiased expected (*He*) heterozygosities, and inbreeding coefficients (*Fis*) with *P*-values obtained based on 1,000 bootstrap replications in each of the three populations.

Principal components analyses (PCA) of CSE, IW and SD, as well as within the CSE population were carried out with the program EIGENSTRAT in the EIGENSOFT v5.0.1 package (Price et al., 2006) to investigate genetic structure. Allele sharing distances between individuals were calculated and used to build a neighbor-joining dendrogram with Populations 1.2.32 software (Langella, 1999). Bayesian clustering analysis was carried out with fastSTRUCTURE v1.0 using default parameters (Raj et al., 2014). We performed 5 independent runs for each k (partition of the dataset) considering k=1 to k=5 to investigate genetic structure of the three canid populations, and within the CSE population.

5.3.3. Y chromosome analysis

Information on 10 Y-chromosome SNPs contained in the Illumina array (Y20p1-35, Y24p1-11, Y28p1-599, Y28p1-619, Y28p1-873, Y29p2-56, Y30p1-655, YBp2-225, YGp1-146 and YNp1-608) was obtained for 27 dogs (SD36 was removed due to >10% missing data) and 5 Iberian wolves. The data was combined with Y-chromosome information available from Pires et al., 2016 for 9 SNPs in common (excluding Y24p1-11) and for an additional 9 CSE and 8 SD dogs, as well as 27 Iberian wolves. For comparison purposes, 46 dogs from

other 8 Portuguese native breeds (6 ‘Cão de Água Português’, 7 ‘Cão de Fila de São Miguel’, 4 ‘Cão de Gado Transmontano’, 8 ‘Cão de Castro Laboreiro’, 3 ‘Perdigueiro Português’, 7 ‘Podengo

Português’, 8 ‘Rafeiro Alentejano’, 3 ‘Cão da Serra de Aires’) were also included in the analysis.

Y-haplotype frequencies were determined with GenAlex 6.5 (Peakall and Smouse, 2006, 2012). Phylogenetic relationships among haplotypes were inferred using the median-joining network algorithm implemented in NETWORK v5 (Bandelt et al., 1999) with default parameters r=2 (reduction threshold) and ε=0. Haplotype components were weighted as SNP transitions w=10 and SNP transversions w=30 so that the component with the lowest expected mutation rate was assigned the highest weight (Bandelt et al., 2000).

5.4. Results

5.4.1. Genetic diversity and population relationships

The average within-population heterozygosity was approximately 0.30 for CSE and IW, and slightly lower for SD (~0.20). Inbreeding coefficients (*Fis*) were not significantly different from zero for any of the populations included in our study (Table 7).

Table 7: Genetic diversity parameters calculated in each canine population from whole-genome SNPs data: observed (*Ho*) and expected (*He*) heterozygosities, inbreeding coefficient (*Fis*) and confidence intervals.

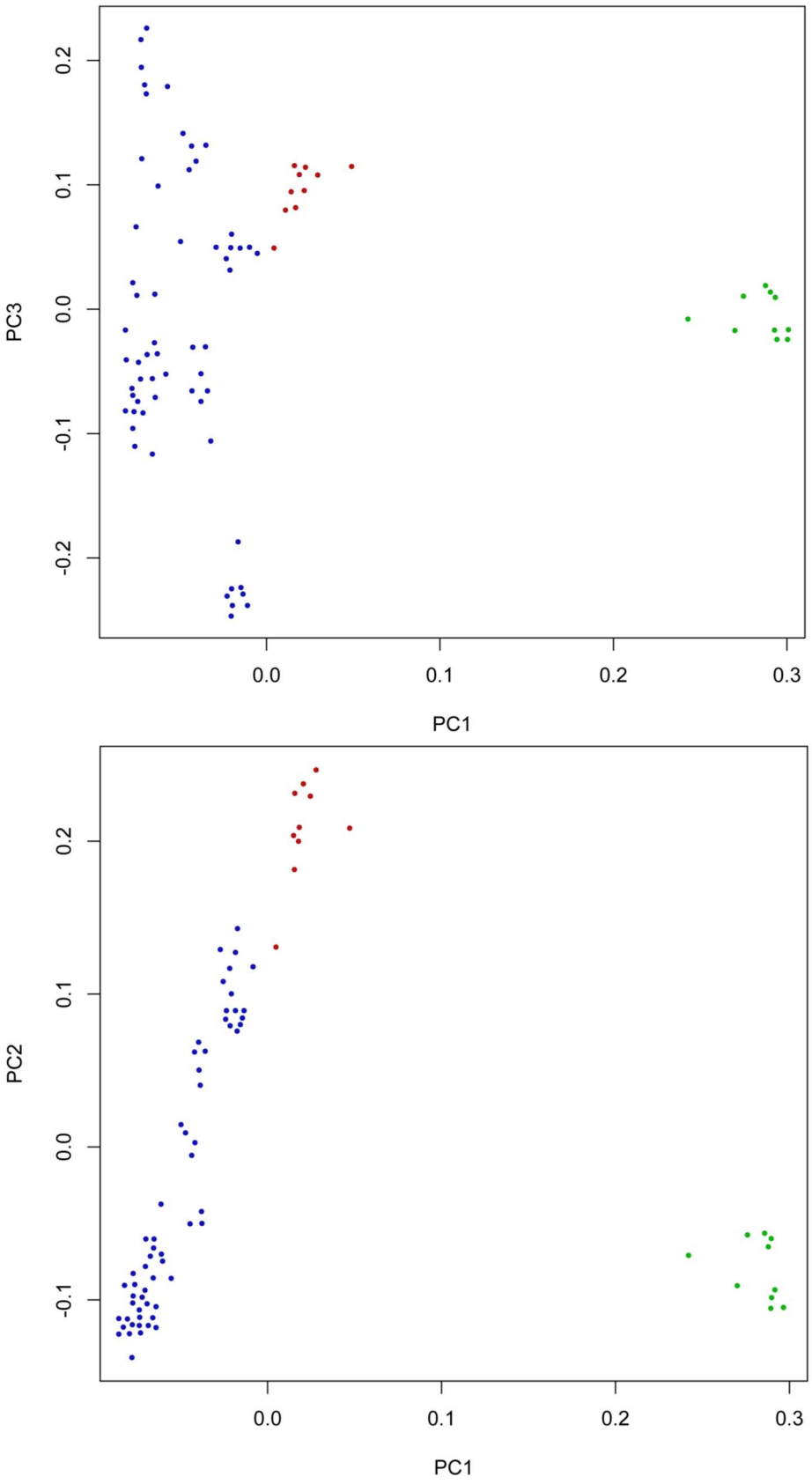
Population	***Ho***	***He***	***Fis***
‘Cão Serra da Estrela’ (shepherd dogs)	0,26	0,26	-0,005 (-0.024 – -0.001)
Portuguese stray dogs	0,17	0,18	-0,066 (-0.172 – -0.067)
Iberian Wolf	0,31	0,30	-0,027 (-0.142 – -0.026)

