Genetic Diversity Testing for English Mastiff

Overview

The Veterinary Genetics Laboratory (VGL), in collaboration with Dr. Niels C. Pedersen and staff, has developed a panel of short tandem repeat (STR) markers that will measure genetic heterogeneity and diversity across the genome and in the Dog Leukocyte Antigen (DLA) class I and II regions for specified dog populations. This test panel will be useful to dog breeders who wish to use DNA-based testing as a supplement to in-depth pedigrees. DNA based information on genetic heterogeneity and diversity, along with DNA testing results for desired phenotypes and health traits, can aid in informing breeding decisions.

A DNA-based genetic assessment of the English Mastiff breed is now in the preliminary results phase with the objective of creating a snapshot of individual- and breed-wide genetic heterogeneity and diversity. This initial testing involved 23 English Mastiff from the USA (n=20) and Australia (n=3). These dogs were tested on a voluntary basis and include closely related pedigrees as well as two littermates rather than the preferred sampling of dogs specifically selected to be as unrelated as possible and most accurately represent the breed as a whole. We are accepting additional dogs from all parts of the world with a goal of 50-75 individuals to complete this preliminary phase. This data base will be progressively expanded as more dogs are added with the goal of characterizing all the known alleles for the breed at 33 STR autosomal loci and existing DLA class I and II haplotypes identified by seven additional STRs.

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Results reported as:
Short tandem repeat (STR) loci: A total of 33 STR loci from across the genome were used to gauge genetic heterogeneity and existing genetic diversity within an individual and across the breed. The alleles inherited from each parent are displayed graphically to highlight heterozygosity and genetic diversity in individuals and breed wide.

DLA haplotypes: Seven STR loci linked to the DLA class I and II genes were used to identify genetic differences in a region that regulates immune responses and self/non-self-recognition. Problems with self/non-self-recognition, along with non-genetic factors in the environment, are responsible for autoimmune disease, allergies, and susceptibility to infectious agents.

Internal Relatedness: The IR value is a measure of the genetic relatedness of an individual's parents. The value takes into consideration both heterozygosity of alleles at each STR loci and their relative frequency in the population. Therefore, IR values heterozygosity over homozygosity and uncommon alleles over common alleles. IR values are unique to each dog and two individuals from different sources may have identical IR values but a very different genetic makeup.

I. Introduction to the English Mastiff

A. Breed history [1-4]

1. Ancient origins- The **English Mastiff,** often simply referred to as Mastiff, are known for their strength and size [1]. Indeed, the greatest weight (155.6 kg) ever officially documented was for an English Mastiff (call name Zorba) in the 1989 edition of the Guinness Book of Records. The contemporary English Mastiff is presumed to have descended from ancient indigenous breeds used as herding, guard and war dogs in Tibet and travelled with migrating tribes through India, Persia, Mesopotamia (what is today Iran and Iraq), Syria and from there to Greece and Europe [2]. These ancient mastiffs were commonly known as Molosser after the Molassians of Epirus [5]. Unfortunately, today among fanciers and breed scholars, the erroneous theory that Mastiffs were in fact introduced by the Phoenicians to the Isles, while navigating into Europe, is widespread, in total disregard of the historical fact that there is no archeological evidence of the Phoenicians ever arriving to the Isles. Mastiff type dogs arrived at the British Isles during the various migrations through the north of Europe, human migrations of the early pro-celtic tribes, who were in fact part of the Alani people (Aryans). [2]. The name Molosser has been also given to several large and solidly built dog breeds that share a common ancestor. English versions of a "British" Molosser were reported by the Roman soldiers during the invasion by the Roman Empire (43 AC) and during battles with Celtic tribes under the lead of the warrior Queen Boadicea. Two distinct types or breeds were reported by the Romans, the smaller but ferocious ***Briton*** and the bigger ***Pugnaces Britanniae***. This last "breed" was pitted against the against the ***pugnaces of Epirus*** and found to be superior to them. Roman historians described them as being superior to their own military dogs, being of great stature, heavy built with broad back and muscular legs, truncated muzzle, loose skin above the brows and light brown eyes [2]. These indigenous Mastiff were exported throughout the Roman empire to fight in their coliseums.

The subsequent history of Mastiff in the British Isles involved a more recent and now extinct type of hunting Molosser known as the Alaunt. The Alaunt were originally bred by the Alani tribes, who rose to prominence in the 1st century CE. A group of Alani subsequently migrated westward to modern day Armenia and then on to Albania in the 5th and 6th centuries BC and later into the rest of Western Europe, including the region known as Gaul (today's France) [7]. Consequently, Alaunt interbred with various indigenous breeds throughout Western Europe in subsequent centuries. The first white Alaunt were brought to Britain with the Normand invasion led by William the Conqueror (11th century CE), where they interbred with the Pugnaces Brittannie and found great use as guard and fighting dogs. Certain ancestors of Pugnaces Brittanniae and Alaunt ultimately became known as the Old English Mastiff. The English Mastiff underwent further refinement in the late 19th century with introgressions from the Tibetan Mastiff, the Alpine Mastiff, the Dogue du Bordeaux and the Spanish Bulldog [8]. The Alpine Mastiff is an extinct breed that belonged to the indigenous Molosser of Southern Europe and itself a progenitor of several modern breeds such as the St. Bernard. The Spanish bulldog also extinct was also a Molosser dog type originating in Spain to catch cattle, hunt, and guard.

Ancient mastiff-type dogs have been used in the creation of many modern breeds [5,9]. Contemporary breeds with large dogs of mastiff type include the Mastiff, Bullmastiff, Spanish Mastiff, Pyrenean Mastiff, Turkish Mastiff (Kangal), South African Mastiff (Boerboel), Neapolitan Mastiff, Tibetan Mastiff, Cane Corso, French Mastiff (Dogue de Bordeaux), Japanese Mastiff (Tosa-Inu), Great Dane, St. Bernard and many others [9]. Mastiff-influenced breeds of medium stature include the Rottweiler, Boxer, Serrano Bulldog, Caucasian Shepherd Dog (Ovtcharka), Brazilian Mastiff (Fila Brasileiro) and Presa Canario, while Mastiff-type breeds of smaller stature include the Bulldog, French Bulldog, Olde English Bulldogge, Ca de Bou, Bulldog Campeiro and Sharpei.

