Comparative Molecular Genetics of the German Shepherd Dog

Natalie June Coutts

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of the requirements for the degree
Magister Scientiae

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Supervised by Professor E.H. Harley
Department of Clinical Laboratory Sciences
Faculty of Health Sciences
University of Cape Town

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Abstract

Microsatellite markers were used to measure genetic diversity and population differentiation within and between domestic dog breeds. The German Shepherd Dog was compared with typical outbred mongrel dogs, Dachshunds, Staffordshire Bull Terriers and a cohort of other pedigreed dogs representing 30 recognised breeds.

Although archaeological records report that grey wolves (*Canis lupus*) were domesticated approximately 14 000 years ago, mtDNA analysis suggests that domestic dogs (*Canis familiaris*) and grey wolves diverged in multiple events over 100 000 years ago. Subsequently, the movement of humans and their dogs resulted in extensive gene flow between dog populations for thousands of years. Breeding practices to obtain distinctive phenotypic uniformity were recently introduced, resulting in purebred dogs becoming essentially closed gene pools. However, further mtDNA analyses have reported unexpectedly high levels of variability, supported by microsatellite loci with heterozygosities of between 36% and 55% being reported for some dog breeds.

Microsatellite analyses of 15 polymorphic canine loci are reported. German Shepherd Dogs and outbred mongrel dogs expressed diversity values of 4.0 alleles per locus in the former and 6.4 in the later (corrected for population size by jack-knifing with 1 000 pseudoreplications), with expected heterozygosities of 62% and 83%, respectively. German Shepherd Dogs showed a moderate loss of genetic diversity relative to outbred dogs, but not sufficient to describe the breed as highly inbred. However, in comparison with other purebred dogs examined, they expressed the least genetic diversity, with Dachshunds having 5.2, Staffordshire Bull Terriers 4.8 and the composite group of pedigreed dogs 6.0 alleles per locus, with expected heterozygosities of 72%, 67% and 80%, respectively. Significant population differentiation (G_{ST} = 0.103; R_{ST} = 0.058) between German Shepherd Dogs and the outbred dogs illustrates the effect of genetic drift since the breed was established just over 100 years ago.

This study would benefit future breeding programs, as management should be facilitated by knowledge of relative measures of inbreeding and differentiation, especially between various separate breeding stocks within the breed.

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List of Symbols and Abbreviations

Symbol	Meaning
[γ^{32}P] dATP	radioactive Phosphate isotope
A_{260}/A_{280}	ratio of light absorbency at 260 and 280nm
aa	amino acid
AB	Applied Biosystems
AKC	American Kennel Club
ANOVA	analysis of variance
APS	ammonium persulphate
ATP	adenosine triphosphate
bp	base pairs
C	cytosine nucleotide
CB	*Canis familiaris*, various pedigreed dogs
CFA	canine chromosome
DH	Dachshund
dNTPs	dinucleotide triphosphates
EDTA	ethylenediaminetetra-acetic acid
F	forward primer
g/M	grams per Molar
GC	guanosine and cytosine content of primers
GSD	German Shepherd Dog
H_E	expected heterozygosity, H-W equilibrium
H_{EQ}	H_E under mutation-drift equilibrium
H_O	observed heterozygosity, H-W equilibrium
H-W	Hardy-Weinberg
IAM	infinite allele model
ISAG	International Society of Animal Genetics
kb	kilo base pairs
KUSA	Kennel Union of South Africa
M	molar
Mya	million years ago
MgCl	magnesium chloride
mtDNA	mitochondrial DNA
MW	molecular weight measured in daltons (g/M)
N_A	number of alleles identified at each locus
NaOH	sodium hydroxide

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nm	nanometer
OBD	outbred mongrel dog
OD 1nM	optical density per 1nM
PAGE	polyacrylamide gel electrophoresis
PCA	Principle Component Analysis
PCR	polymerase chain reaction
PIC	polymorphism information content
PNK	polynucleotide kinase
R	reverse primer
rpm	revolutions per minute
SBT	Staffordshire Bull Terrier
SD	standard deviation
SDS	Sodium dodecyl sulphate
SE	standard error
SNP	single nucleotide polymorphisms
T_A	annealing temperature
Taq	Taq polymerase enzyme
TBE	tris-borate EDTA
TEMED	tetramethylethylene diamine
T_m	melting temperature
TPM	two-phased model of mutation
SMM	stepwise mutation model
UV	ultraviolet light
Var	variance

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Chapter 1

Introduction

1.1. The Evolution of the Family *Canidae*

The order Carnivora originated during the Eocene Epoch (Table 1.1.) and diverged to form two superfamilies, the *Feloidea* and the *Canoidea* (Vilà *et al.* 1999). The *Canidae* family is the most phylogenetically ancient lineage within the *Canoidea*, having diverged from the other carnivore families between approximately 50 and 60 million years ago (Mya), when canids first appeared in the fossil record (Wayne 1993, Wayne and Vilà 2001). A multitude of closely related species of fox, jackal, wolf and other canids (Figure 1.2.) has evolved that last shared a common ancestor approximately 12.5 Mya (Bininda-Emonds *et al.* 1999). The grey wolf (*Canis lupus*) first appeared in Eurasia about one Mya (Bininda-Emonds *et al.* 1999) and spread to North America about 250 000 years ago (Cloete 2001).

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Published phylogenetic analyses of the *Canidae* family (Figure 1.1.) group the "wolf-like" and "fox-like" species into two main clades (Bininda-Emonds *et al.* 1999). The *Canidae* family includes a diverse collection of 36 closely related extant species living in habitats from the tropics to the tundra (Wayne and Vilà 2001). The wolf-like canids include the grey wolf, maned wolf (*Chrysocyon brachyurus*) and African wild dog (*Lycaon pictus*); the fox-like canids include the red (*Vulpes vulpes*), Arctic (*Alopex lagopus*) and Cape foxes (*V. chama*); with the monotypic genera such as the bat-eared fox (*Otocyon megalotis*) and raccoon dog (*Nyctereutes procyonides*) having separate lineages, though clustering with the fox-like and wolf-like species, respectively (Wayne *et al.* 1997, Wayne and Ostrander 1999, Wayne and Vilà 2001).

Chromosome number and structure differs significantly between species belonging to the *Canidae* family, with 78 acrocentric chromosomes in the closely related wolf-like canids, including all breeds of domestic dogs, 74 acrocentric chromosomes in the South American fox-like canids, and between 36 and 64 metacentric chromosomes in the red fox-like canids (Wayne and Vilà 2001). This level of chromosomal variation differs from that found in other carnivore families where chromosome number and structure is well conserved (Wurster-Hill and Centerwall 1982).

Eon	Era	Period	Epoch	Age	Species
Phanerozoic				Present	*Canis familiaris*
	Cenozoic	Quaternary	Holocene	Bronze Age	*Canis familiaris matris-optimae*
				5 800 years ago	*Canis familiaris inostranzewi*
					Canis familiaris poutiatini
				10 000 years ago	*Canis familiaris palustris*
			Pleistocene	1 Mya	*Canis lupus*
				1.5 Mya	*Canis dirus*
		Tertiary	Pliocene	12 Mya	*Amphycyon*
					Tomarctus
			Miocene	26 Mya	*Cynodesmus*
			Oligocene	38 Mya	
			Eocene	50 Mya	*Mesonyx*
					Cynodictus
					Miacis
			Palaeocene	65 Mya	*Loxolophus*
	Mesozoic	Cretaceous		100 Mya	*Creodontus*
		Jurassic		135 Mya	*Eurotherium*
		Triassic		250 Mya	
	Paleozoic	Permian		280 Mya	*Cynognathus*

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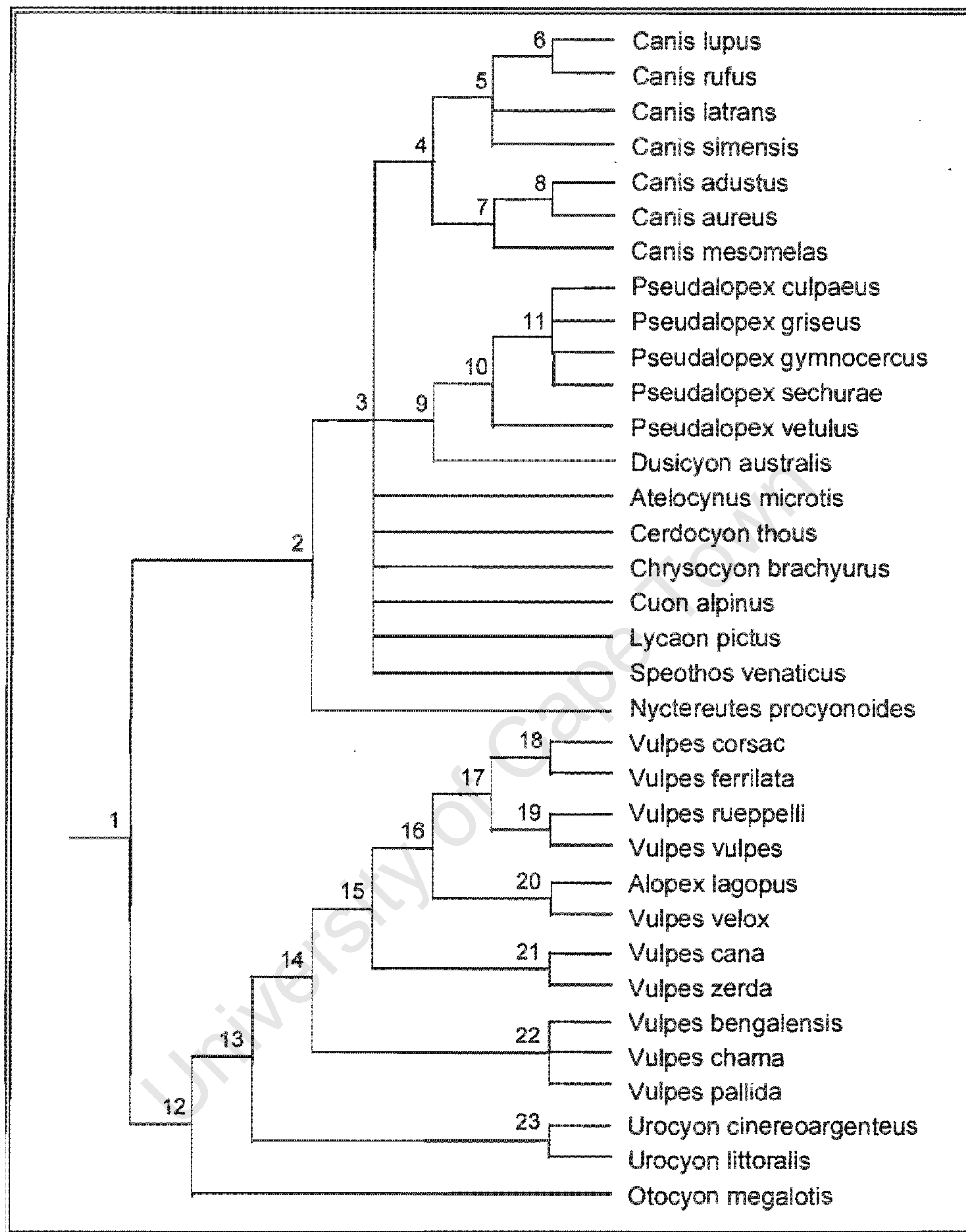
Table 1.1. Earth's History: The Geological Time Scale, the sequence of eons, eras, periods and epochs representing the evolution of the domestic dog (*Canis familiaris*) (Cloete 2001).



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1.2. The Origin of the Domestic Dog (*Canis familiaris*)

"Domestication is a process by which a population of animals becomes adapted to man and the captive environment, by some combination of genetic changes occurring over generations and environmentally induced developmental events recurring during each generation" (Price 1984).

Although the association between humans and dogs has been recorded throughout history, the data acquired from either fossil records or molecular methods do not agree as to when, where or how often this domestication event occurred.

Charles Darwin (1859) suggested that the great diversity of form and function that characterises the domestic dog is indicative of its diverse ancestry, having originated from more than one species. The atypical interspecific breeding ability of the *Canidae*, frequently producing fertile offspring, lent further credibility to this theory. The domestic dog can interbreed with jackal, wolf, dingo and coyote (Hunter *et al.* 1787, Clutton-Brock 1977, Vilà *et al.* 1999, Wilton *et al.* 1999), with all being implicated in the origin of the dog.

Post-Pleistocene Man had "tame canids" that developed into domestic dogs after many generations of isolated breeding (Clutton-Brock 1977). These could have been wolves and coyotes in North America, indigenous foxes in South America, wolves and jackals in Europe and Asia, and wolves, jackals and possibly wild dogs in Africa (Clutton-Brock 1977). However, behavioural, morphological and molecular studies indicate that the domestic dog is most closely related to the wolf (Clutton-Brock 1977, Wayne and O'Brien 1987, Wayne 1993, Morey and Wiant 1992, Tsuda *et al.* 1997, Vilà *et al.* 1997). Primitive people and wolves formed mutually beneficial symbiotic relationships, facilitated by similar hierarchical and complex social structures (Clutton-Brock 1977). Since the grey wolf distribution encompasses the holarctic region with more than 30 recognised subspecies, it is possible that multiple independent domestication events resulted in the evolution of the modern dog (Olsen and Olsen 1977, Morey and Wiant 1992, Leonard *et al.* 2002). Tsuda *et al.* (1997) compared mitochondrial DNA (mtDNA) D-loop sequences of 34 domestic dogs representing 24 breeds (of mostly Asiatic origin) and 19 wolves representing three subspecies (*C. l. lupus*, *C. l. pallipes*, and *C. l. chanco*) and observed that wolves and dogs were not genetically differentiated. This data was the earliest evidence indicating

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that domestic dogs had multiple centres of domestication, and that there was extensive interbreeding between these matriarchal lineages. Sequence divergence values varied within domestic dog breeds between 0% and 3.19%, and within wolf species between 0% and 2.88%. Sequence divergence between wolves and dogs varied between 0.30% and 3.35%, indicating little intraspecific or interspecific divergence, and that the grey wolf is the principal candidate for the matriarchal ancestor of the domestic dog (Tsuda *et al.* 1997).

All domesticated animals serve a purpose; with the concept of having animals merely as pets a comparatively modern aspect of civilisation. Primitive man was a nomadic hunter-gatherer, remaining temporarily at each settlement before moving on to areas of greater food abundance (Montagu 1942). It would appear that the primary function of the dog was the indispensable role of camp scavenger, consuming accumulated human detritus and eradicating not only foul odours and wastes, but also flies, insects and other pests (Montagu 1942). It would have been the progression to a more settled agricultural society that resulted in dogs forming a more integral part of everyday life, being useful for hunting, and guarding and herding domesticated farm animals.

The evolution from wild wolf to domestic dog would have occurred in four distinct phases: contact, acceptance, control and finally selective breeding (Cloete 2001). Wolves would have scavenged food from human settlements, their proximity eventually accepted for the benefits thereof. To perform such functions as guarding and herding, the "tame wolves" had to submit to human control and adopt a new social structure. Selective breeding, in isolation from the ancestral wolf population, improved hunting, herding and guarding instincts (Cloete 2001).

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1.2.1. Archaeological Approach

Evidence unearthed at numerous archaeological excavations has revealed that early domestic dogs and wolves can be identified according to specific morphological features. The process of domestication tends to result in decreased body size, shortening of the rostral or facial area, with larger palates and cranial area, and an overall reduction in tooth size (Olsen and Olsen 1977, Morey and Wiant 1992).

Archaeological sites in different parts of the world, dating back to the beginning of the Holocene Epoch, have yielded skeletal remains identified as early domestic dogs (Clutton-Brock 1977). The most ancient specimens on the North American continent were found in Danger Cave, Utah, and radiocarbon (^{14}C) dated to between 7 000 and 8 000 B.C. (Morey and Wiant 1992). The "Palegawra Dog" found in a cave in Northwest Iraq has been radiocarbon dated to 10 000 B.C. (Olsen and Olsen 1977).

According to Olsen and Olsen (1977), vertebrate remains described as canid in nature were found in close association with *Homo erectus pekinensis* at the Chouk'óutien site in China. Evidently, the lower section of the cave indicated greatest *Homo* activity approximately 500 000 years ago. *Canis lupus variabilis*, found with "Peking Man", was smaller in size, with a more slender muzzle and weaker sagittal crest, than the common wolf of that region. Reference has also been made to a "domestic dog or wolf" from an Upper Paleolithic site (20 900 years ago) on the Yenisei River, Siberia (Vilà *et al.* 1997).

With the skeletal remains of wolves and hominids being found in association from as much as 400 000 years ago, there is a definite chronological period unaccounted for between the most primitive canid remains and later finds officially designated *Canis familiaris* (Olsen and Olsen 1977, Cloete 2001). Although the actual date of domestication is far from resolved, it is accepted that this event did occur prior to the Neolithic revolution (10 000 years ago) when nomadic hunter-gatherer societies gradually gave way to agriculture and the domestication of farm animals. It is possible that the earliest dogs were not yet morphologically distinct from their wolf ancestors and that it was this event that imposed the necessary selective pressures resulting in the observed morphological changes (Vilà *et al.* 1997). If dogs did originate more recently, then they evolved from an unknown extinct species closely related to the grey wolf (Wayne and Ostrander 1999).

1.2.2. Molecular Approach

Archaeologists and anthropologists have been unable to determine whether domestic dogs evolved from a single wolf population, from different populations, or whether there were multiple such occurrences. Vilà *et al.* (1997) applied molecular methods to investigate these questions. A segment, 261bp in size, of the left domain of the mtDNA control region was sequenced from 140 dogs, representing 67 breeds and five crossbred

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dogs, 162 wolves, representing 27 populations from Europe, Asia and North America, and five coyote (*C. latrans*), two golden jackal (*C. aureus*), two black-backed jackal (*C. mesomelas*) and eight Ethiopian wolves (*C. simensis*).

According to Vilà *et al.* (1997), the control region proved to be very polymorphic with 27 wolf haplotypes and 26 dog haplotypes being detected. The wolf haplotypes, differing by an average of 5.31 substitutions (2.10%) and a maximum of ten substitutions (3.95%), were predominantly confined to specific geographic regions. The dog haplotypes, differing by an average of 5.30 substitutions (2.06%) and a maximum of 12 substitutions (4.67%), did not sort according to breed, indicating diverse ancestry and extensive hybridisation of breeds of dogs. Also, the relatively recent origin of most breeds has not allowed sufficient time for the development of unique breed-specific sequences in the mtDNA control region (Wayne and Ostrander 1999). Only a single haplotype (D6/W6) was common to both dogs and grey wolves from Western Russia and Romania. This was attributed to possible recent hybridisation between dogs and wolves. Dog sequences differed from wolf sequences by no more than 12 substitutions and from coyote and jackal by at least 20 substitutions and two insertions, corroborating the wolf ancestry of domestic dogs. Interbreeding between female dogs and male coyote or jackal would not be detected due to the maternal inheritance pattern of mtDNA, but the above interpretation has been corroborated by limited studies on nuclear DNA (Vilà *et al.* 1997).

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Vilà *et al.* (1997) used various methods of phylogenetic analysis which grouped the dog haplotypes into four separate clades: I, II, III and IV (Figure 1.3. A). This suggests either several independent domestication events involving a number of wolf populations or the continued exchange of genetic material between dogs and wolves after the original event. Clade I contained 19 of the 26 dog haplotypes from many common breeds and several ancient ones, but no wolf haplotypes. Clade II consisted of haplotype D8 found only in two Scandinavian breeds, clade III consisted of three dog haplotypes (D7, D19 and D21) from a range of different breeds and clade IV consisted of three haplotyes (D6, D10 and D24) most similar to the wolf haplotype W6. These monophyletic groupings were confirmed by sequencing a further 1 030bp (Figure 1.3. B) from the control region of 24 individuals representing each of the four dog clades (Vilà *et al.* 1997).

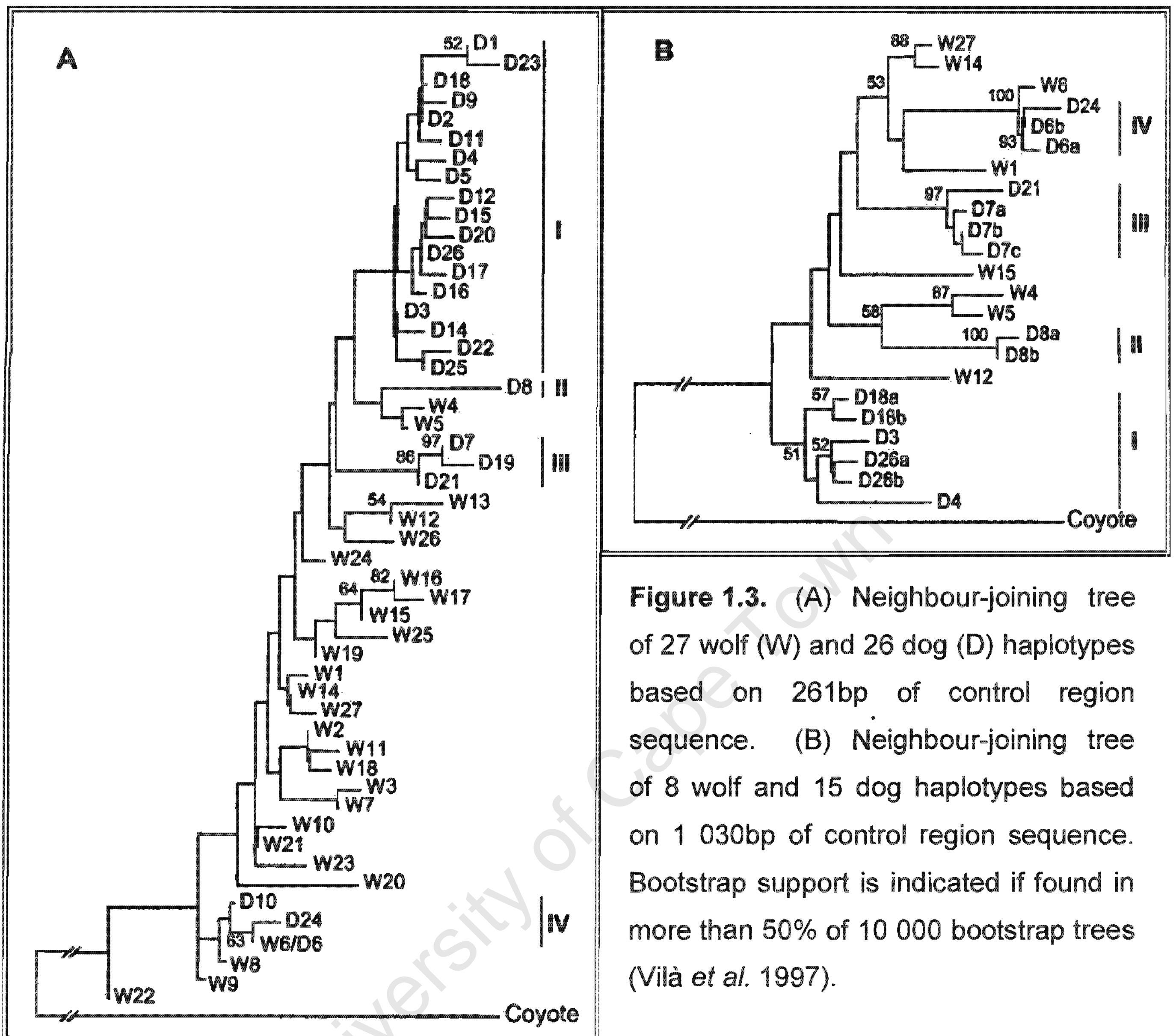

Figure 1.3. (A) Neighbour-joining tree of 27 wolf (W) and 26 dog (D) haplotypes based on 261bp of control region sequence. (B) Neighbour-joining tree of 8 wolf and 15 dog haplotypes based on 1 030bp of control region sequence. Bootstrap support is indicated if found in more than 50% of 10 000 bootstrap trees (Vilà *et al.* 1997).

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Vilà *et al.* (1997) used a "molecular clock" method to determine when dogs and wolves diverged. Evolutionary lineages are gradually altered as mutations cause changes in the nucleotide sequences. The "clock" was calibrated using the sequence divergence of coyotes and wolves that, according to the archaeological record, last shared a common ancestor one million years ago. This corresponds to a sequence divergence of 0.075 (7.5%), and as the divergence between the most varied of the dog haplotypes in clade I is 0.010, it indicates that dogs originated about 135 000 years ago. Although this may be overestimated due to undetected substitutions at hypervariable sites and an uneven mutation rate, Vilà *et al.* (1997) are confident that the domestication event occurred a great deal earlier than the proposed 14 000 years before present.

1.2.3. Old World Origin

The dog was the only domestic animal distributed across Eurasia, Oceania and the Americas before transoceanic voyage in the 15th century (Leonard *et al.* 2002). The Australian dingo, a feral domestic dog, is thought to have originated from Asia approximately 5 000 years ago (Clutton-Brock 1977, Wilton *et al.* 1999). New World dogs either accompanied the Paleo-Indians across the Bering Strait about 20 000 years ago or resulted from independent domestication of North American grey wolves (Vilà *et al.* 1999).

Vilà *et al.* (1999) sought to establish whether mtDNA sequences of native American dogs were similar or identical to North American wolves, implying New World domestication, or similar or identical to European dog breeds, indicating an ancient Old World origin.

The Xoloitzcuintli, a medium-sized, hairless dog, is indigenous to Mexico. Pottery portraying this breed has been discovered in Western Mexico, dating to the Colima culture between 250 B.C. and 450 A.D. (Vilà *et al.* 1999). Due to their religious significance, Mexican tribes concealed the breed to prevent interbreeding with dogs introduced by the Spanish conquistadors. Thus making the Xoloitzcuintli an ideal candidate breed for the investigation of a New World domestication event (Vilà *et al.* 1999).

Vilà *et al.* (1999) sequenced 394bp of the mtDNA control region of 19 Xoloitzcuintlis and detected seven different haplotypes distributed across three of the four dog clades. No haplotype was unique to the breed, or similar to those of North American wolves. The most frequent haplotype (D6) was the one common to both dogs and wolves of Romania and European Russia. These data do not corroborate the New World origin of native North American dogs, especially as the mtDNA sequences were identical to those of Old World dog breeds (Vilà *et al.* 1999).

These data were further substantiated by the research of Leonard *et al.* (2002). As modern New World breeds have probably interbred with dogs of European ancestry, DNA was extracted from 37 dog skeletons from sites in Mexico, Peru, and Bolivia that predate the arrival of Christopher Columbus, and 11 from Alaska before the arrival of European explorers. The control region of a number of samples was successfully sequenced and 11 of the dog haplotypes published by Vilà *et al.* (1997, 1999) were detected. The mtDNA

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sequences of native dog breeds of the Americas, including the Eskimo dog, Xoloitzcuintli, Alaskan husky, Newfoundland and Chesapeake Bay Retriever, and the Australian dingo and New Guinea Singing Dog of Oceania were identical to those of Eurasian dog breeds. Phylogenetic analysis of the ancient New World breeds distributes the haplotypes across two of the four previously defined clades, ten of the pre-Columbian specimens are assigned to clade I and the other to clade IV. These sequences differed from North American wolves by between three and 13bp, but were identical or very similar to Eurasian dogs. These data also indicate that both New and Old World domestic dogs originated from Eurasian wolves, and dogs with multiple Old World lineages accompanied humans across the Bering Strait during the late Pleistocene Epoch (Leonard *et al.* 2002).

1.2.4. East Asian Origin

Several studies have proposed an East Asian origin for modern domestic dogs (Olsen and Olsen 1977, Savolainen *et al.* 2002). The Chinese or Asian wolf (*Canis lupus chanco*), one of the smallest sub-species, could be the forerunner of the early Chinese and Mongolian domestic dogs and probably also those that accompanied man across the Bering Strait to North America (Olsen and Olsen 1977, Leonard *et al.* 2002, Savolainen *et al.* 2002).

An aspect of diagnostic morphology used to differentiate between domestic dogs and other wild canids is the "turned back" apex of the coronoid process of the ascending ramus, this feature is also found in Chinese wolves (Olsen and Olsen 1977). The degree of development of the coronoid apex is also significant. True "meat-eating" carnivores (e.g. Felidae) have no overhang in this area whereas a distinct overhang is evident in omnivores (e.g. Ursidae). The overhang is characteristic of both domestic dogs and Chinese wolves but absent in other wolf subspecies, with no evidence of differences in diet to explain this phenomenon (Olsen and Olsen 1977).

Savolainen *et al.* (2002) used molecular methods to investigate whether dogs were domesticated in a single or in multiple events and approximately when and where the event(s) occurred. The mtDNA sequence variation across 582bp was examined in 38 Eurasian wolves and 654 domestic dogs from Europe, Asia, Africa and Arctic America.

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Cladistic analysis grouped all haplotypes according to the phylogenetic clades previously defined by Vilà *et al.* (1997, 1999), now identified as clades A, B, C, and D, as well as a unique fifth group designated clade E consisting of an isolated haplotype. Dog haplotypes in each clade were interspersed by wolf haplotypes approximately equidistant from a common ancestor of all dogs and wolves. Savolainen *et al.* (2002) concluded that the modern domestic dog population originated from at least five female wolf lineages.

Clade A contained three wolf haplotypes from China and Mongolia, clade B contained two from East European wolves and one from Afghanistan; implying that clade A originated in east Asia and clade B in Europe or Southwest Asia. A total of 95.9% of the dog haplotypes were assigned to clades A, B, and C, therefore these three clades represent a common origin for most genetic variation in modern dog populations. Unless very effective gene flow occurred throughout the Eurasian continent, most dog populations have a common origin from a single gene pool comprising clades A, B, and C.

If an ancestral population was compared with its derived population, more haplotypes and a greater degree of genetic diversity would be expected in the ancestral population. It would follow that clade A had 3.39 substitutions in East Asia, 2.28 in Southwest Asia, and 2.97 in Europe. Also, more haplotypes (corrected for sample size) were detected in the East Asian cohort than in Southeast Asia or Europe; with 20.2 haplotypes in 51 East Asian dogs being significantly greater than the 16 haplotypes in 51 Southwest Asian dogs ($p < 0.05$; 1 000 replications) and no significant differences in Europe. In addition, 30 of the haplotypes in East Asia were unique to that region.

Savolainen *et al.* (2002) further examined the possible East Asian origin by comparing dog populations from the East and West, the regions being defined as east and west of a line from the Himalayas to the Ural Mountains. In clade A, 13 haplotypes were common to both the East and West, while another 35 were unique to the East and 23 to the West, also 51.5% of the individuals in the East had unique haplotypes as compared to 28.1% in the West. These data suggest that the haplotypes of clade A in the western part of the world originated from a subset of East Asian haplotypes, later developing into haplotypes unique to the West.

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Similarly with regards to clade B, more haplotypes were detected in the East than the West, seven haplotypes being unique to the East and only three to the West, and 41.2% of the population having unique haplotypes in the East but only 6.8% in the West.

The haplotype distribution and genetic diversity in Clade C was similar to that of clades A and B, with only the East having two unique haplotypes.

Savolainen *et al.* (2002) concluded that more than 95% of all modern dog populations are grouped in the three phylogenetic clades A, B, and C, indicating a common origin from a single gene pool. The greater genetic diversity in East Asia and the geographic haplotype distribution suggests that these three clades originated in East Asia and the dog populations of Europe and Southwest Asia are derived from the East Asian types.

1.3. Experimental Study of Domestication

> "*How might the contemporary domestic dogs, so very diverse today, have evolved from a uniform wild-type ancestor?*" (Belyaev 1969)

Neoteny, the retention of juvenile traits into adulthood, is considered the principal driving force behind the radical behavioural and phenotypic changes associated with domestication, which occurred at a rate exceeding genetic prediction (Trut 2001). This evolutionary adaptation was most probably driven by selection, with infantile behaviour and appearance best facilitating the co-existence of man and wolf. It has been proposed that a variety of breed-specific traits are essentially retarded juvenile behavioural responses and arrested morphological development (Coppinger *et al.* 1987).

Dmitry Belyaev, a Russian geneticist and evolutionist, considered the nature and origin of these behavioural and morphological changes and proposed to recreate a domestication event with strict selection for amenability to domestication, namely, "tameability" (Trut 2001). This experiment was carried out for more than 40 years at the Institute of Cytology and Genetics of the Siberian Department of the Russian Academy of Sciences, the last 14 of which under the direction of Lyudmila Trut.

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The silver fox (*Vulpes vulpes*) was chosen as the experimental taxon, for although the species is phylogenetically and taxonomically closely related to the dog, it has never been domesticated (Trut 2001). The farm-bred foxes retained their natural behavioural and morphological traits, but approximately 10% exhibited a particularly weak defensive response (Belyaev 1978). Of these individuals, 100 females and 30 males were chosen as the parental generation, with all subsequent progeny selected purely for tameness, being evaluated by their response to human handlers (Trut 2001). It was demonstrated that the variability in defensive behaviour is hereditary and that selection is possible (Belyaev 1978). Individuals in the experimental population that expressed particularly tame characteristics were assigned to the "domestication elite"; and after 47 000 foxes and between 30 and 35 generations of selection, a population of docile "domestic" foxes was produced (Trut 2001). The experimentally bred foxes were not only unafraid of people but exhibited an active positive reaction to human contact, not as a result of training, but rather from continued selection for a tame genotype (Belyaev 1978).