For the PCA analysis of the dataset with CSE, IW and SD (Graphics 3), 31.0% of the total genetic variation is explained by the first three components (15.2%, 8.7% and 7.1%,

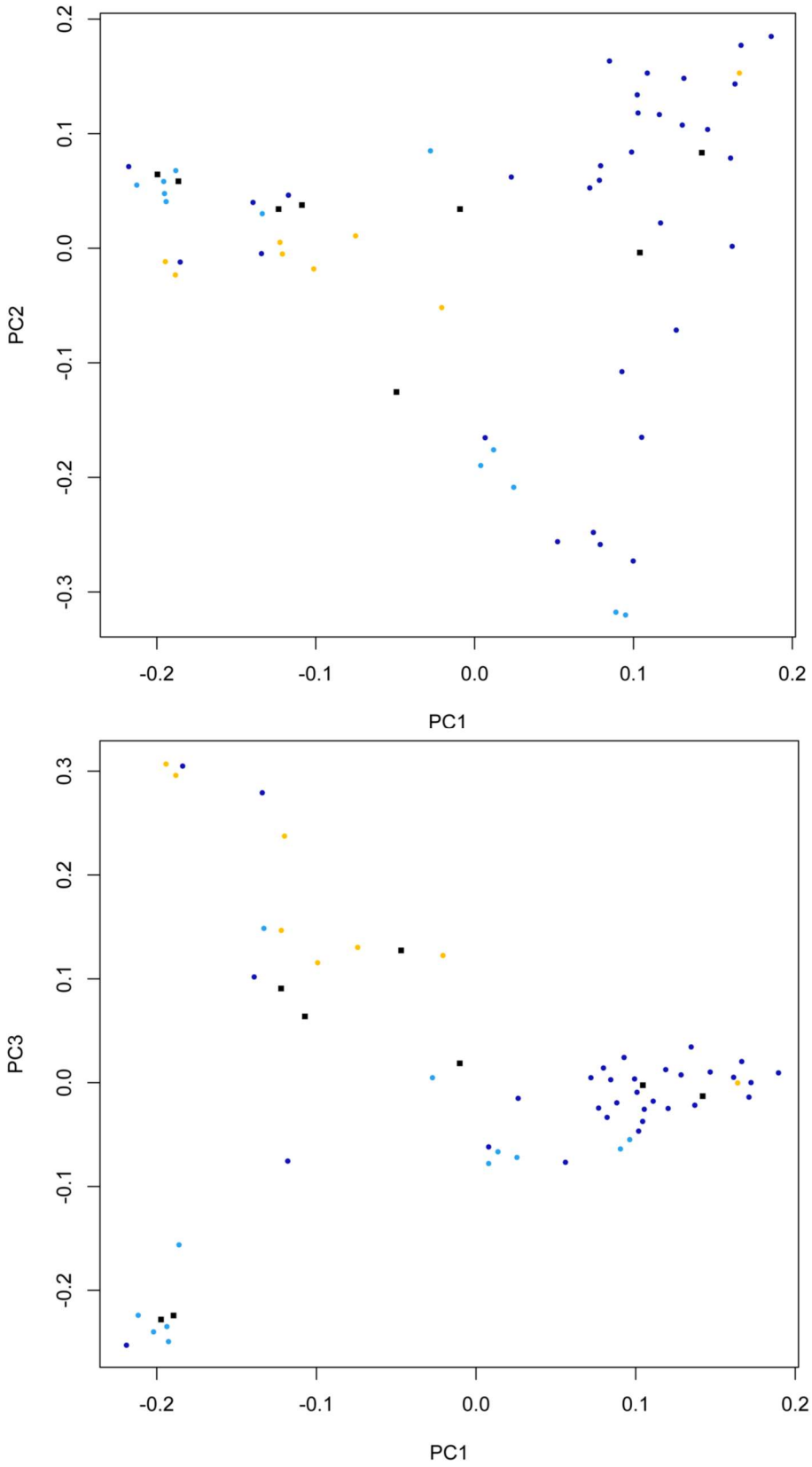
respectively). This analysis allowed to separate the CSE breed (blue) and stray dogs (red) from the IW (green) population (Graphic 3). Although, most of the CSE dogs clustered independently there is a number of animals that grouped closer to the SD population.

For the PCA analysis of the CSE population (Graphic 4), the proportion of the total variance explained by the first three components is 27.4% (10.0%, 9.2% and 8.2%, respectively). Sub-structure related to dog function, namely work vs. pet dogs, was not observed. We found some level of sub-structure within CSE breed, namely PCA allowed to separate between most of the dogs sampled in the Northern region (dark blue) from those of the southern region (light blue, Graphic 4). The majority of the samples collected in the central region of Portugal (orange) clustered with other dogs from several origins (including samples of unknown origin, black squares). Additionally, the sub-population from the south appears to split in two groups in the PCA graphs. These results possibly reflect lineage sorting associated to specific breeders.