2. The contemporary English Mastiff- Bull baiting, dog fighting, and other activities deemed to be cruel were banned by the British Parliament in 1835 [10]. However, Mastiffs continued to be used for guarding and became increasingly popular as household pets and in dog shows. The subsequent history of the breed has been traced by a long series of prominent stud dogs and bitches from 1813 onward. The first Mastiff was shown in 1859, followed by 63 Mastiffs in a single show in 1871. The first Breed clubs were established in Britain (1873) and in the United States (American Mastiff Club in 1879, and the Old English Mastiff Club (1883). No pedigrees were kept at the time as are kept now, but the end result of this type of breeding was the progenitor of the modern breed. These prototypic English Mastiff were of variable form and sound health, reflecting their more haphazard ancestry. A breed standard was created by the Old English Mastiff Club in 1883 and relative points assigned to various desired traits in 1890 [3]. A standard for American dogs was created by the Mastiff Club of America in 1929 and further modified in 1941. The creation of a breed standard necessitated inbreeding to standardize and further refine the breed's appearance. The appearance of these refined English Mastiffs was widely attributed to a popular sire Ch. Crown Prince who was massive, notwithstanding his faults: more brachycephalic and straight-stifled than other members of the early breed, prey eyes (light colored), dudley nose (liver colored), reddish mask and somewhat short tail [8]. Therefore, the breed gained a consistency of type, with leaner, longer-headed specimens being replaced by more massive and short-faced dogs. Unfortunately, this also led to a decrease in soundness and a decline in the popularity. Mastiff numbers in the US declined steadily through the 1890s and only 24 Mastiffs were registered in the United States from 1906 to 1918 and the breed became largely extinct outside of Britain by the end of WWI.

The end of WWI caused an increased interest in the breed. A dog called Beowulf, bred in Canada but of British parents, was registered by the American Kennel Club and re-established the breed in the USA. Several others were imported into the USA in the period between WWI and WWII. However, the total genetic pool of Mastiffs in the country after WWII consisted of 14 Mastiffs.

Mastiff breeding virtually stopped during WWII due to the rationing of meat and many of the puppies born after WWII succumbed to canine distemper. Only a single female of breeding age remained, a bitch named Nydia Of Frithend - who was by the brindle Bullmastiff/Mastiff mix male Templecombe Taurus and out of Sally of Coldblow, both alive after the end of WWII. As a result, several dogs from North America, and predominantly from Canada, were imported into Britain. Therefore, all Mastiffs in the world in the late 1950s were theoretically descended by pedigree from Nydia, the 14 Mastiffs sent back to England from Canada and the USA. All male

bloodline traced back to Ch. Crown Prince. In 1959, a Dogue de Bordeaux bitch was imported to the USA from France and registered as a Mastiff, Fidele de Fenellon, and became the 16th theoretical English Mastiff animal in the post-WWII gene pool [5]. Since that time, the breed has gradually been restored in Britain, has reached 29th most popular breed in the USA [6] and is now found worldwide.

B. Appearance

With a massive body, broad skull and head of generally square appearance, it is the largest dog breed in terms of mass. It is on average slightly heavier than the Saint Bernard, although there is a considerable overlap of size and weight between these two breeds. The Irish Wolfhound and Great Dane can be more than six inches taller but are not nearly as heavy in stature.

The American breed standard has a minimum height of 30 inches (76 cm) at the withers for males and 27.5 inches (70 cm) for females [2]. A typical male can weigh 150–250 pounds (68–113 kg), a typical female can weigh 120–200 pounds (54–91 kg), with very large individuals reaching even more. The typical coat is moderately short and close lying, but long-haired Mastiffs caused by a recessive mutation are occasionally seen. A long coat is considered by all breed standards a fault, but not cause for disqualification. The coat colors accepted today are three: fawn, apricot and brindle (actually a pattern and not a color *per se*). The fawn is generally a light silver but may range up to a golden yellow. The apricot may be a slightly reddish hue up to a deep rich red. The brindle markings should ideally be heavy, even and clear stripes, but may actually be light, uneven, patchy, faint or muddled. Pied Mastiffs occur rarely and even rarer are the Blues - solid blacks have disappeared. A black mask is present in all individuals.

C. Temperament

The American Kennel Club sums up the temperament of the Mastiff as a combination of grandeur and good nature with courage and docility. In short, a Mastiff is powerful and alert, yet gentle and loyal.

II. Genetic diversity studies of contemporary Mastiff

A. Population genetics based on 33 STR loci on 25 chromosomes

STR markers are highly polymorphic and have great power to determine genetic differences among individuals and breeds. The routine test panel contains 33 STRs consisting of those that are recommended for universal parentage determination for domestic dogs by the International Society of Animal Genetics (ISAG) and additional markers developed by the VGL for forensic purposes. Each of these STR loci is known to contain from 7 to 27 different alleles (avg. 15.4 alleles/locus) when tested across many breeds of dogs. Each breed, having evolved from a small number of founders and having been exposed to artificial genetic bottlenecks will end up with only a portion of the total available diversity. Artificial genetic bottlenecks include such things as popular sire effects, geographic isolation, catastrophes, outbreaks of disease, and ups and downs in popularity and resulting increases and decreases in population size. The alleles identified at

each of the 33 STR loci and their relative frequencies were determined for the 23 English Mastiff are listed in Table 1. Link to Table 1

Table 1. Identified alleles and their frequencies for 33 autosomal STR markers in English Mastiff (n=23)

AHT121	AHT137	AHTH130	AHTh171A	AHTh260	AHTk211
94 (0.13)	131 (0.35)	121 (0.41)	219 (0.15)	238 (0.50)	87 (0.24)
96 (0.11)	137 (0.17)	123 (0.02)	223 (0.13)	242 (0.07)	89 (0.67)
100 (0.54)	139 (0.02)	127 (0.28)	225 (0.30)	244 (0.07)	91 (0.04)
102 (0.17)	147 (0.04)	129 (0.20)	227 (0.02)	246 (0.17)	93 (0.02)
104 (0.04)	149 (0.41)	131 (0.09)	229 (0.07)	250 (0.20)	95 (0.02)
			231 (0.09)		
			233 (0.11)		
			235 (0.04)		
			237 (0.09)		

AHTk253	C22.279	FH2001	FH2054	FH2848	INRA21
286 (0.54)	116 (0.24)	128 (0.02)	152 (0.61)	236 (0.07)	93 (0.04)
288 (0.15)	118 (0.04)	132 (0.39)	156 (0.20)	238 (0.37)	95 (0.46)
290 (0.04)	120 (0.46)	140 (0.04)	160 (0.09)	240 (0.39)	97 (0.07)
292 (0.20)	126 (0.26)	144 (0.24)	164 (0.09)	242 (0.09)	101 (0.37)
296 (0.07)		156 (0.30)	168 (0.02)	244 (0.09)	103 (0.07)