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Interestingly, after eight to ten generations of selective breeding, a variety of unselected morphological and physiological changes emerged. These included irregular patches of white incorporated in the previously uniform coat colour, floppy ears, rolled tails, foreshortened legs and tails, elongated bodies, and the feminisation of male craniological dimensions with the loss of natural sexual dimorphism, and a general associated shortening and widening of the face skull and decrease in the width and height of the cerebral skull (Trut 2001). The following domestic characteristics also appeared; altered reproductive cycles with the loss of seasonality and monoestricity, sexual maturity reached at a younger age, larger litters of pups, and the production of higher levels of serotonin, a neurotransmitter thought to inhibit aggressive behaviour (Trut 2001). Belyaev and Trut have motivated that the development of such "phenotypic novelties" are neither the result of inbreeding nor stochastic processes, but rather concordant with the morphological and physiological reorganisation characteristic of domestic dogs (Trut 2001). Most features of the evolutionary pathway were reproducible under domestication as a result of strict selection for the genetic systems governing the behavioural trait for tameability.

1.4.1. Hypotheses

1. German Shepherd Dogs express limited genetic variability as a result of the application of artificial selection and deliberate inbreeding in the formation of the breed.
2. German Shepherd Dog populations are highly differentiated due to the isolation of the breeding stock.

1.4.2. Aims and Objectives

- *To determine the relative genetic diversity and degree of heterozygosity revealed in German Shepherd Dogs in South Africa as compared with those from Germany, and outbred mongrel dogs and other pedigreed dogs.*

- *To determine the extent of population differentiation and substructuring within the breed, with respect to both region of origin and breed type.*

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A population study of the German Shepherd Dog will be completed:

1. Fifteen canine microsatellite markers will be analysed and the data obtained will be quantified by comparison with typically outbred mongrel dogs, Dachshunds, Staffordshire Bull Terriers and a cohort of other pedigreed dogs representing 30 officially recognized breeds.

2. The genetic diversity and degree of heterozygosity will be calculated by determining the number and distribution of allelic variants at each microsatellite locus, and the frequency of heterozygous and homozygous individuals within the population.

3. Population differentiation can be determined by various statistical approaches, with respect to region of origin, as compared with dogs from Germany and the rest of Europe, and type, as compared between "show" and "sport" dogs that are selected for either appearance or performance. These approaches include Hardy-Weinberg Equilibrium, relative G_{ST} and R_{ST} estimates and other F-statistics, and population assignment tests based on allele frequencies.

Chapter 2

Population Genetics

2.1. Microsatellite Markers

Microsatellite markers are polymorphic DNA loci, randomly distributed throughout the mammalian genome, constituting neutral markers under no selective pressure, and exhibiting classic Mendelian inheritance with co-dominant nature of allelic variants (Bruford and Wayne 1993, Zajc and Sampson 1996, Ruzzante 1998). Microsatellites consist of between four to 30 tandem repeats of between two and six nucleotide bases (Figure 2.1.). The number of repeats within individuals of a population is variable as a result of the dynamic rate of mutation, reportedly between 10^{-4} and 5×10^{-6} mutations per chromosome per generation (Bruford and Wayne 1993, Zajc *et al.* 1997). This rapid mutation rate is considered the result of intra-allelic polymerase slippage during DNA replication; although mechanisms may exist that restrict the number of repeat units (Bruford and Wayne 1993).

Figure 2.1. An electropherogram representing a nucleotide sequence incorporating a microsatellite marker consisting of 16 GT repeats.

Microsatellite markers are ubiquitously dispersed across all eukaryotic genomes, the most common motif, $(CA)_n$, distributed in mammalian species approximately every 30kb (Stallings *et al.* 1991, Jouquand *et al.* 2000). These repetitive sequences occur in organisms from yeast to mammals and retain the potential for Z-DNA formation, possibly indicating some functional role. It has been proposed that they participate in chromosomal packaging, genetic recombination and promote gene transcription in plasmid constructs (Stallings *et al.* 1991). Microsatellite sequences are found more frequently in euchromatin than heterochromatin and may differentiate between constitutive heterochromatin and euchromatin or facultative heterochromatin (Stallings *et al.* 1991).

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Laboratory analysis is PCR-based with sequence specific oligonucleotide primers, designed to recognise and anneal to the flanking regions, amplifying the microsatellite locus. Polyacrylamide gel electrophoresis (PAGE) allows the resolution of alleles differing in size by only 1bp. The amplified PCR product (Figure 2.2.) is visualised by either fluorescent or radioactive [γ^{32}P] dATP-labelled primers and are accurately sized by comparison with a size standard. This approach is extremely sensitive; microsatellite loci can be amplified from minute quantities of target DNA or from significantly degraded DNA, such as forensic material or ancient samples (Bruford and Wayne 1993). PCR primers are relatively species-specific, but often function for other closely related taxa, e.g. microsatellite markers isolated in domestic dogs will often amplify other canid species like wolf, coyote, jackal and African wild dog (Bruford and Wayne 1993).

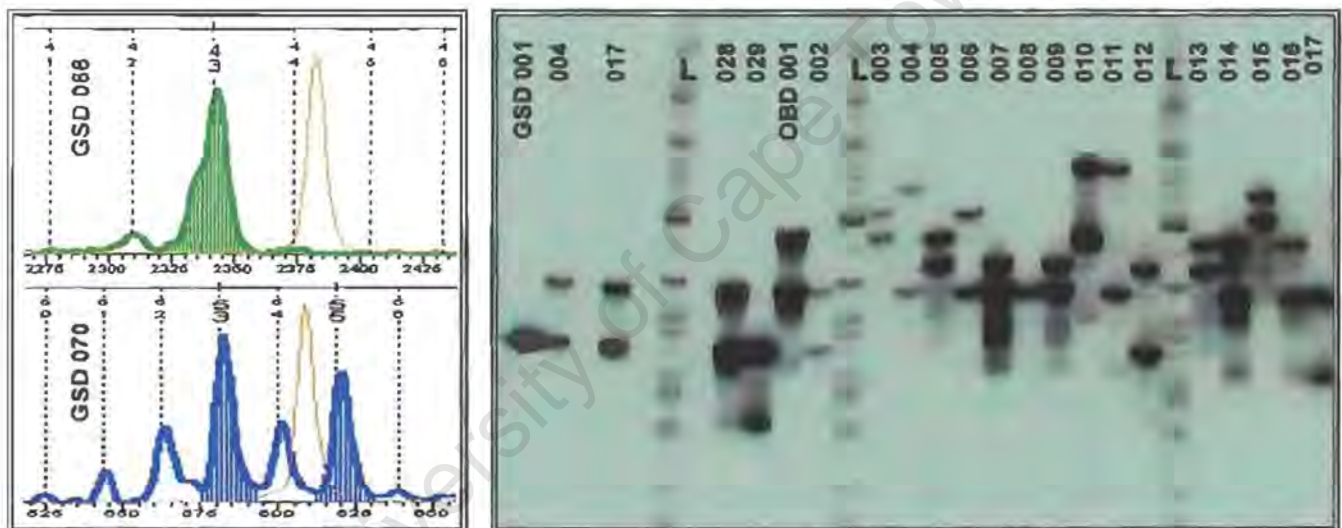

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Figure 2.2. Fluorescent-labelled microsatellite markers (left), FH2289 (green, homozygous) and AHT121 (blue, heterozygous), were sized according to an internal size standard (orange). Radioactive [γ^{32}P] dATP-labelled microsatellite marker FH2328 (right) was sized by comparison with standard A-T ladders (L).

The highly polymorphic nature of microsatellite markers renders them useful tools for genetic analyses, and an extensive array has been described in the canine genome. These have been employed for genome mapping, genetic linkage analysis, parentage verification, population studies, examining evolutionary and filial relationships, forensic identity testing, and conservation genetics (Bruford and Wayne 1993, Ostrander *et al.* 1993, Gotelli *et al.* 1994, Roy *et al.* 1994, Fredholm and Winterø 1995, Francisco *et al.* 1996, Zajc and Sampson 1996, Müller *et al.* 1999, Zajc and Sampson 1999).

2.2. Genetic Diversity

"Individuals can carry only two different alleles at a given locus, whereas a group of individuals can carry a larger number of alleles, giving rise to a reservoir of genetic diversity" (Klug and Cummings 1994).

The level of genetic diversity expressed by a population can be estimated by measuring allele frequency distribution across a number of loci. Allele frequencies, the proportion of individuals in a population carrying particular alleles, fluctuate at a rate relative to population size as a result of the following modifying factors: (i) random genetic drift, (ii) mutation, (iii) migration, (iv) selection, and (v) inbreeding.

Genetic drift is the result of random changes in allele frequencies from one generation to the next because the progeny comprise a finite sample of the parental gene pool (Brewer and Sing 1983). The rate at which this process occurs is inversely related to population size, having critical consequences for conservation purposes because the effect is compounded in populations passing through a bottleneck. Genetic drift serves to limit genetic diversity and heterozygosity, and promote differentiation as isolated populations eventually express divergent allele frequencies.

Although mutation is the ultimate source of genetic diversity and can modify allele frequencies in a population, its ability to expand the gene pool is relatively gradual (Brewer and Sing 1983). The mutation process is random and would require multiple generations before the allele frequency in a population is affected. Various models of mutation have been proposed; these include the Infinite Allele Model (IAM), where mutations create new allele at rate μ, the Stepwise Mutation Model (SMM), mutations add or delete a single unit to/from the current allele, and the Two-Phased Model of Mutation, which incorporates mostly one-step mutations, but also a small percentage (5 - 10%) of multi-step mutations (Cornuet and Luikart 1996, Samadi *et al.* 1998).

Migration introduces genetic diversity into a population; the extent of isolation from other populations determines the importance of the role of gene flow. The gradual exchange of genes between populations homogenises the genetic diversity between separate populations (Wayne 1993). Various factors promote the fixation of some alleles and loss of others, such that a population can become divided into genetically distinct subgroups. If

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these subgroups are also geographically or physiologically isolated then gene flow is reduced and they may become evolutionarily divergent. An estimate of the rate of migration can be determined by the equation $F_{ST} = 1 / (1 + 4Nm)$, where N is the population size and m the rate of migration (Gotelli *et al.* 1994). Theoretically, only one migrant per generation is sufficient to thwart divergence due to random genetic drift in finite populations (Vucetich and Waite 2000).

Natural selection results in the most advantageous adaptations becoming more common in a population. Individuals may not all survive equally well in a particular habitat and their reproductive success depends on a combination of viability and fertility, i.e. genetic fitness (Brewer and Sing 1983). If individuals carrying a particular allele were more likely to produce progeny as a result of better adaptation to the environment, then the frequency of that allele, and the adaptation, would tend to increase in the population. This process has been intuitively understood for millennia, with man endeavouring to change the nature of plants and animals to fulfil particular functions. In this case, the parental generation is artificially controlled and the process is described as artificial selection.

The consanguineous mating between closely related individuals, or inbreeding, results in the proliferation of only part of the total gene pool, as the parental generation expresses identical alleles more frequently than predicted by chance, with a resultant increase in homozygous offspring and associated decrease in genetic fitness (Klug and Cummings 1994). The inbreeding coefficient measures the probability that two alleles at a locus are identical in origin as inherited from a common ancestor, and not two identical alleles uniting at random in the population (Brewer and Sing 1983). Brouillette and Venta (2002) calculated the inbreeding coefficient with the equation: $F = (H_E - H_O)/H_E$, being the observed and expected heterozygosity values.

Loss of genetic diversity reduces the evolutionary potential of a population because it limits response to habitat changes, e.g. newly introduced pathogens or parasites (Garza and Williamson 2001). Heterozygosity (H) is an informative evaluation of genetic diversity, being the probability of a random individual expressing two different allelic forms at a particular locus. The level of heterozygosity can be calculated according to the equation: $H = 1 - \Sigma p_i^2$, where p_i is the frequency of the ith allele at that locus (Ostrander *et al.* 2001).

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Significant reductions in effective population size (bottlenecks) result in the rapid loss of genetic diversity, especially when post-bottleneck recovery is slow, with the result that homogeneity is increased and deleterious alleles become fixed (Luikart *et al.* 1998b). The effects of random genetic drift intensify, resulting in altered allele frequencies and the loss of some alleles. Recently bottlenecked populations exhibit transient heterozygosity excess relative to that expected from the observed number of alleles, due to the more rapid loss of heterozygosity than alleles (Cornuet and Luikart 1996). Another estimate of reduced effective population size is the mean ratio (M) of the total number of alleles (k) to the range in allele size (r) as calculated from microsatellite loci (Garza and Williamson 2001). When a population experiences a bottleneck event, the loss of any allele would decrease k, but only the loss of the largest or smallest allele would affect r, thus the ratio M = k/r would be smaller in recently reduced populations (Garza and Williamson 2001).

Godfrey H. Hardy and Wilhem Weinberg independently developed the Hardy-Weinberg (H-W) Principle, a mathematical model that calculates allele frequencies under the assumption that the population is infinitely large, undergoes random mating, and that no modifying factors are involved (Klug and Cumming 1994). A population is described as being in equilibrium if the allele frequencies remain constant from generation to generation, with the expected and observed allele frequencies remaining virtually identical (Klug and Cumming 1994). Genetic diversity will be conserved provided the population remains in H-W equilibrium; allele frequencies of 0.5 indicate higher proportions of heterozygotes than values nearer one or zero (Brewer and Sing 1983). If a population is in disequilibrium with the expected allele frequencies predicted by H-W deviating significantly from the observed values, it indicates that the population is being influenced by one or more modifying factor(s).

The polymorphic information content (PIC) can be considered a more accurate measure of genetic diversity than the levels of heterozygosity, for both the number and frequency of alleles at a particular locus are incorporated (Zajc *et al.* 1997). The PIC values is calculated according to the equation: $PIC = (1 - \Sigma_{i=1}^{n} p_i^2 - 2) \times (\Sigma_{l=1}^{n-1} \Sigma_{j=l+1}^{n} p_i^2 p_j^2)$, where p_i is the frequency of the ith allele and n the number of individuals in the population (Ostrander *et al.* 2001). The PIC value was initially devised for use in genome scanning to measure the informativeness of markers, but is now commonly used in population studies.

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2.3. Population Differentiation

"The process of speciation divides an originally homogenous gene pool into two or more reproductively separate gene pools. This division may be accompanied by changes in morphology, physiology, and adaptation to the environment" (Klug and Cummings 1994).

Populations distributed across extensive geographic areas encompassing varied habitats often become isolated and genetically differentiated, as these subpopulations no longer comprise a single randomly mating population (Klug and Cummings 1994). Genetic drift, inbreeding, mutation, migration, and selection are factors that can separately or collectively modify allele frequency distribution and initiate evolutionary divergence with eventual speciation. The degree of differentiation between subpopulations depends on population size, the extent of migration, and the period of separation. Population differentiation can be measured by various statistical analyses of allele frequencies in order to reject, with a level of statistical confidence, the null hypothesis that there is no differentiation amongst the populations examined (Raymond and Rousset 1995a).

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Wright (1965) introduced the F-statistic parameters (F_{ST}, F_{IT} and F_{IS}) for the analysis of population structure. The fixation indices are related according to the following equation: $1 - F_{IT} = (1 - F_{IS})(1 - F_{ST})$, for neutral diallelic loci (Jordana *et al.* 1992), although Nei (1977) expanded this concept to multiple alleles. Masatoshi Nei proposed that the F_{ST} statistical measurement was appropriate for use with molecular data to estimate genetic differentiation between populations, assuming the "Infinite Allele Model" whereby all mutations generate new alleles (Nei 1977, Nei and Chesser 1983). The F_{ST} parameter varies between 0 (absence of differentiation) and 1 (complete differentiation), with values less than 0.05 implying insignificant population differentiation and those greater than 0.1 indicating the existence of substantial population differentiation. F_{ST} is usually symbolised as G_{ST} if the locus expresses more than two alleles (Irion *et al.* 2003). The F_{IS} and F_{IT} parameters provide a measure of the excess or deficit of average heterozygotes within each subpopulation or the global estimate of the total population, respectively (Goudet 1995, Rousset and Raymond 1995, Morera *et al.* 1999).

The R_{ST} estimator of population differentiation was introduced by Chakraborty and Nei (1982) and independently defined and promoted by Slatkin (1995). This measure was designed specifically for the microsatellite "single-step" model of evolution, assuming an increase or decrease of one repeat unit per mutation and allowing for the rapid mutation rate that generates the multitude of alleles characteristic of most microsatellite markers. The R_{ST} estimate can be calculated by the equation: $R_{ST} = (S - S_W) / S$, where S is the mean estimated variance of allele sizes in the populations, and S_W the mean estimate within each population (Slatkin 1995).

Both F_{ST} and R_{ST} estimates are used to ascertain population structure, but frequently differ. For example, R_{ST} has a greater variance than F_{ST}, and requires more loci to obtain accurate results. Balloux and Goudet (2002) used computer simulations to compare these statistical values, using different conditions of gene flow, mutation rate, population size and number under a finite island model restricted to an exact symmetrical stepwise mutation model. It was found that there is no single best estimator of population differentiation, with both experiencing bias and variance to differing degrees under different conditions.

H-W equilibrium is also an effective gauge of differentiation between subpopulations, for if the combined population is significantly out of H-W proportions then disequilibrium results from genetic drift altering allele frequencies in the subpopulations. The significance of the deviations from H-W equilibrium is calculated by chi-square analysis: $\chi^2 = (1 - H_O/H_E)^2 \times n$, where H_O and H_E are the observed and expected heterozygosities and n the population size. The degrees of freedom are determined by: $df = r \times (r - 1) / 2$, with r the number of alleles expressed by the locus (Irion *et al.* 2003).

Divergent populations may differ sufficiently in allele frequency distribution, for assignment tests to either group individuals into or exclude them from predefined reference populations, with a degree of statistical confidence (Koskinen 2003). Assignment values are determined as a ratio of the likelihood of the genotype of the individual corresponding with each of the possible source populations. The proportion of individuals correctly assigned to their own population is another measure of population differentiation, and can also be used to determine the hybrid status of individuals or to categorise individuals of uncertain origin.

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2.4. Canine Molecular Genetics

In conflict with the archaeological record, mtDNA analysis suggests that dogs and wolves diverged in multiple events over 100 000 years ago (Vilà *et al.* 1997, 1999). Regardless of the exact date, man has long intervened in the breeding of domestic dogs to produce diverse characteristics that serve to support human society (Figure 2.3.). Additional mtDNA analysis of polymorphisms at 21 enzyme restriction sites has revealed that domestic dogs and grey wolves differ at the genomic level by just 0.2% (Wayne *et al.* 1992), thus diversity under domestication is the result of only a few changes in the DNA sequence of the founding populations.

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Figure 2.3. From the 60kg Great Dane to the 2kg Chihuahua, the extravagant diversity of body size, conformation, pelage, temperament, and behaviour characterised by the domestic dog is indicative of the power of artificial selection (© Nouvelles Images 2002).

The movement of humans and their dogs has resulted in extensive gene flow between domestic dog populations for many thousands of years, with mtDNA analysis revealing that dogs display a high degree of polymorphism that is neither breed-specific nor defined by geographical distribution (Tsuda *et al.* 1997). With modern breeding practices striving to achieve distinctive phenotypic uniformity, purebred dogs have become essentially closed gene pools (Vilà *et al.* 1999, Parker *et al.* 2004). Significant levels of inbreeding increase the proportion of homozygotes with the accompanying increased risk of genetic disorders if deleterious recessive alleles coalesce (Koskinen and Bredbacka 2000).

The modern domestic dog population consists of approximately 350 partially inbred genetic isolates (breeds) and a multitude of heterogeneous outbred mongrel dogs (Ostrander *et al.* 2000). The pedigree barrier usually restricts gene flow between these subpopulations to such an extent that 46% of the known genetic disorders associated with dogs occur predominantly or exclusively in one or a few breeds (Ostrander and Kruglyak 2000). This phenomenon is the result of the chance occurrence of recessive disease alleles in one breed but not another, mostly due to the small number of founding individuals and extensive breeding with "popular sires" and other closely related individuals (Ostrander *et al.* 2000). For example, the modern Irish Wolfhound is believed to have arisen from six individuals, and only five of the Leonburger breed survived in Europe after World War I (Wayne and Ostrander 1999). The increased prevalence of a particular disease condition in a breed can be further exaggerated by the inadvertent co-selection of a disease gene with a breed-specific trait (Brooks and Sargan 2001).

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Thus, in parallel with the extraordinary variation displayed by domestic dogs, breeds commonly express specific genetic defects; for example, Dalmatians have deafness, Yorkshire Terriers have hydrocephalus and collies a series of ocular defects (Galibert *et al.* 2001). More than 370 canine diseases have been identified as either inherited or with significant inherited components, and many more are detected each year (Ostrander *et al.* 2000). The prevalence of recessively inherited disease traits is a result of the high levels of inbreeding associated with intensive artificial selection and the absence of strong selection against disease phenotypes (Richman *et al.* 2001). Outbred mongrels express inherited diseases fairly infrequently, due to the increased heterozygosity in the population whereby the unaffected gene copy provides a masking effect (Brooks and Sargan 2001).

However, further mtDNA analysis has revealed unexpectedly high levels of variability within certain breeds of dogs, probably due to the heterogeneous outbred nature of the founding individuals, and the relatively recent origin of many breeds during the 19th century (Vilà *et al.* 1999, Wayne and Ostrander 1999, Irion *et al.* 2003). Microsatellite marker heterozygosities of between 36% and 55% have been reported, reflecting the diverse gene pool of the founding populations and that breeds of dogs do not necessarily express high levels of genetic homogeneity (Wayne and Vilà 2001).

Significant levels of inbreeding and artificial selection would have been required for the rapid establishment of multiple breeds of dogs; the following studies have used microsatellite markers to investigate genetic diversity and population differentiation.

Fredholm and Winterø (1995) examined the similarities and divergence within and between different populations of *Canidae,* the Flat-coated Retriever and Dachshund (Table 2.4.), and the red fox (*Vulpes vulpes*) and Arctic fox (*Alopex lagopus*). The two dog breeds had significantly different allele distribution frequencies and heterozygosities at individual loci, but total breed heterozygosities were comparable. The size range and number of alleles were similar, and breed-specific alleles were observed in all but one locus. Intrabreed diversity was only slightly less than that between wild canid species, but again significantly less than the total diversity in domestic dog breeds.

Pihkanen *et al.* (1996) examined two populations of German Shepherd Dogs (GSD), selected for either appearance or performance since the 1960's, and a composite of 34 other breeds as a reference population (Table 2.4.). The average allelic diversity in the combined GSD population was half that of the composite sample group, with the alleles of the former a subset of the latter, containing only a single unique allele. The average heterozygosity in each GSD subpopulation was similar, but the total less than that of the composite breed group. The homozygote-heterozygote proportions in the two subpopulations were in balance, indicating a relatively stable intrapopulation genetic structure. The genetic profile of the two subpopulations differed greatly from the composite breed population, with significant population differentiation (F_{ST} = 0.30) between the combined GSD and composite breed populations. Such interbreed differentiation supports the concept of small founder populations and intense inbreeding in the early

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stages of breed establishment. However, these findings were based on limited molecular data, with less than 30 individuals per population analysed for only three microsatellite markers. The relative measures of differentiation between the divergent GSD subpopulations were not investigated. The genetic diversity revealed by this study could have been influenced by the breeding strategies employed in different countries, with dogs selected for performance sampled from Germany (the ancestral population) and dogs selected for appearance, from Finland (a derived population).

Zajc *et al.* (1997) analysed the genetic variability within and between the Greyhound, Labrador Retriever and German Shepherd Dog (Table 2.4.). Relative measures of heterozygosity were less than that of humans or mice but similar to horses and cattle. The mean PIC value across all breeds, 0.5, was similar to the 0.52 calculated for domestic dogs by Ostrander *et al.* (1993). When compared with a composite group of other dog breeds, a substantial decrease in genetic variation was observed within all three breeds, probably due to the inbreeding associated with purebred dogs. Although a few breed-specific alleles were observed, a better measure of breed identification was the relative frequency and distribution of alleles across all the microsatellite loci. The H-W proportions of each breed were tested and significant allele frequency deviations were observed, indicating that none of the populations were in equilibrium, attributed to the limited sample size and non-random mating. Microsatellite genetic distances were used to determine the evolutionary relationships between breeds. Labrador Retrievers were approximately equidistant from the other breeds ($D_{L,GH}$ = 0.028, $D_{L,GSD}$ = 0.031); with Greyhounds and German Shepherd Dogs significantly separate ($D_{GH,GSD}$ = 0.054), indicating that the former was established more recently and that the latter two breeds diverged at an earlier stage.

Morera *et al.* (1999) studied genetic variation within and between five Spanish dog breeds: the Spanish Alano, Spanish Greyhound, Andalusian Hound, Spanish Water Dog and Maneto (Table 2.4.). The results indicated similar levels of genetic diversity between the breeds, with comparable allele counts and heterozygosities. Significant deviations from H-W equilibrium were detected at six of the 20 possible loci with an average heterozygote deficit at all loci for each breed. The levels of population differentiation between the breeds were relatively low, but still significant and thus used as estimates of genetic distance between the breeds. The shortest genetic distance was between the Spanish

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Greyhound and Andalusian Hound (0.067 ± 0.014), with the Alano most separate from this group for it belongs to a different morphological group, the Moloses. The molecular data described indicates substantial variation within breeds of Spanish dogs, but less significant interbreed genetic variation than that reported between other breeds of dogs.

Koskinen and Bredbacka (2000) examined the population structure of five Finnish dog breeds: Golden Retrievers, German Shepherd Dogs, wire-haired Dachshunds, Pembroke Welsh Corgis and Bedlington Terriers (Table 2.4.). The Golden Retriever and German Shepherd Dog breeds had more than 18 000 registrations during the previous decade, the wire-haired Dachshunds and Pembroke Welsh Corgis had about 4 000 registrations, but only 138 Bedlington Terriers were registered during that period. This German Shepherd Dog sample group randomly included individuals from both the appearance and performance selection strategies. The frequency and distribution of alleles varied between breeds with the greatest level of genetic diversity usually found in the larger populations. However, the wire-haired Dachshunds displayed the highest levels of variation, indicating that this breed has the most extensive gene pool. Significant deviations from H-W equilibrium were detected at eight of the 50 loci, with the average H-W disequilibrium being statistically significant across all breeds. Positive F_{IS} estimates coincided consistently with the deviations from H-W equilibrium, indicating that disequilibrium was the result of a total heterozygote deficit (global F_{IS} = 0.058). The Bedlington Terrier was a surprising exception with every locus examined being congruent with H-W proportions. Therefore, despite low levels of genetic diversity and a small population size, negligible inbreeding has occurred over recent generations. None of the breeds displayed a significant excess of H_E over H_{EQ}, thus there was no indication of recent bottlenecks in any of the populations. All F_{ST} estimates between breeds were highly significant, indicating extensive population differentiation. Also, genetic distances suggested significant divergence between dog breeds, with the largest distance (0.975 ± 0.229) only slightly less than the lowest between humans and chimpanzees (D_S between 1.334 and 1.901). The molecular data obtained supports both the genetic isolation of dog breeds and the use of intensive inbreeding and artificial selection in the history of these breeds.

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Altet *et al.* (2001) investigated the loss of microsatellite variability in an inbred population by examining three generations of closely related Rottweilers (inbreeding coefficient of 16%) as compared with three populations of unrelated dogs (no common grandparents): Golden Retrievers, Labrador Retrievers and a composite sample of purebred dogs (Table 2.4.). The mean PIC estimate for the Rottweiler population remained similar to those of the other breeds, it was suggested that this sustained polymorphism might be explained in two ways. Either that the animals used for breeding happened to express relatively high levels of heterozygosity or that enough animals were used for breeding to maintain the variability. The mean PIC estimate for the composite sample of 24 dog breeds was significantly greater than that of each of the breeds examined, for it incorporated several breed-specific alleles and expressed an artificially high level of genetic diversity. While breed-specific alleles were revealed in most purebred dog populations, a better measure of breed identification was the relative PIC estimate. For example, at locus CXX366 both the Golden Retriever and Labrador Retriever populations had the same four alleles, but the PIC values (0.427 and 0.085, respectively) were due to the different distribution of these alleles. It was suggested that the microsatellite data acquired from heterogeneous populations could not always be extrapolated to individual breeds.

Irion *et al.* (2003) analysed intrabreed diversity and interbreed differentiation in 28 recognised breeds, representing all seven of the American Kennel Club (AKC) breed groups (Table 2.4.). The German Shepherd Dog, Dachshund and Staffordshire Bull Terrier were not included in the breeds examined. The approximate population size of each breed was calculated according to the number of registrations over the previous five years, with the largest populations more than 100-times greater than the smallest. The approximate age of each breed was determined as the period since official recognition by the breed registry. The molecular data revealed a considerable degree of genetic variation, with the smaller populations tending towards 6% fewer alleles per breed than the larger populations and the older breeds tending towards 7% fewer alleles. Across all the breeds, there was a 3% correlation between average breed heterozygosity (H_B) and estimated population size. There was a more robust correlation between H_B and time since breed recognition, with the more recently recognised breeds having 19% higher H_B than the older breeds. Tests for H-W equilibrium showed that an average of 27% of the

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loci per breed were in disequilibrium. The number of loci in H-W equilibrium was plotted for each breed against estimated population size and time since breed recognition, and indicated a 10% increase in the larger populations and 4% increase in the more recently recognised breeds. Thus the highest levels of genetic polymorphism would be expected in the breed with the largest population size and most recent origin. Phylogenetic analysis, measured by genetic distances from allele frequencies, implied two significant relationships between the 28 breeds. Bull Terriers and Miniature Bull Terriers were grouped together in all trees generated for the final consensus tree, and Australian Shepherds diverged significantly from the remaining 27 breeds (95.9% confidence), possibly due to the geographic isolation of this breed. The extreme polymorphic nature of the microsatellite panel, with additional allelic forms generated at a relatively high rate, chosen specifically for use in genome screening, must be taken into account for these factors may cause breeds to appear more heterogeneous than implied by their pedigrees.

Parker *et al.* (2004) investigated genetic relationships and classification in domestic dog breeds (Table 2.4.). SNP data was used to estimate average genetic distances between breeds (F_{ST} = 0.33), which verified the intense isolation of breeds. A Bayesian model-based clustering algorithm was used to identify genetically diverse subpopulations according to patterns of microsatellite allele frequencies. Individual dogs from nearly all the breeds clustered together, and direct assignment tests accurately grouped 99% of the dogs to each breed. Microsatellite typing combined with phylogenetic analysis identified four genetic clusters that included breeds of similar geographic origin, morphology, and/or functional role. One cluster included breeds from Central Africa, the Middle East, Tibet, China, Japan and the Arctic. It has been suggested that early pariah dogs originated in Asia and migrated with nomadic people southwards to Africa and northwards to the Arctic, therefore these breeds may be representative of the true ancestral gene pool. However, most breeds radiated more recently from Europe and probably correspond to the explosion of well-defined breeds in the mid-19th century. Further analysis of this group revealed a clustering of Mastiff-like breeds, another of herding breeds, and a third of the hunting breeds. This genetic classification substantiates some conventional breed categories and exposes other previously unidentified groupings.

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Reference	Number of Loci	Breeds	Country of Origin	Sample Size	Alleles / Locus	H_T	PIC	F_{IS}	F_{ST}
Fredholm and Winterø (1995)	20	Flat-coated Retrievers	Denmark	33	4.5	0.52	-	-	-
		Dachshunds	Denmark	32	5.6	0.55	-	-	
Pihkanen *et al.* (1996)	3	Show German Shepherd Dogs	Finland	25	5.0	0.58	-	-0.03	0.30
		Sport German Shepherd Dogs	Germany	23	5.0	0.63	-	0.03	
		Composite of 34 Breeds	Finland	28	10.0	0.86	-	0.29	
Zajc *et al.* (1997)	19	Greyhounds	Ireland	50	2.5	0.36	0.31	-	-
		Labrador Retrievers	U.K./ Slovenia	50	3.3	0.48	0.43	-	
		German Shepherd Dogs	U.K./ Slovenia	50	3.3	0.43	0.38	-	
		Composite of 15 Breeds	U.K./ Slovenia	22	4.2	0.58	0.50	-	
Morera *et al.* (1999)	4	Spanish Alano	Spain	35	5.0	0.70	0.64	0.09	0.11
		Spanish Greyhounds	Spain	30	6.5	0.74	0.69		
		Andalusian Hounds	Spain	37	6.3	0.71	0.67		
		Spanish Water Dogs	Spain	28	5.8	0.77	0.72		
		Maneto	Spain	32	7.0	0.76	0.71		

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Reference	Number of Loci	Breeds	Country of Origin	Sample Size	Alleles / Locus	H_T	PIC	F_{IS}	F_{ST}
Koskinen and Bredbacka (2000)	10	Golden Retrievers	Finland	50	5.6	0.61	-	0.01	0.23
		German Shepherd Dogs	Finland	50	6.4	0.62	-	0.04	
		Wire-haired Dachshunds	Finland	50	8.0	0.67	-	0.07	
		Pembroke Welsh Corgis	Finland	50	5.9	0.55	-	0.13	
		Bedlington Terriers	Finland	50	5.2	0.55	-	0.02	
Altet *et al.* (2001)	10	Rottweilers	Spain	360	3.8	0.45	0.40	-	-
		Golden Retrievers	Spain	33	4.1	0.59	0.52	-	
		Labrador Retrievers	Spain	23	4.4	0.52	0.45	-	
		Composite of 24 Breeds	Spain	95	7.3	0.55	0.71	-	
Irion *et al.* (2003)	100	28 Breeds	U.S.A.	29 - 45	4.0 - 8.1	0.39 - 0.76	-	-	0.23
Parker *et al.* (2004)	96	85 Breeds	U.S.A.	384	-	-	0.65	-	-

Table 2.4. The results of molecular genetic studies of domestic dogs, indicating the number of microsatellite markers, the breeds investigated, their country of origin, sample size, degree of breed heterozygosity (H_T), PIC values, coefficients of inbreeding (F_{IS}) and the mean standardised variance in allele frequencies among populations (F_{ST}).