Relationships among canid populations were also investigated by means of a neighbor-joining dendogram shown in Figure 10. These results were in agreement with those of the PCA analysis in separating IW (green), SD (red) and the CSE breed. Sub-structure within CSE was more difficult to infer but we could still identify dogs from the north (dark blue), the center (orange), and the south of Portugal (light blue).



Graphic 3: Principal components analyses of 'Cão da Serra da Estrela' (blue), Iberian wolf (green) and stray dogs (red) populations. The first 3 components explain 31.0% of the total genetic variation (15.2%, 8.7% and 7.1%, respectively).



Graphic 4: Principal components analyses showing within-breed sub-structure of 'Cão da Serra da Estrela' splitting the northern (dark blue), the southern (light blue) and center populations (orange). Individuals of unknown origin are represented by black squares. The first 3 components explain 27.4% of the total genetic variation (10.0%, 9.2% and 8.2%, respectively).

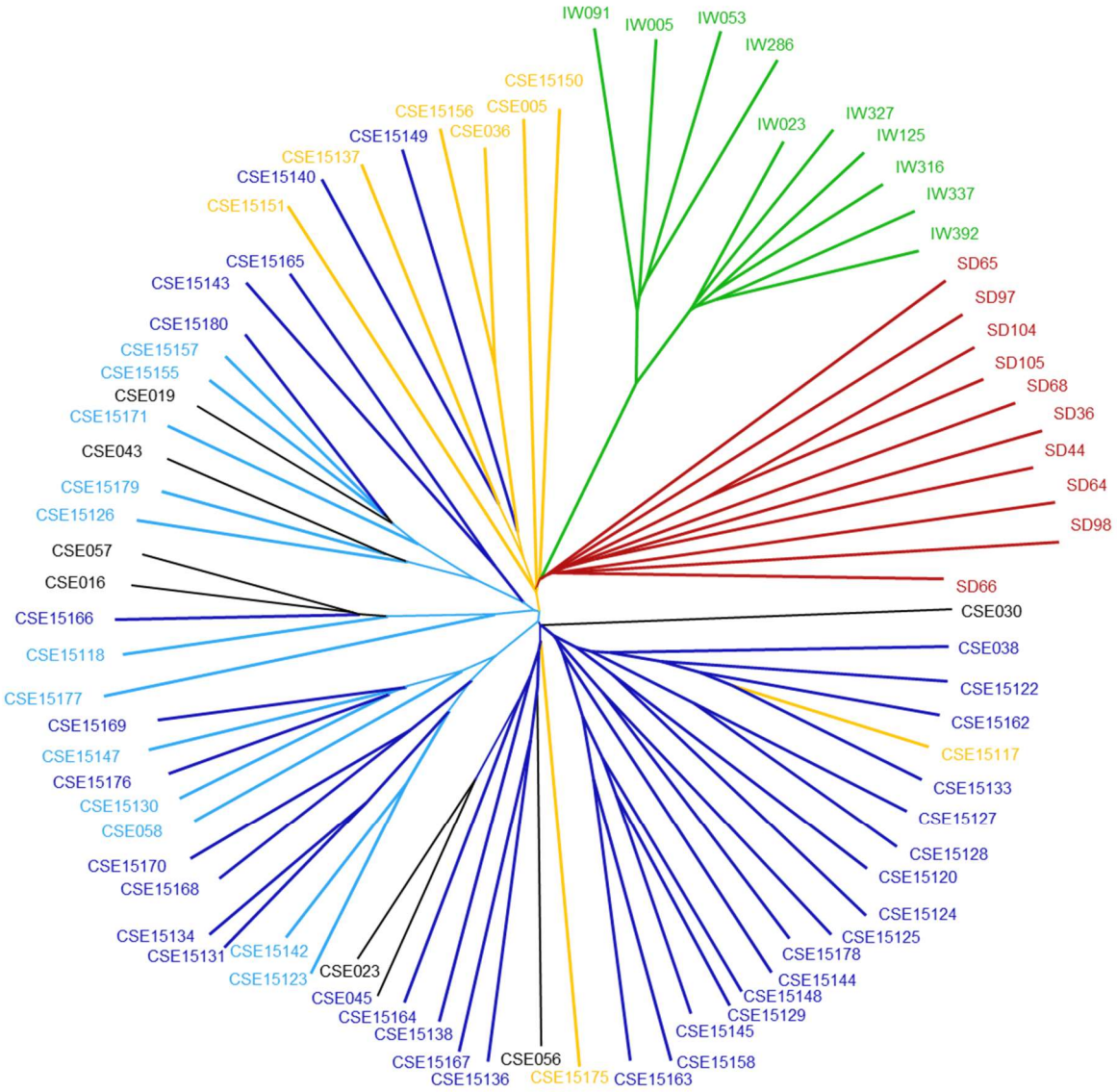


Figure 10: Neighbor-joining dendogram of allele sharing distances depicting genetic relationships between individuals of the 'Cão da Serra da Estrela' breed, Iberian wolf and stray dogs populations. Geographic origin of 'Cão da Serra da Estrela' is colored coded according to the following: north – dark blue; south – light blue; center – orange; unknown origin – black.

5.4.2. Population structure

The results of the Bayesian cluster analysis of fastStructure for the three canid populations are shown in Figure 11 and Table S1. The maximum marginal likelihood of the data was obtained at k=3, but 4 possible model components can be used to explain the genetic structure of these populations. For k=2 (Figure 11, top), IW was clearly separated from the dog populations, but the heterogeneous SD population shared some genetic diversity with the IW population (~20% genotype membership in the IW cluster). The CSE breed showed a high mean genotype membership coefficient in the dog cluster (Q=0.994±0.018).

For k =3 (Figure 11, center), CSE formed a single cluster (dark blue) with high genotype membership coefficients (mean Q=0.990±0.027). Stray dogs (red color) had low individual genotype membership coefficients (mean Q=0.210±0.023), with all animals displaying high genotype memberships within CSE. One SD sample (SD36) had a high percentage of missing genotypes (~20%), and showed ~7% IW ancestry (Table S1).