INU005	INU030	INU055	LEI004	REN105L03	REN162C04
110 (0.04)	144 (0.30)	210 (0.17)	85 (0.11)	233 (0.39)	202 (0.59)
122 (0.07)	148 (0.07)	214 (0.02)	95 (0.13)	235 (0.20)	204 (0.15)
124 (0.83)	150 (0.63)	218 (0.80)	105 (0.17)	237 (0.41)	208 (0.26)
126 (0.04)			107 (0.59)		
132 (0.02)					

REN169D01	REN169O18	REN247M23	REN54P11	REN64E19	VGL0760
202 (0.15)	168 (0.07)	268 (0.89)	226 (0.04)	145 (0.02)	12 (0.11)
212 (0.28)	170 (0.09)	270 (0.02)	232 (0.09)	147 (0.07)	13 (0.28)
216 (0.57)	172 (0.85)	272 (0.09)	234 (0.17)	149 (0.04)	20.2 (0.02)
			236 (0.61)	153 (0.87)	21.2 (0.39)
			238 (0.09)		22.2 (0.15)
					23.2 (0.04)

VGL0910	VGL1063	VGL1165	VGL1828	VGL2009	VGL2409
12 (0.17)	13 (0.04)	15 (0.39)	15 (0.15)	9 (0.17)	13 (0.04)
16.1 (0.02)	14 (0.80)	16 (0.02)	16 (0.33)	11 (0.07)	17 (0.39)
17.1 (0.11)	15 (0.11)	18 (0.09)	17 (0.02)	12 (0.02)	18 (0.33)
18.1 (0.20)	19 (0.04)	19 (0.20)	19 (0.37)	13 (0.52)	19 (0.22)
19.1 (0.28)		21 (0.02)	20 (0.13)	14 (0.22)	22 (0.02)
20.1 (0.20)		23 (0.02)			
21.1 (0.02)		26 (0.04)			
		27 (0.22)			

VGL2918	VGL3008	VGL3235
12 (0.07)	14 (0.07)	13 (0.17)
13 (0.30)	17 (0.04)	14 (0.24)
14 (0.07)	18 (0.35)	16 (0.04)
18.3 (0.04)	19 (0.37)	17 (0.54)
19.3 (0.50)	20 (0.11)	
20.3 (0.02)	21 (0.07)	

B. Assessment of population diversity using standard genetic parameters

Allele and allele frequencies at each of the 33 STR loci are listed in Table 1 and used to determine basic genetic parameters (Table 2), such as the number of alleles found at each STR locus (Na); the number of effective alleles (Ne) per locus (i.e., the number of alleles that contribute most to genetic differences); the observed or actual heterozygosity (Ho) that was

found; and the heterozygosity that would be expected (He) if the existing population is being randomly bred. The value F is a coefficient of inbreeding derived from the Ho and He values. A value of +1.0 would occur only if every individual were genetically indistinguishable at each of the 33 STR loci, while a value of -1.0 would be seen when all of the dogs were completely different at each of the 33 loci. A value of 0.00 would be seen if the selection of sires and dams was entirely random in reference to the existing gene pool.

The allele frequency data obtained from the 33 STR panels can be used to assess heterozygosity within a population (Table 2). Using the 33-marker panel, the 23 English Mastiffs had an average of 4.79 alleles/loci (Na). However, the average number of alleles is less important than the number of alleles that have the greatest genetic influence on heterozygosity, a figure known as average effective alleles/loci or Ne. The Ne in this group of dogs averaged 2.78 effective alleles per locus. This means that 2.78/4.79=58% of the alleles were the same in virtually all English Mastiff. This figure is close to the average percentage of homozygous single nucleotide polymorphisms (SNPs) seen in the genomes of most pure breeds of dogs, i.e., 60% [10].

The observed (actual) heterozygosity of this group of 23 dogs was 0.57, while the expected heterozygosity (He) for a population in Hardy-Weinberg equilibrium (HWE) was similar at 0.59, yielding a coefficient of inbreeding (F) of 0.035, i.e., this group of dogs was only 3.5% more inbred than predicted for HWE. This suggests that English Mastiff breeders have been able to maintain breed-wide genetic diversity in a relatively random state.

Table 2: Genetic assessment of 23 English Mastiff based on allele frequencies at 33 genomic STR loci on Table 1.

	N	Na	Ne	Ho	He	F
Mean	23	4.79	2.78	0.57	0.59	0.035
SE		0.24	0.18	0.03	0.03	0.03

On average, the alleles identified in this group of 23 dogs represented 4.79/15.4=31% of alleles known to exist in all canids tested at the VGL. This is similar to retained canid-wide genetic diversity of the Swedish Vallhund (31.9%); somewhat lower than retained diversity for the Irish Red and White Setter (34.8%) and Flat Coated Retriever (38.6%); and significantly lower than retained canid genetic diversity of the most genetically diverse breeds - the Golden Retriever (54.5%), Toy Poodle (55.6%) and Standard Poodle (58%).

B. Standard genetic assessment values for individual STR loci

The allele frequencies can be also used to do a standard genetic assessment of heterozygosity at each STR locus (Table 3). This provides an estimate of genetic similarities in the specific regions of the genome that are associated with each STR marker. Phenotypic differences equate to genotypic differences. Therefore, alleles that are widely shared across the population are indicators that positive selection is occurring for certain desired traits. The Na values for an

individual STR locus for this population of 23 English Mastiff ranged from a low of 3 to a high of 8 alleles per locus, while the Ne ranged from 1.31 to 6.01 alleles per locus. The observed heterozygosity (Ho) for an individual STR locus ranged from 0.13 to 0.87, while He ranged from 0.25 to 0.83 (Table 3). Loci with the lowest Ho and He values contributed the least to heterozygosity and involved with traits that are most important in maintaining standard breed characteristics. Loci with high Ho and He values are more genetically variable and associated with phenotypic variation within the breed.

Eleven of the 33 loci had values of $F > 0.10$ and 5 were negative with $F < -0.10$. The loci with positive F values were under a greater degree of positive selection than those with negative F values and therefore areas of the genome that are more strongly associated with desired breed-specific traits. However, the influences of these various inbred, neutral and outbred regions of the genome defined by these 33 STR loci have been kept in good balance by breeder as evidenced by only a slightly positive F value (Table 2).