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2.5. Population History of the Breeds

2.5.1. The German Shepherd Dog

Figure 2.5. Xavier von der Kahler Heide (left, S. von Kraayenburg), South African Sieger 2001 and 2003, illustrates the conformation and appearance of a typical show dog. Amos vom Chantian (right, S. Lombard), South African Schutzhund Champion 2002 and 2003, is representative of a typical sport dog.

The ancestors of the German Shepherd Dog (Figure 2.5.) can be traced back to the collection of dogs used to herd and guard flocks of sheep in 19th century Germany. On the 3rd of April 1899, Captain Max von Stephanitz attended one of the first dog shows held in Karlsrühe, Germany where he purchased a grey herding dog named Hektor Linksrhein for it conformed greatly to his ideal of utility and intelligence (Willis 1977). On the 22nd of April of the same year he formed the *Verein für Deutscher Schäferhunde* or SV (Club for German Shepherd Dogs), ushering in the second era of the breed; before 1899 there were German sheepdogs, thereafter German Shepherd Dogs (Kern 1994).

The German Shepherd Dog Breed Standard was drawn up at the first membership meeting of the S.V. in Frankfurt on the 20th of September 1899 (www.gsdfederation.co.za), and official tests of performance called *Schutzhund* (protection dog) that encompass three disciplines; tracking, obedience and protection work, were introduced. These competitions emphasised the working abilities of the breed in keeping with the development of a dog with *"a highly developed sense of smell, enormous courage, intrepidness, agility and, despite its aggressiveness, great obedience"*. (www.cluebus.com/holly/gsdfaq.html).

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The S.V. controlled and directed breeding throughout Germany by exercising authority over which dogs and bitches could be bred and which of the offspring could be kept and raised from each litter (Willis 1977). The dog purchased by von Stephanitz, renamed Horand von Grafrath, was designated S.Z. 1 as the first dog registered with the S.V. Bitches were sought that would perfectly complement his conformation, temperament and utilitarian type and he is regarded as the founding sire of the breed (Pihkanen *et al.* 1996).

The role of the pastoral sheepdog was to become virtually obsolete with the growing industrialisation of the 20th century. But the German Shepherd Dog remained in demand for the breed could adapt to other work, for instance being the first breed trained as Guide Dogs for the blind (Kern 1994). During World War I, they were used both by the German military and the Red Cross, and at the end of the war servicemen from America and Europe returned home with stories of these dogs, catapulting the breed into the limelight.

Following the Great War anything German fell out of popular favour and breeds of dogs suffered together with Beethoven and Bach. The German Shepherd Dog was renamed simply Shepherd Dog in the United States of America and Alsatian in Great Britain after the German-French border area of Alsace-Lorraine (Kern 1994). However, the name in German is *Deustche Schaferhund*, the "German Shepherd Dog", and after numerous campaigns the name of the breed was officially re-instated in 1977 (Kern 1994).

The German Shepherd Dog Breed Standard has remained relatively unchanged since its inception a century ago, however this does not apply to the dogs themselves. German Shepherd Dogs across the world looked quite similar up to the 1940's, but today there are, amongst others, West German and East German show and sport dogs, English Alsatians and American show dogs. These types are distinct and easily distinguishable due to varying physical construction and temperament as a result of alternative interpretations of the breed guidelines, and fashion trends in different countries. Functional preferences have also played an important role, with breed purists placing the emphasis on the show-ring and focussing on correct conformation and movement, and dog-sport enthusiasts focusing on the dog's innate desire to perform, and its intelligence and trainability.

Show dogs (Figure 2.5.) are preferentially black and tan in colour and have a distinctive shape, with a sloping topline, being slightly long in comparison to the height, the construction enabling an effortless and long-reaching trotting gait. The topline should curve gently from the tip of the ears over the wither and back to the end of the tail. The overall impression of a typical show dog is that of a powerful, well-balanced dog that is substantial but not heavy or clumsy.

Sport dogs (Figure 2.5.) are often sable in colour (black overlay on grey or light-brown), but little attention has been is given to specific physical conformation. Selection for those mental attributes that comprise the ideal sport dog has resulted in a more square shape with a flatter topline from wither to croup, and usually smaller in stature and more agile. Breeding programmes focus on temperament and the ability to perform willingly and tirelessly, these dogs must be capable of learning and remembering, determined and self-confident in order to be successful in dog-sport competition.

The German Shepherd Dog has proven popular with 7 990 individuals registered with the Kennel Union of South Africa (KUSA) and 7 415 with the German Shepherd Dog Federation of South Africa for the years 1993 to 2003. However, with little or no compromise on either side, a near complete division exists worldwide within the breed.

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2.5.2. The Dachshund

Figure 2.6. Red and black and tan standard short-haired Dachshunds (left, J. Arthur), and a red long-haired miniature Dachshund (right, Scanziani 1988).

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"*Dachs*" is the German word for badger for the standard Dachshund (Figure 2.6.) was bred specifically for the purpose of hunting these animals (Van der Lyn 1995). This sport required a short-legged hound with a well-developed sense of smell, great courage and perseverance that would burrow underground in pursuit of its quarry (Palmer 1981).

The Dachshund is derived from the oldest German hunting breeds, and was first mentioned in the book *La Venerie* (The Hunt) written by Jaques du Fouilloux in 1561 (Van der Lyn 1995). Towards the end of the 17th century, the "Badger Fighter" was described as "*a peculiar, low, crook-legged species*" (Raine 1989).

In the mid-1700's, refugees of the French Revolution arrived in Germany and Austria, often accompanied by French Basset hounds (Nicholas and Foy 1987). Crossbreeding between these dogs and the native Dachshund resulted in shortened ears and a more pointed foreface (Nicholas and Foy 1987).

The miniature Dachshund was also established for a specific purpose, for towards the end of the 19th century German sportsmen wanted a hunting dog that could go to ground after rabbits and foxes (Palmer 1981). The smallest standard Dachshunds were crossed with toy terriers and Miniature Pinschers to produce this miniature type (Palmer 1981).

The first Dachshund Club was founded in Germany in 1840, with the official Breed Standard being characterised in 1879 (Adler 1975). Today, there are three varieties of coat-type, the long, short and wire-haired Dachshunds of both the standard and miniature breed types (Figure 2.6.). The long-haired type was acquired by selectively crossing the original short-haired Dachshund with the spaniel and the old German gundogs, the Stöberhund and Wachtel, and received official recognition in 1886 (Scanziani 1988, Raine 1989). The wire-haired type resulted from selective crossing of the short-haired variety with the Scottish and Dandie Dinmont terriers and Miniature Wire-haired Pinschers and received official recognition in 1898 (Palmer 1981, Scanziani 1988, Raine 1989).

After World War I, anti-German sentiments caused the breed to be reviled and detested to such an extent that dogs were even stoned in the street (Van der Lyn 1995). Similarly, after World War II, political prejudice caused the number of individuals in the breed to reach close to obscurity (Palmer 1981). The Dachshund was chosen as the mascot for the 1972 Olympic games held in Munich, Germany, helping to advertise and promote the breed (Adler 1975).

Dachshunds are still bred for hunting, but today their function is mostly as companion dogs (Palmer 1981). The breed has become popular due to the retention of their inherent characteristics of bravery and loyalty, with 655 standard Dachshunds and 6 070 miniature Dachshunds registered in the Hound Group with the Kennel Union of South Africa for the years 1993 to 2003.

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2.5.3. The Staffordshire Bull Terrier

Figure 2.7. An example of a tan (left, www.donellas.co.uk) and a brindle (right, www.staffordshirebullterrierdogs.com) Staffordshire bull terrier.

The Staffordshire Bull Terrier (Figure 2.7.) was specifically developed for the once fashionable sports of bull-baiting and dog-fighting (Palmer 1981). Once these blood sports were prohibited, enthusiasts began to promote the breed as a companion dog. Official recognition by the British Kennel Club was received in the mid-1930's with a Breed Standard being drawn up and a Breed Club formed in Cradley Heath, South Staffordshire (Palmer 1981).

Bull-baiting was first endorsed by the Earl Warrenne, Lord of Stamford, in Lincolnshire when on the 13th of November 1209, he happened to see an enraged bull being tormented by a pack of dogs on the village green (Palmer 1981). Dogs previously used for bear-baiting proved too cumbersome and thus vulnerable to goring and tossing, and faster, more nimble, lower-to-ground dogs were required for bull-baiting (Gordon 1986). The bulldog, as evolved from the Old English Mastiff, was crossbred with various English terrier breeds of that time to produce the forerunner of the modern Staffordshire Bull Terrier (Gordon 1986).

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An appeal was made in 1802 to prohibit both bear and bull-baiting, but it was in 1835 before the Society for the Prevention of Cruelty to Animals (now the RSPCA) was able to organize a successful test case at Lincoln Assizes (Gordon 1986). Thereafter, small Bulldogs and the Bulldog terrier crosses were used for dog-fighting, a sport made popular when the more sensational bear and bull-baiting were removed from the public scene (Gordon 1986). Although the Prohibitions Act of 1835 also outlawed dog and cock-fighting, these events continued for they were more adaptable to secret and underground staging (Gordon 1986).

Mr Joseph Dunn of Cradley Heath should receive most of the credit for saving the Staffordshire Bull Terrier from obscurity. He and other admirers of the breed eventually received permission to display the breed in the Any Variety Terrier Class at a show held in Cradley Heath in the spring of 1935, and in July of the same year they founded the first Staffordshire Bull Terrier Club to sponsor and promote the breed (Gordon 1986).

Although the primary function of the Staffordshire Bull Terrier has been rendered null and void, the breed has since proven extremely popular as companion dogs, with 20 472 individuals registered in the Terrier Group with the Kennel Union of South Africa for the years 1993 to 2003. The Staffordshire Bull Terrier has been described as having "*indomitable courage, high intelligence and tenacity, he is affectionate with friends and his character is one of trustworthiness and stability*" (Gordon 1986). The breed can be characterised as "honest" for their true nature should be one that is positive and straightforward, being intelligent rather than shrewd (Gordon 1986).

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Chapter 3

Materials and Methods

3.1. Sample Selection

Whole blood or tissue samples from 156 outbred mongrel dogs were collected from the Society for the Prevention of Cruelty to Animals (SPCA) in Cape Town, Johannesburg and Pretoria, the Animal Welfare at Bellville South, Cape Town and the Port Elizabeth Community Centre. Whole blood samples or buccal cell swabs of 101 German Shepherd Dogs registered with the German Shepherd Dog Federation of South Africa were collected from various provinces in South Africa and from dogs imported from Germany or other European countries. Buccal cell swabs from 26 Dachshunds, 18 Staffordshire Bull Terriers, and 37 pedigreed dogs representing 30 breeds registered with the Kennel Union of South Africa, were collected from dog shows, veterinary practices, boarding kennels, and private dog owners.

Samples were collected from different geographic areas, for a particular breed might be more inbred within a limited region. First order relatives (parent-offspring and full siblings) were excluded from this data set to reduce the possibility of sampling bias, but the structure of the breed prevented the exclusion of paternal half-siblings. Large samples sizes were selected, where possible, to minimise the effect of second-generation relatives.

The composite group included the following breeds: Airedale, Beagle, Belgian Tervueren Shepherd, Border Collie, Bernese Mountain Dog, Bouvier des Flandres, Boxer, Bulldog, Cocker Spaniel, Dobermann, Elkhound, German Short-haired Pointer, Giant Schnauzer, Golden Retriever, Great Dane, Greyhound, Irish Setter, Jack Russell Terrier, Labrador Retriever, Mastiff, Miniature Schnauzer, Rhodesian Ridgeback, Rough Collie, Scottish Terrier, Siberian Husky, Standard French Poodle, Standard Schnauzer, Weimaraner, Whippet and Wire-haired Fox Terrier.

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3.2. DNA Isolation Protocols

DNA was isolated from either whole blood, tissue, or buccal cell samples for each of the individuals included in this research. Reagent blank controls were included and processed through each DNA extraction and quantification procedure.

3.2.1. Whole Blood Extraction

Approximately 10ml of whole blood were collected in two 7ml Vacutainer® K3 EDTA Tubes (Becton Dickinson Vacutainer Systems) using either 20 or 22 gauge Vacutainer® Needles (Becton Dickinson Vacutainer Systems). The samples were stored at -20°C to initiate red blood cell lysis before undergoing DNA extraction.

Red blood cell lysis buffer (Appendix III) was added to the defrosted blood and centrifuged at 3 000rpm for 10 minutes (Sigma®-302K Centrifuge), the supernatant discarded. The process was repeated until the supernatant was clear, indicating that all red blood cells had lysed, the pellets were transferred to 1.5ml Eppendorf tubes (B&M Scientific).

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The following DNA salt precipitation process was employed (Miller *et al.* 1988):

The pellets were resuspended in 450µl saline EDTA solution (Appendix III), 50µl 10% SDS (BDH Laboratory Supplies) and 60µl 20mg/ml Proteinase K (Roche Diagnostics) and incubated overnight in a water bath (Shinko GCS) at 56 °C, being periodically agitated on a vortex machine (Supermixer). Thereafter, each sample was aliquoted 225µl 6M (Saturated) NaCl (Saarchem-Holpro Analytic) and centrifuged at 10 000rpm for 10 minutes at 5 °C (Sigma® - 2MK Centrifuge). The supernatant was collected in fresh sterile 1.5ml Eppendorf tubes (B&M Scientific) and precipitated with approximately 1ml ice-cold isopropanol (BDH Laboratory Supplies). The samples were placed at -20°C for two hours, and then centrifuged at 10 000rpm for 10 minutes at 5°C, discarding the supernatant. The pellets were washed twice with 70% ethanol (Saarchem-Holpro Analytic) by centrifugation at 10 000rpm for 10 minutes at 5°C and discarding the supernatant.

The DNA pellets were air-dried and resuspended with between 100µl and 500µl 1× TE buffer (Appendix III) and incubated in a water bath (Shinko GCS) at 56°C for two hours.

3.2.2. Tissue Extraction

Tissue samples were either dissected from uteri and epididymides during sterilisation procedures, or as muscle biopsies from euthanased dogs, using sterile surgical blades (Paramount). These samples were stored at -20°C to prevent cell degradation before undergoing DNA extraction.

Tissue samples were finely chopped and rinsed in several washes of saline EDTA and transferred to sterile 1.5ml Eppendorf tubes (B&M Scientific) for the DNA salt precipitation process described above, except that these samples were incubated at 56 °C with 60µl 20mg/ml Proteinase K (Roche Diagnostics) for several days, until the solution had reached a homogenous state.

3.2.3. Buccal Swab Extraction

Two ear-buds were applied to the gums and cheeks of each dog to collect buccal cells. The swabs were placed in a 450µl saline EDTA (Appendix III) and 50µl 10% SDS (BDH Laboratory Supplies) solution in sterile 1.5ml Eppendorf tubes (B&M Scientific) and kept at room temperature for 24 to 36 hours. The swab was removed from the solution, and each sample was aliquoted 40µl 20mg/ml Proteinase K (Roche Diagnostics), continuing with the DNA salt precipitation process described above.

3.3. DNA Quantification and Dilution

The DNA concentration (µg/µl) of each extracted sample was calculated by measuring the absorbency of light through the solution at both 260 and 280nm, either with a Gene Quant II RNA/DNA Calculator (Pharmacia Biotech) or an 8450 A Diode Array Spectrophotometer (Hewlett Packard). The ratio of protein contamination, with an optimum value of 2, was calculated by the absorbency ratio of A_{260}/A_{280}. A waterblank control sample was included in every process.

DNA stock solutions of each sample were diluted with sterile distilled water (sdH_2O) to produce working solutions of each sample with a final DNA concentration of 100µg/µl. DNA stock solutions were stored at -20°C and working dilutions at 5°C.

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3.4. Microsatellite Markers

Four polymorphic canine microsatellite markers (Microsatellite loci 1 - 4, Table 3.1. and Table 3.2.) were selected from the Fred Hutchinson Cancer Research Centre Dog Genome Project (www.fhcrc/org/science/dog_genome/). The oligonucleotide primer pairs were synthesised by Beckman Instruments Inc. (Oligo 1 000M DNA Synthesizer). Each microsatellite marker was analysed by the manual end-labelling of the forward primer with a standard [γ^{32}P] dATP (Amersham) and T4 polynucleotide kinase (BioLabs) reaction. The amplified PCR product was visualised by standard polyacrylamide gel electrophoresis. A nonrecombinant M13 control sequence (Appendix III) was run on each gel adjacent to the reaction samples as an absolute size standard for comparisons between different gels.

A further eleven microsatellite markers (Microsatellite loci 5 - 15, Table 3.1. and Table 3.2.) were selected from the panel for canine parentage verification as voted for at the 28th conference of the International Society of Animal Genetics (ISAG) in August 2002 in Göttingen, Germany (www.vgl.ucdavies.edu/research/canine/ISAG/index.html). Each forward primer was labelled with a fluorescent dye (Applied Biosystems) for the visualisation of the amplified PCR product by automated fragment size analysis on an ABI Prism™ 310 Genetic Analyzer. A number of these fluorescent-labelled loci have characteristic peaks that show two parts separated by only a single base pair. This phenomenon is the result of Taq polymerase attaching a non-template A nucleotide to the end of the amplification product, and the resultant biphasic peak represents the true allele and the allele-plus-A peak. Therefore, to ensure reliable genotyping, the INRA21, AHTh171, and FH2001 loci have been consistently sized according to the typically large allele-plus-A peaks.

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	Locus	Size (bp)	T_m (°C)	GC (%)	MW (g/M)	OD 1nM	Nucleotide Sequence
1	DTRCN1 F	20	50	45	6098	4552	AATGCTGACACCAGTAGCTT
	DTRCN1 R	20	48	40	6038	5252	TTCTGCCTGTTTATCTGTCA
2	FH2137 F	21	52	48	6339	4630	GCAGTCCCTTATTCCAACATG
	FH2137 R	20	50	45	6032	5115	CCCCAAGTTTTGCATCTGTT
3	FH2140 F	20	50	45	6176	4376	GGGGAAGCCATTTTTAAAGC
	FH2140 R	20	54	55	6089	4810	TGACCCTCTGGCATCTAGGA
4	FH2328 F	22	51	41	6098	4552	ACCAGGTAGTTTTCAGAAATGC
	FH2328 R	20	52	50	6038	5252	AGTTATGGGACTTGAGGCTG
5	AHT121 F	20	48	40	6095	4808	TATTGCGAATGTCACTGCTT
	AHT121 R	20	50	45	6011	4895	ATAGATACACTCTCTCTCCG
6	INRA 21 F	24	54	42	7369	3912	ATGTAGTTGAGATTTCTCCTACGG
	INRA 21 R	22	49	36	6820	4188	TAATGGCTGATTTATTTGGTGG
7	AHTh171F	22	57	55	6670	4177	CTCACCAGGCATAGACACTCAG
	AHTh171R	19	51	53	5674	5531	CTCATTTGTTCACGCACCC
8	AHTk253F	22	57	55	6829	4255	ACATTTGTGGGCATTGGGGCTG
	AHTk253R	22	59	59	6772	3982	TGCACATGGAGGACAAGCACGC
9	CXX279 F	21	50	43	6459	4063	TGCTCAATGAAATAAGCCAGG
	CXX279 R	20	54	55	6041	5079	GGCGACCTTCATTCTCTGAC
10	FH2001 F	21	52	48	6264	5400	TCCTCCTCTTCTTTCCATTGG
	FH2001 R	24	54	42	7453	3413	TGAACAGAGTTAAGGATAGACACG
11	FH2164 F	22	53	45	6748	3946	GATTATGACTCGAACCAAAGGC
	FH2164 R	22	53	45	6818	3912	TGGAGGAAGTTCATTAAGCAGC
12	FH2611 F	20	52	50	6131	4363	GAAGCCTATGAGCCAGATCA
	FH2611 R	22	51	41	6679	4598	TGTTAGATGATGCCTTCCTTCT
13	FH2247 F	24	51	33	7213	4112	TTCCCACTTACTTTATCATAGCAT
	FH2247 R	21	50	43	6468	3957	CAAATGCAGATTAGGGACACA
14	FH2289 F	21	54	52	6441	4392	CATGGTCTCAGGATCCTAGGA
	FH2289 R	24	54	42	7282	4163	CTAAGCATTCTCTCTGATGGTCTT
15	PEZ08 F	20	48	40	6095	4808	TATCGACTTTATCACTGTGG
	PEZ08 R	20	52	50	6095	4878	ATGGAGCCTCATGTCTCATC

Table 3.1. Forward (F) and reverse (R) primer sequences for PCR amplification of microsatellite markers, indicating locus name, primer length (bp), melting temperature (T_m), GC content, molecular weight (MW) and optical density (OD) (Oligocalculator).

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	Locus	Chromo-some	Label	T_A (°C)	Allele size range (bp)	N_A	Type	Repeat Motif
1	DTRCN1	CFA 17	^{32}P	60	94 - 148	13	tetra	$(GATA)_n$
2	FH2137	CFA 03	^{32}P	63	150 - 194	11	tetra	$(GAAA)_n$
3	FH2140	CFA 05	^{32}P	58	129 - 169	11	tetra	$(GAAA)_n$
4	FH2328	CFA 29	^{32}P	52	180 - 220	11	tetra	$(GAAA)_n$
5	AHT121	CFA 13	Fam	60	79 - 113	15	di	$(CA)_n$
6	INRA21	CFA 21	Vic	60	86 - 102	9	di	$(TG)_n$
7	AHTh171	CFA 06	Vic	60	122 - 142	11	di	$(GT)_n$
8	AHTk253	CFA 23	Fam	60	279 - 297	10	di	$(TG)_n$
9	CXX279	CFA 22	Ned	60	113 - 223	11	di	$(CA)_n$
10	FH2001	CFA 23	Fam	60	120 - 160	11	tetra	$(GATA)_n$int
11	FH2164	CFA 06	Ned	60	246 - 354	16	tetra	$(GAAA)_n$
12	FH2611	CFA 36	Vic	60	185 - 229	11	tetra	-
13	FH2247	Unlinked	Fam	60	171 - 263	22	tetra	-
14	FH2289	CFA 27	Vic	60	239 - 351	22	tetra	$(GAAA)_n$
15	PEZ08	CFA 17	Ned	60	215 - 247	9	tetra	-

Table 3.2. Microsatellite markers indicating their location on different canine chromosomes (CFA), whether labelled with radioactive [$\gamma^{32}P$] dATP (^{32}P) or one of three different coloured fluorescent dyes, the annealing temperature (T_A) of each primer pair, the range of allele sizes (bp) and number of alleles at each locus (N_A) identified in this research, and the di- or tetranucleotide nature of the repeat motif.

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3.5. Radioactive [γ^{32}P] dATP-Labelled Microsatellite Markers

3.5.1. PCR Protocol

Dilutions of both forward and reverse oligonucleotide primers were prepared with a final concentration of 50pmol/µl, according to the following equation:

$$\text{Concentration (pmol/µl)} = \text{Optical density (OD)} / \varepsilon$$

Where $\varepsilon = \Sigma C + \Sigma G + \Sigma T + \Sigma A$,

and $\Sigma C = C_n \times 7.3$ ml/µmol

$\Sigma G = G_n \times 11.7$ ml/µmol

$\Sigma T = T_n \times 8.8$ ml/µmol

$\Sigma A = A_n \times 15.4$ ml/µmol

Microsatellite analysis was performed with [γ^{32}P] dATP-labelled primers for the visualisation of the amplified PCR product. All Polymerase Chain Reactions (PCR) were set up under sterile conditions in a separate laboratory from the processing laboratory, in order to eliminate the possibility of DNA amplicon contamination. A negative control and at least one positive control were included in every reaction.

Each forward primer was end-labelled by a standard [γ^{32}P] dATP (Amersham) and T4 polynucleotide kinase (Biolabs) reaction, whereby ATP molecules including the radioactive ^{32}P isotope were incorporated in the oligonucleotide primer sequence. The reaction reagents were aliquoted together in a 0.5ml Eppendorf tube (B&M Scientific) to make up the primer end-labelling mix (Table 3.3.). All steps utilising radioactivity were completed under the necessary safety conditions. The reaction was accomplished by incubation for at least 90min at 37°C and then 80°C for 2min (Hybaid Thermal Reactor) and then centrifuged briefly (Beckman Microfuge E™) and stored at -20°C.

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Reagent	Stock Concentration	Required Concentration	Quantity per 10 Samples
sdH_2O			6µl
Forward primer	50µM	7.5µM	1.5µl
PNK buffer (BioLabs)	10×	1×	1µl
T4 PNK (BioLabs)	10U/µl	1U/µl	1µl
[γ^{32}P] dATP (Amersham)	20µCi/µl	1µCi/µl	0.5µl
Final Volume			**10µl**

Table 3.3. The primer end-labelling mix used for a standard [γ^{32}P] dATP (Amersham) and T4 polynucleotide kinase (PNK) (Biolabs) reaction for ten samples.

The reagents of the PCR reaction mix were aliquoted aseptically in a 0.5ml Eppendorf tube (B&M Scientific) and added to the end-labelled primer mix to make up the final master mix (Table 3.4.). The DNA samples were aliquoted, 1µl of 100ng/µl DNA solution, added to 9µl of the PCR master mix. All PCR reactions were carried out in a final volume of 10µl using 0.2ml Thin Wall PCR® Tubes (Whitehead Scientific), with one drop (~20µl) of mineral oil (Kimix) added to each sample to prevent evaporation during the PCR reaction.

The amplification reactions were performed in a PCR Thermal Cycler (Stratagene® RoboCycler® 96) using the following programme: 35 cycles of denaturation at 94°C for 45sec, annealing at the specific T_A for 45sec, extension at 72°C for 45sec and then a final extension at 72°C for 30min to complete the reaction. The specific annealing temperatures (T_A) for each microsatellite marker are listed in Table 3.2.

The post-PCR samples were each aliquoted 4µl loading buffer (Appendix III) and stored at -20°C before undergoing polyacrylamide gel electrophoresis.

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Reagent	Given Concentration	Required Concentration	Quantity per 10 Samples
End-labelled forward primer			10µl
sdH_2O			50.07µl
Reverse primer	50µM	7.5µM	1.5µl
Mg-free buffer (Promega)	10×	1×	10µl
$MgCl_2$ (Promega)	25mM	15mM	6µl
dNTPs (Amersham)	100mM	8mM	0.8µl
Taq polymerase (Promega)	5U/µl	0.5U/µl	1µl
Final Volume			**90µl**

Table 3.4. The above reagents in the PCR reaction mix were added to the end-labelled forward primer and used to amplify the microsatellite marker for ten DNA samples.

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3.5.2. Polyacrylamide Gel Electrophoresis

The radioactive [$\gamma^{32}P$] dATP-labelled amplified PCR products were visualised by polyacrylamide gel electrophoresis (PAGE), with alleles being separated according to size. The standard PAGE protocol was followed, using a 6% acrylamide solution (Appendix III) and 1× TBE running buffer (Appendix III), prepared in a purpose-built vertical buffer tank electrophoresis apparatus, approximately 40cm in length. An M13 control sequencing reaction, the A-T ladder (Appendix III), was run adjacent to the samples as an absolute size marker for comparative analysis to determine individual allele sizes (bp).

Post-PCR samples and A-T ladder, combined with loading buffer, were heat denatured at 90°C for 3min and then held at 6°C (Stratagene® RoboCycler® 96) to prevent reannealing of the DNA strands. Electrophoresis was performed at 65W for approximately 30min to allow the gel apparatus and running buffer to reach optimum temperature. Five microlitres of the denatured product was loaded per sample well. Gel electrophoresis continued at 65W for approximately 6 hours, depending on ambient conditions.

After electrophoresis, gels were fixed to Replica Blotting paper (Waltons Stationery), covered with cling-wrap (Klinga), and dried under vacuum for an hour at 80°C in a Drygel Slab gel dryer (Hoffer Scientific Instruments) attached to a vacuum freeze-dryer (VirTis). Microsatellite marker alleles were visualised by exposure to autoradiographic film (AGFA) for between 12 and 72hrs at -70°C (Revco Freezer).

The autoradiographs were developed in X-ray developer solution (Matalex) for a few minutes until allele bands were visible, placed in a 2% acetic acid (BDH) stop solution before being fixed in a 20% solution of Rapid Fixer (Ilford Hypam). The gel was well rinsed in running water and dried in a gel dryer (Durst UT100) before being analysed manually on a lightbox (Kodak Coldlight Illuminator).

3.6. Fluorescent-Labelled Microsatellite Markers

3.6.1. PCR Protocol

All PCR reactions were prepared aseptically in a laminar flow hood (Laminar Flow Systems Ltd Lamarflo) in a laboratory separate from the DNA isolation laboratory, oligonucleotide primers were stored and aliquoted in a separate room, and post-PCR samples were prepared under ultraviolet light in a laminar flow hood (Laminar Flow Systems Ltd Lamarflo) to prevent contamination of either reagents or DNA samples.

The forward primers (Applied Biosystems) of all eleven microsatellite markers, labelled with FAM™, VIC™ or NED™ fluorescent dyes, were aliquoted with the corresponding reverse primers to make up the primer mix for the multiplex PCR. The concentration of each primer pair varied between 0.02 and 0.67µM, according to the published protocol (www.vgl.ucdavis.edu/research/canine/ISAG/CurrentISAGPanelsAug2002.htm). The total primer volume was 34.8µl, to which 4.5µl 10× PCR Gold Buffer (Applied Biosystems) and 5.7µl sdH_2O (Eppendorf AG) were added, and the final volume of the primer mix was 45µl.

The reagents of the PCR reaction mix were aliquoted aseptically in a 0.5ml Eppendorf tube (Whitehead Scientific) and added to the primer mix to make up the final master mix (Table 3.5.). After aliquoting 1µl of the DNA sample solution (100ng/µl), 15µl of the master mix was added to each sample, all PCR reactions were carried out in a final volume of 16µl using 0.2ml Thin Wall PCR® Tubes (Whitehead Scientific),

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The amplification reactions were performed in a Gene Amp® PCR System 9700 (PE Applied Biosystems) using the following programme: initial denaturation at 95°C for 10min followed by 30 cycles of denaturation at 95°C for 30sec, annealing at 60°C for 30sec, extension at 72°C for 1min and then a final extension at 72°C for 60min to complete the reaction. Once the amplification reaction was complete, the post-PCR samples were stored at 4°C until undergoing automated capillary electrophoresis.

Reagent	Stock Concentration	Required Concentration	Quantity per 10 Samples
Primer Mix			42µl
sd H_2O (Eppendorf AG)			49.5µl
PCR Gold Buffer (AB)	10×	1×	15µl
$MgCl_2$ (AB)	25mM	3.34mM	20µl
dNTPs with dTTP (AB)	10mM	1.47mM	22µl
AmpliTaq Gold (AB)			1.5µl
Final Volume			**150µl**

Table 3.5. The above reagents of the multiplex PCR reaction mix were added to the primer mix and used to amplify the microsatellite marker for ten DNA samples, with all components added in the above order.

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3.6.2. Capillary Electrophoresis

Automated fluorescent fragment size analysis for the visualisation of the PCR product was carried out by capillary electrophoresis on an ABI Prism™ 310 Genetic Analyser (Applied Biosystems) according to the manufacturer's specifications. The fluorescent-labelled fragments are separated according to size and a laser reader generates a profile based on fragment lengths. Reproducibility tests were performed based on repeated analysis of standard samples. Every sample run included both a positive and negative reagent control and each sample was sized relative to the internal size standard.

After aliquoting 1µl of PCR product, 10µl of formamide (Applied Biosystems) and 0.25µl internal size standard (Prism Genescan-500™ LIZ™) were added to each sample. All DNA samples were denatured at 95°C for 2min on a Gene Amp® PCR System 9700 (PE Applied Biosystems) before being processed through the Genetic Analyser for 26min per sample. Capillary electrophoresis was performed in a 36cm microcapillary tube filled with POP-4 Performance Optimising Polymer (Applied Biosystems).