At k=4 (Figure 11, bottom), sub-structure was detected for CSE (light blue) with mean Q=0.885±0.361, which included mostly dogs sampled in the south of Portugal (Table S1).

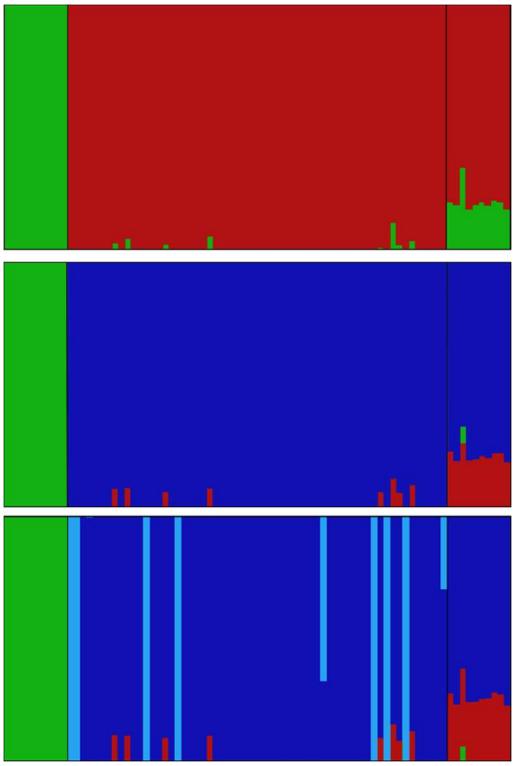


Figure 11: Representation of individual genotype membership coefficients obtained with fastStructure in each canid population for k=2 (top), k=3 (center) and k=4 (bottom). Iberian wolves are represented in green, stray dogs and 'Cão da Serra da Estrela' populations in red and blue, respectively.

The results of the Bayesian cluster analysis of fastStructure for the CSE breed are shown in Figure 12 and Table S2. The maximum marginal likelihood of the data was obtained at k=1, but 3 possible model components can be used to explain genetic structure within the CSE breed. For k=2 (Figure 12, top), differentiation between CSE dogs sampled in the north (dark blue) and south (light blue) of Portugal could be inferred, with mean Q=0.893±0.251 and mean Q=0.476±0.342, respectively, whereas dogs sampled in the center split between these two clusters.

For k=3 (Figure 12, bottom), the southern CSE group split in cluster 1 and 3. Cluster 1 (light blue) comprised 5 dogs sampled in the south and 6 dogs sampled in the north. The latter group included: dogs with either one (CSE15169 and CSE15170) or both (CSE15131 and CSE15134) progenitors from the south; CSE15176 (q=0.980), which could also have ancestors from the southern region of the country; and CSE15168 (q=0.646) of admixed origin. Cluster 3 (grey), included the following: 6 dogs sampled in the south of Portugal (q≥0.8); 6 dogs sampled in the center with ancestry in cluster 1 and 2 (CSE005, CSE15150,

CSE15137, and CSE15151) and in cluster 2 (CSE036 and CSE15156); 5 dogs sampled in the north (CSE15140, CSE15143, CSE15149, CSE15166, and CSE15180), of which the latter two have at least one progenitor from the south; and 4 dogs of unknown origin (CSE019, CSE043, CSE016, and CSE057), of which CSE043 has both progenitors from the south, and the latter 2 dogs had ancestry in cluster 2. Cluster 2 (dark blue), included mostly dogs sampled in the northern region, but also 1 dog sampled in the center whose female progenitor is from the north (CSE15117), and 1 dog of unknown origin (CSE023) which could also have ancestors from the northern region. Several dogs classified within cluster 2 ($q \geq 0.5$), showed some ancestry from cluster 1 (4 dogs), cluster 3 (5 dogs) or both (2 dogs). Among these and besides the 3 dogs sampled in the northern region (CSE15120, CSE15136 and CSE15167), 1 dog of unknown origin (CSE045) also showed ancestry in Cluster 1. As for the dogs with ancestry in the southern cluster 3, there are 3 individuals sampled in the north (CSE15138, CSE15144, and CSE15165), 1 of unknown origin (CSE030) and 1 sampled the south (CSE15177), which could also have ancestors from the northern region. The 2 dogs with admixed ancestry of clusters 1 and 3 were CSE15175 and CSE15178 sampled in the center and the north, respectively.

Although no further sub-structure was supported by the data, at k=4 CSE dogs collected in the central region of Portugal clustered together (data not shown). However, their individual q values were low (Q=0.665±0.276), and some dogs appear admixed with the CSE dogs group sampled in the north. Three CSE dogs of unknown origin were within this geographic group (CSE016, CSE056, and CSE057) and also showed ancestry from the northern cluster.

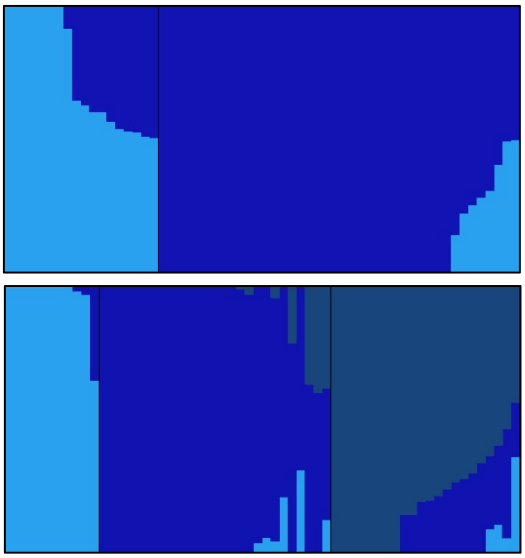


Figure 12: Representation of individual genotype membership coefficients obtained with fastStructure for the within-breed analysis of 'Cão da Serra da Estrela' for k=2 (top) and *k*=3 (bottom). There were two clusters of dogs sampled in the southern region (light blue and grey), and one cluster comprising mainly dogs sampled in the north of the country (dark blue).