Table 3: Standard Genetic Assessment for English Mastiff using 33 STR loci

#	Locus	N	Na	Ne	Ho	He	F
1	**AHT121**	23	5	2.81	0.57	0.64	0.122
2	**AHT137**	23	5	3.09	0.57	0.68	0.164
3	**AHTH130**	23	5	3.37	0.70	0.7	0.011
4	**AHTh171-A**	23	9	6.01	0.83	0.83	0.009
5	**AHTh260**	23	5	3.06	0.70	0.67	-0.034
6	**AHTk211**	23	5	1.95	0.44	0.49	0.105
7	**AHTk253**	23	5	2.76	0.65	0.64	-0.024
8	**C22.279**	23	4	2.98	0.52	0.66	0.215
9	**FH2001**	23	5	3.28	0.70	0.70	-0.001
10	**FH2054**	23	5	2.36	0.35	0.58	0.396
11	**FH2848**	23	5	3.24	0.70	0.69	-0.007
12	**INRA21**	23	5	2.81	0.61	0.65	0.056
13	**INU005**	23	5	1.45	0.26	0.31	0.156
14	**INU030**	23	3	2.02	0.44	0.51	0.140
15	**INU055**	23	3	1.48	0.35	0.32	-0.079
16	**LEI004**	23	4	2.48	0.61	0.6	-0.021
17	**REN105L03**	23	3	2.76	0.87	0.64	-0.363
18	**REN162C04**	23	3	2.30	0.48	0.56	0.152
19	**REN169D01**	23	3	2.37	0.61	0.58	-0.054
20	**REN169O18**	23	3	1.37	0.13	0.27	0.516
21	**REN247M23**	23	3	1.25	0.22	0.20	-0.100
22	**REN54P11**	23	5	2.39	0.65	0.58	-0.120
23	**REN64E19**	23	4	1.31	0.22	0.24	0.084
24	**VGL0760**	23	6	3.70	0.52	0.73	0.285
25	**VGL0910**	23	7	5.01	0.87	0.80	-0.086
26	**VGL1063**	23	4	1.51	0.30	0.34	0.098

27	**VGL1165**	23	8	4.01	0.78	0.75	-0.043
28	**VGL1828**	23	5	3.53	0.65	0.72	0.090
29	**VGL2009**	23	5	2.82	0.83	0.65	-0.280
30	**VGL2409**	23	5	3.24	0.61	0.69	0.119
31	**VGL2918**	23	6	2.83	0.74	0.65	-0.143
32	**VGL3008**	23	6	3.57	0.78	0.72	-0.087
33	**VGL3235**	23	4	2.60	0.70	0.62	-0.131

D. Differences in population structure as determined by principal coordinate analysis (PCoA)

PCoA measures the genetic relatedness of individuals in a population. The data is computed in a spherical form, but it is often presented in the two dimensions that most closely represent its multi-dimensional form (usually coordinates 1 and 2). The more closely individuals cluster together around the XY axis, the more related they are to each other.

The 23 English Mastiffs formed a single population (i.e., breed) in PCoA (Fig. 1). Individual dogs in the group were reasonably dispersed across all four quadrants of the graph, with the exception of three pairs of dogs (upper left quadrant) that appeared to be closely related. Several individuals were also outliers from the main population (periphery of lower left and right quadrants). It can be assumed, therefore, that this group of 23 dogs, with the exception of three pairs, were reasonably unrelated and representative of the breed.

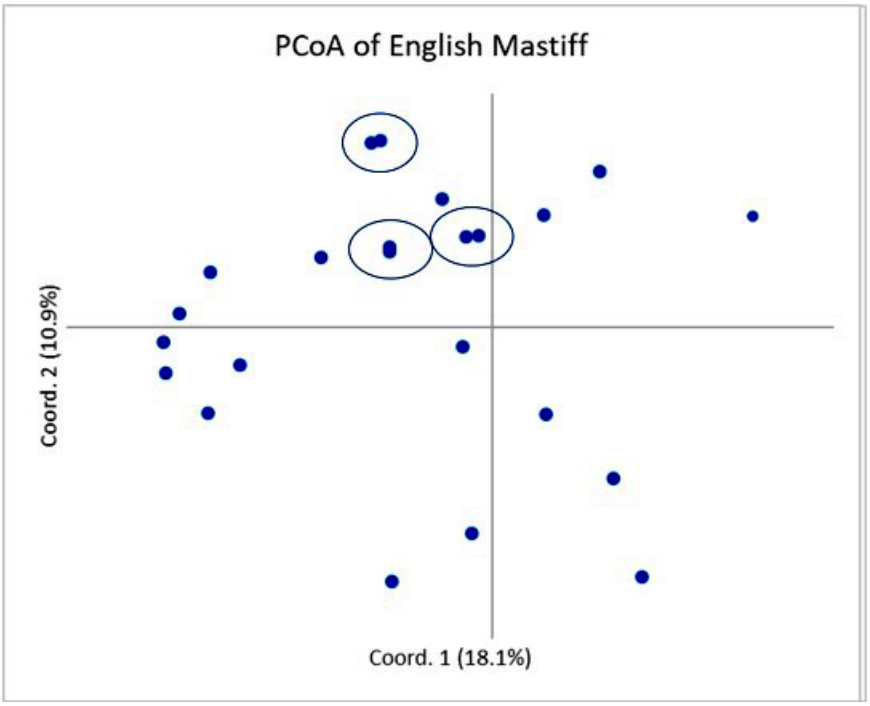


Figure 1. PCoA graph portraying the genetic relatedness of 23 English Mastiffs. Three pairs of dogs (circled) appear to be closely related.

The degree of relatedness of individuals within a breed can be further emphasized by comparing the 23 English Mastiffs with genetically distinct breeds, such as the Golden Retriever (Fig. 2). These breeds have parallel histories and are from the same region of the world. They also share a common DLA class I/II haplotype (Fig. 6). This comparison shows the two breeds to be genetically distinct, as would be expected (Fig. 2). However, this type of comparison accentuates the relatedness of individuals within a breed. A group of 6 English Mastiffs (mid-right), representing the dogs circled in Figure 1, remain more related to each other than to other dogs in the group. However, the outlier English Mastiffs identified in Fig. 1 still remain dispersed. This indicates that many of these dogs are quite genotypically (and possibly phenotypically) diverse at the breed level. However, this diversity is not to the level seen in the Golden Retrievers (Fig. 2), where two performance bred dogs are clear outliers at the level of variety.