3.7. Morphological Measurements of Breed Types

Morphological measurements (Appendix II) were taken of dogs representative of each breed type, i.e. show, sport and crossbred GSDs, in order to quantify a phenotypic trait that differentiated the morphology of typical show dogs and sport dogs. Head length (hl), shoulder height (sh), pelvic height (ph) and body length (bl) were measured as indicated in Figure 3.6. The author took multiple measurements with the use of a single tape measure, in order to ensure that these measurements were consistent between animals.

Principle component analysis (PCA) and analysis of variance (ANOVA) of the morphological data were used to determine whether significant differentiation existed between the GSD breed types.

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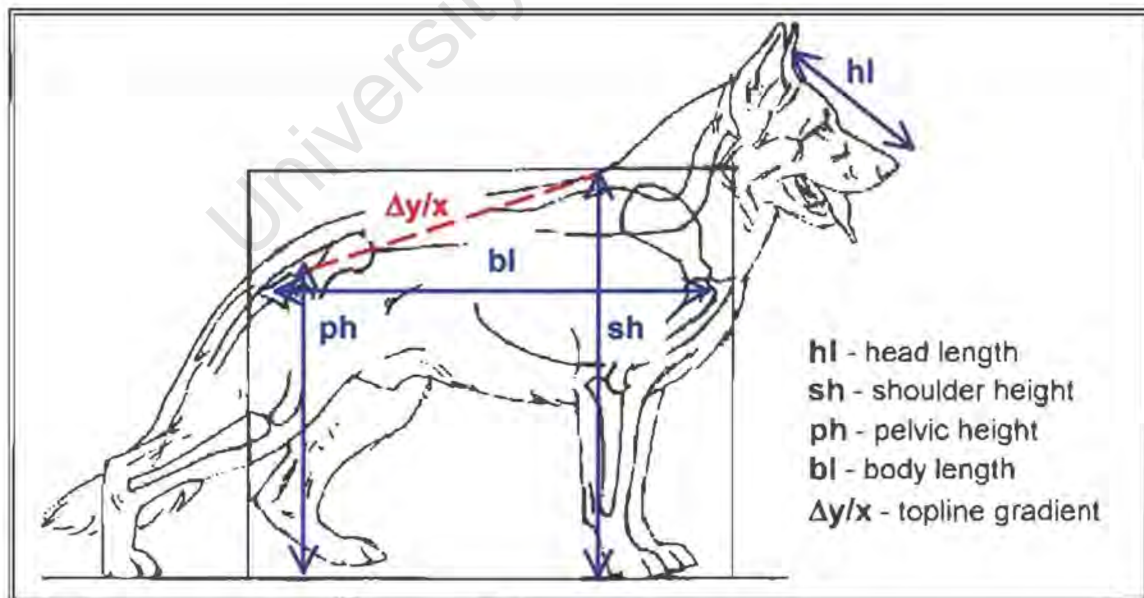

Figure 3.6. A diagrammatic representation of the morphological measurements used to calculate the GSD phenotypic trait quantified as the gradient of the slope of the back (topline).

3.8. Computational Analysis

Oligo Calculator (http://mbcf.dfci.harvard.edu/docs/oligocalc.html), a web-based program hosted by Harvard University, was used to confirm the melting temperatures (T_M), GC-content and various other parameters of the primer pairs (Table 3.1.).

The molecular data from the ABI Prism™ 310 Genetic Analyser was gathered with the 310 Data Collection Software version 3.0.0. DNA fragment analysis was then performed with STRand software version 2.2.195. (Hughes 1998) and fluorescent-labelled PCR products were sized relative to an internal size standard. These data, together with the manually sized [γ^{32}P] dATP-labelled microsatellite data, were transferred to an Excel (Microsoft®) spreadsheet for further analyses.

Microsatellite Toolkit version 3.1. (Park 2001), an Excel (Microsoft®) add-in, was used to calculate allele numbers and frequencies at each locus by direct counting, for detecting identical genotypes in a population, checking data integrity, and converting data to formats compatible with GENEPOP and BOTTLENECK.

AGARst version 3.1. (Harley 2001) was used for calculating allele number and frequency, G_{ST} and R_{ST} values, population assignment tests and "M" ratios for detecting reductions in effective population size (Garza and Williamson 2001) from microsatellite data, as well as converting data to formats compatible with GENEPOP and BOTTLENECK.

GENEPOP version 3.3., an updated version of the population genetic software package presented by Raymond and Rousset (1995b), was used to calculate allele numbers, observed and expected heterozygosities. This program was also used to test for deviations from Hardy-Weinberg (H-W) equilibrium, and to assign F_{IS} and F_{ST} estimates (Weir and Cockerham 1984). Exact p values, along with their standard errors, were calculated using a Markov chain algorithm with 1 000 dememorisation steps for 100 batches and 1 000 iterations per batch. Corrections for multiple significance tests were performed by the application of sequential Bonferroni tests (Rice 1989). This program was also used to convert data to a format compatible with FSTAT.

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The Polymorphism Information Content Calculator (www.agri.huji.ac.il/~weller/Hayim/parent/PIC.htm), a web-based program, was used to determine all PIC values.

FSTAT version 2.9.1. was presented by Goudet in 1995 and was used for the calculation of Weir and Cockerham's (1984) estimators of F statistics. The F_{IT}, F_{ST} and F_{IS} parameters were estimated per allele, locus, and globally over all samples, including tests of significance and confidence intervals based on resampling schemes.

BOTTLENECK version 1.2.02. (Piry *et al.* 1999) was used to detect recent reductions in effective population size by implementing three statistical tests, the "sign test", "Wilcoxon sign-rank test" and "standardised differences test". The "standardised differences test" requires at least 20 polymorphic loci, so only the first two tests were applied. Allele distribution was measured by simulating three mutation models, the "Infinite Allele Model" (IAM), the "Stepwise Mutation Model" (SMM), and the "Two-phased model of mutation" (TPM). TPM, intermediate to the IAM and SMM, was recommended for microsatellite loci with 95% one-step mutations and 30% variance (Luikart *et al.* 1998b). A qualitative description of the distribution of allele frequencies, the "modal-shift" indicator, separated bottlenecked populations from stable populations (Luikart *et al.* 1998a).

StatSoft, Inc. (2003) STATISTICA (data analysis software system), version 6.1. (www.statstoft.com) was used to identify significant variation in the morphological data. The data were subjected to both multivariate (ANOVA) and univariate (ANOVA) analyses for comparative purposes.

The critical significance level applied to all statistical analyses was 0.05.

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Chapter 4

Results

4.1. The Outbred Dog according to region of origin

A total of 156 outbred dogs (OBDs), 46 from Cape Town, 42 from Port Elizabeth, 30 from Johannesburg and 38 from Pretoria (Appendix I), were analysed at 15 polymorphic canine microsatellite markers. Of these microsatellite loci, four were labelled with radioactive [γ^{32}P] dATP (Figure 4.1.) and 11 with fluorescence (Figure 4.2.). This data revealed the heterozygous nature of the locus and allele sizes for each individual in the population.

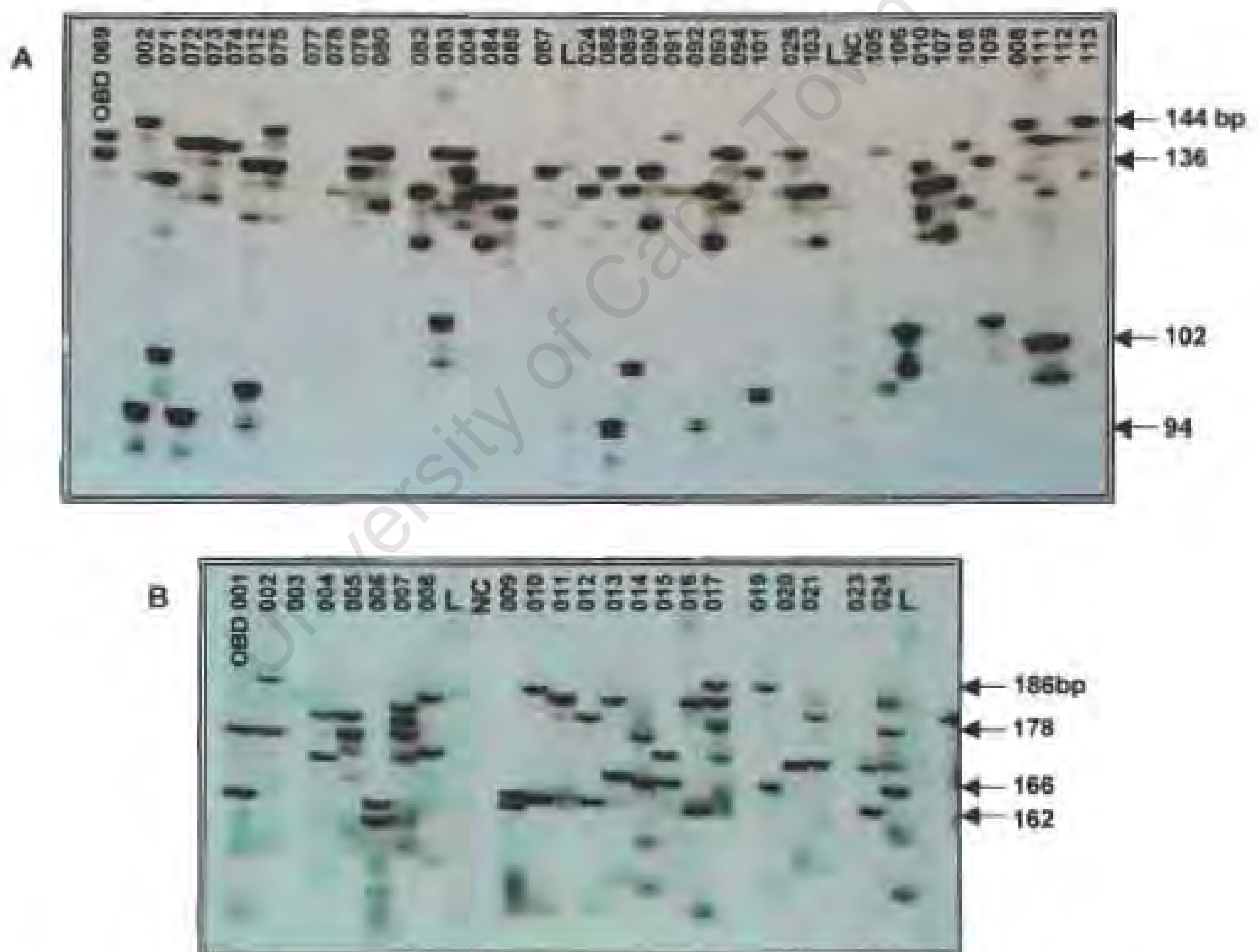

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Figure 4.1. Polyacrylamide gel electropherograms of some representative OBDs at radioactive [γ^{32}P] dATP-labelled microsatellite loci, DTRCN1 (A) and FH2137 (B), visualised autoradiographically. Alleles were sized vertically by comparison with a standard A-T ladder (L), and include a negative control (NC).

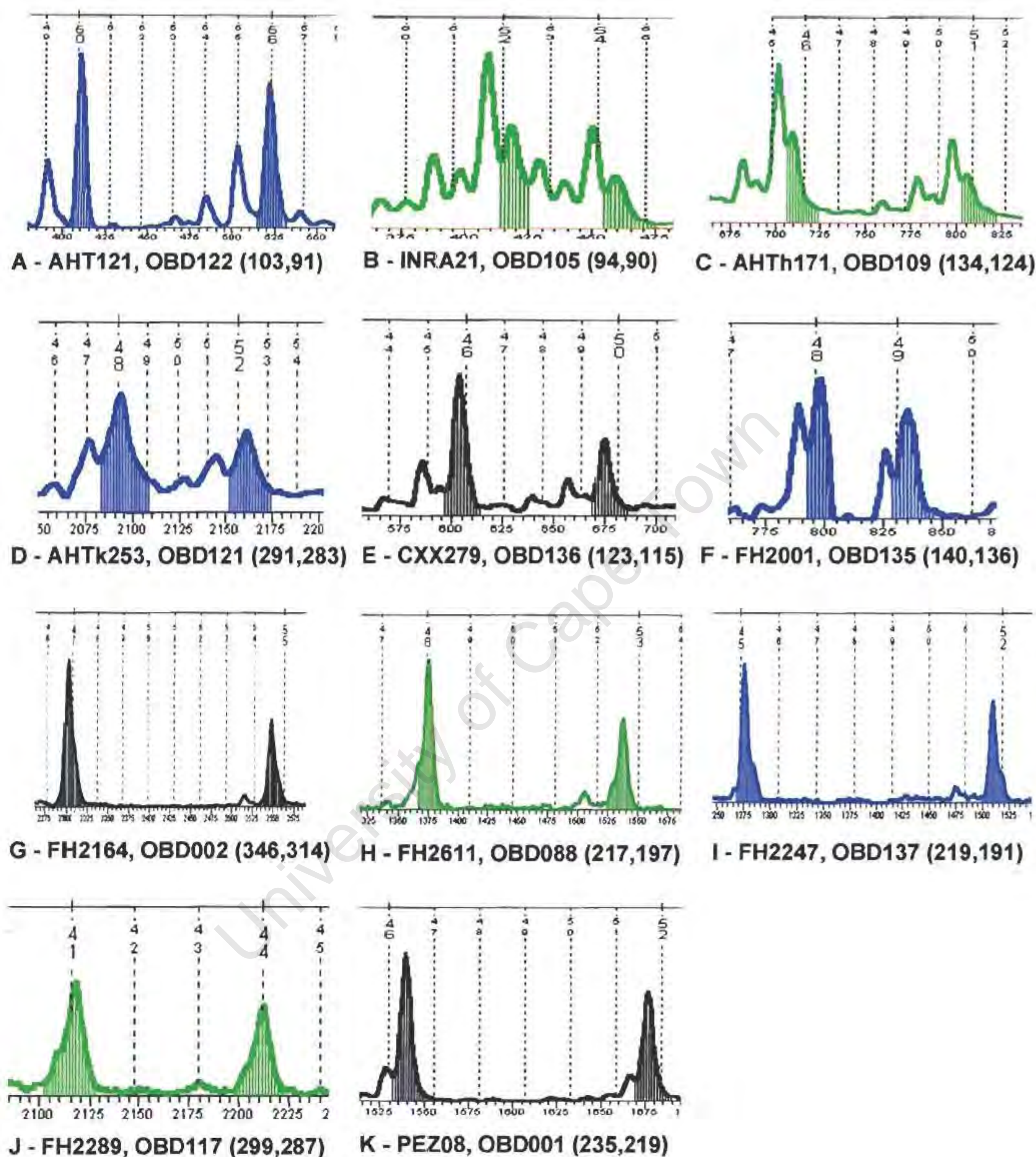

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Figure 4.2. Electropherograms representing fluorescent-labelled microsatellite alleles (shaded peaks) in some representative OBDs, sized from left to right according to an internal size standard (STRand). Microsatellite marker name, individual sample number, and allele sizes (bp) are indicated.

The genetic diversity or polymorphism expressed by a population can be estimated by measuring allele distribution frequencies across a number of loci (Appendix II). An example of one such locus (Figure 4.3.) graphically illustrates the comparable allele frequencies of representative samples selected from Cape Town, Port Elizabeth, Johannesburg and Pretoria, as characteristic of the general South African mongrel population. This data indicated extensive genetic diversity and non-significant differentiation between the regional subpopulations.

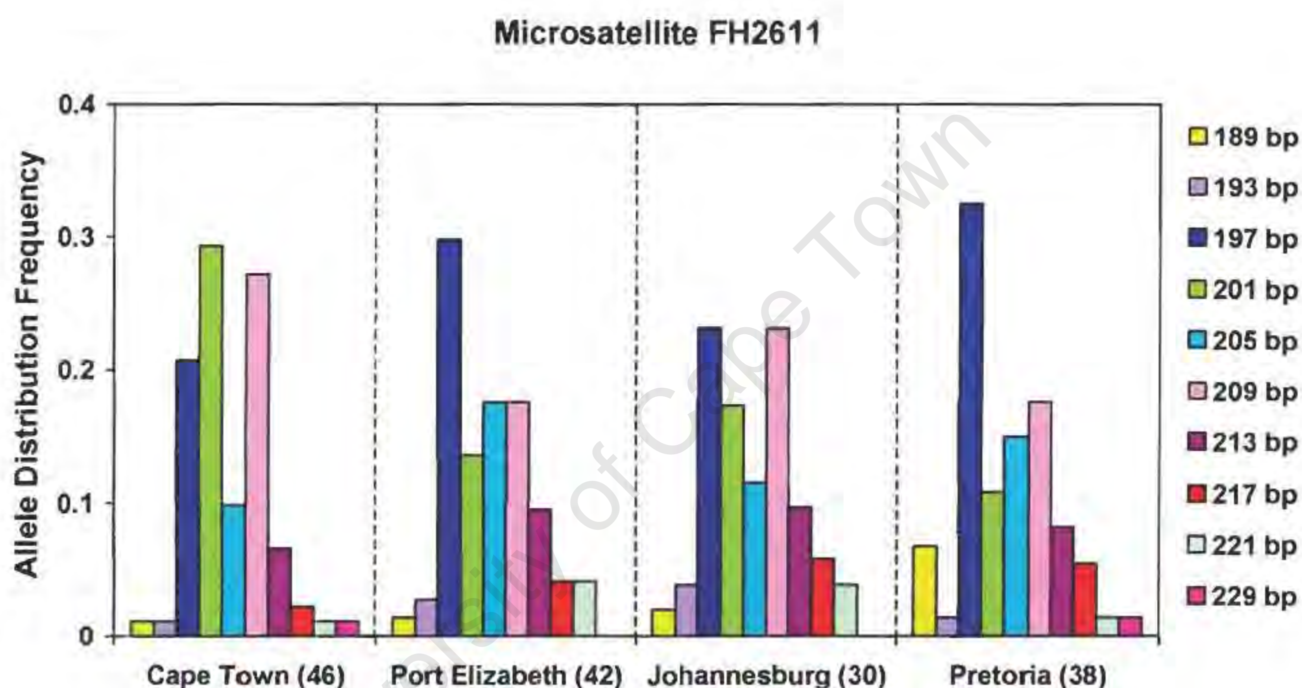

Figure 4.3. A graphic representation of the allele distribution frequencies of each regional OBD subpopulation at the FH2611 microsatellite locus. Sample sizes are indicated in parentheses.

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Table 4.4. represents the average genetic diversity of the OBD subpopulations as compared with the combined population across all 15 microsatellite loci. These data include the number of individuals in each population, the total number of alleles, the mean number of alleles per locus corrected for differing sample size with 1 000 pseudoreplications by both the bootstrap and jacknife methods, the observed and expected heterozygosity values, and mean PIC values (AGARst, PIC Calculator).

The corrected number of alleles per locus (between 8.66 and 9.67), expected heterozygosities (between 81.8% and 82.5%), and PIC values (between 0.796 and 0.802), effectively illustrated the comparable and extensive levels of genetic diversity expressed by the OBD subpopulations. Although, evidence of homozygous excess was suggested by the smaller observed heterozygosity values for each subpopulation. The extent of the genetic diversity of the combined OBD population was such that no identical genotypes were detected (Microsatellite Toolkit).

Population	Sample Size	Allele Number	Alleles / Locus (Bootstrap)	Alleles / Locus (Jacknife)	Heterozygosity		PIC
					Obs	Exp	
Cape Town	46	157	8.95	9.50	0.776	0.822	0.800
Port Elizabeth	42	155	9.06	9.80	0.752	0.818	0.796
Johannesburg	30	145	8.66	9.67	0.720	0.819	0.797
Pretoria	38	159	8.96	9.80	0.730	0.825	0.802
OBD	156	185	9.59	9.79	0.748	0.831	0.811

Table 4.4. The genetic diversity expressed by each OBD subpopulation and the combined population, as illustrated by comparative allele counts, degrees of heterozygosity, and PIC values (AGARst, PIC Calculator).

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Bottleneck events result in the rapid loss of genetic diversity in the population, especially when post-bottleneck recovery is gradual. The effects of random genetic drift intensify as effective population size decreases, resulting in altered allele frequencies and loss of alleles. A recently bottlenecked population would display excess heterozygosity (H_E) relative to a population at equilibrium (H_{EQ}). Microsatellite allele frequency data of each OBD subpopulation (Table 4.5.) was tested for evidence of such heterozygosity excess using the program BOTTLENECK (Piry et al. 1999). The results of both the "sign test" and "Wilcoxon sign-rank test" under TPM are indicated, with the expected number of alleles exhibiting excess H_E over H_{EQ} and p-value for the former, and the probability of either significant excess (H_{EXC}) or deficit (H_{DEF}) of H_E over H_{EQ} for the latter.

The OBD subpopulations and the combined population did not exhibit statistically significant excess H_E over H_{EQ}, the populations appear to be in mutation-drift equilibrium in both statistical tests ($P > 0.05$). There is also no indication of a modal shift in allele frequencies, with all populations having normal L-shaped allele distributions. There was no evidence of recent reductions in effective population size in these populations (BOTTLENECK).

Garza and Williamson's "M" is the mean ratio of the total number of alleles observed in a population (k) to the range in allele size (r), where $M = k/r$, and post-bottleneck populations would indicate a reduction in the number of alleles relative to the total allele size range (Garza and Williamson 2001). A population with $M < 0.680$ can be assumed to have experienced a recent bottleneck event (Garza and Williamson 2001). All M ratios calculated for the OBD subpopulations and the combined population (Table 4.6.) exceeded this critical value, indicating no evidence of bottleneck events in the recent histories of these subpopulations (calculated using AGARst).

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Population	"Sign Test" TPM		"Wilcoxon Test" TPM		Modal Shift
	$H_E > H_{EQ}$	p-value	P of H_{EXC}	P of H_{DEF}	
Cape Town	8.9	0.578	0.467	0.555	L-shaped
Port Elizabeth	8.8	0.570	0.381	0.640	L-shaped
Johannesburg	8.8	0.191	0.104	0.906	L-shaped
Pretoria	8.9	0.236	0.511	0.511	L-shaped
OBD	8.7	0.447	0.555	0.467	L-shaped

Table 4.5. A summary of the results of the "sign test" and " Wilcoxon sign-rank test" that detect bottlenecks in the history of each OBD subpopulation (BOTTLENECK).

Population	Garza & Williamson's "M"	Variance	Monomorphic Loci
Cape Town	0.904	0.024	0
Port Elizabeth	0.878	0.026	0
Johannesburg	0.849	0.027	0
Pretoria	0.787	0.065	0
OBD	0.832	0.057	0

Table 4.6. Garza & Williamson's "M" ratios for the detection of bottleneck events in each OBD subpopulation, the variance and number of monomorphic loci are also indicated (calculated using AGARst).

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F-statistic and Rho-statistic estimates were used to measure genetic differentiation within and between populations. The homozygote-heterozygote proportions of the Cape Town, Port Elizabeth, Johannesburg, and Pretoria OBD subpopulations were F_{IS} = 0.068, 0.095, 0.136, and 0.128, respectively (FSTAT). There was an average global heterozygote deficit of 10.3% across all 15 microsatellite loci for each OBD subpopulation (F_{IS} = 0.103), with a heterozygote deficit of 10.4% (F_{IT} = 0.104) in the combined population. There was non-significant average differentiation between these subpopulations (G_{ST} = 0.002, R_{ST} = 0.003; FSTAT, GENEPOP).

Table 4.7. represents the pairwise comparisons of G_{ST} and R_{ST} values between the OBD subpopulations (GENEPOP). These data consistently indicated non-significant differentiation, indicating that sufficient gene flow has occurred across the country during past generations to result in a homogenous OBD population.

Population	Cape Town	Port Elizabeth	Johannesburg	Pretoria
Cape Town	-	0.002	0.002	0.002
Port Elizabeth	0.009	-	0.003	0.001
Johannesburg	0.001	0.010	-	0.001
Pretoria	0.002	0.004	0.001	-

Table 4.7. The mean pairwise G_{ST} and R_{ST} estimates between OBD subpopulations across all 15 micosatellite loci (G_{ST} values above the diagonal and R_{ST} below; GENEPOP).

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The number of loci in H-W equilibrium was another method of detecting levels of population differentiation. Table 4.8. indicates that the deviations across all 15 loci remained highly significant ($p < 0.001$) and thus H-W disequilibrium was statistically supported for all OBD subpopulations (GENEPOP).

Population	χ^2	Df	p-value	H-W Equilibrium
Cape Town	49.8	30	0.013	disequilibrium
Port Elizabeth	66.3	30	<0.001	disequilibrium
Johannesburg	84.6	30	<0.001	disequilibrium
Pretoria	85.8	30	<0.001	disequilibrium
OBD	infinity	30	<0.001	disequilibrium

Table 4.8. The results of probability tests verifying whether populations deviate significantly from Hardy-Weinberg equilibrium, indicating χ^2, degrees of freedom (Df) and p-values (GENEPOP).

The proportion of individuals in a population correctly assigned to its own source population was another useful measure of population differentiation (Table 4.9.). Only between 80% and 92% of the OBDs were correctly assigned to each subpopulation, indicating minimal genetic differentiation between the regional OBD subpopulations.

Population	Correctly Assigned	Median Value Likelihood Ratios	Range of Values Likelihood Ratios
Cape Town	80%	2.60×10^{1}	1.22×10^{0} to 7.30×10^{3}
Port Elizabeth	83%	1.58×10^{1}	1.11×10^{0} to 3.25×10^{3}
Johannesburg	87%	6.14×10^{1}	1.16×10^{0} to 1.26×10^{5}
Pretoria	92%	2.41×10^{1}	1.83×10^{0} to 1.48×10^{3}

Table 4.9. The results of assignment tests indicated the percentage and likelihood ratios of individuals correctly assigned to their own source OBD subpopulation (AGARst).

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Figure 4.10. (A to D) is a graphic representation of the pairwise plots of the negative log likelihood of individuals being assigned to either their own or another of the regional OBD subpopulations (AGARst). The diagonal represents the probability that an individual is equally likely to be assigned to its own or another population. With each comparison between subpopulations, most individuals were clustered across this diagonal, further indication of minimal differentiation between the regional OBD subpopulations.

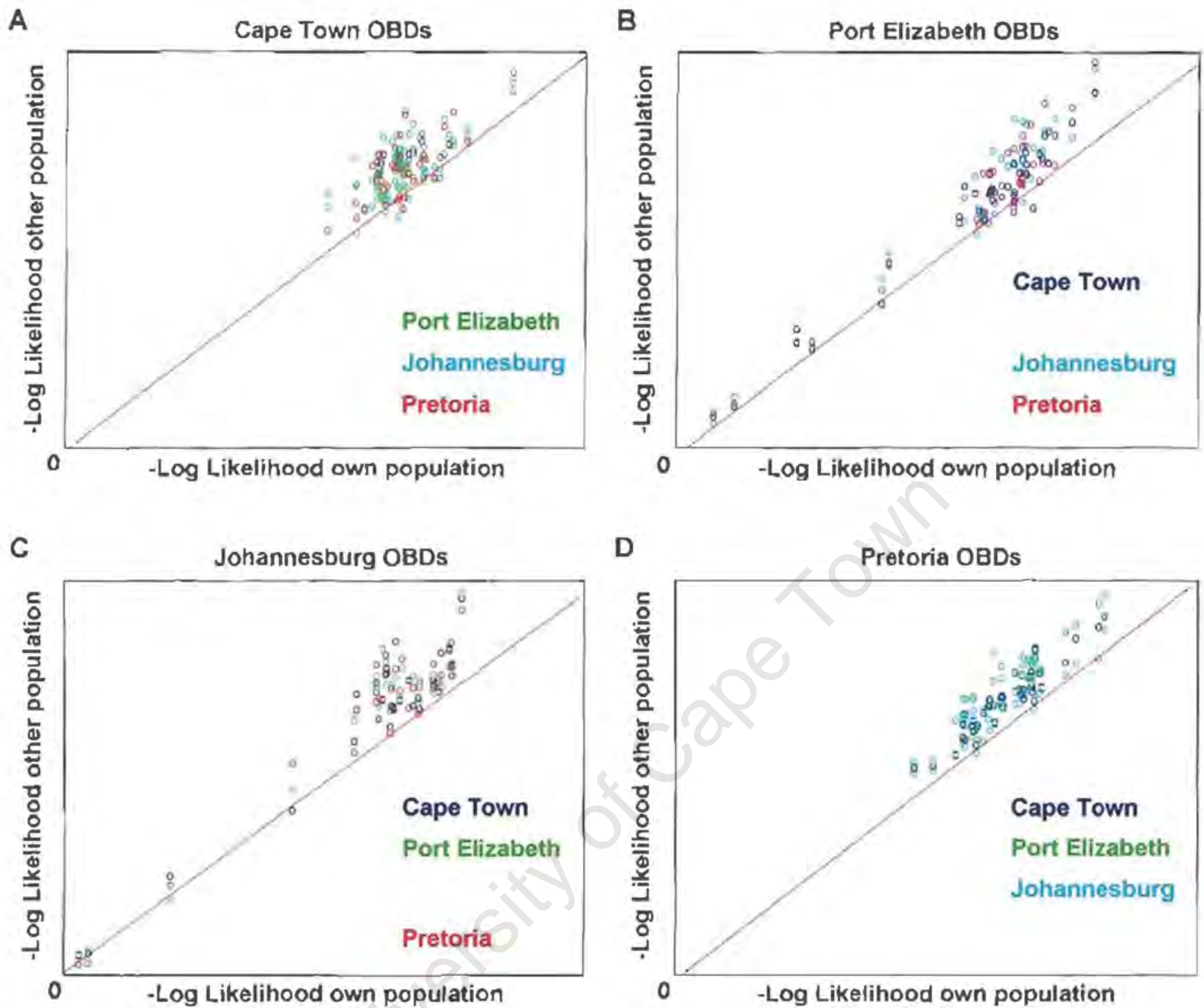

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Figure 4.10. The pairwise plots of the negative log likelihood of each regional OBD subpopulation (A to D) being assigned to its own source population as compared with the other OBD subpopulations (AGARst).

4.2. The German Shepherd Dog

A total of 101 German Shepherd Dogs (GSDs), 56 South African show dogs, eight South African KUSA-bred show dogs, nine South African crossbred show and sport dogs, ten German show dogs, and 18 German sport dogs, were analysed at 15 polymorphic canine microsatellite markers (Appendix I). Four of the microsatellite loci were labelled with radioactive [γ^{32}P] dATP (Figure 4.11.) and 11 with fluorescence (Figure 4.12.).

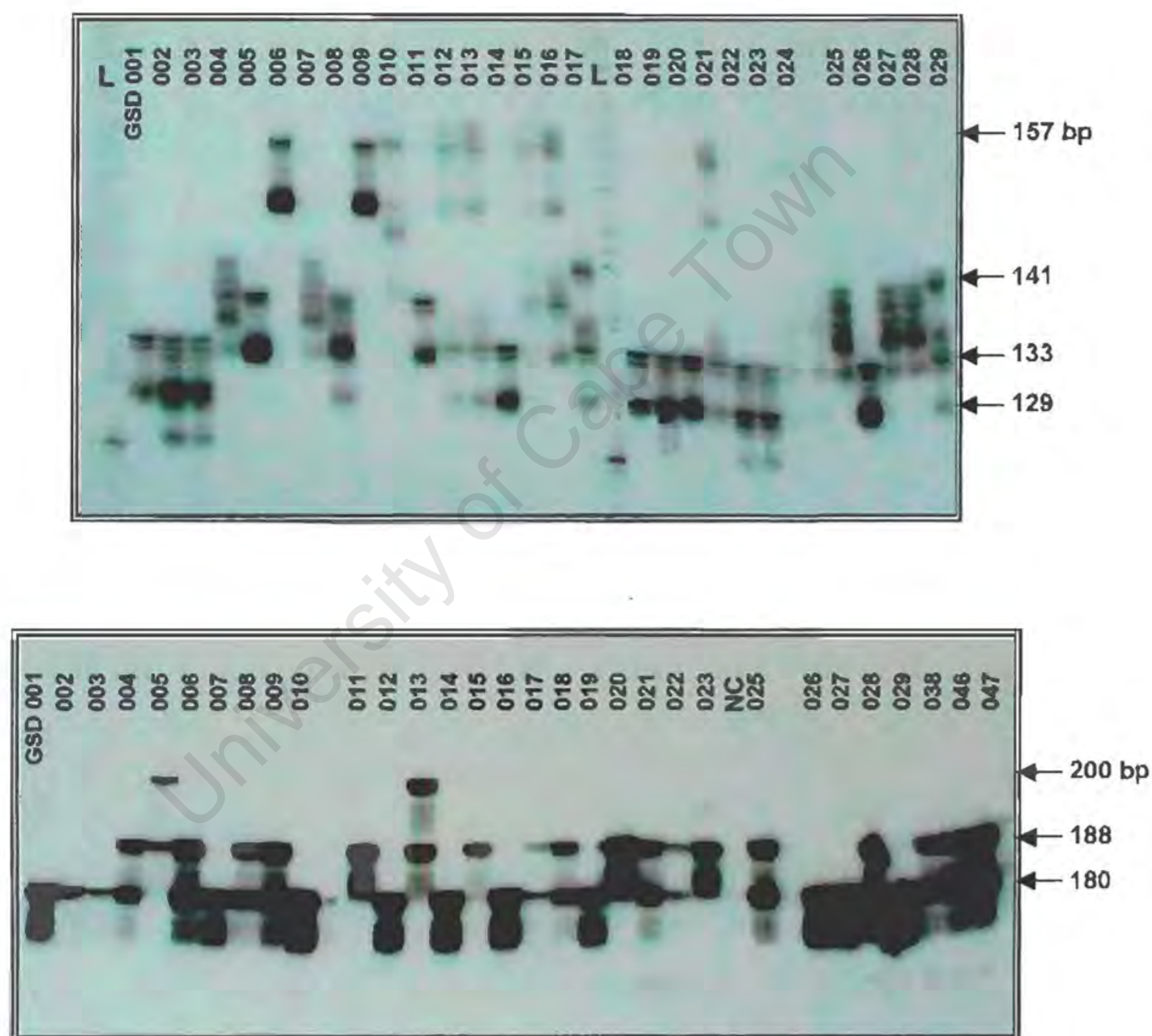

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Figure 4.11. Polyacrylamide gel electropherograms of some representative GSDs at radioactive [γ^{32}P] dATP-labelled microsatellite loci, FH2140 (A) and FH2328 (B), visualised autoradiographically. Alleles were sized vertically by comparison with a standard A-T ladder (L), and included a negative control (NC).