5.4.3. Y chromosome analysis

The following SNPs were monomorphic in these populations and thus not informative: *Y20p1-35, Y24p1-11, Y29p2-56, Y30p1-655* and *YNp1-608*. Haplotype composition and frequencies are shown in Table 8. Y-chromosome diversity was low with a single haplotype (H1) detected in 32 CSE (*H*=0.00±0.00) and 46 males from other Portuguese native breeds (*H*=0.00±0.00). Two Y-haplotypes (H1 and H7/H10/H23) were found in the SD population (*H*=0.068±0.027). Iberian wolves showed two distinct haplotypes (H32 and H33/H34) from those found in the domestic dogs (*H*=0.013±0.013).

Relationships among Y-haplotypes as defined by 9 SNPs are depicted in the median-joining network of Figure 13. The CSE dogs clustered with all other Portuguese native dogs and the majority of SD within the H1 lineage. Iberian wolf patrilines (H32 and H33/H34) were separated from the domestic dog group by one median vector, and clustered closer to the other SD haplotype (H7/H10/H23).

Table 8: Frequencies of Y-chromosome haplotypes observed in 90 males of the following populations: 32 'Cão da Serra da Estrela'; 46 dogs from other Portuguese native breeds; 12 stray dogs; and 32 Iberian wolves.

Population	T-A-A-C-T-G-C-G-G H1	T-G-C-T-T-G-C-T-G H7/H10/H23	T-G-C-C-T-G-T-T-G H32	T-G-C-T-T-G-T-T-G H33/H34
'Cão da Serra da Estrela'	32			
Other dogs	46			
Stray dogs	11	1		
Iberian Wolf			30	2

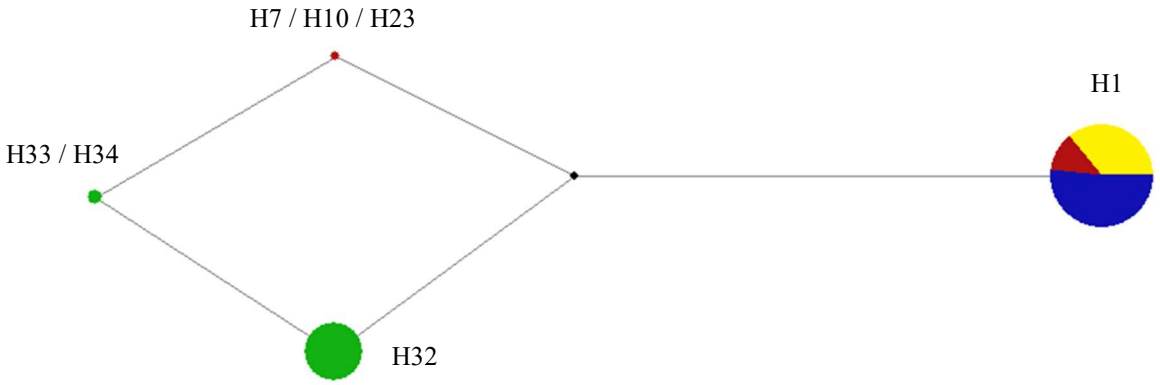


Figure 13: Median-joining network of Y-chromosome haplotypes obtained with 9 SNPs. A total of 90 males were included in the analysis, as follows: 32 'Cão da Serra da Estrela' (blue); 46 dogs from other Portuguese native breeds (yellow); 12 stray dogs (red); and 32 Iberian wolves (green).

5.5. Discussion

The SNPs panel genotyped was useful to investigate genome-wide genetic diversity of the Portuguese native CSE breed in comparison to stray dogs and the Iberian wolf. Additionally, these markers allowed to infer genetic structure within the CSE shepherd dog breed. The genetic diversity detected in this study was within the range estimated with the Illumina CanineHD Beadchip for other dog and wolf populations. The CSE breed showed somewhat high diversity (*Ho*=0.26), when compared to other pedigree dogs for whole-

genome SNPs (0.15<*Ho*<0.29) (Quignon et al., 2007; Mortlock et al., 2016). Inbreeding appears to be negligible (*Fis*=-0.005), and considerably lower than the described for a large range of dog breeds (-0.10 in Bull terrier to 0.13 in the Whippet) (Mortlock et al., 2016). Studies of STRs and mtDNA markers also confirmed CSE as one of the Portuguese breeds that holds greatest genetic diversity (Pires et al., 2006, 2009). We would expect SD to show higher diversity than the CSE and the IW populations, but the low value found (*Ho*=0.17) might be due to their low sample size and also to ascertainment bias resulting from the development of the SNP array in specific dog breeds (probably inbred and showing lower levels of genetic diversity than SD). The Iberian wolf population appears to retain significant genetic diversity showing higher heterozygosity (*Ho*=0.31) for whole-genome SNPs than the observed for wolves from North America and Europe (0.12<*Ho*<0.28) (vonHoldt et al., 2011; Cronin et al., 2015).

The results of PCA, allele-sharing distances, and Bayesian clustering analyses were mostly consistent. The CSE dogs formed an independent cluster, and subtle within-breed sub-structure was detected. The genetic structure inferred is consistent with geographical differentiation of dogs sampled in the north of Portugal and the two groups of dogs sampled in the southern region. Interestingly, CSE samples collected in the central region of the country reflect influences from dogs sampled in the north and south of Portugal. This appears to be indicative of lineage sorting associated to different breeders in each region. Because breeders often acquire CSE dogs of different origins, it is expected that some samples collected in a particular region may indeed have ancestors from other geographic areas. This explains the fact that a number of dogs were assigned to a cluster other than the sample source, or showed admixture from more than one cluster, as determined with fastStructure. Understanding the patterns of within-breed genetic structure may help to develop appropriate management and conservation programs in CSE, to maintain genetic diversity and investigate the genetic basis for disease-associated traits (e.g. related to canine hip dysplasia, a prevalent condition in this breed).