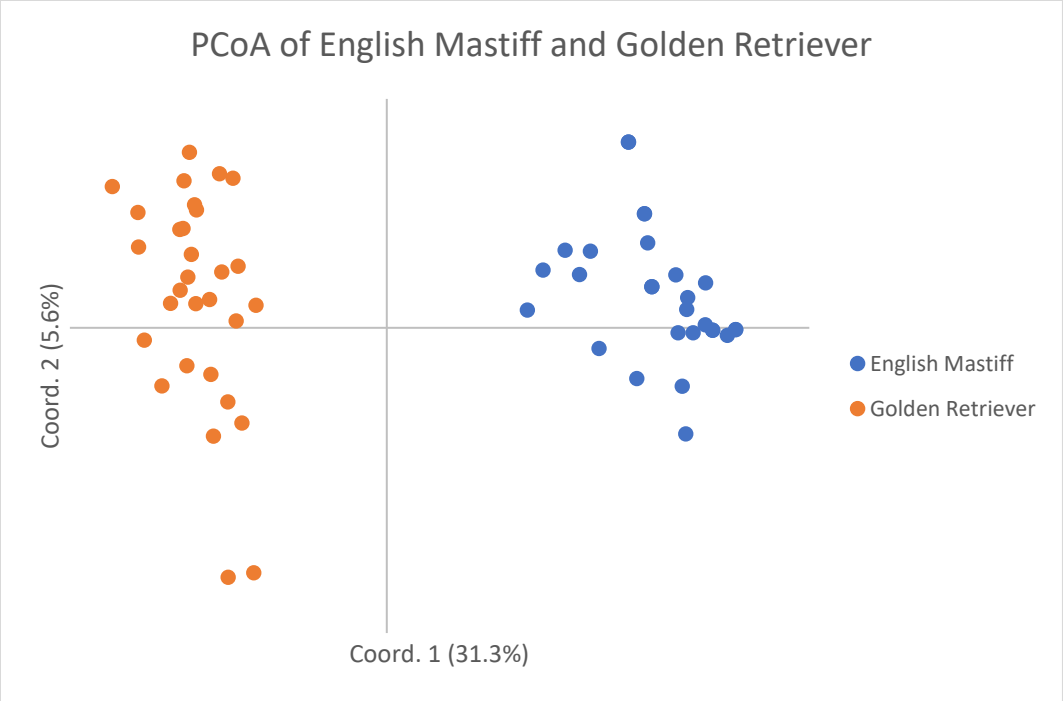


Fig. 2. PCoA of English Mastiff (n=23) and Golden Retriever (n=30, selected at random) based on the 33 STRs

D. Internal relatedness (IR) of individuals and the population as a whole

1. IR testing

Genetic assessments such as those presented in Tables 1-3 are indicators of population-wide heterozygosity and do not reflect the genetic diversity being provided to individuals by their parents. Internal Relatedness (IR) is a calculation that has been used to determine the degree to which the two parents of an individual dog were related. The IR calculation takes into consideration homozygosity at each locus and gives more importance to rare and uncommon alleles. Rare and uncommon alleles would presumably be present in less related individuals. IR scores of all individuals in a population can be graphed to form a curve ranging from -1.0 to +1.0. A dog with a value of -1.0 would have parents that were totally unrelated at all 33 STR loci, while a dog with an IR value of +1.0 has parents that were genetically identical at all loci. An IR value of +0.25 would be found among offspring of full sibling parents from a random breeding population. IR values >0.25 occur when the parents of the full sibling parents were themselves highly inbred. The higher the IR value above 0.25 the more closely related were the parents and grandparents of the siblings.

Table 4 summarizes the IR values for the 23 English Mastiff that were initially tested. The most outbred dog in the population had an IR score of -0.169, while the most inbred dog in the group had an IR score of 0.244, while the mean (average) IR score for the group was 0.026. Therefore, one or more dogs were almost as inbred as offspring of full sibling parents. However, inbred dogs (IR >0.026) are balanced by more outbred dogs (IR <0.26), giving the impression that all of the dogs are products of random breeding.

The IR/IRVD curve created from this data was unique compared to other breeds (Fig. 4). The graph is biphasic, with the bulk of dogs having IRVD scores >0.20 and a small second population with IRVD scores <0.20 (Fig. 4). Therefore, if these 23 English Mastiff existed as village dogs, most of them would be related to a level near or greater than offspring of full sibling village dog parents. The fact that the dogs represented by the darkened region fall entirely under the IR curve, while the remainder of the dogs fall entirely outside the IR curve, has not been observed in such a clear-cut manner in other breeds. It suggests that most of the shared genetic diversity exists only in a small subset of the more outbred dogs, while lost diversity has occurred almost entirely in dominant and more inbred population.

Table 4. Internal relatedness (IR) values calculated using allele numbers and frequencies 23 English Mastiffs. The IR values can be adjusted to reflect how these same dogs would score if they were to exist in a large population of village dogs (IRVD).

	IR	IRVD
Min	-0.169	0.021
1st Qu	-0.052	0.312
Mean	0.026	0.355
Median	0.014	0.366
3rd Qu	0.083	0.430
Max	0.244	0.528

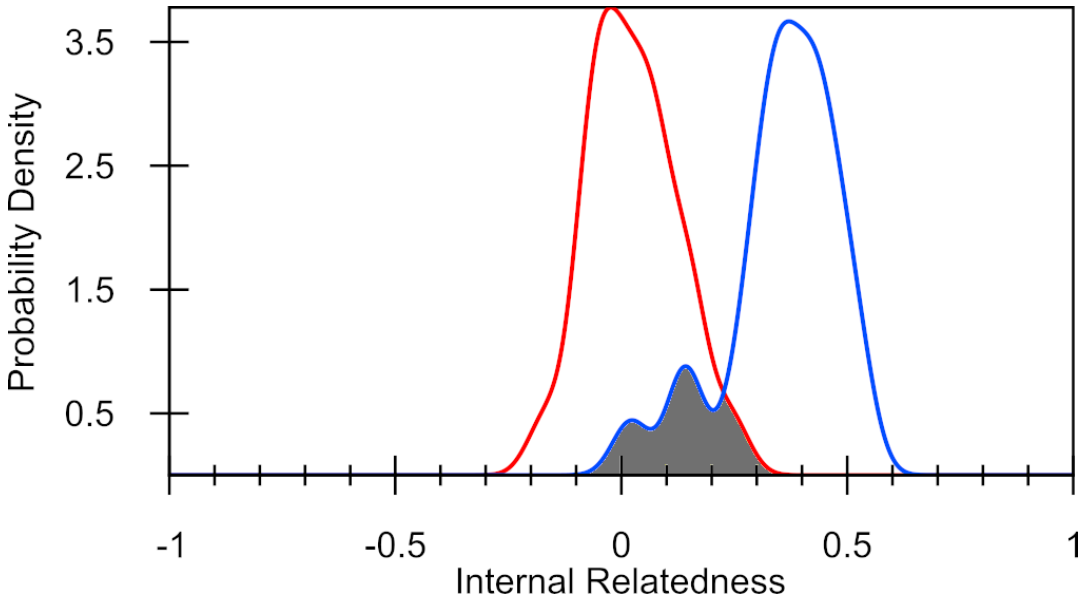


Fig. 4. Distribution of IR (red line) and IR-village dog (IRVD) (blue line) values for English Mastiff (n=23) The area under the curve (black) represents the degree of allele sharing (16.8%) between English Mastiffs and village dogs.

2. Adjusted IR values (IRVD) as a measure of genetic diversity lost during breed evolution from time of origin to the present time.