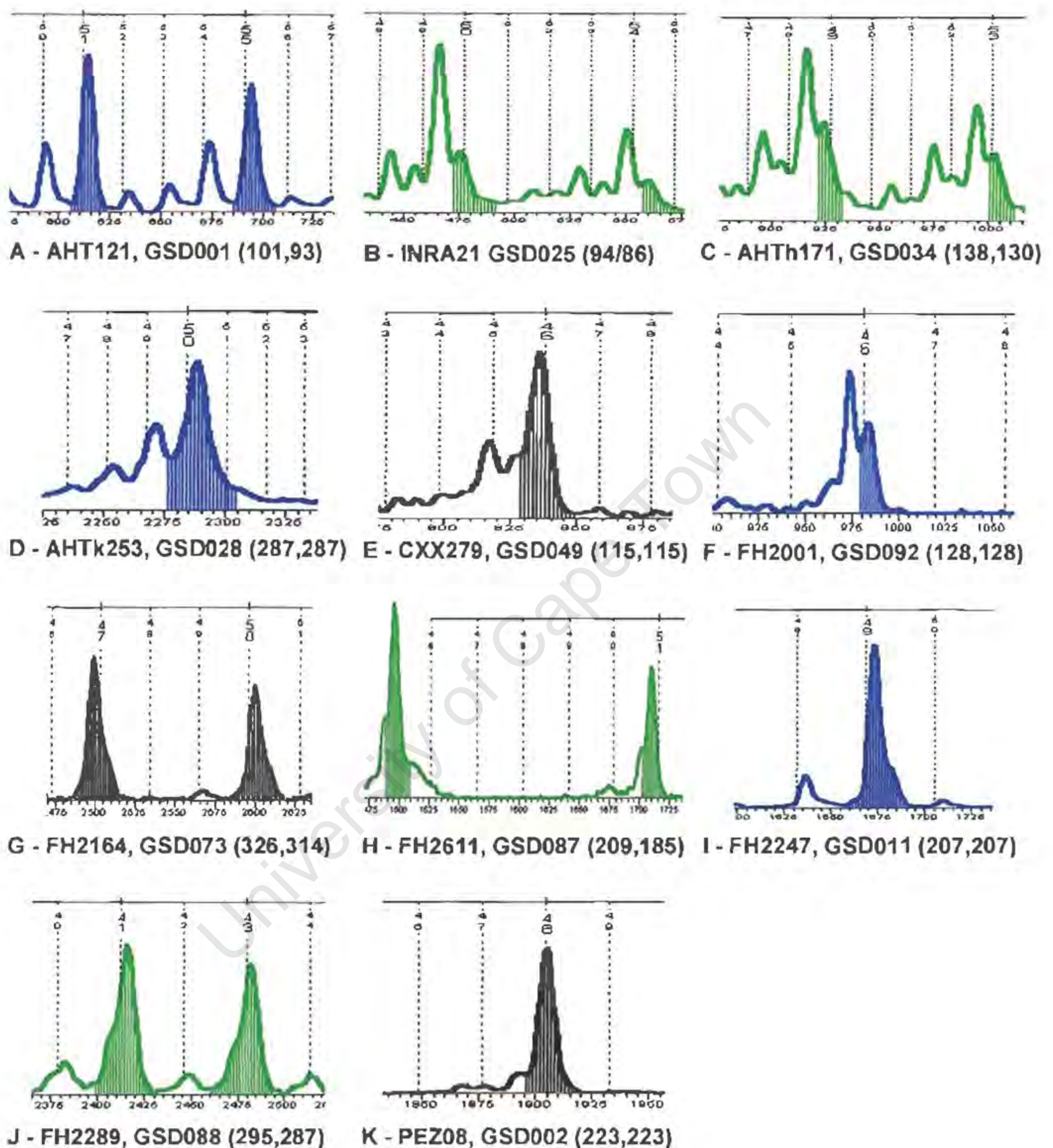

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Figure 4.12. Electropherograms representing fluorescent-labelled microsatellite alleles (shaded peaks) in some representative GSDs, sized from left to right according to an internal size standard (STRand). Microsatellite marker name, individual sample number, and allele sizes (bp) are indicated.

4.2.1. According to country of origin and breed type

Pedigree analysis revealed that it was impossible to classify South African bred GSDs according to region of origin due to the breeding strategy commonly employed. Breeders regularly transport dogs across the country for matings, usually maintaining their own bitch-lines and using imported stud dogs. Dogs with at least one locally bred parent were designated as part of the South African subpopulation and all the imported dogs and those with imported parents as part of the German subpopulation.

However, pedigree analysis permitted the unambiguous classification of breed lines for distinct types, namely show-lines, KUSA-bred show-lines, sport-lines and crossbred show and sport dogs. The show-lines included both locally bred and imported dogs, all the KUSA show dogs have been locally bred for several generations, and all the sport dogs were imported from Germany.

The genetic diversity or polymorphism expressed by a population can be estimated by measuring allele distribution frequencies across a number of loci (Appendix II). Examples of two such loci (Figure 4.13.) graphically illustrated the differing allele distribution frequencies, with lower allele numbers indicating loss of diversity in the GSD subpopulations and significant differentiation between these and the OBD population. At the AHT121 microsatellite locus, the same allele (101bp) was most common in all populations, but with a very high frequency in the GSD subpopulations, and the German sport dog subpopulation expressed a private allele (79bp allele found otherwise only in the CB group). At the AHTh171 microsatellite locus, a single common allele (138bp) was expressed by all GSD subpopulations and interestingly, the most common allele (130bp) in the crossbred show and sport dogs was fairly infrequent in both the show and sport subpopulations.

Table 4.14. represents the average relative genetic diversity of the GSD subpopulations as compared with the OBD population across all 15 microsatellite loci. These data include the population sample size, total number of alleles, mean number of alleles per locus corrected for differing sample size by 1 000 pseudoreplications with both the bootstrap and jacknife methods, the observed and expected heterozygosity values, and mean PIC values (AGARst, PIC Calculator).

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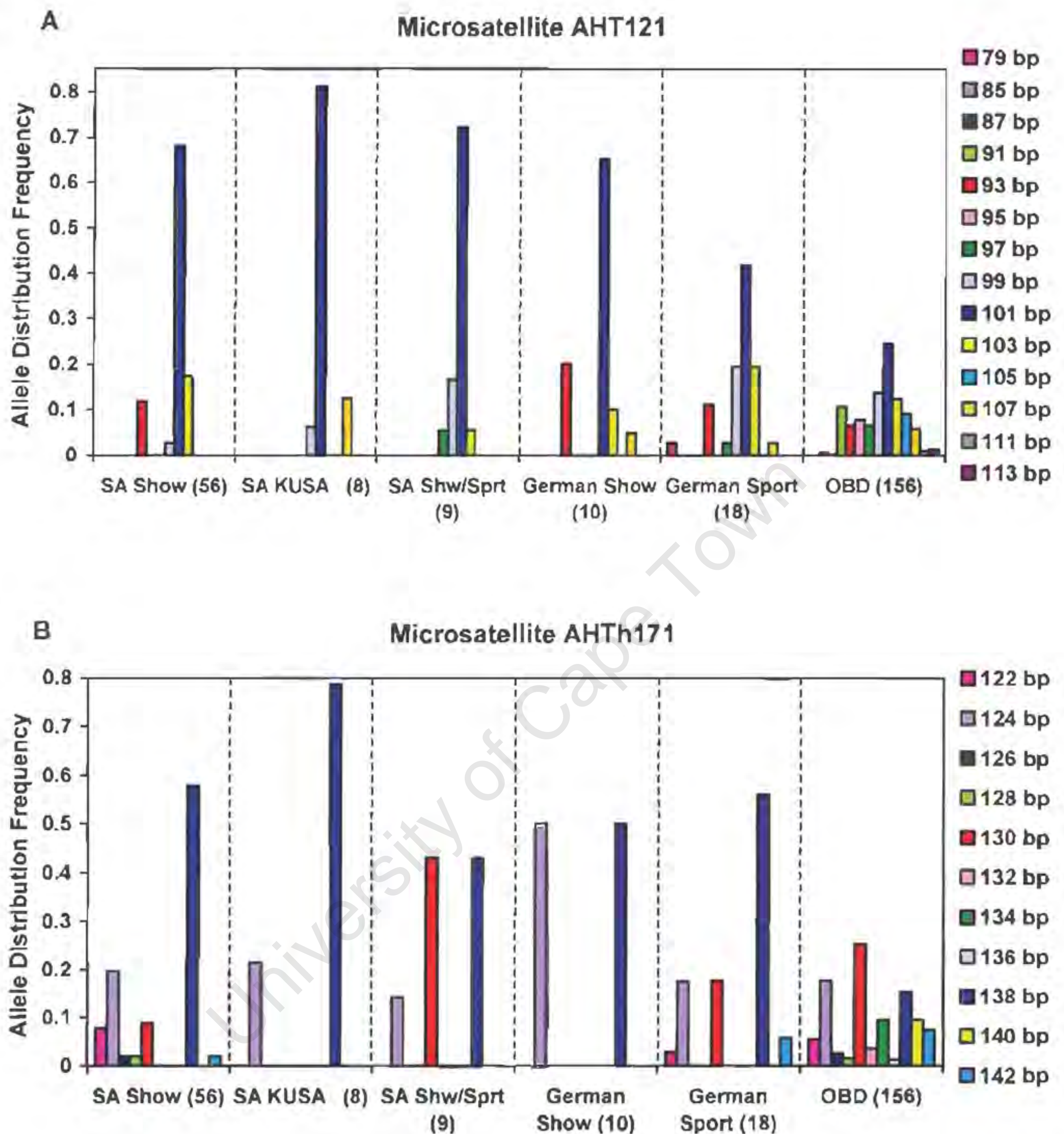

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Figure 4.13. Graphic representations of the allele distribution frequencies for the South African show, KUSA-bred show and crossbred subpopulations, and the German show and sport subpopulations, as compared with the OBD population at the AHT121 (A) and AHTh171 (B) microsatellite loci. Sample sizes are indicated in parentheses.

The relative loss of genetic diversity in the GSD population was effectively illustrated by the corrected number of alleles per locus, with the subpopulations expressing approximately half (between 3.45 and 3.72) that of the OBD population (6.71 and 6.80). The average observed and expected heterozygosities for the combined GSD population (57.5% and 57.1%) were approximately three quarters that of the OBD population (74.8% and 83.1%). However, the genetic diversity in the GSD population was sufficiently extensive that no identical genotypes were detected (Microsatellite Toolkit).

The corrected number of alleles per locus effectively illustrated the loss of genetic diversity in all GSD subpopulations relative to the OBD population. However, the sport dogs consistently expressed the highest corrected number of alleles per locus, observed and expected heterozygosities, and PIC values in comparison with the other subpopulations. The South African show dogs were more genetically diverse than the imported German show dogs, in terms of corrected numbers of alleles, heterozygosities, and PIC values. The crossbred dogs, despite their hybrid status, and the KUSA-bred show dogs expressed the least genetic diversity.

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Population	Sample Size	Allele Number	Alleles / Locus (Bootstrap)	Alleles / Locus (Jacknife)	Heterozygosity		PIC
					Obs	Exp	
SA Show	56	92	3.86	3.93	0.589	0.610	0.569
KUSA Show	8	52	3.08	3.47	0.597	0.536	0.474
SA Shw/Sprt	9	54	3.23	3.53	0.549	0.557	0.502
German Show	10	53	3.11	3.38	0.548	0.537	0.481
German Sport	18	80	3.98	4.28	0.621	0.615	0.575
OBD	156	185	6.71	6.80	0.748	0.831	0.811

Table 4.14. The genetic diversity expressed by the South African show, KUSA-bred show and crossbred dogs and German show and sport subpopulations, relative to the OBD population, as illustrated by comparative allele counts, degrees of heterozygosity, and PIC values (AGARst, PIC Calculator).

Table 4.15. summarises the results of both the "sign test" and Wilcoxon sign-rank test" (BOTTLENECK). The South African show and KUSA-bred show subpopulations did not exhibit statistically significant excess H_E over H_{EQ}, and had normal L-shaped allele distributions. The crossbred and German show subpopulations indicated a modal-shift in allele frequencies. The German sport subpopulation, while having 9.0 alleles ($p = 0.010$) exhibiting statistically significant excess H_E over H_{EQ}, still had a normal L-shaped allele distribution.

Table 4.16. summarises the Garza and Williamson's "M" values (calculated using AGARst). The ratios calculated for the South African show and crossbred GSD subpopulations exceeded the critical value, indicating no detectable recent bottleneck event. The KUSA-bred show and both imported German subpopulations had ratios less than 0.680, indicating recent reductions in effective population size.

Except for the South African show dog subpopulation, there was evidence of recent bottleneck events in the histories of all the other GSD subpopulations.

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Population	**"Sign Test" TPM**		**"Wilcoxon Test" TPM**		**Modal Shift**
	$H_E > H_{EQ}$	**p-value**	**P of H_{EXC}**	**P of H_{DEF}**	
SA Show	8.9	0.109	0.924	0.084	L-shaped
KUSA Show	8.8	0.560	0.230	0.719	L-shaped
SA Shw/Sprt	8.2	0.560	0.179	0.837	Shifted
German Show	8.8	0.245	0.598	0.423	Shifted
German Sport	9.0	0.010	0.976	0.028	L-shaped

Table 4.15. A summary of the results of the "sign test" and " Wilcoxon sign-rank test" that detect bottleneck events in the recent histories of the South African show, KUSA-bred show and crossbred dogs and the German show and sport subpopulations (BOTTLENECK).

Population	Garza & Williamson's "M"	Variance	Monomorphic Loci
SA Show	0.774	0.046	0
KUSA Show	0.630	0.056	0
SA Shw/Sprt	0.736	0.052	1
German Show	0.629	0.063	0
German Sport	0.657	0.063	0

Table 4.16. Garza & Williamson's "M" ratios for the detection of bottleneck events in the South African show, KUSA-bred show and crossbred dogs and the German show and sport subpopulations, the variance and number of monomorphic loci are also indicated (calculated using AGARst).

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The homozygote-heterozygote proportions of the combined GSD population and OBD population were F_{IS} = 0.054 and 0.104, respectively (FSTAT). There was an average global heterozygote deficit of 8.7% (F_{IS} = 0.087) across all 15 microsatellite loci for each population, with a heterozygote deficit of 18.1% (F_{IT} = 0.181) in the combined population. There was significant average differentiation between the two populations (G_{ST} = 0.103, R_{ST} = 0.058; FSTAT, GENEPOP).

The homozygote-heterozygote proportions of the South African show, KUSA-bred show, crossbred, and German show and sport GSD subpopulations were F_{IS} = 0.046, -0.040, 0.081, 0.046, and 0.020, respectively (FSTAT). There was an average global heterozygote deficit of 3.8% (F_{IS} = 0.038) across all 15 microsatellite loci for each subpopulation, and the heterozygote deficit in the combined population was 6.3% (F_{IT} = 0.063). There was non-significant differentiation between the GSD subpopulations (G_{ST} = 0.026, R_{ST} = 0.026; FSTAT, GENEPOP).

Table 4.17. represents the pairwise comparisons of G_{ST} and R_{ST} values between the GSD subpopulations and OBD population (GENEPOP). These data indicated varying levels of differentiation between the GSD subpopulations and significant differentiation between each GSD subpopulation and the OBD population. There was relatively significant differentiation between the isolated KUSA-bred show dogs and all other GSD subpopulations, with mean G_{ST} and R_{ST} values of 0.046 and 0.063. There was non-significant differentiation between the South African and German show subpopulations, with G_{ST} and R_{ST} values of 0.007 and 0.021, and relatively significant differentiation between the German show and sport subpopulations, with G_{ST} and R_{ST} values of 0.054 and 0.069.

Population	SA Show	KUSA Show	SA Shw/Sprt	German Show	German Sport	OBD
SA Show	-	0.024	0.021	0.007	0.025	0.104
KUSA Show	0.028	-	0.037	0.065	0.059	0.109
SA Shw/Sprt	0.002	0.083	-	0.040	0.025	0.098
German Show	0.021	0.137	0.002	-	0.054	0.110
German Sport	0.023	0.002	0.027	0.069	-	0.080
OBD	0.053	0.032	0.048	0.083	0.042	-

Table 4.17. The mean pairwise G_{ST} and R_{ST} estimates between GSD subpopulations and the OBD population across all 15 micosatellite loci (G_{ST} values above the diagonal and R_{ST} below; GENEPOP).

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Table 4.18. indicates that the deviations from H-W equilibrium across all 15 loci remained highly significant for the South African show subpopulation ($p < 0.001$) and that H-W disequilibrium was statistically supported. However, the deviations were not significant for the remaining GSD subpopulations and these were in equilibrium (BOTTLENECK).

Population	χ^2	Df	p-value	H-W Equilibrium
SA Show	infinity	30	<0.001	disequilibrium
KUSA Show	20.1	30	0.914	equilibrium
SA Shw/Sprt	24.3	28	0.668	equilibrium
German Show	38.1	30	0.146	equilibrium
German Sport	34.1	30	0.279	equilibrium

Table 4.18. Probability tests verified whether the South African show, KUSA-bred show and crossbred dogs and the German show and sport subpopulations deviated significantly from Hardy-Weinberg equilibrium; χ^2, degrees of freedom (Df) and p-values are indicated (GENEPOP).

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Assignment tests correctly grouped 100% of the individuals in the combined GSD population (mean likelihood ratio of 8.89×10^7), whereas only 96% of the OBDs (mean likelihood ratio of 8.14×10^8) were correctly assigned (AGARst). This indicated that gene flow was unidirectional, only from the purebred dog population to the mongrel population because of the "breed barrier" rule; a proportion of OBD individuals would have GSD ancestry.

Analysis of each GSD subpopulation correctly assigned between 70% and 94% of individuals to their own source subpopulation, indicating non-significant to moderate levels of differentiation (Table 4.19.). The imported German sport subpopulation was most differentiated from the remaining show dog subpopulations (AGARst).

Population	Correctly Assigned	Median Value Likelihood Ratios	Range of Values Likelihood Ratios
SA Show	70%	1.16×10^1	1.17×10^0 to 3.25×10^3
KUSA Show	88%	3.09×10^4	1.63×10^1 to 6.51×10^5
SA Shw/Sprt	89%	4.33×10^4	1.2×10^1 to 2.36×10^5
German Show	90%	1.26×10^2	1.33×10^1 to 2.72×10^4
German Sport	94%	6.66×10^2	2.17×10^0 to 3.38×10^5

Table 4.19. The results of assignment tests indicating the percentage and likelihood ratios of individuals correctly assigned to the South African show, KUSA-bred show, and crossbred subpopulations, and the German show and sport subpopulations (AGARst).

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The pairwise plots of the negative log likelihood of individuals in a subpopulation being assigned to its own source population were graphic representations of the assignment test results (AGARst). The diagonal represents the probability that an individual was equally likely to be assigned to its own or to the other population. These results indicated significant differentiation between the GSD and OBD populations (Figure 4.20. A and B), a number of OBDs had a greater likelihood of being assigned to the GSD population, suggesting that these dogs had GSD ancestry. In addition, minimal differentiation between the South African and German-bred show dogs (Figure 4.20. C and D), and moderate differentiation between the show and sport dog subpopulations (Figure 4.20. E and F), was suggested by this data.

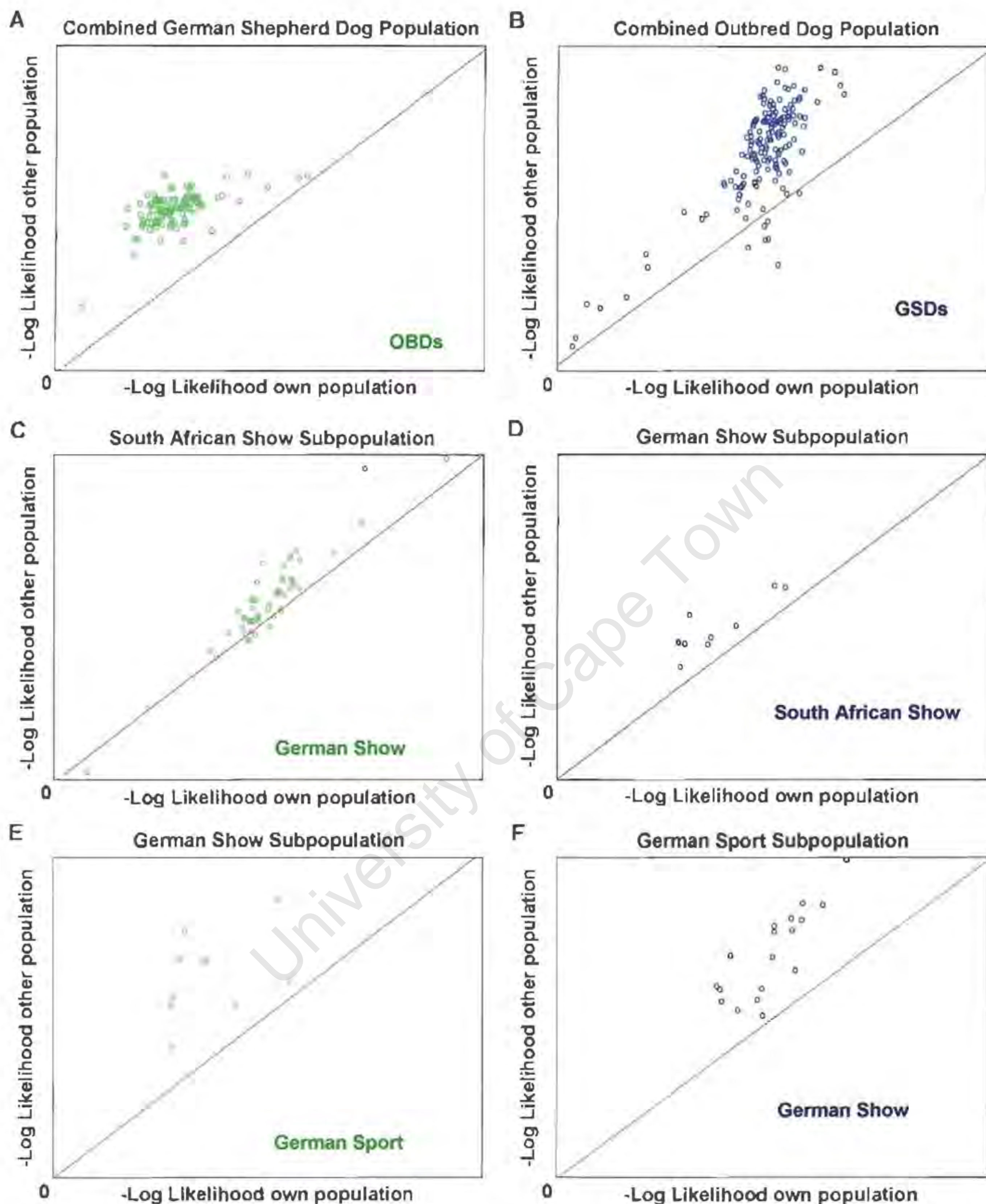

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Figure 4.20. The pairwise plots of the negative log likelihood of the GSD population (A and B) being assigned to its own source population as compared with the OBD population, the South African show subpopulation (C and D) compared with German show dogs, and the German show subpopulation (E and F) compared with German sport dogs (AGARst).

Morphological measurements (Appendix II) were taken of dogs representative of each breed type in order to quantify a phenotypic trait that differentiated the morphology of typical show dogs and sport dogs. These morphological measurements were averaged across each breed type, and the gradient ($\Delta y/x$) of the slope of the back, the ratio of body length to height and the ratio of head to body length were calculated (Table 4.21.). The GSD Breed Standard states that the height at the wither should be between 60 and 65cm, the body should be slightly long in comparison to the height, approximately 110 to 117% of the height, and the head length should be approximately 40% of the height (Willis 1977).

Population	Sample Size	Head length	Shoulder height	Pelvic height	Body length	Length: height	Head: body	Topline gradient
Show	30	27.8	64.1	43.7	74.0	115.6	37.5	0.28
Sport	20	27.1	62.8	53.4	72.9	116.2	37.2	0.13
Show/Sport	7	28.0	65.9	51.4	71.8	109.2	39.0	0.20

Table 4.21. A summary of the average morphological measurements (cm) and ratios (%) calculated for show, sport and crossbred GSDs.

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PCA is a method of ordination that extracts factors from correlations or covariances between variates in order to discern inherent patterns, and involves the eigenanalysis of a symmetric matrix of similarities to calculate eigenvalues and corresponding eigenvectors (Fry 1993). The first two PCA factors (Figure 4.22.) accounted for 86.37% of the variance in the data (eigenvalues: F1 = 2.476, F2 = 0.979, F3 = 0.369, and F4 = 0.176). Pelvic height had a high factor score on the F2 axis; the other three variables had low factor scores, therefore pelvic height was responsible for most of the variation in this data. The graphical plot of the eigenvectors of the first two factors demonstrated the relationship between them, with mean component scores for each breed type separating primarily along the y-axis (Figure 4.23.). The show and sport dogs were most separate, with the crossbred dogs situated between the other two groups.

ANOVA confirmed the existence of different morphological breed types. Univariate tests of significance produced probability values for pelvic height ($p < 0.001$), shoulder height ($p = 0.212$), and body length ($p = 0.342$). Shoulder height and body length were similar in all breed types, but significant differences in pelvic height result in variation of the gradient of the topline in the GSD breed types.

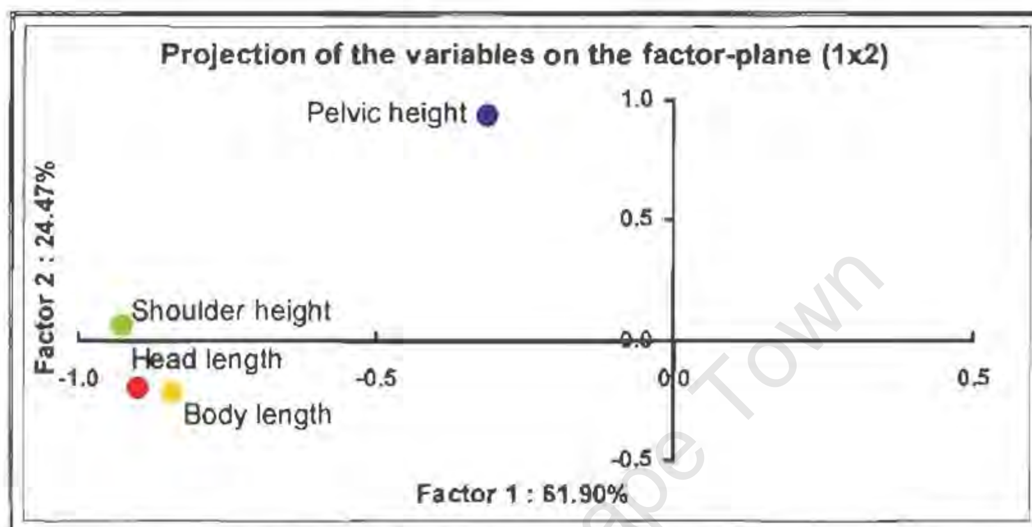

Figure 4.22. PCA of the GSD breed type differentiation, the first two factors plotted accounted for 86.37% of the variance in the morphological data (STATISTICA).

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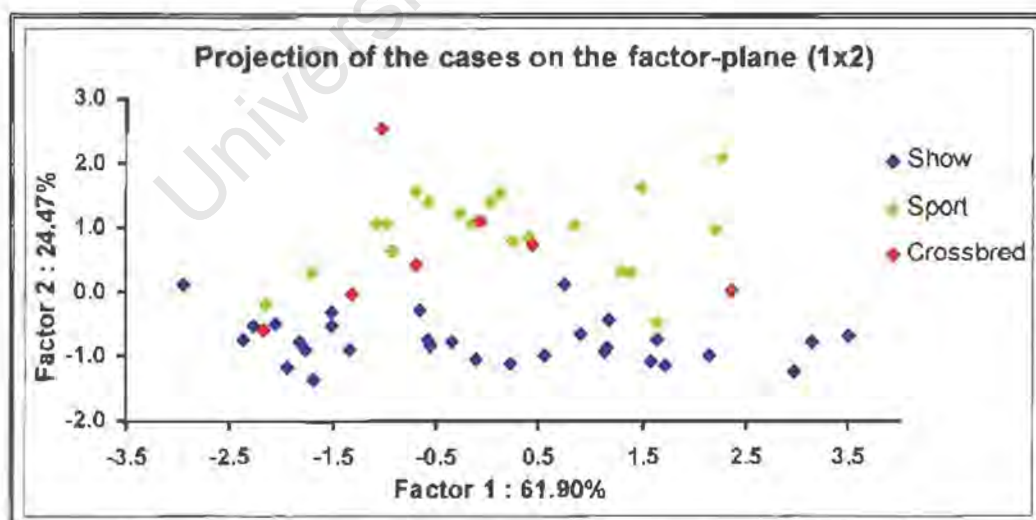

Figure 4.23. PCA mean component scores separated breed types primarily along the y-axis (F2), indicating that morphological variation was predominantly due to differences in shape rather than size (STATISTICA).

4.2.2. Comparisons with DHs, SBTs, and other purebred dogs

A total of 26 Dachshunds (DHs), eight standard short-coat, 13 miniature short-coat and five miniature long-coat (Appendix I), were analysed at four polymorphic canine microsatellite markers labelled with radioactive [γ^{32}P] dATP (Figure 4.24.). Of these, only three standard short-coat, three miniature short-coat and two miniature long-coat DHs were analysed at the 11 microsatellite markers labelled with fluorescence (Figure 4.25.).

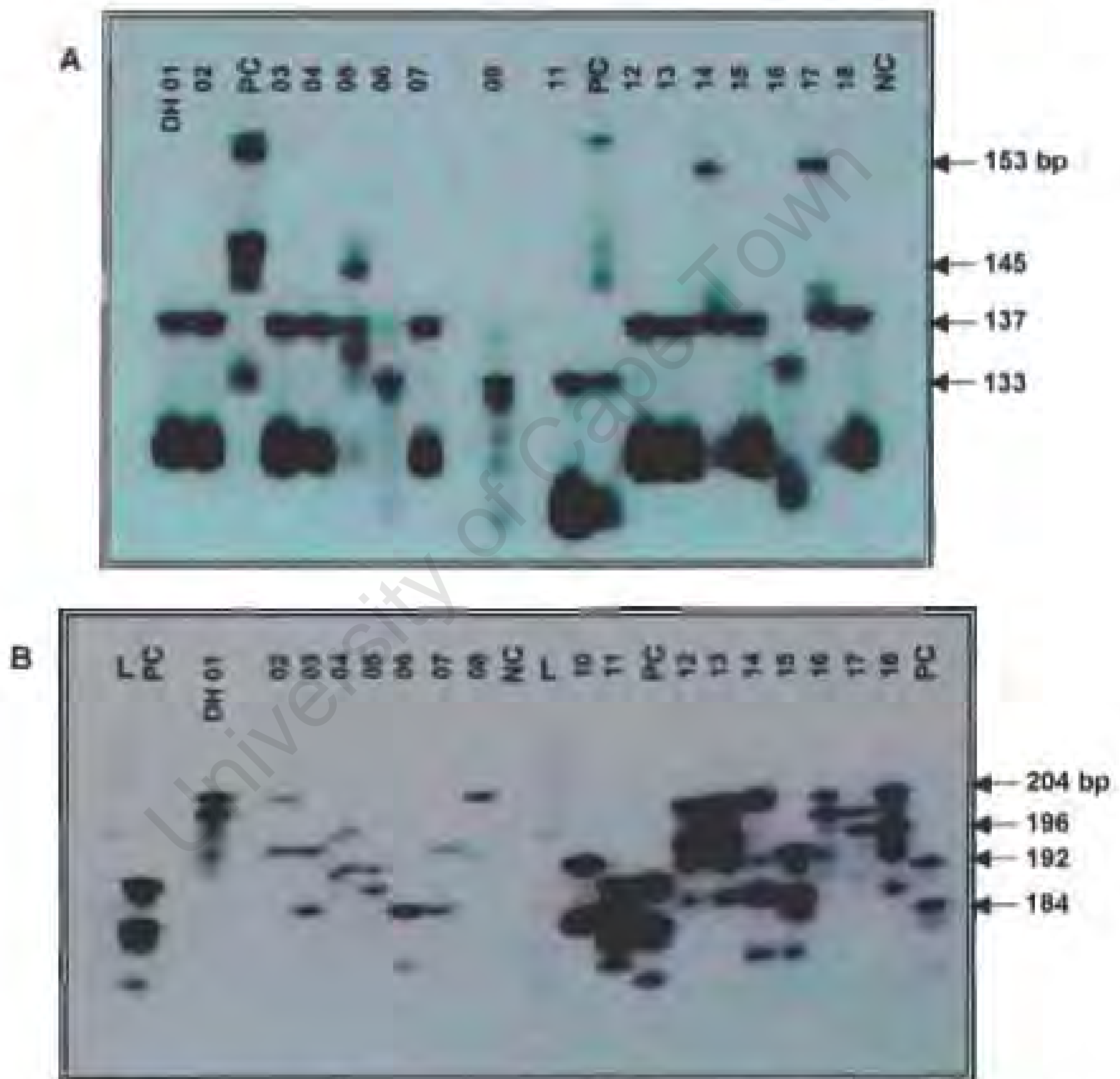

Figure 4.24. Polyacrylamide gel electropherograms of some representative DHs at radioactive [γ^{32}P] dATP-labelled microsatellite loci, FH2140 (A) and FH2328 (B), visualised autoradiographically. Alleles were sized vertically by comparison with both standard A-T ladders (L) and positive controls (PC), and included a negative control (NC).