In the neighbor-joining dendrogram of allele sharing distances between individuals, SD appear well differentiated. However, in the PCA and the more refined Bayesian clustering analysis the SD population emerges as admixed and entangled with the CSE breed. Rather than genetic proximity with the CSE breed, such result might imply that populations that could have influenced the heterogeneous SD group (including other Portuguese native breeds) are missing. Bayesian clustering is sensitive to model assumptions, namely the method will

assign admixture proportions for each individual across all the populations represented in the dataset, even when the ancestral population is missing (Raj et al., 2014).

Regarding the IW and consistently across methods, it was clearly differentiated from all dog populations corroborating the results of previous studies with other markers (Godinho et al., 2011; Pires et al., 2016). This result is relevant concerning IW conservation, as it represents a biodiversity reservoir for the species without strong signals of dilution from admixture with their domestic counterparts. Despite, IW and the heterogeneous SD population shared some genetic diversity (~20% as inferred with fastStructure for k=2).

Y-chromosome markers are useful to investigate recent demographic events, namely founder effects, population expansions and bottlenecks. Our results confirm that unlike the observed for STRs, Y-chromosome diversity detected with Y-SNPs is low (Brown et al., 2011; Sacks et al., 2013; Pires et al., 2016). The CSE samples analyzed here belong to a single patriline (H1) together with most SD males, as determined in a previous study of Y-haplotypes in Portuguese native dogs (Pires et al., 2016). This patriline belongs to the major Y-haplogroup HG1/HG3 described by Ding et al. 2012, which is shared with domestic dogs distributed worldwide (Sacks et al., 2013). One SD carried a distinct haplotype (H7/H10/H23), which is shared by dogs and wolves from several geographic origins. This lineage belongs to the major haplogroup HG23 which is highly frequent in dogs from Asia (Ding et al., 2012). Iberian wolves were well-differentiated from the dog populations included in this study, and belong to distinct Y-haplotypes (H32 and H33/H34) from those found in wolves from other European regions, Asia and North America (Brown et al., 2011; Ding et al., 2012; Pires et al., 2016).

5.6. Conclusion

In our study, a dog breed native to Portugal was analyzed for the first time with whole-genome SNP markers. We carried out a comprehensive analysis of the within-breed diversity and genetic structure of one of the most ancient shepherd dog breed from Portugal, 'Cão da Serra da Estrela'. Our results indicate that CSE breed retains high genetic diversity and is well differentiated, supporting the maintenance of management and conservation programs to preserve key domestic animal genetic resources. Although weak, the within-breed structure observed in CSE appears to be related to the geographic origin of the samples, which is probably indicative of lineage sorting associated to different breeders, and not to dog function (work vs. pet). This information is useful regarding breed management, and can reflect the

recent abandonment of the use of these dogs for their primary function, i.e. to protect the herds from Iberian wolf attacks and to lead sheep during transhumance. This study sets the basis for an understanding of the genetic mechanisms underlying complex disease traits that affect CSE breed, namely canine hip dysplasia, although more thorough genomic analyses, of a larger number of dogs with good quality phenotypic registries, is necessary.

5.7. Acknowledgements

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6. Concluding remarks and future perspectives

In the present dissertation it was proposed to investigate radiographic approaches for the diagnosis of CHD, and to analyze the genetic diversity and population structure of the CSE breed with newly developed markers, namely the Illumina canine SNP array.

The international collaboration between the different Institutions involved in this project was very enriching both at the personal and professional levels. It indorsed the discussion and knowledge broadening of different techniques, work methodologies and the development of a contacts network.

The published review article (third chapter) allowed for an overall perspective on what it is now being done regarding CHD diagnosis giving special attention to both radiographic and molecular aspects. We pointed out that, due to the complex genetic architecture behind CHD, new research is always being produced and there is still an extensive unknown area to be discovered and investigated.

Pelvic radiographic evaluation remains the primordial and most commonly diagnostic method used. In this project three different techniques were assessed, the NA measurement in the SVDV, the DI in the PennHIP method and the DLS score (the latter was developed at Cornell University by R Todhunter, with whom I had the pleasure to work and learn from his large experience and expertise). It is known that the methods used to estimate hip joint laxity, PennHIP and DLS, are the most accurate predictors of the indication if a hip is non-dysplastic. But these are not accessible to every veterinarian, because additional formation and/or specific equipment are needed. The SVDV with NA measurement is generally used in practice and with our analysis we could assess that it is possible to maximize its specificity using a different cut-off, ~112° instead of the regular 105°, to obtain equivalent precision in our results as given by DI or DLS.

Originally, it was proposed to do a genome-wide association study in the CSE breed to investigate the genetic basis for CHD traits. This was impaired both due to lack of funding and an insufficient number of individuals with high quality phenotypic data. Because such analysis also demands for a good knowledge of the within-breed genetic structure, it was decided to carry out a comprehensive analysis of the genetic variation and population structure of CSE. The results obtained are innovative and not yet described in CSE, which is one of the most important Portuguese native dog breeds. We found a weak level of sub-structure in CSE which appears to relate to lineage sorting associated to specific breeders in

each region, and not to function (i.e. work vs. pet dogs) as one would probably expect. These results warrant further research namely to determine molecular coancestry coefficients, and also to include a larger sample of work dogs. These data provide the basis for a future genome-wide association study using a larger set of CSE purebred dogs, by taking into account population structure and considering that high heritability has been confirmed at least for some traits (e.g. hip laxity) related to CHD.

Ultimately, it would be useful to detect the genome sections responsible for the undesired traits and select markers to include in a commercial DNA-based test specific for this breed. This would be an important finding for CHD diagnosis: breeders would be able to know if their puppies are carriers of CHD traits with a simple drop of blood. It is possible that, at first, the cost of this test would be high but with the continued use one would expect it to become more accessible to everyone. This would facilitate to remove susceptible dogs from reproduction programs and to reduce prevalence of CHD in this Portuguese native dog breed.