The darkened area in Figure 4 is an estimate of the amount (16.8%) of genetic diversity available in present-day randomly breeding village dogs still existing in contemporary English Mastiffs. A similar measure can be obtained by comparing the average Ne for the breed with the average number of known canid alleles (Na) (Table 2), or 2.78/15.4=18.2%. This figure is not significantly different than the 16.8% calculated from the IR/IRVD. The first and greatest loss of diversity probably occurred when founders were selected and after the registry was closed. Further loss of diversity may have occurred from a range of artificial genetic bottlenecks such as geographic isolation, natural and man-made catastrophes, breed refinement, popular sire and dam effects, change in interpretation of breed standard, etc. All of these types of genetic bottlenecks have been documented at one time or another in the history of the English Mastiff.

E. DLA Class I and II Haplotype frequencies and genetic diversity

The DLA consists of four gene rich regions making up a small part of canine chromosome 12. Two of these regions contain genes that help regulate normal cell- (Class I) and antibody-mediated (Class II) immunity. Polymorphisms in these regions have also been associated with abnormal immune responses responsible for autoimmune diseases, allergies, and resistance/susceptibility to infectious diseases.

The Class I region contains several genes, but only one, DLA-88, is highly polymorphic (with many allelic forms) and is therefore most important for immune regulation. Specific alleles at the four STR loci associated with the DLA88 are linked together in various combinations, forming specific haplotypes (Table 4). Groups of genes and their alleles inherited as a block, rather than singly, are called haplotypes.

The class II region also contains several genes, three of which are highly polymorphic, DLA-DRB1, DLA-DQB1 and DLA-DQA1. Specific alleles at the three STR loci associated with the three Class II genes are strongly linked and are often inherited as a single block or haplotype (Table 6). One haplotype comes from each of the parents. The STR-based haplotype nomenclature used in this breed diversity analysis is based on numerical ranking with the first haplotypes identified in Standard Poodles being named 1001, 1002, ... for class I haplotypes and 2001, 2002, ... for class II haplotypes. It is common for various dog breeds to share common and even rare haplotypes, depending on common ancestry.

1. DLA class I and II haplotypes existing in the English Mastiff

The 23 English Mastiffs possessed 6 DLA class I and 6 DLA class II haplotypes (Table 5). One class I (1221) and two class II (2117, 2118) were unique to the breed and the rest shared with a number of other breeds (Table 6). One class I (1066) and one class II (2046) haplotypes occurred in 54% of the dogs tested. All of the remaining haplotypes occurred at incidences ranging from 2-17%.

The number of DLA class I and II haplotypes found in these 23 English Mastiff was low compared to many other breeds studied to date. The numbers of DLA class I (n=6) and II (n=6) haplotypes found in these English Mastiffs was similar to the Swedish Vallhund (6, 4) and Shiloh shepherd (7, 6); somewhat lower than Giant Schnauzer (14, 15), Samoyed (13, 12) and Shiba Inu (16, 15); and much lower than Golden Retriever (26, 23) and Miniature Poodle (33, 23). If these 23 dogs are representative of the breed as a whole, it would suggest that four founders or closely related bloodlines have played dominant roles in the breed. However, the fact that several low incidence haplotypes appear to be unique to the breed, also that dogs of these ancestries were also important to the breed's standard and closely preserved.

Table 6. DLA class I and Class II haplotypes in English Mastiff and their frequencies (n=23)

DLA1 #	STR types	English Mastiff (n=23)
1006	387 375 293 180	0.02
1016	382 371 277 178	0.17
1030	380 373 293 178	0.11
1056	386 373 289 190	0.13
1066	376 375 277 178	0.54
1221	380 365 293 180	0.02
DLA2 #		
2014	339 322 284	0.17
2017	343 322 280	0.13
2023	341 323 282	0.11
2046	339 329 280	0.54
2117	353 327 280	0.02
2118	347 327 280	0.02

2. DLA haplotype sharing with other dog breeds

DLA haplotypes are much more conserved than most other regions of the genome and each DLA region is inherited as a block of linked genes from each parent and passed on by descent. Therefore, the number and incidence of DLA haplotypes found in a breed can be used to estimate the founder/founder lines that were used to create a breed and the importance of the various lines in subsequent breed evolution. The DLA class I and II regions are frequently shared between breeds, reflecting common distant ancestry and inheritance by descent (Table 6).

The 1066 and 2046 haplotypes were in strong linkage disequilibrium (LD), forming a larger 1066/2046 haplotype spanning the entire DLA class I and II regions. Interestingly, the 1066/2046 haplotype in English Mastiff is also common in the Golden Retriever, while the 1030/2023 and 1030/2023 haplotypes are shared by many other breeds. The strong sharing of the 1030/2023 haplotypes with the Biewer and Yorkshire Terriers is intriguing. The 1056 haplotype is found only in the Italian Greyhound, but not in linkage with 2017. The 2017 haplotype is found in linkage with other class I haplotypes in a number of other breeds.

Table 6. Sharing of specific DLA class I and II haplotypes between English Mastiff and various breeds.