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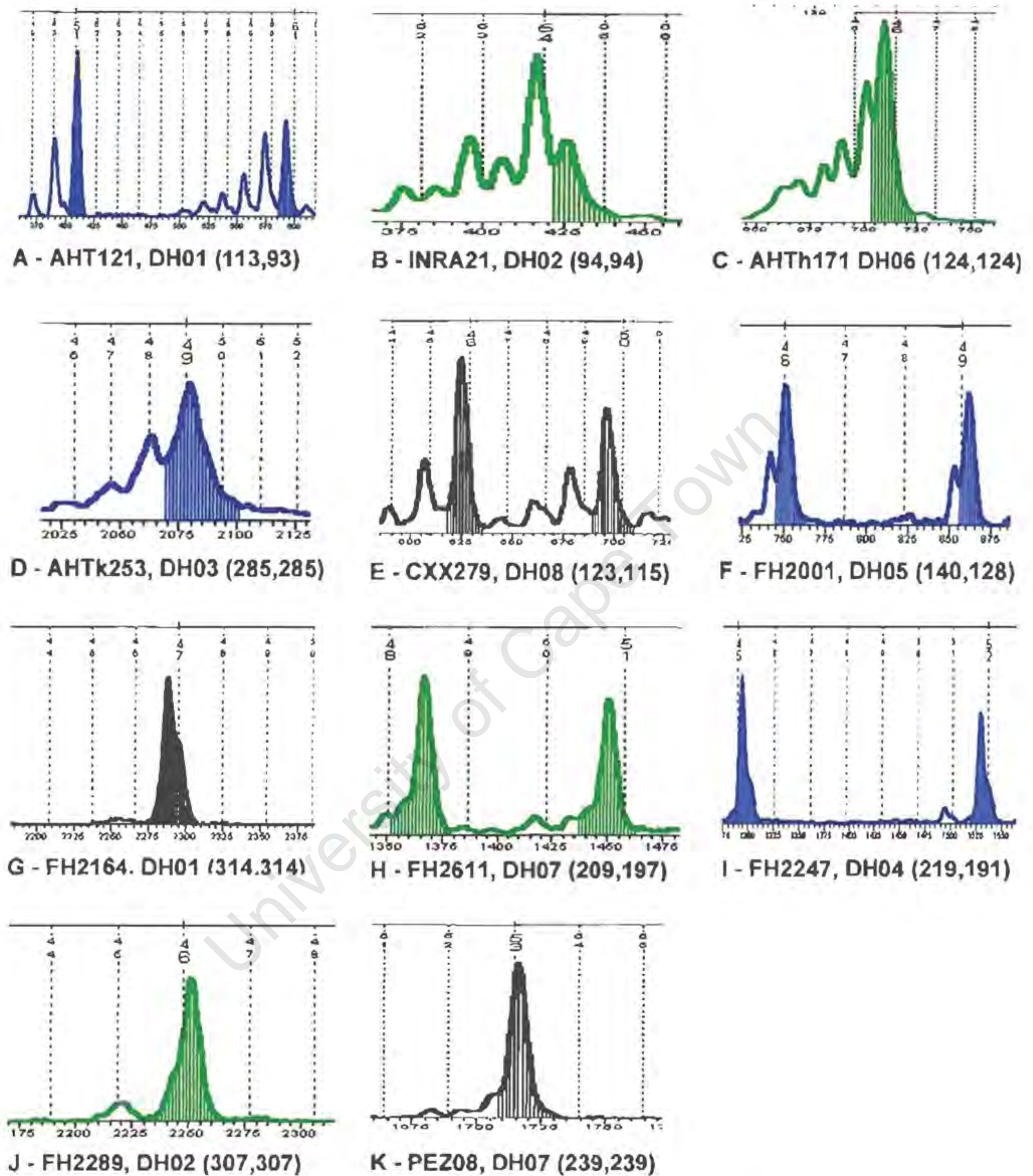

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Figure 4.25. Electropherograms representing fluorescent-labelled microsatellite alleles (shaded peaks) in some representative DHs, sized from left to right according to an internal size standard (STRand). Microsatellite marker name, individual sample number, and allele sizes (bp) are indicated.

A total of 18 Staffordshire Bull Terriers (SBTs) were analysed at four polymorphic canine microsatellite markers (Appendix I) labelled with radioactive [γ^{32}P] dATP (Figure 4.26.). Of these, only six individuals were analysed at the 11 microsatellite markers labelled with fluorescence (Figure 4.27.).

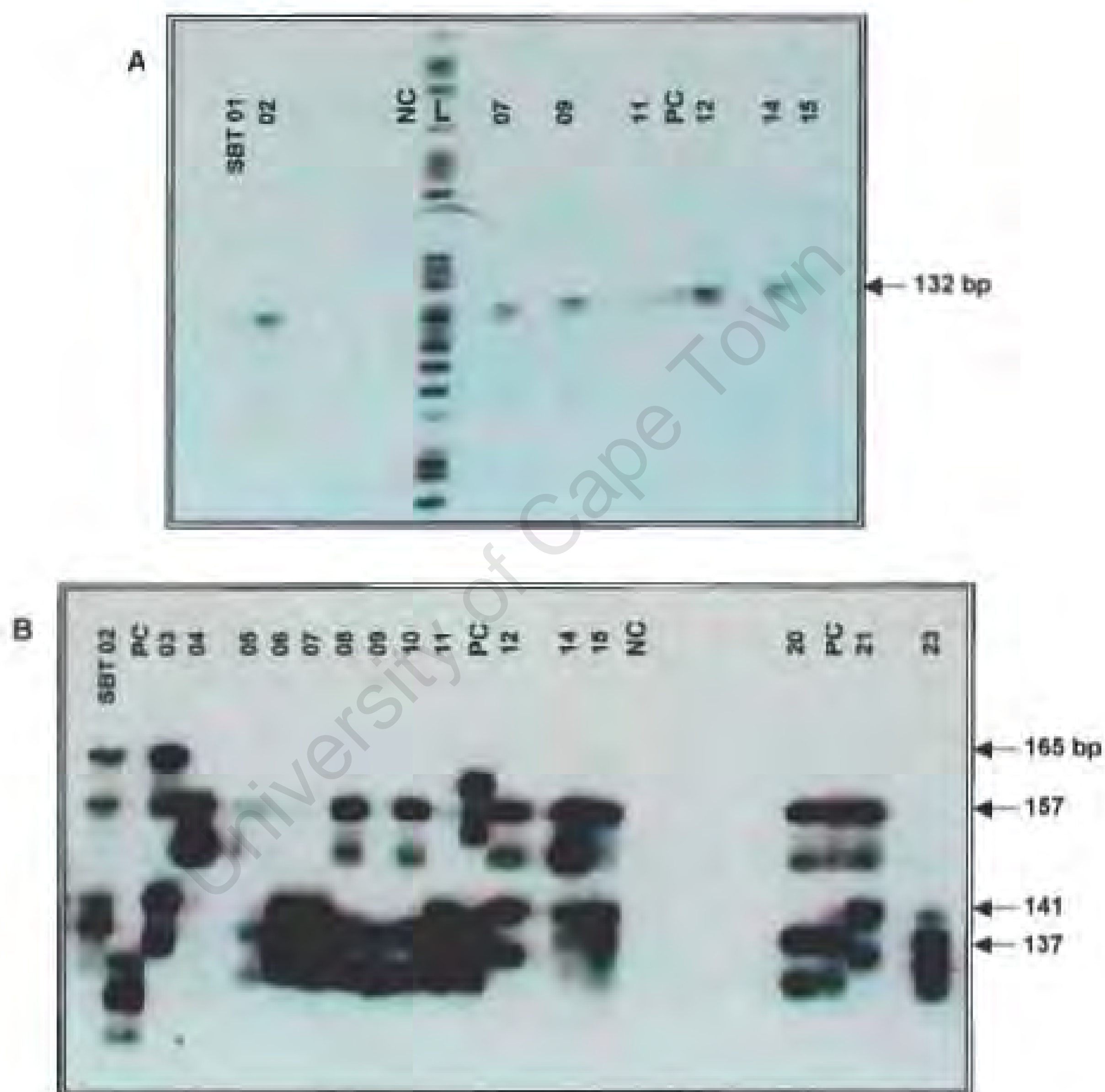

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Figure 4.26. Polyacrylamide gel electropherograms of some representative SBTs at radioactive [γ^{32}P] dATP-labelled microsatellite loci, DTRCN1 (A) and FH2140 (B), visualised autoradiographically. Alleles were sized vertically by comparison with standard A-T ladders (L) and positive controls (PC), and included a negative control (NC).

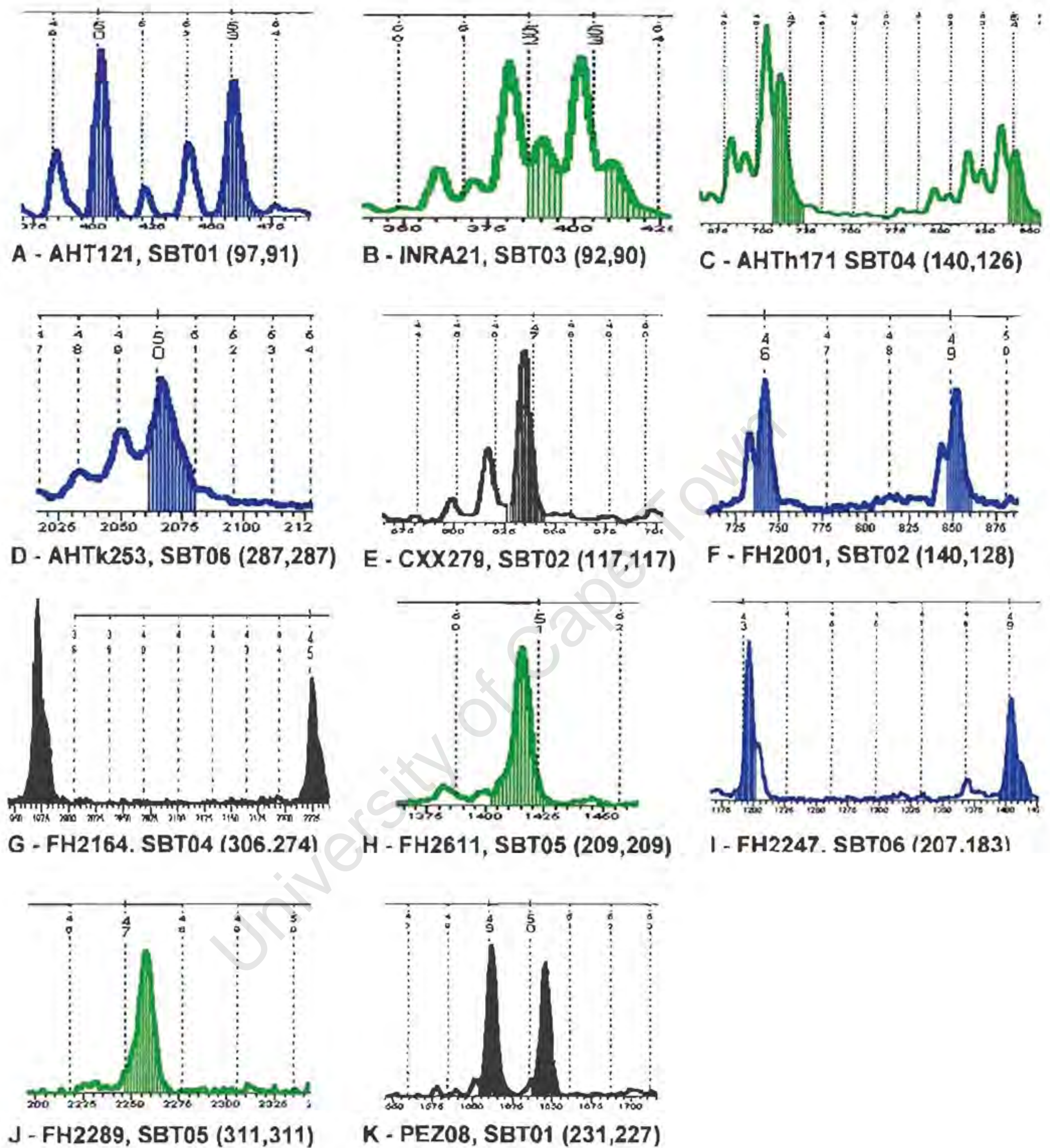

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Figure 4.27. Electropherograms representing fluorescent-labelled microsatellite alleles (shaded peaks) in some representative SBTs, sized from left to right according to an internal size standard (STRand). Microsatellite marker name, individual sample number, and allele sizes (bp) are indicated.

A total of 37 purebred dogs, forming a composite breed (CB) group representing 30 officially recognised breeds, were analysed at four polymorphic canine microsatellite markers (Appendix I) labelled with radioactive [γ^{32}P] dATP (Figure 4.28.). Of these, only 12 individuals representing 11 of the breeds were analysed at the 11 microsatellite markers labelled with fluorescence (Figure 4.29.).

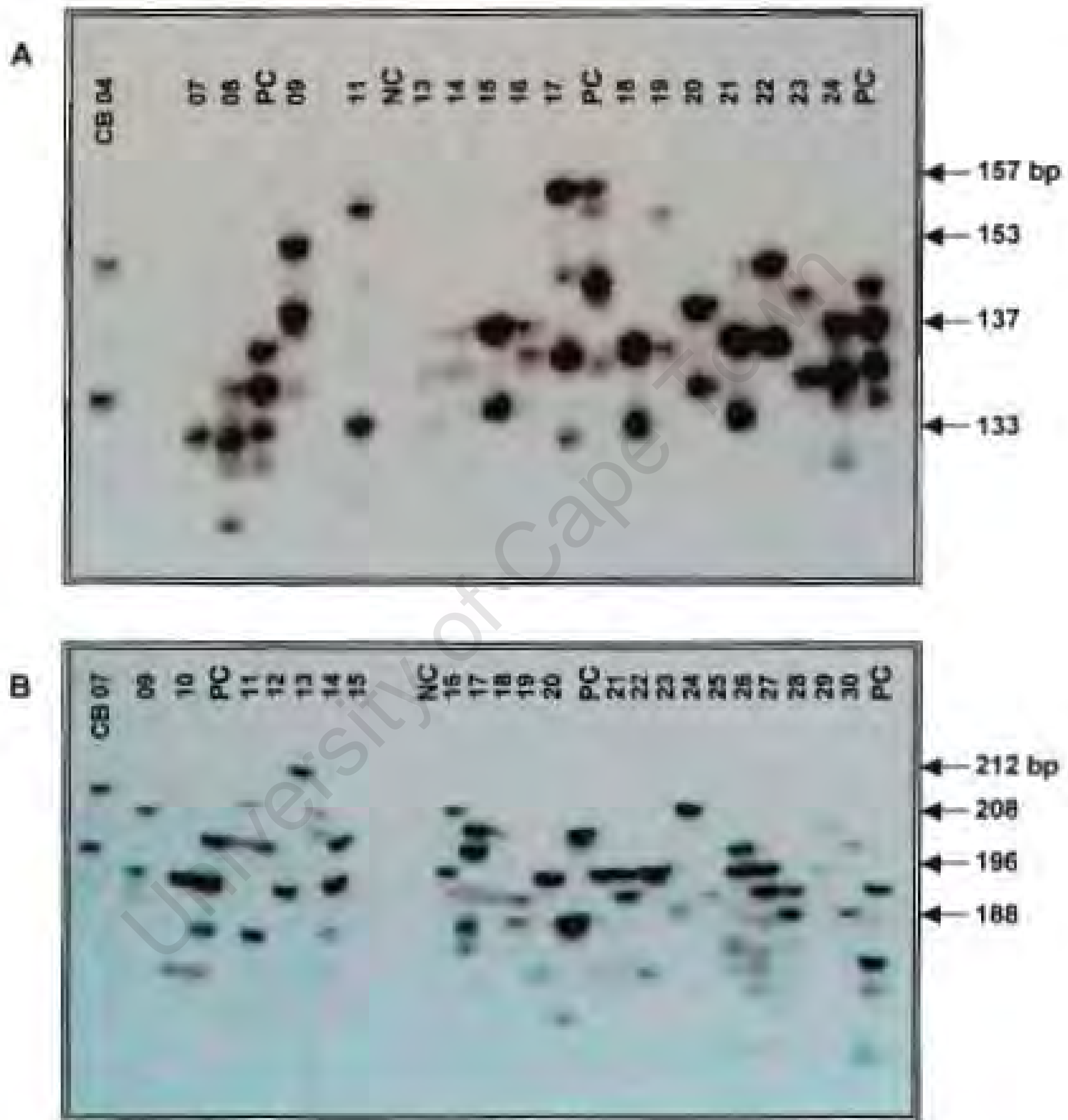

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Figure 4.28. Polyacrylamide gel electropherograms of some representative dogs of the composite breed group at radioactive [γ^{32}P] dATP-labelled microsatellite loci, FH2140 (A) and FH2328 (B), visualised autoradiographically. Alleles were sized vertically by comparison with standard A-T ladders (L) and positive controls (PC), and included a negative control (NC).

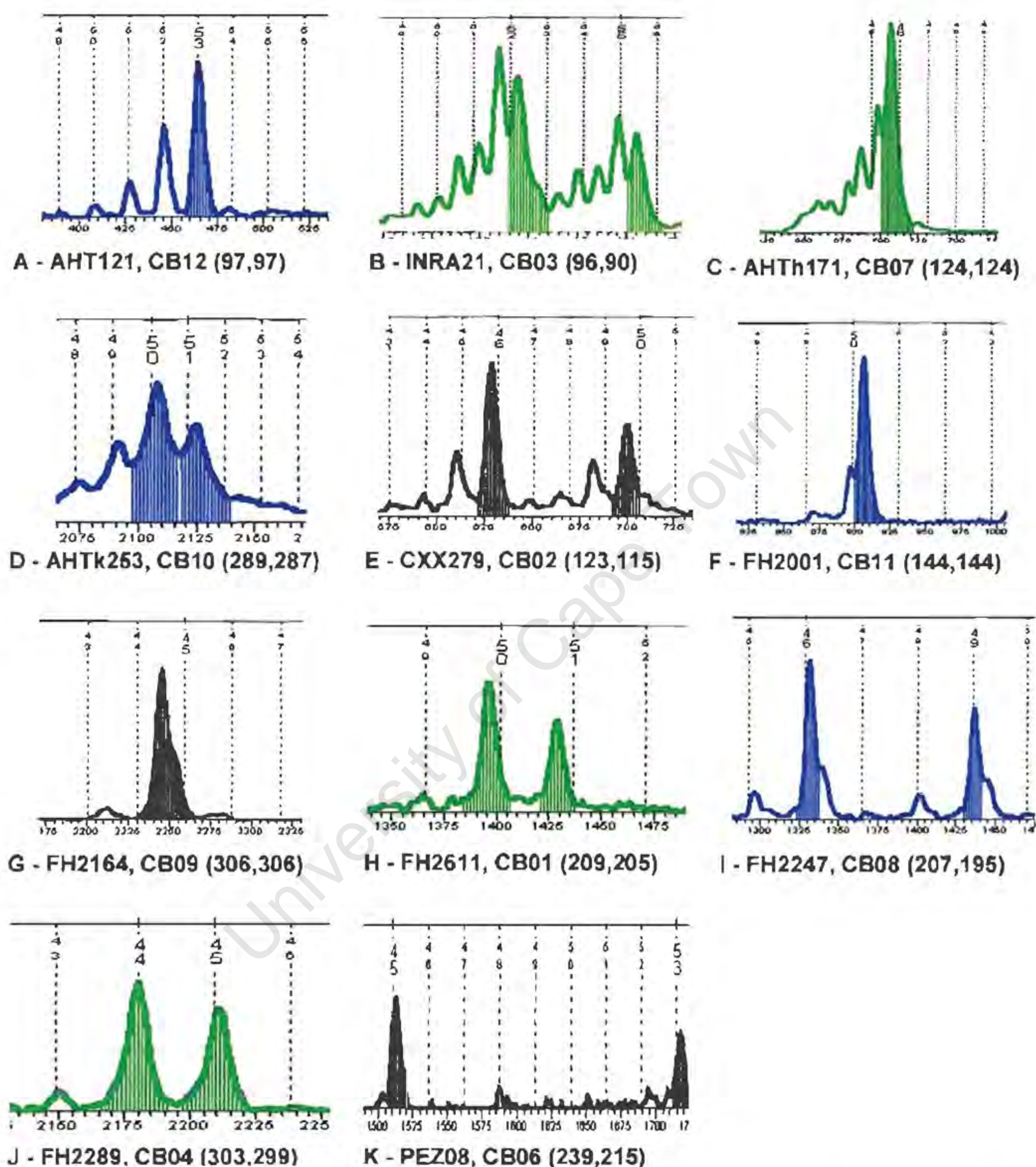

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Figure 4.29. Electropherograms representing fluorescent-labelled microsatellite alleles (shaded peaks) in some representative dogs of the composite breed group, sized from left to right according to an internal size standard (STRand). Microsatellite marker name, individual sample number, and allele sizes (bp) are indicated.

The genetic diversity or polymorphism expressed by a population can be estimated by measuring allele frequency and distribution across a number of loci (Appendix II). An example of one such a locus (Figure 4.30.) graphically illustrated the allele distribution frequencies of GSDs relative to DHs, SBTs, and the CB group, as compared with the OBD population. At the FH2328 microsatellite locus, the GSD population had two common alleles (180bp and 188bp) with frequencies of 46.05 and 50.50, and the DH population expressed a private allele (184bp). Whereas the allele size range was similar for the DH, SBT, and CB populations, the heterozygosity and allele frequency distributions within the 15 microsatellite loci varied considerably, indicative of diverse genetic variation.

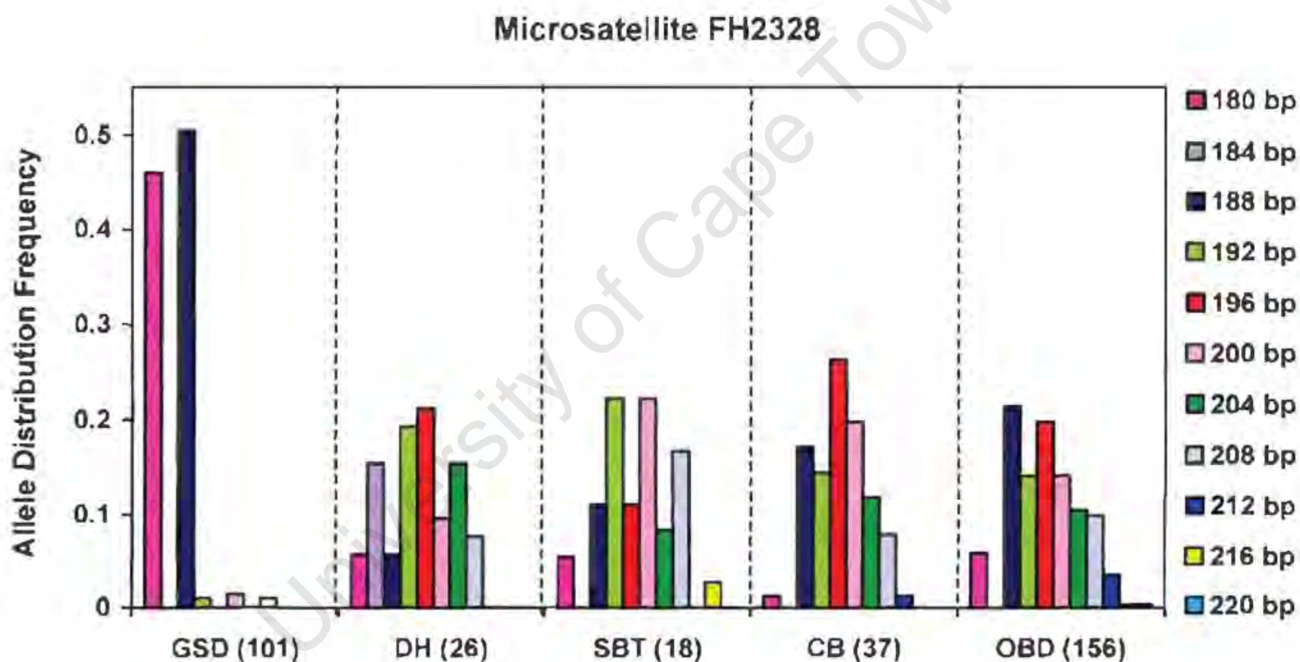

Figure 4.30. A graphic representation of the allele distribution frequencies for each purebred dog population, as compared with a population of OBDs, at the FH2328 microsatellite locus. Sample sizes are indicated in parentheses.

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Table 4.31. represents the average genetic diversity of the combined GSD population relative to two other purebred dog populations and the CB group across all 15 microsatellite loci. These data include the population sample size, total number of alleles, mean number of alleles per locus corrected for differing sample size by 1 000 pseudoreplications with both the bootstrap and jacknife methods, the observed and expected heterozygosity values, and mean PIC values (AGARst, PIC Calculator).

In comparison with the other purebred dogs and the OBD population, the GSD population consistently expressed the least genetic diversity in terms of corrected number of alleles per locus, heterozygosities, and PIC values. The DH population had both the highest corrected number of alleles and PIC, although the observed and expected heterozygosity values suggested evidence of homozygous excess. While the SBT population expressed both fewer corrected number of alleles and PIC, it did have high levels of heterozygosity. The CB group expressed the highest levels of genetic diversity, and if this can be estimated as the total diversity in all dog breeds and representative of the ancestral or average population composition, then it suggests extensive variation within different breeds.

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Population	Sample Size	Allele Number	Alleles / Locus (Bootstrap)	Alleles / Locus (Jacknife)	Heterozygosity		PIC
					Obs	Exp	
GSD	101	106	3.89	3.94	0.588	0.616	0.570
DH	26	87	4.62	5.14	0.589	0.718	0.676
SBT	18	72	4.10	4.80	0.725	0.669	0.620
CB	37	115	5.55	6.05	0.625	0.795	0.764
OBD	156	185	6.35	6.42	0.748	0.831	0.811

Table 4.31. The genetic diversity expressed by each purebred dog population relative to the OBD population, as illustrated by comparative allele counts, degrees of heterozygosity, and PIC values (AGARst, PIC Calculator).

Table 4.32. summarises the results of both the "sign test" and Wilcoxon sign-rank test" (BOTTLENECK). The GSD, DH, and CB populations exhibited statistically significant excess H_E over H_{EQ}, and there was no indication of a modal-shift in allele frequencies with a normal L-shaped allele distribution. The SBT population had a modal-shift in allele frequencies indicating a recent bottleneck event.

Table 4.33. summarises the Garza and Williamson's "M" values (calculated using AGARst). The ratios calculated for the GSD, DH, and CB populations exceeded the critical value, indicating no detectable recent bottleneck event. However, the SBT population had a ratio less than 0.680, indicating recent reductions in effective population size.

Bottlenecks in the recent histories of the GSD, DH and CB populations were not statistically supported, but there was evidence of reduced effective population size in the SBT population.

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Population	"Sign Test" TPM		"Wilcoxon Test" TPM		Modal Shift
	$H_E > H_{EQ}$	p-value	P of H_{EXC}	P of H_{DEF}	
GSD	8.85	0.012	0.011	0.991	L-shaped
DH	9.07	0.375	0.681	0.339	L-shaped
SBT	8.57	0.315	0.906	0.104	shifted
CB	8.94	0.390	0.906	0.104	L-shaped

Table 4.32. A summary of the results of the "sign test" and " Wilcoxon sign-rank test" that detect bottleneck events in the recent history of each purebred dog population (BOTTLENECK).

Population	Garza & Williamson's "M"	Variance	Monomorphic Loci
GSD	0.756	0.051	0
DH	0.750	0.066	0
SBT	0.668	0.062	0
CB	0.796	0.042	0

Table 4.33. Garza & Williamson's "M" ratios for the detection of bottleneck events in each purebred dog population, the variance and number of monomorphic loci are also indicated (calculated using AGARst).

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The homozygote-heterozygote proportions of the GSD, DH, SBT, and CB populations were F_{IS} = 0.054, 0.248, 0.053, and 0.252, respectively (FSTAT). There was a global average heterozygote deficit of 9.5% (F_{IS} = 0.095) across all 15 microsatellite loci for each population, and a heterozygote deficit of 23.8% (F_{IT} = 0.238) in the combined purebred dog population. There was a significant differentiation between the populations (G_{ST} = 0.158, R_{ST} = 0.160), however these estimates were less when the purebred dog populations were compared with the OBD population (G_{ST} = 0.092, R_{ST} = 0.069; FSTAT, GENEPOP).

Table 4.34. represents the pairwise comparisons of G_{ST} and R_{ST} values between the GSD, DH, SBT, CB, and OBD populations (GENEPOP). These data consistently indicated significant differentiation between the purebred dog populations and between these populations and the CB group and the OBD population. There was non-significant differentiation between the CB group and the OBD population (G_{ST} = 0.010, R_{ST} = 0.005).

Population	GSD	DH	SBT	CB	OBD
GSD	-	0.215	0.177	0.145	0.103
DH	0.196	-	0.143	0.060	0.043
SBT	0.228	0.112	-	0.061	0.063
CB	0.126	0.051	0.052	-	0.010
OBD	0.068	0.080	0.028	0.005	-

Table 4.34. The mean pairwise G_{ST} and R_{ST} estimates between purebred dog populations and the OBD population across all 15 microsatellite loci (G_{ST} values above the diagonal and R_{ST} below; GENEPOP).

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Table 4.35. indicates that the deviations from H-W equilibrium were significant for the GSD, DH, and CB populations ($p < 0.001$) and that disequilibrium was statistically supported. However, the deviation was not significant ($p = 0.465$) for the SBT population and was thus in H-W equilibrium.

Population	χ^2	Df	p-value	H-W Equilibrium
GSD	infinity	30	<0.001	disequilibrium
DH	77.4	30	<0.001	disequilibrium
SBT	30.0	30	0.465	equilibrium
CB	infinity	30	<0.001	disequilibrium

Table 4.35. Probability tests verified whether the purebred dog populations deviated significantly from Hardy-Weinberg equilibrium; χ^2, degrees of freedom (Df) and p-values are indicated (GENEPOP).

Assignment tests correctly grouped almost all individuals in the GSD, DH, and SBT populations, indicting significant differentiation among these breeds (Table 4.36.). However, only 81% of the individuals in the CB group were correctly assigned, indicating a certain degree of homogeneity within the founding individuals of the various breed populations (AGARst).

Population	Correctly Assigned	Median Value Likelihood Ratios	Range of Values Likelihood Ratios
GSD	99%	9.20×10^{7}	2.02×10^{1} to 1.95×10^{12}
DH	100%	1.41×10^{2}	2.17×10^{0} to 2.81×10^{10}
SBT	94%	3.67×10^{2}	6.26×10^{0} to 5.96×10^{14}
CB	81%	1.18×10^{1}	1.07×10^{0} to 5.68×10^{8}
OBD	84%	4.56×10^{2}	1.05×10^{0} to 2.35×10^{7}

Table 4.36. The results of assignment tests indicating the percentage and likelihood ratios of individuals correctly assigned to each purebred dog population or to the OBD population (AGARst).

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The pairwise plots of the negative log likelihood of individuals in each purebred dog population being assigned to its own source population was graphic representations of the assignment test results (AGARst). These results suggested significant differentiation between the GSD, DH, SBT, CB, and OBD populations (Figure 4.37. A to E).