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Supplementary information

Table S1: Results of the Bayesian cluster analysis done with fastStructure and showing individual genotype membership coefficients (*q*) in each cluster obtained for *k*=2, *k*=3 and *k*=4 (partitions of the dataset) for the 'Cão da Serra da Estrela' (CSE), stray dogs (SD) and Iberian wolf (IW) populations. Details on the animals sampled are also shown, namely population of origin, individual identification, gender (coded as F for female and M for male), geographic location of sampling, phenotypes for canine hip dysplasia and CSE breed use (work vs pet). †Phenotypes for canine hip dysplasia as judged by pelvic radiograph, unaffected - 1, affected - 2.

						Individual *q*, *k*=2		**Individual *q*, *k*=3**			**Individual *q*, *k*=4**			
Population of origin	**Individual identification**	**Gender**	**Region**	**Phenotype†**	**CSE Breed use**	**Cluster 1**	**Cluster 2**	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 4**
CSE	CSE15117	F	Center	1	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15118	F	South	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15120	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15122	M	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15123	F	South	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15124	M	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15125	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15126	M	South	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	0,328	0,672
CSE	CSE15127	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15128	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15129	M	North	1	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15130	F	South	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15131	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15133	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15134	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15136	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15138	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000

Population of origin	Individual identification	Gender	Region	Phenotype†	CSE Breed use	Individual *q*, *k*=2		Individual *q*, *k*=3			Individual *q*, *k*=4			
						Cluster 1	Cluster 2	Cluster 1	Cluster 2	Cluster 3	Cluster 1	Cluster 2	Cluster 3	Cluster 4
CSE	CSE15140	M	North	2	work dog	0,022	0,978	0,059	0,000	0,941	0,000	0,086	0,914	0,000
CSE	CSE15142	M	South	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15143	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	0,703	0,297
CSE	CSE15144	M	North	1	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15145	F	North	1	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15147	M	South	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15148	F	North	1	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15149	M	North	2	work dog	0,039	0,961	0,091	0,000	0,909	0,000	0,125	0,875	0,000
CSE	CSE15150	F	Center	1	work dog	0,113	0,887	0,117	0,000	0,883	0,000	0,154	0,846	0,000
CSE	CSE15155	F	South	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	0,000	1,000
CSE	CSE15156	M	Center	1	pet dog	0,011	0,989	0,062	0,000	0,938	0,000	0,097	0,903	0,000
CSE	CSE15157	F	South	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	0,000	1,000
CSE	CSE15158	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15162	F	North	1	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15163	M	North	1	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15164	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15165	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15166	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15167	M	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15168	M	North	1	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15169	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15170	M	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15171	F	South	1	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	0,000	1,000
CSE	CSE15175	F	Center	1	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,002	0,998	0,000

Population of origin	Individual identification	Gender	Region	Phenotype[†]	CSE Breed use	Individual *q*, *k*=2		Individual *q*, *k*=3			Individual *q*, *k*=4			
						Cluster 1	Cluster 2	Cluster 1	Cluster 2	Cluster 3	Cluster 1	Cluster 2	Cluster 3	Cluster 4
CSE	CSE15176	M	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15177	F	South	1	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	0,995	0,005
CSE	CSE15178	F	North	2	work dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE15179	F	South	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	0,000	1,000
CSE	CSE15180	F	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	0,000	1,000
CSE	CSE15137	M	Center	2	work dog	0,031	0,969	0,076	0,000	0,924	0,000	0,108	0,892	0,000
CSE	CSE15151	M	Center	2	work dog	0,048	0,952	0,079	0,000	0,921	0,000	0,106	0,894	0,000
CSE	CSE005	F	Center	1	work dog	0,058	0,942	0,077	0,000	0,923	0,000	0,106	0,894	0,000
CSE	CSE016	M	0	0	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE019	F	0	0	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	0,000	1,000
CSE	CSE023	F	0	0	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE030	M	0	0	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE036	M	Center	1	pet dog	0,025	0,975	0,063	0,000	0,937	0,000	0,097	0,903	0,000
CSE	CSE038	M	North	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE043	M	0	0	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	0,000	1,000
CSE	CSE045	F	0	0	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE056	F	0	0	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE057	M	0	0	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
CSE	CSE058	F	South	2	pet dog	0,000	1,000	0,000	0,000	1,000	0,000	0,000	1,000	0,000
SD	SD104	F	-	0	-	0,205	0,795	0,221	0,000	0,779	0,000	0,282	0,718	0,000
SD	SD105	M	-	0	-	0,183	0,817	0,201	0,000	0,799	0,000	0,258	0,742	0,000
SD	SD36	M	-	0	-	0,337	0,663	0,261	0,067	0,671	0,064	0,316	0,620	0,000
SD	SD44	M	-	0	-	0,196	0,804	0,222	0,000	0,778	0,000	0,276	0,724	0,000
SD	SD64	M	-	0	-	0,197	0,803	0,228	0,000	0,772	0,000	0,279	0,721	0,000

Population of origin	Individual identification	Gender	Region	Phenotype†	CSE Breed use	Individual *q*, *k*=2		Individual *q*, *k*=3			Individual *q*, *k*=4			
						Cluster 1	Cluster 2	Cluster 1	Cluster 2	Cluster 3	Cluster 1	Cluster 2	Cluster 3	Cluster 4
SD	SD65	F	-	0	-	0,185	0,815	0,189	0,000	0,811	0,000	0,234	0,766	0,000
SD	SD66	F	-	0	-	0,166	0,834	0,184	0,000	0,816	0,000	0,228	0,772	0,000
SD	SD68	F	-	0	-	0,167	0,833	0,192	0,000	0,808	0,000	0,244	0,756	0,000
SD	SD97	M	-	0	-	0,185	0,815	0,196	0,000	0,804	0,000	0,246	0,754	0,000
SD	SD98	F	-	0	-	0,196	0,804	0,209	0,000	0,791	0,000	0,256	0,744	0,000
IW	IW005	F	-	0	-	1,000	0,000	0,000	1,000	0,000	1,000	0,000	0,000	0,000
IW	IW023	F	-	0	-	1,000	0,000	0,000	1,000	0,000	1,000	0,000	0,000	0,000
IW	IW053	M	-	0	-	1,000	0,000	0,000	1,000	0,000	1,000	0,000	0,000	0,000
IW	IW091	F	-	0	-	1,000	0,000	0,000	1,000	0,000	1,000	0,000	0,000	0,000
IW	IW125	M	-	0	-	1,000	0,000	0,000	1,000	0,000	1,000	0,000	0,000	0,000
IW	IW286	M	-	0	-	1,000	0,000	0,000	1,000	0,000	1,000	0,000	0,000	0,000
IW	IW316	M	-	0	-	1,000	0,000	0,000	1,000	0,000	1,000	0,000	0,000	0,000
IW	IW327	M	-	0	-	1,000	0,000	0,000	1,000	0,000	1,000	0,000	0,000	0,000
IW	IW337	F	-	0	-	1,000	0,000	0,000	1,000	0,000	1,000	0,000	0,000	0,000
IW	IW392	F	-	0	-	1,000	0,000	0,000	1,000	0,000	1,000	0,000	0,000	0,000