DLA1 #	STRtypes	Black Russian Terrier (n=131)	Lakeland Terrier (n=63)	Labrador Retriever (n=178)	Irish Red and White Setter (n=59)	Doberman Pinscher (n=587)	Flat Coated Retriever (n=541)	Havanese (n=423)	Samoyed (n=189)	Giant Schnauzer (n=210)	Polish Lowland Sheepdog (n=16)	Borzoi (n=31)	English Bulldog (n=163)	Biewer (n=120)	Biewer Yorshire Terrier (n=53)	Biewer Terrier (n=105)	Yorkshire Terrier (n=16)	Italian Greyhound (n=826)	Alaskan Klee Kai (n=537)	Shiloh Shepherd, ISSA (n=179)	Magyar Agar (n=59)	English Mastiff (n=23)	Llewellin Setter (n=91)	American Aleta (n=99)	Japanese Aleta (n=353)	Golden Retriever (n=709)	Miniature Poodle (n=282)	Barbet (n=60)	Swedish Vallhund (n=220)	Poodle (n=2770)	Toy Poodle (n=142)
1006	387 375 298 180	0.042		0.039	0.059			0.047	0.005	0.052		0.18	0.008									0.02			0.056		0.0141	0.004		0.261	0.046	0.004
1016	382 371 277 178	0.008	0.016	0.011		0.0136		0.212		0.086			0.095	0.025	0.019	0.019	0.03	0.0393				0.17	0.005			0.0014	0.023			0.0179	0.082
1030	380 373 298 178			0.031		0.0928		0.001						0.458	0.349	0.467	0.31	0.0242				0.11	0.027				0.0007		0.025		0.0027	
1056	386 373 289 190																	0.0061				0.13									
1066	376 375 277 178			0.008			0.0009														0.076	0.54				0.2793					
1221	380 365 298 180																					0.02									
DLA Class II Haplotype Frequencies (Updated Aug 22, 2019)																															
DLA2 #	STRtypes	Black Russian Terrier (n=131)	Lakeland Terrier (n=63)	Labrador Retriever (n=178)	Irish Red and White Setter (n=59)	Doberman Pinscher (n=587)	Flat Coated Retriever (n=541)	Havanese (n=423)	Samoyed (n=189)	Giant Schnauzer (n=210)	Polish Lowland Sheepdog (n=16)	Borzoi (n=31)	English Bulldog (n=163)	Biewer (n=120)	Biewer Yorshire Terrier (n=53)	Biewer Terrier (n=105)	Yorkshire Terrier (n=16)	Italian Greyhound (n=826)	Alaskan Klee Kai (n=537)	Shiloh Shepherd, ISSA (n=179)	Magyar Agar (n=59)	English Mastiff (n=23)	Llewellin Setter (n=91)	American Aleta (n=99)	Japanese Aleta (n=353)	Golden Retriever (n=709)	Miniature Poodle (n=282)	Barbet (n=60)	Swedish Vallhund (n=220)	Poodle (n=2770)	Toy Poodle (n=142)
2014	339 322 284		0.016	0.008			0.0305	0.008		0.002	0.59		0.092						0.0717			0.17					0.023			0.0168	0.028
2017	343 322 280		0.04				0.0009	0.008			0.38	0.02	0.215					0.2203		0.363	0.407	0.13		0.015	0.006	0.0409		0.033		0.0027	0.004
2023	341 323 282	0.004		0.031		0.0928		0.001						0.458	0.349	0.467	0.31	0.0242				0.11	0.016			0.0007		0.025		0.0027	
2046	339 329 280			0.008			0.0009															0.54				0.268					
2117	353 327 280																					0.02									
2118	347 327 280																					0.02									

3. Heterozygosity in the DLA region

The 7 loci that define the DLA class I and II haplotypes are in stronger linkage disequilibrium that other parts of the genome that are measured by the 33 autosomal STR markers. However, the expectation is that these loci have achieved an equilibrium with other loci in the genome through random mating and over enough time. This can be tested by doing a standard genetic assessment of each locus (Table 7), as well as all of the loci taken together (Table 8). Standard genetic assessment of each of the 7 loci demonstrates a range of Ho/He values, with more heterogeneous loci contributing disproportionately to Ne and therefore genetic variation/heterogeneity. The values for F (inbreeding coefficient) ranged from somewhat negative (-0.02 to -0.09) to positive (0.13 to 0.24) (Table 7). The F values tend to balance out in the overall standard genetic assessment of all 7 loci, where F is only slightly positive at 0.05 (Table 8). These standard genetic assessment values for the population as a whole closely paralleled those shown for the 33 genomic STRs (Table 2), as did the values obtained for each of the genomic loci (Table 3). This indicates that the DLA region is in equilibrium with the rest of the genome and that the proportionately high incidence of certain haplotypes occurred at the foundation of the breed and has come into equilibrium over the breed's existence. In other words, the dominance of certain DLA haplotypes has become a fixed feature of the breed. This feature is common in many pure breeds of dogs.

Table 7. Standard Genetic Assessment for English Mastiff using 7 STRs in the DLA region

#	Locus	N	Na	Ne	Ho	He	F
1	**DLA I-3CCA**	23	5	2.78	0.65	0.64	-0.02
2	**DLA I-4ACA**	23	4	2.46	0.61	0.59	-0.03
3	**DLA I-4BCT**	23	3	1.80	0.35	0.45	0.22
4	**DLA1131**	23	3	1.43	0.26	0.3	0.13
5	**5ACA**	23	5	1.84	0.35	0.46	0.24
6	**5ACT**	23	4	2.49	0.65	0.6	-0.09
7	**5BCA**	23	3	1.8	0.48	0.44	-0.08

Table 8. Summary of Standard Genetic Assessment for English Mastiff using 7 STRs in the DLA region

	N	Na	Ne	Ho	He	F
Mean	23	3.86	2.08	0.48	0.5	0.05
SE		0.32	0.17	0.06	0.04	0.05

III. What does this assessment of genetic diversity tell us about contemporary English Mastiffs

The English Mastiffs tested constituted a single breed based on allele and allele frequencies for the 33 autosomal STR loci, albeit with limited genetic diversity and some intra-breed variation. Although it is not possible at this point to provide a definitive assessment of genetic diversity. The low amount of canid genetic diversity in these first 23 English Mastiff that were tested speaks to a number of potential artificial genetic bottlenecks. The first, and possibly most important, was a small number of founders or founder lines. However, the breed history also contains examples of many other types of genetic bottlenecks that could have led to bouts of intense inbreeding and loss of founder diversity after the registry was closed. Although DNA alone cannot provide answers to the importance of various potential bottlenecks, it does allow for several assumptions on breed origin. The breed originated from dogs that shared many of their genomic alleles with village dogs currently found in the Middle East, SE Asia and Pacific Island nations. Their DLA haplotypes are also shared with a number of other common breeds that evolved in this same region of Europe and not in Asia.

DNA analysis of the DLA regions of English Mastiff also mirrors the findings for lost diversity in other regions of the genome. Four DLA class I and II haplotypes occurred in 95% of individuals tested, and the extended DLA 1016/2046 haplotype was found in over half of the dogs. This is evidence that only four founders, or closely related founder lines, have contributed disproportionately to the breed's evolution up to modern times. This is in line with historical

evidence indicating that all modern English Mastiff have evolved from a single female (Nydia), 14 dogs from North America, and a single Dogue de Bordeaux. It could also be speculated that the dominant 1016/2046 haplotype was inherited from a single popular sire. However, the fact that these haplotypes are in equilibrium with the rest of the genome indicates that this imbalance occurred at the time of the founding and has become fixed/equilibrated over the time, and not from some more recent genetic bottleneck.

Although it appears that contemporary English Mastiff have a very narrow genetic base, a lack of genetic diversity is not in itself bad, providing the founder population was relatively free of deleterious genetic traits and breeders have been judicious in avoiding any artificial genetic bottlenecks that may cause either a loss or imbalance in the original diversity. The breed is surprisingly clear of simple breed-specific heritable disease traits and enjoys a reasonable lifespan compared to other large dog breeds. The health problems that exist are of a complex genetic basis and are common to many dog breeds and even mongrels [12]. These traits common to modern dogs have most likely been inherited from generation to generation as dogs underwent progressively more intense human-directed artificial selection.