Table 4.38. summarises the average number of alleles per locus, expected heterozygosity and PIC value across all 15 microsatellite loci for each of the breeds of dogs and OBDs examined in this study. These values were averaged across the entire domestic dog population to reveal seven alleles per locus, an expected heterozygosity of 0.726, and PIC value of 0.688.

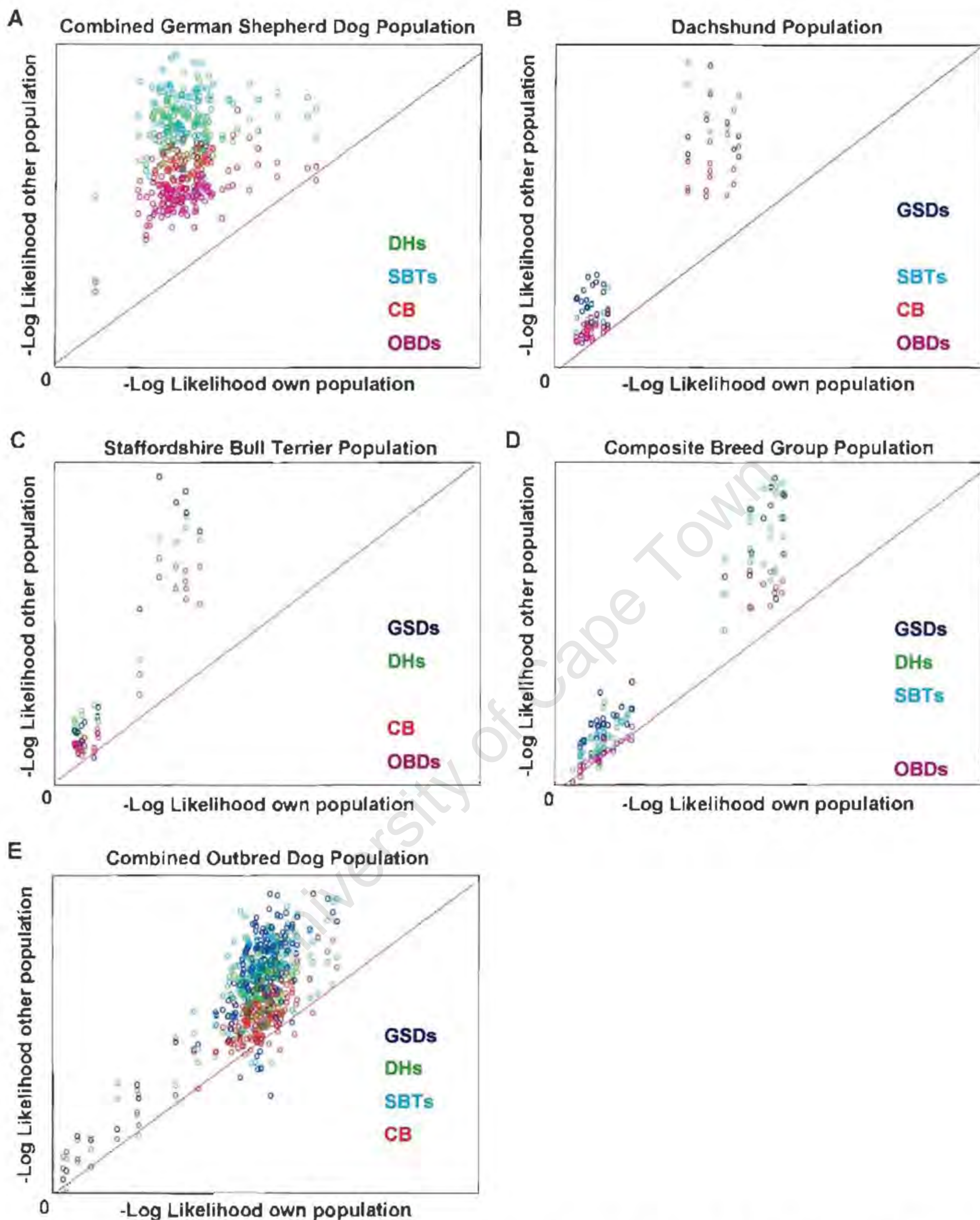

Figure 4.37. The pairwise plots of the negative log likelihood of the combined GSD population being assigned to its own source population as compared with the other purebred dog populations (AGARst).

Locus	GSD			DH			SBT			CB			OBD			Mean		
	n	H_E	PIC	n	H_E	PIC	n	H_E	PIC	n	H_E	PIC	n	H_E	PIC	n	H_E	PIC
DTRCN1	8	0.230	0.226	8	0.820	0.797	4	0.250	0.238	8	0.762	0.729	13	0.839	0.820	**8**	**0.580**	**0.562**
FH2137	9	0.727	0.689	5	0.680	0.625	7	0.750	0.719	10	0.865	0.850	10	0.857	0.840	**8**	**0.776**	**0.745**
FH2140	8	0.763	0.728	6	0.490	0.460	5	0.714	0.660	8	0.774	0.745	11	0.783	0.764	**7**	**0.705**	**0.671**
FH2328	5	0.533	0.425	8	0.849	0.831	8	0.838	0.818	8	0.825	0.802	10	0.850	0.832	**8**	**0.779**	**0.742**
AHT121	7	0.546	0.515	8	0.851	0.833	4	0.680	0.622	9	0.802	0.778	13	0.869	0.856	**8**	**0.750**	**0.721**
INRA21	7	0.736	0.696	6	0.734	0.693	4	0.680	0.622	5	0.719	0.623	8	0.813	0.790	**6**	**0.736**	**0.685**
AHTh171	7	0.609	0.565	4	0.601	0.525	5	0.750	0.708	7	0.816	0.791	11	0.850	0.835	**7**	**0.725**	**0.685**
AHTk253	6	0.355	0.327	4	0.656	0.605	2	0.480	0.365	5	0.764	0.726	9	0.723	0.686	**5**	**0.596**	**0.542**
CXX279	3	0.609	0.529	5	0.695	0.642	3	0.625	0.555	5	0.722	0.674	11	0.800	0.772	**5**	**0.690**	**0.634**
FH2001	5	0.677	0.607	6	0.672	0.630	4	0.580	0.535	5	0.729	0.683	11	0.772	0.737	**6**	**0.686**	**0.638**
FH2164	8	0.602	0.543	3	0.656	0.582	7	0.840	0.820	8	0.835	0.815	15	0.848	0.831	**8**	**0.756**	**0.718**
FH2611	6	0.674	0.622	6	0.781	0.748	4	0.700	0.645	7	0.729	0.701	10	0.821	0.798	**7**	**0.741**	**0.703**
FH2247	14	0.825	0.804	7	0.773	0.740	7	0.820	0.798	11	0.858	0.845	22	0.928	0.923	**12**	**0.841**	**0.822**
FH2289	5	0.604	0.553	7	0.788	0.758	4	0.660	0.596	12	0.896	0.887	22	0.894	0.886	**10**	**0.768**	**0.736**
PEZ08	8	0.755	0.718	4	0.726	0.677	4	0.660	0.596	7	0.833	0.812	9	0.821	0.797	**6**	**0.759**	**0.720**
Mean	**7**	**0.616**	**0.570**	**6**	**0.718**	**0.676**	**5**	**0.668**	**0.620**	**8**	**0.795**	**0.764**	**12**	**0.831**	**0.811**	**7**	**0.726**	**0.688**

Table 4.38. The number of alleles (n), expected heterozygosity (H_E), and polymorphism information content (PIC) for 15 microsatellite loci.

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Chapter 5

Discussion

5.1. Introduction

"The breeding of fine dogs has not advanced as rapidly as it should have, due to a lack of knowledge of many breeders of the hidden faults in the available breeding stock. Perhaps we breeders will soon outgrow our adolescence - perhaps the time is near when we will be able to discuss the faults of our dogs and the reasons for them. We acknowledge that all dogs have faults, but it is 'bad form' to speak openly about them. Let us hope that the near future will bring an end to such a childish attitude so that we may progress towards the ideal in sureness and light, whereas now we creep in semi-darkness". Goldbecker and Hart "This is the German Shepherd" (Elliott 1968).

A breed is defined as an intraspecies group of individuals with uniformly similar physical characteristics developed and directed by human control (Irion *et al.* 2003). Artificial selection has generated an array of phenotypically distinct breeds of dogs; this diversity cannot be equalled by any other animal species (Richman *et al.* 2001). The breeding strategies used to develop these breeds is associated with the inherent risk of losing genetic diversity, although certain breeds would have potentially lost more than others. The mtDNA genomes of the domestic dog and grey wolf differ by just 0.2%, and a limited number of generations have elapsed since the origin of many breeds, implying that uniformity is probably limited to only a small number of genes affecting breed-specific physiological or behavioural characteristics (Aguirre *et al.* 1999, Irion *et al.* 2003).

The explosion in breed variety began in the mid-19th century as a result of public demand for unique and unusual dogs, whereby breeds became fashionable for their novelty value rather than the ability to fulfil a particular function. Even traditionally functional breeds are now bred primarily for exhibition. Such breeding programmes are controlled by the demands of the show ring with specific physical traits tending to be most important, rather than sound behaviour and temperament. Breed registries and kennel clubs became commonplace towards the end of the 19th century for the regulation of breeding and exhibition. Breed integrity was ensured by the practice of only including progeny in breed databases if both parents had been registered and conformed to certain minimum criteria.

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Levels of inbreeding were previously calculated according to pedigree analysis, but these estimations were frequently incorrect. Only a few generations are represented and earlier matings between related individuals are not included, and before the use of molecular genetic techniques to verify parentage, pedigrees were frequently incorrect (Koskinen and Bredbacka 2000).

Molecular genetic techniques have been used to investigate numerous breeds of dogs, with most reportedly expressing moderate to high levels of genetic diversity, with the degree of population differentiation indicated being primarily the result of differing allele distribution frequencies (Fredholm and Winterø 1995, Pihkanen 1996, Zajc *et al.* 1997, Morera *et al.* 1999, Koskinen and Bredbacka 2000, Altet *et al.* 2001, Irion *et al.* 2003 and Parker *et al.* 2004). These data were indicative of diverse founding populations with much interbreeding prior to the relatively recent origin of many modern breeds of dogs.

Dogs registered with the German Shepherd Dog Federation of South Africa are only eligible for breeding if certain minimum criteria are met. Every dog and bitch must conform to the Breed Standard and receive a grading of at least "good" at a breed show under an accredited judge, they must be x-rayed and passed free of hip dysplasia, they must have had their parentage verified, and all parents must have been registered with the Federation. As of February 2003, preliminary pedigree analysis revealed that only about 200 of the 1 130 parentage verified dogs were not first-degree relatives. This breeding stock included large numbers of individuals either sired or grand-sired by one of three dogs; the German-bred imported show dogs, Lasso von Descharo, Quando vom Bohawald, and Harto von Sendling. According to their pedigrees (Figure 5.1.), these dogs shared seven common ancestors and four sets of littermates in the four generations examined. Any recessive genetic disorder carried by one of these dogs could have been passed to their progeny to result in future widespread disease conditions. These three dogs have been retired from stud but a large proportion of the population now carries their bloodlines. This cycle is perpetuated; breed politics and success at the annual national breed show will result in a couple of dogs assuming the role of "popular sire". Further analysis of 250 dogs that were not first-degree relatives revealed only eight individuals that did not share any common ancestors for the four generations examined, this being representative of the levels of inbreeding and relatedness in the GSD breeding stock.

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Breeders of show dogs tend to focus on the importance of bloodlines, with high levels of inbreeding existing in these lineages (Figure 5.1.). These breeders often maintain their own bitch-lines for breeding with the best available stud dogs, usually imported dogs. Current breed regulations allow for progeny to have multiple common ancestors, although the closest inbreeding allowed is for any particular individual to be the parent of the potential stud dog and grandparent of the bitch, or vice versa. However, it is possible to obtain special permission for mating combinations of father/daughter, mother/son or full-siblings. This permission is usually only granted when the dogs concerned have been graded VA (excellent select) at the national breed show, for it is a commonly held belief that close inbreeding, the ultimate mating of "like to like", will produce an exceptional dog.

Pedigree analysis of sport dogs revealed much lower levels of inbreeding, with three of the most popular stud dogs, the German imports, Ari vom Eckgrund and Canto von Neumis Flucht, and the Belgian import, Vasco van Salenshof, sharing no common ancestors in the four generations examined. Bloodlines are still important, but breeders tend to focus on the performances of the potential stud dog and bitch, and that of the bitch sire. Both bitches and stud dogs are usually imported; possibly because this breed type has only been in South Africa for about ten years and no local bloodlines have been established.

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Kennel Union of South Africa registered show dogs have been bred in almost complete isolation for nearly twenty years, with little regulation regarding levels of inbreeding and minimum breeding criteria. Crossbred show and sport dogs would have no ancestors common to both sides of their pedigrees, although it is generally inexperienced amateur breeders that experiment with mating such "unlike" dogs. Only a small number of these crossbred dogs are produced because they usually do not inherit the best of each parent, but are rather a mixture of the characteristic physical and mental traits, and are not typically suited to either breed type.

With the advent of more specialised requirements, it would appear as though two distinct types of dogs are required to completely fulfil the demands of the breed standard. It would be the ultimate aspiration of any breed enthusiast to produce the "perfect" GSD, having the physical conformation of a show dog and mental abilities of a sport dog, unfortunately this seems unlikely to be achieved with the existing politics and breeding strategies.

Name of Dog	1st Generation	2nd Generation	3rd Generation	4th Generation
Lasso von Descharo	Cim vom Ecknachtal	Cello von der Römerau	Natz vom Hasenborn	Dax von der Wienerau
				Yanka vom Hühnegrab
			Quana von Arminius	Xaver von Arminius
				Palme vom Wildsteiger Land
		Tigrise vom Wildsteiger Land	Ingo vom Haus Vögele	Warro vom Asterplatz
				Eve vom Haus Vögele
			Palme vom Wildsteiger Land	Nick von der Wienerau
				Fina vom Badsee
	Joga von Descharo	Fanto vom Hirschel	Tell vom Groβen Sand	Sonny vom Badener-Land
				Jenny vom Groβen Sand
			Ica vom Haus Reiterland	Reza vom Haus Beck
				Afra vom Haus Reiterland
		Fina von Arminius	Lasso vom Wiederbrücker Land	Zorro vom Haus Beck
				Ora vom Wiederbrücker Land
			Fee vom Weihertürchen	Xando von Arminius
				Anja von Restrauch
Quando vom Bohawald	Darius aus Wattenscheid	Rony von Arminius	Fedor von Arminius	Lasso vom Wiedenbrücker Land
				Fee vom Weihertürchen
			Nati vom Grafenhain	Quino von Arminius
				Ira vom Weihertürchen
		Xena aus Wattenscheid	Uran vom Wildsteiger Land	[illegible] von Arminius
				Palme vom Wildsteiger Land
			Kralle aus Wattenscheid	Harko von der Bayernwaldperk
				Rinda aus Wattenscheid
	Milli vom Bohawald	Zasko vom Klostermoor	Uran vom Wildsteiger Land	[illegible] von Arminius
				Palme vom Wildsteiger Land
			Ilja vom Maggenheim	Pass di ca San Marco
				Nelke van Noort
		Indy vom Bohawald	Dando aus Nordrheinland	Dingo vom Haus Gero
				Amanda aus Nordrheinland
			Ery vom Bohawald	Kim vom Wildpferdbruch
				Aida vom Roruper Waldschlöβchen
Harto von Sendling	Vitus vom Haus-Farrenkopf	Visum von Arminius	Jeck vom Noricum	Odin von Tannenmeise
				Anett vom Noricum
			Ratta von Arminius	Fedor von Arminius
				Nati vom Grafenhain
		Yanka aus Agrigento	Asswan von Altkircher Wild	Pütz von Arjakjo
				Liane von der Bargefenne
			Bonnie vom Hühnegrab	Quando von Arminius
				Moni vom Hühnegrab
	Cina von der Wienerau	Jello von der Wienerau	Fedor von Arminius	Lasso vom Wiedenbrücker Land
				Fee vom Weihertürchen
			Ussi von der Wienerau	Uran vom Wildsteiger Land
				Xinte von der Wienerau
		Venja von der Wienerau	Zamb von der Wienerau	Odin von Tannenmeise
				Ica von der Wienerau
			Xandra von der Wienerau	Cello von der Römerau
				Ussi von der Wienerau

Figure 5.1. The four generational pedigrees of the "popular sires" Lasso von Descharo, Quando vom Bohawald, and Harto von Sendling, common ancestors and sets of littermates are indicated by corresponding colours (German Shepherd Dog Federation of South Africa and www.pedigreedatabase.com/gsd/search.html).

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5.2. The Outbred Dog according to region of origin

The comparison of outbred mongrel dogs from Cape Town in the Western Cape, Port Elizabeth in the Eastern Cape, Johannesburg in southwestern Gauteng and Pretoria in northeastern Gauteng, indicated comparable levels of extensive genetic diversity as well as non-significant population differentiation. This was clearly illustrated by the allele frequency distribution at locus FH2611, two subpopulations expressed the same ten alleles and the other two expressed nine of these alleles. All subpopulations had the same four most common alleles, with the highest individual allele frequency of 0.324. The corrected numbers of alleles per locus (between 8.66 and 9.80), the observed heterozygosity values (between 72% and 77.6%), expected heterozygosity values (between 81.8% and 82.5%), and PIC values (between 0.796 and 0.802) further demonstrated the comparable levels of genetic diversity. The discrepancy in the values of the observed and expected heterozygosities was indicative of slight homozygous excess.

Microsatellite marker data have been successfully tested for heterozygosity excess, modal shifts in allele size distributions, and ratios of allele number to range in allele size, for the detection of recent bottleneck events in an array of natural populations (Luikart and Cornuet 1998, Luikart et al. 1998a, Garza and Williamson 2001). These tests indicated that there was no statistical support for significant bottleneck events in the history of any of the OBD subpopulations, indicating that no recent reductions in effective population size have occurred.

F-statistic and Rho-statistic estimates were used to measure genetic differentiation within and between the OBD subpopulations. The homozygote-heterozygote proportions of the Cape Town, Port Elizabeth, Johannesburg, and Pretoria OBD subpopulations were all positive (F_{IS} = 0.068, 0.095, 0.136, and 0.128, respectively), with an average global heterozygote deficit for each subpopulation of 10.3%, indicating the existence of significant levels of homozygous excess. There was no indication of non-amplifying or silent alleles in the parentage verification analyses and is unlikely to be affecting these statistical estimates, thus the homozygous excess indicated in all four OBD subpopulations could be the result of internal substructuring due to local inbreeding.

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The pairwise G_{ST} and R_{ST} estimates of between 0.001 and 0.011 and an average global differentiation of 0.2% among the OBD subpopulations was indicative of non-significant levels of population differentiation. Substantial migration and gene flow across the country during past generations has resulted in genetic homogeneity within the subpopulations.

The combined OBD population was not congruent with H-W proportions, most likely due to the substructuring caused by the geographic isolation of the subpopulations. H-W disequilibrium was also statistically supported for each of the subpopulations; these deviations could have resulted from any infringement of the assumptions made for populations in equilibrium. As there was no evidence of allele non-amplification, and the mongrel population is extremely large, these deviations are most probably due to the occurrence of non-random mating and extensive gene flow. In addition, these deviations from H-W equilibrium occurred consistently with positive F_{IS} values, indicating that homozygous excess influenced this state of disequilibrium.

The proportion of individuals in a population correctly assigned to its own source population was another useful measure of differentiation. Assignment tests determined the ratio of the likelihood of the genotype of each dog in a population being actually drawn from that population. Between 8% and 20% of the individuals in each OBD subpopulation could be statistically assigned to another of the subpopulations, indicating the occurrence of substantial gene flow resulting in minimal differentiation in the mongrel populations.

The pairwise plots of the negative log likelihood of individuals being assigned to either their own or another of the OBD subpopulations were graphic representations of the assignment test results. Each pairwise comparison revealed that many individuals were clustered across the diagonal; suggesting an equal likelihood of being assigned to another subpopulation, further indication of the minimal differentiation between the OBD subpopulations.

There was a significant degree of genetic homogeneity between the OBD regional subpopulations, such that the combined South African population was representative of a genetically diverse control population, used in this study for comparative analyses with purebred dog populations.

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5.3. The German Shepherd Dog

5.3.1. According to country of origin and breed type

Comparative analyses between the GSD subpopulations indicated a moderate loss of genetic diversity relative to the OBD population, comparable levels of genetic diversity in the various breed types, non-significant differentiation between the South African and German-bred show dog subpopulations, and moderate differentiation between the German show and sport dog subpopulations.

At the AHT121 microsatellite marker, the South African show, KUSA show, crossbred, German show and sport subpopulations expressed different combinations of four, three, four, four, and seven alleles, respectively, in comparison with the 13 alleles of the OBD population. The German sport dogs had a private allele (79bp) at this locus, with the 101bp allele being most common in all subpopulations and reaching a highest frequency of 0.813 in the KUSA show subpopulation. At the AHTh171 microsatellite marker, the South African show, KUSA show, crossbred, German show and sport subpopulations expressed seven, two, three, two, and five alleles, respectively, in comparison with the 11 alleles of the OBD population. The 138bp allele was most common in all GSD subpopulations, reaching a highest frequency of 0.786 in the KUSA show subpopulation. Interestingly, the most common allele (130bp) in the crossbred dog subpopulation was fairly infrequent in both the show and sport subpopulations.

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The combined GSD population showed a moderate loss of genetic diversity relative to the OBD population. The average number of alleles per locus is a more sensitive measure of genetic diversity, with the GSD population expressing almost half that of the OBDs (6.48 and 7.07 compared with 11.48 and 11.90 according to the bootstrap and jacknife methods, respectively). The average observed and expected heterozygosities and PIC values were both approximately three quarters that of the OBDs (58.8% and 60.6% compared with 74.8% and 83.1%, respectively, and 0.570 compared with 0.811).

The German sport dog subpopulation consistently expressed the highest levels of genetic diversity, with the most corrected alleles per locus (3.96 and 4.02 according to the bootstrap and jacknife methods), observed and expected heterozygosities (62.1% and 61.5%), and PIC value (0.575). All but one of the private alleles detected in the GSD

subpopulations were restricted to these German-bred sport dogs (alleles 79bp at AHT121, 88bp at INRA21 and 354bp at FH2164). The South African show dog subpopulation expressed the second highest levels of genetic diversity, with respect to the corrected alleles per locus (3.86 and 3.93 according to the bootstrap and jacknife methods), observed and expected heterozygosities (61.0% and 56.9%), and PIC value (0.569). This subpopulation had a single private allele (185bp) at locus FH2611. The German-bred show, crossbred and KUSA show subpopulations expressed the least genetic diversity with the lowest numbers of corrected alleles per locus, observed and expected heterozygosities, and PIC values. The crossbred show and sport dog subpopulation was monomorphic at a single microsatellite marker, the DTRCN1 locus.

Greater genetic diversity would have been expected in the ancestral (German) population in comparison with a derived (South African) population. However, even those show dogs classified as locally bred have imported ancestors only one or two generations back in their pedigrees, especially on the sire-line. Importation (migration and gene flow) over a number of decades has resulted in the continuous accumulation of genetic diversity in the South African show dog subpopulation. However, the effective population size of the breeding stock is much smaller than that of the household pet population and includes many closely related individuals. As a result, the show dogs expressed less genetic diversity than that of the much smaller sport dog subpopulation, representative of the large and diverse population of sport dogs in Germany. The first sport dogs arrived in South Africa approximately ten years ago and the subpopulation size has remained much smaller in comparison with the show dog subpopulation as this breed type is usually only sold to a small community of dog-sport enthusiasts and not generally as household pets. It was surprising that despite a "hybrid" status, the crossbred show and sport dog subpopulation, along with the KUSA show dogs, expressed the least genetic diversity. The crossbred subpopulation had a small sample size, but is also a very small population, resulting in limited genetic diversity. KUSA-bred show dogs have been bred in near isolation for many generations and inbreeding and restricted levels of migration and gene flow have resulted in depleted genetic diversity.

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There was no evidence of a bottleneck event in the recent history of the South African show dog subpopulation, however there was some statistical support for reductions in effective population size in the other GSD subpopulations. Heterozygosity excess in post-bottleneck populations is temporary and lasts only a few generations, whereas the ratio of allele number to range in allele size range (M) usually remains for many generations (Luikart and Cornuet 1998, Garza and Williamson 2001).

The GSD breed has remained popular both as show stock and household pets since first arriving in this country many decades ago, with the large population size frequently further expanded by the importation of additional breeding stock. This immigration from Germany could have expanded the number of rare alleles in the South African show dog subpopulation without influencing the levels of heterozygosity, thereby concealing any heterozygosity excess that may have existed in the population (Cornuet and Luikart 1996). The KUSA-bred show dog subpopulation would have experienced a reduction in population size when the German Shepherd Dog Federation of South Africa was established in 1984. Prior to that date, all GSDs were registered and bred under the auspices of KUSA, but nearly all breed enthusiasts transferred their membership to the Federation. The crossbred show and sport dogs have had a constantly small effective population size, for only a small number of this type is produced. The bottleneck events detected in the two German-bred subpopulations would be reflecting founder effects resulting from the migration of a limited number of individuals from a much larger population, due to the continuous importation of both breed types for exhibition and breeding purposes.

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The homozygote-heterozygote proportions of the combined GSD population and the OBD population (F_{IS} = 0.054 and 0.104, respectively) revealed that homozygous excess in the OBD population was nearly twice that of the GSDs. The significant level of homozygous excess in the OBD population was purportedly due to internal substructuring, as there was no indication of non-amplifying or silent alleles in the parentage verification analyses. It could therefore be reasoned that this factor had less effect on the GSDs as a result of the greater degree of movement of dogs across the country. There was significant average differentiation between these two populations (G_{ST} = 0.103, R_{ST} = 0.058).

Statistical pairwise G_{ST} and R_{ST} comparisons between each GSD subpopulation and the OBD population (mean G_{ST} and R_{ST} values of 0.060 and 0.084) reflected significant levels of population differentiation. These data illustrated the effects of genetic drift since the GSD breed was established just over 100 years ago.

F-statistic and Rho-statistic estimates were used to measure genetic differentiation within and between the GSD subpopulations. The homozygote-heterozygote proportions of the South African show, KUSA-bred show, crossbred, and German show and sport subpopulations (F_{IS} = 0.046, -0.040, 0.081, 0.046, and 0.020, respectively) revealed that only the KUSA show dogs had no evidence of homozygous excess. There was non-significant average differentiation between the subpopulations (G_{ST} = 0.026, R_{ST} = 0.026), although pairwise G_{ST} and R_{ST} comparisons between the subpopulations revealed varying levels of differentiation. There was non-significant differentiation between the South African and German show dogs (G_{ST} = 0.007, R_{ST} = 0.021), as sufficient migration has occurred during past generations to provide gene flow resulting in genetic homogeneity. There was significant differentiation between the KUSA-bred show dogs and the two German imported subpopulations (mean G_{ST} = 0.062, R_{ST} = 0.070), as the relatively small population has been bred in near isolation for 20 years, accelerating the effects of random genetic drift. There was significant differentiation between the German-bred imported show and sport dogs (G_{ST} = 0.054, R_{ST} = 0.069), as distinct show and sport dogs have evolved from separate breeding programmes since the 1960s, although both subpopulations have experienced extensive levels of migration and gene flow.

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The number of loci in H-W equilibrium was another method of detecting population differentiation. H-W disequilibrium was statistically supported for the combined GSD population, indicating intrabreed substructuring. Deviations from H-W equilibrium across all 15 loci were significant for the South African show subpopulation ($p < 0.001$), statistically supporting disequilibrium. Deviations from H-W equilibrium were not significant for the other subpopulations and were thus in equilibrium. There was no evidence of allele non-amplification and the GSD population is relatively large in size, therefore these deviations were probably due to non-random mating, inbreeding and extensive gene flow.

Assignment tests correctly grouped 100% of the individuals in the combined GSD population, whereas only 96% of the OBDs were correctly assigned. This indicated that gene flow was unidirectional, from the purebred dog population to the mongrel population. The "breed barrier" rule prevents the inclusion of dogs in the breed database unless both parents were registered members, although a proportion of the individuals in the OBD population would have GSD ancestry. Only 70% of the South African show subpopulation was correctly assigned, indicating minimal levels of differentiation due to substantial migration and gene flow from Germany to South Africa. The German sport subpopulation had 94% of its individuals correctly assigned, indicating a moderate degree of differentiation during the 40 years since the breed types separated.

The pairwise plots of the negative log likelihood of individuals in a subpopulation correctly assigned to its own source population were graphic representations of the assignment test results. The diagonal represented the probability that an individual was equally likely to be assigned to its own or another population. Significant differentiation was indicated between the GSD and OBD populations, although a number of OBDs had a greater likelihood of being assigned to the GSD population, suggestive of GSD ancestry. These data also suggested minimal differentiation between the South African and German-bred show dogs, and moderate levels of differentiation between the show and sport dog subpopulations.

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Morphological measurements where taken of 30 representative GSD show dogs, 20 sport dogs and seven crossbred dogs, from which the average gradient of the slope of the back of each breed type was calculated. These data indicated that show dogs conformed best to the Breed Standard in all respects, with sport dogs tending to be smaller at the shoulder but with flatter topline gradients due to higher pelvic height measurements. Crossbred dogs were tallest at the shoulder coupled with the shortest body length and an average topline gradient halfway between the show and sport dog types. Principle component analysis (PCA) and analysis of variance (ANOVA) of the morphological data were used to determine the significance of the differentiation between the GSD breed types.

The total variance in the data set was effectively summarised by the first two PCA components (86.37%). The pelvic height measurements had a high factor score on the y-axis (F2); with the other three variables having low factor scores on this axis. This

indicated that pelvic height was responsible for most of the variation between the breed types. The graphical plot of the mean component scores for each type separated primarily along the y-axis (F2), indicating that morphological variation was predominantly due to differences in shape rather than size. The show and sport dogs were most separate, with the crossbred dogs situated between the other breed types.

ANOVA tested the significance of differences between means in the data set of each breed type, with probability values calculated for shoulder height ($p = 0.212$), body length ($p = 0.342$), and pelvic height ($p < 0.001$) measurements. These results indicated that shoulder height and body length were similar in all breed types, but significant variation existed in pelvic height measurements.

The gradient of the topline ($\Delta y/x$) of each GSD breed type was calculated according to the average change in y-axis (difference between shoulder height and pelvic height) in relation to the average change in x-axis (body length). Thus, significant differences in pelvic height resulted in significantly different topline gradients. Show dogs had the lowest average pelvic height resulting in the steepest topline gradient (0.28) and sport dogs had the highest average pelvic height resulting in the flattest topline gradient (0.13). The crossbred dogs had an average pelvic height between the show and sport dogs resulting in a topline gradient (0.20) halfway between that of the two main breed types.

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PCA and ANOVA confirmed the existence of GSD breed types, thus morphological analysis revealed significant differentiation between show dogs and sport dogs with regards to the phenotypic trait characterised as the gradient of the slope of the back. However, molecular genetic techniques indicated only moderate levels of differentiation between these breed types. This discrepancy could be influenced by the microsatellite markers used not being linked to the genes responsible for the morphology and/or temperament characteristic of each breed type. In addition, it is possible that the 40 years since the demand for specialised dogs initiated the division in the breed has not been sufficient for significant divergence in allele frequency distributions or for the accumulation of private alleles in these neutral markers. Alternatively, the observed morphological differences could be merely the result of founder effects and random genetic drift influencing these phenotypic determinants.

5.3.2. Comparisons with DHs, SBTs, and other purebred dogs

Comparative analyses indicated a moderate loss of genetic diversity in the GSD population relative to other purebred dog populations. Significant differentiation between these breeds of dogs was indicative of genetic isolation and resultant random drift since the establishment of each breed.

At the FH2328 microsatellite marker, lower number of alleles indicated loss of diversity in the GSD population, with five alleles compared with eight alleles in the DH, SBT, and CB populations, and ten in the OBD population. Two of the GSD alleles were very common, with frequencies of 0.460 and 0.505, and the remaining three alleles were quite rare.

In comparison with other purebred dog populations, the GSD population consistently expressed the least genetic diversity in terms of corrected number of alleles per locus, heterozygosities, and PIC values. The DH population had both the highest corrected number of alleles and PIC value, although the large discrepancy in observed and expected heterozygosity suggested homozygous excess. This population consisted of three distinct breed types, the standard short-coat, miniature short-coat and miniature long-coat DHs. The miniature DH and other coat types were derived from the original standard short-coat DH by outcrosses with other breeds in the last 100 years to obtain the desired phenotypic traits, introducing much genetic diversity into the DH population. In addition, Koskinen and Bredbacka (2000) reported that wire-haired DHs had the highest level of genetic diversity compared with four other breeds of dogs examined. While the SBT population had both fewer corrected number of alleles and PIC value, it did express high levels of heterozygosity.