SD samples were collected in a small village in Viana do Castelo (north of Portugal) and the Municipal Animal Shelter in Porto; IW samples were collected in Viana do Castelo and Bragança (north of Portugal).

Table S2: Results of the Bayesian cluster analysis done with fastStructure and showing individual genotype membership coefficients (*q*) in each cluster obtained for *k*=2, *k*=3 and *k*=4 (partitions of the dataset) for the 'Cão da Serra da Estrela' breed (CSE). Individual identification and geographic location of sampling are also shown.

Individual identification	Region	Individual *q*, *k*=2		Individual *q*, *k*=3		
		Cluster 1	Cluster 2	Cluster 1	Cluster 2	Cluster 3
CSE005	Center	0,504	0,496	0,106	0,298	0,596
CSE016	0	0,496	0,504	0,000	0,266	0,734
CSE019	0	1,000	0,000	0,000	0,000	1,000
CSE023	0	0,000	1,000	0,000	1,000	0,000
CSE030	0	0,220	0,780	0,000	0,632	0,368
CSE036	Center	0,645	0,355	0,000	0,144	0,856
CSE038	North	0,000	1,000	0,000	1,000	0,000
CSE043	0	1,000	0,000	0,000	0,000	1,000
CSE045	0	0,000	1,000	0,058	0,942	0,000
CSE056	0	0,305	0,695	0,361	0,201	0,438
CSE057	0	0,527	0,473	0,000	0,238	0,762
CSE058	South	0,000	1,000	1,000	0,000	0,000
CSE15117	Center	0,000	1,000	0,000	1,000	0,000
CSE15118	South	0,524	0,476	0,000	0,215	0,785
CSE15120	North	0,000	1,000	0,038	0,962	0,000
CSE15122	North	0,000	1,000	0,000	1,000	0,000
CSE15123	South	0,000	1,000	1,000	0,000	0,000
CSE15124	North	0,000	1,000	0,000	1,000	0,000
CSE15125	North	0,000	1,000	0,000	1,000	0,000
CSE15126	South	0,912	0,088	0,000	0,000	1,000
CSE15127	North	0,000	1,000	0,000	1,000	0,000
CSE15128	North	0,000	1,000	0,000	1,000	0,000
CSE15129	North	0,000	1,000	0,000	1,000	0,000
CSE15130	South	0,000	1,000	0,966	0,034	0,000
CSE15131	North	0,000	1,000	1,000	0,000	0,000
CSE15133	North	0,000	1,000	0,000	1,000	0,000
CSE15134	North	0,000	1,000	1,000	0,000	0,000
CSE15136	North	0,000	1,000	0,212	0,788	0,000
CSE15138	North	0,000	1,000	0,000	0,987	0,013
CSE15140	North	0,508	0,492	0,000	0,299	0,701
CSE15142	South	0,000	1,000	1,000	0,000	0,000
CSE15143	North	0,600	0,400	0,000	0,278	0,722
CSE15144	North	0,000	1,000	0,000	0,965	0,035
CSE15145	North	0,000	1,000	0,000	1,000	0,000
CSE15147	South	0,002	0,998	1,000	0,000	0,000
CSE15148	North	0,000	1,000	0,000	1,000	0,000
CSE15149	North	0,599	0,401	0,000	0,197	0,803
CSE15150	Center	0,536	0,464	0,089	0,274	0,636
CSE15155	South	1,000	0,000	0,000	0,000	1,000
CSE15156	Center	0,626	0,374	0,000	0,143	0,856
CSE15157	South	1,000	0,000	0,000	0,000	1,000
CSE15158	North	0,000	1,000	0,000	1,000	0,000
CSE15162	North	0,000	1,000	0,000	1,000	0,000
CSE15163	North	0,000	1,000	0,000	1,000	0,000
CSE15164	North	0,000	1,000	0,000	1,000	0,000
CSE15165	North	0,139	0,861	0,000	0,784	0,216
CSE15166	North	0,565	0,435	0,000	0,192	0,808
CSE15167	North	0,000	1,000	0,311	0,689	0,000

Individual identification	Region	Individual *q*, *k*=2		Individual *q*, *k*=3		
		Cluster 1	Cluster 2	Cluster 1	Cluster 2	Cluster 3
CSE15168	North	0,000	1,000	0,646	0,354	0,000
CSE15169	North	0,000	1,000	1,000	0,000	0,000
CSE15170	North	0,000	1,000	1,000	0,000	0,000
CSE15171	South	1,000	0,000	0,000	0,000	1,000
CSE15175	Center	0,251	0,749	0,126	0,490	0,384
CSE15176	North	0,000	1,000	0,980	0,020	0,000
CSE15177	South	0,279	0,721	0,000	0,600	0,400
CSE15178	North	0,000	1,000	0,046	0,908	0,046
CSE15179	South	1,000	0,000	0,000	0,000	1,000
CSE15180	North	1,000	0,000	0,000	0,000	1,000
CSE15137	Center	0,490	0,510	0,004	0,332	0,664
CSE15151	Center	0,404	0,596	0,057	0,408	0,535