The lack of genetic diversity in the DLA class I and II region of these 23 English Mastiff is troublesome, but it is uncertain what it means. Certain DLA class I and II haplotypes have been associated with specific autoimmune diseases in certain breeds [13], but autoimmune disorders other than hypothyroidism have not been documented as serious problems in English Mastiff. Nevertheless, it is important that breeders maintain as much diversity and heterozygosity in the DLA region as possible.

Breeds that lack genetic diversity must be managed much more closely to avoid further loss of genetic diversity and have less leeway in dealing with simple recessive or complex polygenic disorders that might arise. Elimination of deleterious traits may result in loss of genetic diversity, especially when diversity is limited.

IV. Heritable disorders of the English Mastiff [11]

Major health problems can include hip dysplasia and gastric torsion, which are common complex genetic disorders that are more common in large dogs in general [12]. Minor problems include obesity, osteosarcoma, and cystinuria. Problems only occasionally found include cardiomyopathy, allergies, vaginal hyperplasia, cruciate ligament rupture, hypothyroidism, OCD, entropion, progressive retinal atrophy (PRA), and persistent pupillary membranes (PPM).

It is difficult to find incidence figures on cancer in the English Mastiff, but it appears to be similar to dogs in general, i.e., around 30%. Lymphoma may be the most common tumor in Mastiffs, as in dogs in general, accounting for about 25% of all cancer. The second most common tumor is osteosarcoma, a problem shared by many larger breeds of dogs.

A. Lifespan

The lifespan of the Mastiff is 10-11 years (average 7 years) [1].

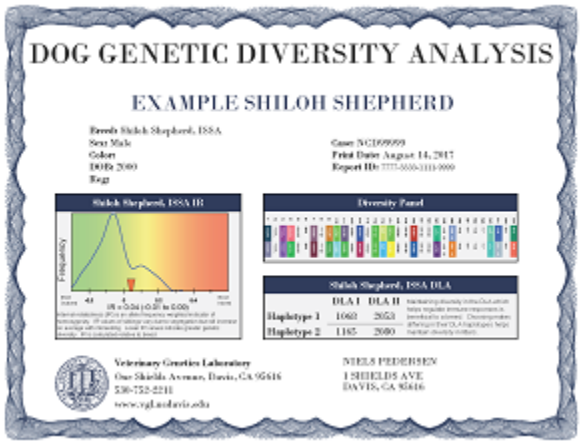
B. Testing for heritable conditions

DNA based tests are available for several disease mutations that have been identified in the breed. These diseases and their mutations include: autosomal dominant progressive retinal atrophy (ADPRA), canine multi-focal retinopathy 1 (CMR1), canine multi-focal retinopathy 3 (CMR3), degenerative myelopathy (SOD1), coat color dilution alopecia, hyperuricosuria (HUU), and cystinuria type III [11].

V. Results of Diversity Testing

A. How will you be given the results of DNA-based genetic diversity testing on your dog?

After a sample is submitted for genetic testing, the identity of the dog and owner will be replaced by a laboratory barcode identifier. This identifier will be used for all subsequent activities and each owner will be provided with a certificate that reports the internal relatedness, genomic STR genotypes and DLA class I and II haplotypes for the dog(s) tested. The internal relatedness value for the dog being tested is related to the population as a whole. The alleles at each of the 33 STR loci are presented as numbers that correspond to those found in Table 1. Each locus will have two alleles, which are either different (heterozygous) or the same (homozygous). Each allele is inherited from each of the parents. More of the alleles at each locus will be homozygous in dogs from closely related parents or that in regions of the genome that are under strong positive selection for some favored phenotypic trait or traits. Dogs with a predominance of rarer (i.e., low incidence) alleles will be more distantly related to the bulk of the population than dogs that have a predominance of common (i.e., high incidence) alleles.


DOG GENETIC DIVERSITY ANALYSIS

EXAMPLE SHILOH SHEPHERD

Breed: Shiloh Shepherd, ISSA
Sex: Male
Color:
DOB: 2010
Reg:

Case: NG109999
Print Date: August 14, 2017
Report ID: ????-????-????-????

Shiloh Shepherd, ISSA DLA

	DLA I	DLA II
Haplotype 1	1002	2053
Haplotype 2	1185	2080

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B. What should you do with this information?

The use of DNA for testing genetic diversity in the English Mastiff has confirmed that the breed lacks genetic diversity genome-wide and in the DLA region, most likely from a small number of founder individuals/lines. It is more important, therefore, to closely monitor existing diversity into the future. We believe that this can be most accurately done with DNA testing as a supplement to in-depth pedigrees. If the breed were to consider increasing genetic diversity by

further genetic introgressions, DNA testing of dogs intended for such introgressions would also be essential.

The goal for breeders should be to continue to produce puppies with IR scores less than 0, and with time, even lower scores. Although most of the individuals tested were randomly bred, there were small subpopulations of dogs that were much more inbred or outbred than the rest of the population. Therefore, there is a possibility to better balance genetic diversity in the breed. Mates should be selected to avoid homozygosity at any genomic loci or DLA class I and II haplotype and encourage the use of dogs with less common genomic alleles or DLA haplotypes. Maintaining existing genomic diversity will require using IR values of potential mates based on the 33 STR loci to assure puppies of equal or greater overall diversity, similar to what is being done by many Standard Poodle breeders. However, IR values, because they reflect the unique genetics of each individual, cannot be used as the criteria for selecting ideal mates. Mates with identical IR values may produce puppies significantly more or less diverse than their parents. Conversely, a mating between dogs with high IR values, providing they are genetically different, may produce puppies having much lower IR scores than either parent. A mating between a dog with a high IR value and a low IR value, providing the latter has few alleles and DLA haplotypes in common, will produce puppies much more diverse than the highly inbred parent. Breeders should also realize that a litter of puppies may have a wide range of IR values, depending on the comparative contributions of each of the parents. The more genetically diverse and different the parents, the greater the range of IR values in their offspring.

The next step is to compare the DLA class I and II haplotypes. You want to avoid breeding pairs that will produce puppies that will be homozygous for the same haplotypes, and once again, less common haplotypes may offer more diversity than common ones.

Breeders who do not have access to computer programs to predict the outcome of matings based on IR values of sire and dam can also compare values by manual screening. Potential sires and dams should be first screened for genetic differences in alleles and allele frequencies for the 33 genomic STR loci. Some extra weight should be given to rare v*s* common alleles. This information is included on all certificates and on the breed-wide data on the VGL website.

Puppies, once born, should be tested for their actual IR values, which will reflect the actual genetic impact of each parent on internal diversity. Considerations of mate choices for genetic diversity should be balanced with other breeding goals but maintaining and/or improving genetic diversity in puppies should be paramount.

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