The alleles expressed by the purebred dog populations across all 15 microsatellite loci were effectively a subset of those in the OBD population, with 36 alleles unique to the OBDs. All the private alleles detected in purebred dog populations were found in those breeds that originated in Germany. Two of the GSD subpopulations, the German sport dogs (alleles 88bp at INRA21 and 354bp at FH2164) and South African show dogs (alleles 297bp at AHTk253 and 185bp at FH2611), and the DH population (alleles 150bp at FH2137, 184bp at FH2328, and 109bp at AHT121) expressed unique alleles that would probably be more common in the German OBD population.

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These results indicated no significant correlation between levels of genetic diversity and either population size or length of time since official breed recognition. This contradicted the data reported by Irion *et al.* (2003), whereby the oldest and smallest breeds of dogs tended to express the least amount of genetic diversity. The GSD breed received official recognition with the establishment of the S.V. in 1899, with a population size of 7 990 individuals registered with KUSA and 7 415 registered with the GSD Federation of South Africa for the period 1993 to 2003 (the German population would be much larger). The standard short-coat DH was recognised in 1840, and the other breed types between 1886 and 1898, with a population size of 655 standard and 6 070 miniature DHs registered with KUSA for the same period. The SBT breed was recognised in the mid 1930's, and has a population size of 20 472 individuals registered with KUSA for the same period. In terms of corrected numbers of alleles and PIC values, the DH population expressed the highest levels of genetic diversity despite being both the oldest and smallest breed. The SBT population expressed the second highest levels of genetic diversity, even though it was both the youngest and largest breed. The GSD population, intermediate in terms of population size and time since breed recognition, consistently expressed the least genetic diversity.

With the exception of the OBD population, the CB group expressed the highest levels of genetic diversity. If this can be estimated as the total diversity in all purebred dog breeds and representative of the ancestral or average population composition, then it suggested extensive variation within different breeds. Despite relatively high levels of inbreeding and intense selection for phenotypic uniformity, genetic diversity within the GSD, DH, SBT, and CB populations was not considerably less than that of the OBD population, indicating only moderate loss of genetic diversity in purebred dogs.

A mean PIC value of 0.69 (Table 5.39.), across all 15 microsatellite markers for the five domestic dog populations, was revealed by this study. This value was greater than the 0.52 reported by Ostrander *et al.* (1993) or the 0.50 reported by Zajc *et al.* (1997). It was possible that the microsatellite markers used for this research were unusually polymorphic in nature, as 11 were chosen specifically for parentage verification. It must be taken into consideration that microsatellite marker data can be confounded by high mutation rates (Irion *et al.* 2003).

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There was statistical support for a bottleneck event in the recent history of the SBT population, however there was no evidence of reductions in effective population size in the other purebred dog populations. The GSD breed has remained widespread throughout the world since gaining popularity after World War I, and it could be deduced that the breed has had little or no reduction in population size. The DH breed was presumed to experience bottleneck events after each World War, although these may not have been detected because a new equilibrium would be attained after a number of generations. In addition, if the population numbers increased rapidly after the event, then surprisingly little diversity can have been lost. The modern SBT only developed as a companion dog after receiving official recognition in the 1930's, and becoming extremely popular in the latter half of the 20th century. This popularity has ensured a consistently large effective population size to date.

The homozygote-heterozygote proportions in the GSD, DH, SBT, and CB populations (F_{IS} = 0.054, 0.248, 0.053, and 0.252, respectively) indicated homozygous excess in all purebred dogs, most especially the DHs and CB group. There was a significant average differentiation between these populations (G_{ST} = 0.158, R_{ST} = 0.160); however, the estimates were less when the purebred dogs were compared with the OBD population (G_{ST} = 0.092, R_{ST} = 0.069). This would be indicative of the outbred heterogeneous nature of the founding individuals of many breeds of dogs. The pairwise G_{ST} and R_{ST} values consistently indicated significant differentiation (mean G_{ST} = 0.178, R_{ST} = 0.179) between the GSD, DH, and SBT populations, and between these populations and the OBDs (mean G_{ST} = 0.070, R_{ST} = 0.059). However, non-significant differentiation between the CB group and the OBD population (G_{ST} = 0.010, R_{ST} = 0.005) was indicative of the diverse nature of the founding populations with much interbreeding prior to the relatively recent origin of many modern breeds of dogs. The degree of population differentiation (G_{ST} = 0.092) among the purebred and mongrel dog populations reported in this study was similar to that detected (G_{ST} = 0.088) among the three main races of man, the Negroid, Mongoloid, and Caucasoid (Jordana *et al.* 1992). These high values of G_{ST} and R_{ST} are indicative of the occurrence of rapid genetic drift due to small population sizes.

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The number of loci in H-W equilibrium was another method of detecting population differentiation. Deviations from H-W equilibrium were statistically supported ($p < 0.001$) for the GSD, DH, and CB populations. This disequilibrium was indicative of intrabreed substructuring possibly due to the effects of inbreeding, the genetic isolation of individual breed types in the GSD and DH populations, and non-random mating associated with the breeding strategies used by dog breeders, and limited sample sizes. The deviations from equilibrium were not significant for the SBT population, and the breed was thus in H-W equilibrium. Therefore, despite moderately low levels of genetic diversity, there has been negligible inbreeding over recent generations and the breeding strategy used has been optimal. The SBT breed has a very large population size in South Africa, and originated relatively recently from diverse founding individuals bred primarily for performance.

Assignment tests correctly grouped almost all individuals in the GSD, DH, and SBT populations, indicting significant differentiation among these breeds. However, only 81% of the individuals in the CB group were correctly assigned, indicating a certain degree of homogeneity within the founding individuals of the various breed populations. The "breed barrier" rule ensures that gene flow is unidirectional, from the purebred dog populations to the mongrel population, with a proportion of OBD individuals having GSD, DH, or SBT ancestry, as indicated by the fact that only 84% of the OBD population was correctly assigned to its own source population.

The pairwise plots of the negative log likelihood of individuals in each purebred dog population being assigned to its own source population were graphic representations of the these assignment test results, and indicated moderate to significant levels of differentiation between the GSD, DH, SBT, CB, and OBD populations.

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5.4. Conclusions

The GSD breed could not be described as highly inbred, and does not appear to be in imminent danger of inbreeding depression or drastic loss of variation. Although, care must be taken in the future not to further decrease the current levels of genetic diversity.

Dog breeders would obviously intend mating their bitches with the best available stud dogs, however it must be taken into consideration that overusing a single dog could be detrimental to the breed as a whole. More effective levels of communication would perhaps result in the imported dogs being as unrelated as possible, many different bloodlines being brought into the country would provide a broader base from which breeders could choose a stud dog. It is a considerable financial investment to import good quality breeding stock and, considering the exchange rate, the purchase of a single dog could run to several hundred thousand rands. To receive a return on this investment, stud dog owners tend to accept as many matings as possible and are not likely to turn down enquiries for the use of their dog.

It must therefore be the responsibility of the breeder to take levels of genetic diversity into consideration when choosing a stud dog. One possible approach for the management of the genetic health of the breed would be to make use of the already available molecular data, with 12 microsatellite markers being routinely analysed for parentage verification. That is to say, if the breeder were to choose a selection of potential stud dogs according to the mental and physical traits that would best complement their bitch, but then permitted the final deciding factor to be the dog that contributed the most genetic diversity to the offspring as determined by these neutral markers. This would facilitate the preservation of genetic diversity in the breed.

The results of this study demonstrated that purebred dog populations are complex in nature. Many factors contribute to the observed genetic diversity, for although breeds of dogs are strictly selected for phenotypic traits, the breeds investigated were more heterogeneous than would have been expected from the analysis of their pedigrees.

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Chapter 6

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Appendix I

DNA Samples and Raw Data

A summarised table of the DNA samples included in this study, and the tabulated raw molecular data obtained from microsatellite marker analysis of 15 polymorphic loci.

Population	Sample Size	Region of Origin (South Africa)
Outbred Dogs	46	Cape Town, Western Cape
	42	Port Elizabeth, Eastern Cape
	30	Johannesburg, south-west Gauteng
	38	Pretoria, north-east Gauteng

Population	Sample Size	Country of Origin	Breed Type
German Shepherd Dogs	56	South Africa	Show
	8	South Africa	KUSA
	9	South Africa	Show / Sport (Shw/Sp)
	10	Germany	Show
	18	Germany	Sport
Dachshunds	8	South Africa	Standard short-coat
	13	South Africa	Miniature short-coat
	5	South Africa	Miniature long-coat
Staffordshire Bull Terriers	18	South Africa	-
Composite Breed Group	8	South Africa	Gundogs
	3	South Africa	Herding
	5	South Africa	Hounds
	5	South Africa	Terriers
	6	South Africa	Utility
	10	South Africa	Working

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Sample	Origin	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
OBD001	Cape Town	140	106	174	162	137	137	196	188	99	99	102	98	138	138	293	287	127	115	136	128	322	318	201	201	203	203	295	283	235	219
OBD002	Cape Town	144	94	186	174	141	137	188	180	101	93	98	98	138	130	287	287	125	117	144	128	346	314	209	209	207	187	295	287	231	223
OBD003	Cape Town	144	98	182	182	149	145	200	196	103	101	100	94	130	124	291	285	123	117	144	128	322	298	209	205	243	207	347	275	235	231
OBD004	Cape Town	140	136	178	170	145	141	204	188	107	95	98	98	138	130	289	287	119	117	128	128	326	314	213	209	251	243	303	295	235	231
OBD005	Cape Town	140	110	178	174	137	137	196	192	103	103	100	96	130	124	291	287	123	117	144	140	322	314	217	197	227	203	295	271	227	227
OBD006	Cape Town	140	132	162	158	145	137	200	188	101	93	90	90	136	130	291	291	125	115	156	140	326	322	209	209	243	207	319	295	231	227
OBD007	Cape Town	136	102	182	174	141	137	192	188	101	95	92	90	138	130	289	287	125	115	144	128	322	306	209	201	231	203	331	295	227	223
OBD008	Cape Town	144	102	182	170	137	133	188	188	101	97	98	98	132	122	291	287	117	115	148	128	322	322	205	201	207	207	299	283	235	227
OBD009	Cape Town	140	132	162	162	137	137	192	188	105	97	90	90	126	124	287	283	117	115	144	128	328	314	229	201	223	191	295	275	239	223
OBD010	Cape Town	136	132	188	162	165	137	208	196	101	99	94	94	130	124	287	287	125	113	144	128	322	322	201	201	223	223	291	287	231	231
OBD011	Cape Town	144	144	182	182	137	137	208	188	103	91	98	90	140	128	289	287	129	117	140	136	322	314	209	209	243	207	295	291	231	231
OBD012	Cape Town	136	98	178	162	137	137	192	180	107	91	96	96	138	124	291	287	129	115	152	144	314	274	205	201	243	199	331	295	231	231
OBD013	Cape Town	144	110	182	166	137	137	196	192	107	95	92	90	142	132	287	287	117	117	144	136	334	314	205	201	207	203	303	291	239	231
OBD014	Cape Town	144	136	174	166	141	137	196	188	105	101	92	90	132	130	287	285	125	125	144	128	318	318	213	197	243	211	291	291	235	227
OBD015	Cape Town	132	106	170	166	157	129	204	200	107	103	96	92	134	134	289	287	127	121	144	144	322	318	201	197	203	199	295	283	239	235
OBD016	Cape Town	144	94	182	162	157	137	196	188	101	101	96	96	140	130	291	283	123	123	144	144	318	310	221	197	235	203	295	287	227	223
OBD017	Cape Town	140	98	188	182	137	137	188	188	105	101	96	90	142	142	291	291	117	117	136	128	322	314	201	197	243	231	291	287	223	223
OBD018	Cape Town	106	106	182	166	165	133	204	196	105	91	94	92	138	134	289	287	117	117	144	140	322	314	209	197	231	183	343	283	235	227
OBD019	Cape Town	98	98	186	166	137	137	208	208	107	91	96	94	134	134	289	287	123	117	140	140	322	314	209	201	239	227	299	295	235	219
OBD020	Cape Town	132	106	170	170	137	133	212	180	101	97	90	86	134	132	287	287	117	115	144	128	322	318	209	209	251	215	295	283	235	223
OBD021	Cape Town	140	140	178	170	141	137	208	192	105	101	94	86	124	124	291	287	117	115	148	128	330	314	209	201	203	199	295	295	223	223
OBD022	Cape Town	144	106	186	178	165	137	192	188	103	101	92	92	0	0	0	0	117	115	0	0	318	318	197	197	215	215	291	283	239	239
OBD023	Cape Town	136	132	170	162	133	133	196	196	101	91	102	96	134	130	289	289	117	115	144	144	318	310	209	197	203	203	343	291	243	235
OBD024	Cape Town	132	132	178	166	161	129	208	188	99	97	96	90	140	124	287	287	125	115	140	128	322	322	213	213	251	227	307	299	227	223
OBD025	Cape Town	132	132	186	186	137	137	208	196	105	105	100	92	140	130	291	287	123	117	144	128	346	322	205	201	207	203	335	283	235	231
OBD026	Cape Town	140	132	186	170	149	141	204	200	99	97	94	90	134	130	287	287	123	123	140	140	318	298	213	201	235	203	303	291	231	227
OBD027	Cape Town	106	106	186	166	165	137	208	196	103	95	96	92	142	130	291	291	123	119	140	140	342	314	209	197	231	227	335	299	243	235
OBD028	Cape Town	136	132	186	166	157	133	196	192	103	97	102	88	122	122	291	287	123	115	144	128	314	302	201	183	231	187	347	283	227	223
OBD029	Cape Town	136	102	194	188	137	137	208	200	99	99	90	90	130	124	291	289	123	117	144	140	318	274	209	205	247	195	279	279	235	227
OBD030	Cape Town	136	106	186	166	141	137	200	188	107	99	100	90	130	124	289	289	131	117	152	144	314	314	209	201	207	203	351	295	235	223
OBD031	Cape Town	144	144	170	158	157	133	208	200	101	95	90	88	140	132	291	287	123	123	140	128	318	318	201	201	239	231	299	299	235	223
OBD032	Cape Town	144	136	178	162	145	137	208	188	97	93	100	94	138	124	291	283	117	113	140	132	326	318	213	197	247	215	291	279	235	235

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Sample	Origin	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
OBD071	Port Elizabeth	132	102	170	162	161	137	188	188	99	91	98	90	142	130	287	285	123	115	140	128	330	314	201	197	231	215	315	287	235	223
OBD072	Port Elizabeth	140	94	182	166	133	133	192	192	103	99	98	86	130	124	291	289	123	119	140	120	338	314	209	197	235	223	307	299	247	235
OBD073	Port Elizabeth	140	140	178	162	145	133	196	192	105	103	98	92	140	140	293	289	123	117	140	128	322	314	209	205	231	211	299	291	235	231
OBD074	Port Elizabeth	140	140	186	166	137	137	200	188	103	95	98	90	138	138	287	287	115	115	152	148	318	306	213	193	199	183	343	315	231	227
OBD075	Port Elizabeth	144	136	170	166	141	129	200	200	97	93	92	90	130	122	291	287	123	115	140	128	322	306	201	197	243	239	339	331	235	223
OBD076	Port Elizabeth	136	132	186	182	137	133	204	188	103	91	98	92	138	134	291	291	123	119	140	140	322	274	221	217	207	191	315	299	231	227
OBD077	Port Elizabeth	136	102	182	182	137	137	188	188	103	97	98	98	134	134	289	287	117	115	0	0	322	322	205	201	207	195	275	275	231	215
OBD078	Port Elizabeth	132	132	132	170	166	153	137	188	188	101	99	92	90	138	134	287	287	123	123	128	128	338	322	213	205	231	223	295	283	239
OBD079	Port Elizabeth	140	136	182	178	149	145	208	200	103	95	94	90	136	128	289	287	123	117	140	132	334	322	201	197	243	219	291	275	235	235
OBD080	Port Elizabeth	140	140	182	182	149	137	208	204	107	103	94	90	136	132	287	287	123	117	140	128	342	334	201	197	243	219	287	275	235	219
OBD081	Port Elizabeth	144	136	182	166	149	137	204	200	99	97	98	98	142	142	287	287	129	115	140	140	326	298	205	205	227	215	295	291	231	231
OBD082	Port Elizabeth	132	132	182	170	141	133	188	180	101	87	96	94	138	124	287	287	115	115	144	144	326	318	209	213	219	207	295	295	239	219
OBD083	Port Elizabeth	140	110	178	162	165	133	200	188	99	95	90	90	140	134	287	287	119	117	144	132	322	318	205	197	203	191	331	319	231	231
OBD084	Port Elizabeth	132	132	182	174	157	133	220	208	113	105	98	90	138	122	287	287	123	123	128	128	322	314	213	197	195	191	295	291	223	223
OBD085	Port Elizabeth	132	128	166	162	141	137	200	188	95	91	96	90	130	124	289	287	117	115	144	140	322	306	197	197	247	191	291	279	243	223
OBD086	Port Elizabeth	136	132	178	170	141	137	212	200	99	99	94	90	126	122	291	287	123	117	140	140	326	326	197	189	195	187	303	291	231	219
OBD087	Port Elizabeth	136	136	182	162	157	141	204	200	101	101	92	90	130	130	291	283	125	117	128	128	322	322	213	209	243	199	343	295	227	223
OBD088	Port Elizabeth	136	94	182	166	141	141	196	196	107	101	98	90	134	130	291	287	123	117	140	132	346	318	217	197	231	195	295	291	235	231
OBD089	Port Elizabeth	132	102	162	162	157	137	196	196	105	101	90	90	130	130	289	287	119	119	144	140	318	306	209	197	231	207	311	279	227	223
OBD090	Port Elizabeth	136	136	174	162	149	149	204	196	103	95	98	90	140	140	287	287	119	115	144	144	318	318	209	201	223	215	287	287	223	223
OBD091	Port Elizabeth	144	132	166	162	145	137	192	188	101	101	98	90	138	124	287	285	121	115	144	140	322	318	213	205	227	223	299	295	235	227
OBD092	Port Elizabeth	132	94	182	178	165	161	188	180	101	101	90	86	142	138	287	287	123	115	140	128	314	314	201	197	231	203	331	295	227	223
OBD093	Port Elizabeth	132	132	174	174	145	137	192	192	101	101	102	98	138	138	291	285	123	119	148	140	326	322	205	197	195	195	279	279	235	223
OBD094	Port Elizabeth	140	140	182	162	157	145	200	200	103	99	98	90	140	130	291	291	123	117	140	128	302	302	197	197	223	203	295	295	227	227
OBD101	Johannesburg	136	98	170	170	153	137	200	196	99	97	90	90	124	124	291	287	125	119	144	132	338	318	209	205	247	187	311	279	235	227
OBD102	Johannesburg	0	0	0	0	0	0	0	0	99	95	92	90	130	124	0	0	125	115	0	0	0	0	0	0	0	0	0	0	0	0
OBD103	Johannesburg	132	132	178	162	137	137	204	204	105	91	92	90	134	134	291	287	125	117	140	136	314	274	213	201	223	195	335	279	231	227
OBD104	Johannesburg	144	98	178	170	157	137	208	192	101	99	98	94	140	130	291	281	117	115	132	128	322	314	221	197	191	191	347	279	235	219
OBD105	Johannesburg	140	98	182	182	157	133	180	180	105	101	94	90	142	142	287	287	119	117	140	128	326	314	201	193	255	251	295	283	231	227
OBD106	Johannesburg	106	106	174	166	157	133	188	188	101	101	90	90	138	130	295	287	117	117	140	140	314	314	209	209	195	195	295	283	235	223
OBD107	Johannesburg	132	132	174	166	153	137	196	192	101	101	96	90	124	124	285	285	125	119	144	144	322	322	205	205	211	203	339	279	223	223

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Sample	Origin	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
OBD108	Johannesburg	140	128	170	162	157	137	204	180	97	93	96	92	130	124	291	287	127	123	144	144	314	302	201	197	231	207	299	271	235	223
OBD109	Johannesburg	136	106	182	162	153	137	212	200	101	97	96	90	134	124	289	287	123	119	140	136	322	302	209	201	255	203	295	291	231	223
OBD110	Johannesburg	140	136	166	162	137	133	204	188	101	101	94	90	138	138	0	0	119	117	0	0	0	0	0	0	0	0	0	0	0	0
OBD111	Johannesburg	140	102	102	162	158	169	161	196	196	103	103	94	90	130	124	289	287	119	117	140	140	326	318	209	205	199	195	311	291	235
OBD112	Johannesburg	140	140	162	162	133	129	196	192	107	93	96	90	138	124	291	289	123	119	152	144	314	314	201	197	207	195	291	279	235	231
OBD113	Johannesburg	144	144	182	166	153	153	212	192	91	91	96	90	130	130	291	291	117	117	136	136	326	318	217	217	227	187	319	299	227	227
OBD114	Johannesburg	136	132	182	182	153	141	188	188	107	101	96	96	130	124	287	287	117	115	140	128	318	318	213	209	239	239	295	283	235	223
OBD115	Johannesburg	132	102	166	158	137	137	196	196	101	99	96	90	124	124	289	287	129	117	140	128	330	318	197	193	231	199	295	287	239	223
OBD116	Johannesburg	132	132	166	166	153	137	196	196	101	101	96	90	124	124	287	285	125	119	144	144	322	322	221	209	227	203	299	275	227	223
OBD117	Johannesburg	108	106	166	162	137	133	192	192	97	97	96	96	124	124	289	287	117	117	140	140	346	322	209	197	191	191	299	287	231	223
OBD118	Johannesburg	140	140	182	170	137	129	212	192	101	95	100	90	134	130	287	285	125	115	140	128	322	302	213	201	235	227	327	311	239	219
OBD119	Johannesburg	140	140	186	186	145	137	208	196	105	101	98	98	130	130	287	283	123	117	140	128	318	314	213	197	227	223	315	307	231	231
OBD120	Johannesburg	136	98	178	178	137	137	192	192	103	93	96	90	130	122	285	285	123	123	148	128	318	310	209	197	243	199	307	287	235	223
OBD121	Johannesburg	132	132	166	162	137	137	200	200	95	91	96	96	124	122	291	283	123	123	140	128	322	322	217	201	203	203	279	279	227	227
OBD122	Johannesburg	140	110	166	166	137	133	208	196	103	91	94	92	140	138	291	287	123	117	136	128	346	318	205	197	223	203	295	279	239	231
OBD123	Johannesburg	136	110	166	162	153	133	212	188	101	97	96	90	134	124	291	289	123	119	140	132	322	322	201	189	255	227	315	291	227	223
OBD124	Johannesburg	144	140	182	166	145	141	196	188	105	103	92	92	124	124	289	285	125	123	128	128	334	330	209	197	243	243	295	279	235	219
OBD125	Johannesburg	98	98	178	170	157	137	196	196	111	99	96	92	0	0	291	291	125	117	144	144	326	318	209	197	239	191	335	311	231	231
OBD126	Johannesburg	110	98	186	186	137	137	208	188	99	93	102	92	138	138	291	287	123	123	144	128	0	0	213	209	199	191	0	0	227	227
OBD127	Johannesburg	136	110	166	162	153	137	208	196	99	91	98	90	138	138	287	285	123	123	136	128	346	334	205	197	227	203	295	279	239	231
OBD128	Johannesburg	0	0	0	0	0	0	0	0	99	91	92	90	142	130	0	0	125	115	0	0	0	0	0	0	0	0	0	0	0	0
OBD129	Johannesburg	136	136	166	162	137	137	204	200	105	99	98	92	130	130	291	285	125	117	140	140	322	322	201	197	239	179	339	307	243	239
OBD130	Johannesburg	148	136	186	182	149	137	192	192	107	93	92	86	132	132	0	0	131	117	0	0	0	0	0	0	0	0	0	0	0	0
OBD131	Pretoria	144	140	178	174	149	137	188	180	103	93	96	90	140	138	287	287	123	117	144	144	322	302	209	189	227	191	319	283	227	227
OBD132	Pretoria	140	98	178	170	137	133	196	196	95	93	94	90	124	124	287	287	123	117	148	128	326	318	209	197	203	195	335	295	235	223
OBD133	Pretoria	140	140	170	170	157	137	204	196	99	95	94	90	142	142	0	0	123	117	152	152	0	0	0	0	0	0	0	0	215	215
OBD134	Pretoria	132	132	166	162	157	145	204	200	99	95	100	96	134	130	285	285	123	117	144	132	314	314	205	197	231	183	343	275	239	223
OBD135	Pretoria	136	102	182	162	157	141	208	208	99	99	96	96	140	140	291	291	119	119	140	136	302	274	213	197	203	199	307	307	235	223
OBD136	Pretoria	132	132	162	158	153	137	208	188	105	93	92	90	140	130	287	287	123	115	144	128	0	0	201	197	199	199	0	0	219	219
OBD137	Pretoria	136	136	190	186	137	133	192	188	111	91	100	96	142	130	291	289	127	123	144	140	322	314	205	201	219	191	347	291	235	227
OBD138	Pretoria	136	136	190	162	137	137	200	192	91	91	96	96	136	130	287	285	125	115	132	128	322	310	201	197	239	187	319	299	231	223

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Sample	Origin	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
OBD139	Pretoria	132	132	166	166	153	141	208	196	101	91	102	92	138	130	291	285	117	117	128	128	322	318	209	197	203	203	291	291	227	223
OBD140	Pretoria	136	98	186	178	137	137	204	200	99	95	92	92	138	122	295	287	123	117	148	128	322	306	189	189	263	187	295	295	235	231
OBD141	Pretoria	144	136	186	178	153	145	192	188	101	93	96	92	130	124	291	283	123	123	144	128	322	322	197	189	251	243	335	283	227	227
OBD142	Pretoria	144	132	190	162	141	137	196	188	103	101	96	96	140	124	291	287	117	115	140	140	318	314	205	197	203	171	351	283	239	227
OBD143	Pretoria	136	136	162	158	145	137	204	188	91	91	100	98	130	124	289	285	127	117	144	128	334	318	209	201	247	227	283	283	231	231
OBD144	Pretoria	132	132	170	166	165	133	188	188	101	101	102	92	124	124	287	287	125	117	128	128	314	306	213	213	227	191	303	283	243	223
OBD145	Pretoria	132	132	186	166	141	137	204	196	105	103	96	94	142	134	289	285	129	125	148	128	326	326	209	197	223	223	307	299	231	227
OBD146	Pretoria	136	136	178	162	157	141	200	196	103	95	98	98	138	130	287	283	123	115	140	140	326	322	229	197	247	203	239	291	239	231
OBD147	Pretoria	140	140	178	162	169	145	204	196	101	97	102	90	142	134	289	287	123	115	128	124	322	314	217	205	231	215	327	275	235	215
OBD148	Pretoria	132	132	186	182	133	133	188	180	103	101	94	90	138	130	289	285	125	123	140	128	318	306	201	197	247	195	295	295	223	223
OBD149	Pretoria	140	136	186	166	145	141	196	180	103	97	100	98	134	130	289	287	123	117	148	148	334	330	213	197	235	203	295	283	231	227
OBD150	Pretoria	136	136	178	178	157	133	212	192	103	101	92	92	138	130	291	287	125	113	128	128	322	314	205	197	231	203	343	287	243	231
OBD151	Pretoria	114	102	182	162	149	145	188	188	105	99	90	90	130	130	289	287	129	223	148	144	246	246	209	201	191	191	343	295	239	219
OBD152	Pretoria	136	98	174	166	141	137	196	188	103	91	98	90	142	138	287	285	121	117	140	128	322	318	213	197	227	195	291	291	231	231
OBD153	Pretoria	98	98	178	162	137	137	204	204	93	93	100	98	138	138	287	285	123	123	140	140	326	326	205	205	215	203	323	291	239	235
OBD154	Pretoria	140	102	190	162	137	133	192	192	105	91	96	90	130	126	291	287	117	115	140	140	318	306	213	209	203	199	295	291	235	231
OBD155	Pretoria	132	132	166	162	137	137	200	188	99	91	100	98	130	124	289	285	129	117	144	128	334	318	209	201	247	227	283	283	231	231
OBD156	Pretoria	132	102	182	182	137	133	188	180	105	101	90	90	140	140	289	287	125	123	144	128	326	318	205	197	239	207	295	283	235	231
OBD157	Pretoria	132	102	182	162	137	133	192	180	99	91	98	98	142	130	289	289	123	123	148	144	322	318	205	197	223	191	295	287	223	223
OBD158	Pretoria	132	102	182	162	137	133	204	180	101	101	90	90	140	124	289	287	125	125	160	144	318	318	209	209	207	207	295	295	235	235
OBD159	Pretoria	140	136	170	166	137	137	216	212	103	101	92	92	130	124	289	287	125	123	144	128	314	314	201	197	247	203	343	307	243	231
OBD160	Pretoria	136	102	178	178	145	137	196	196	101	97	94	92	130	130	287	287	123	119	140	140	338	298	197	197	207	195	291	283	239	227
OBD161	Pretoria	140	136	186	170	141	137	200	200	99	95	96	96	138	130	287	285	123	115	140	140	318	298	217	197	223	215	295	283	223	223
OBD162	Pretoria	144	144	186	162	137	133	200	196	103	101	92	90	124	124	291	285	125	115	152	152	322	318	217	197	203	199	319	291	231	231
OBD163	Pretoria	136	132	162	162	157	153	208	208	101	99	98	90	130	130	291	287	123	119	144	140	314	274	217	205	207	195	307	295	239	231
OBD164	Pretoria	110	110	182	170	141	137	200	196	107	91	92	92	130	130	287	285	115	115	140	124	314	306	209	189	231	227	291	291	235	231
OBD165	Pretoria	132	132	190	166	145	141	204	180	107	101	90	92	134	132	295	287	115	115	144	144	322	322	209	197	231	199	307	299	235	231
OBD166	Pretoria	140	132	170	162	165	153	204	200	105	101	90	96	138	138	291	285	123	123	144	140	314	306	221	205	239	207	311	283	235	227
OBD167	Pretoria	132	102	162	158	141	141	200	188	113	85	90	90	138	134	287	287	117	115	140	140	342	306	209	193	207	203	283	279	227	227
OBD168	Pretoria	140	136	182	166	141	137	200	200	99	95	96	90	138	130	293	293	123	123	140	128	318	318	197	197	223	215	295	295	235	223

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Sample	Origin	Type	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
GSD001	South Africa	Show	132	132	170	170	133	133	180	180	101	93	98	94	138	138	293	287	125	115	140	140	318	314	209	201	199	195	295	295	243	243
GSD002	South Africa	Shw/Sp	132	132	182	170	133	129	180	180	101	101	94	92	0	0	287	287	115	115	140	140	318	314	201	197	211	207	299	295	223	223
GSD003	South Africa	Shw/Sp	132	132	182	162	133	129	180	180	101	101	92	90	138	130	287	287	125	115	140	128	318	314	201	197	203	199	299	295	223	223
GSD004	South Africa	KUSA	132	132	182	170	141	137	188	180	101	101	98	94	138	138	287	287	125	115	144	128	318	314	209	209	203	199	299	291	239	223
GSD005	Germany	Sport	132	94	182	170	137	137	200	188	101	99	90	90	138	124	287	287	115	115	128	128	314	314	209	201	207	203	295	295	239	223
GSD006	South Africa	Show	132	132	170	170	157	157	188	180	101	101	94	90	138	130	293	287	125	115	140	140	326	314	205	205	207	207	295	291	227	223
GSD007	South Africa	Shw/Sp	132	132	170	170	141	137	180	180	99	99	98	90	138	130	293	287	115	115	144	140	314	314	209	205	207	207	299	291	239	231
GSD008	Germany	Show	132	132	170	166	137	133	188	180	101	93	94	90	124	124	287	287	125	115	144	140	326	314	209	209	195	195	295	291	227	223
GSD009	South Africa	KUSA	132	132	182	170	157	157	188	180	101	101	94	94	138	138	293	287	117	115	144	140	318	318	209	201	243	243	299	299	239	235
GSD010	Germany	Sport	132	132	186	186	157	145	180	180	101	99	90	90	142	130	287	287	125	115	140	128	314	314	201	201	243	207	295	291	235	235
GSD011	Germany	Sport	132	132	186	182	137	137	188	188	103	101	94	90	138	130	287	287	117	115	144	128	326	318	209	197	207	207	295	283	235	223
GSD012	South Africa	Show	132	132	182	170	157	133	180	180	101	101	94	94	124	124	287	287	125	125	144	140	318	314	209	201	243	203	299	291	239	235
GSD013	South Africa	Show	132	132	182	170	157	133	200	188	103	101	94	90	130	130	293	293	125	115	144	140	318	314	209	205	243	207	295	291	235	223
GSD014	South Africa	Show	132	132	170	166	133	133	180	180	101	101	94	92	138	138	287	287	125	115	140	140	318	318	205	197	211	199	299	295	235	223
GSD015	South Africa	KUSA	132	102	190	182	157	137	188	188	107	101	98	90	138	138	287	287	125	115	144	128	314	314	209	209	243	199	299	291	235	235
GSD016	South Africa	KUSA	132	132	182	170	157	137	180	180	101	101	94	94	138	124	293	287	125	115	144	128	318	314	209	201	243	199	299	299	239	235
GSD017	South Africa	Show	132	132	182	166	141	133	188	180	101	101	96	94	0	0	287	287	125	115	140	128	318	318	201	197	211	199	299	295	235	235
GSD018	Germany	Sport	132	94	182	170	133	133	188	180	99	99	90	90	124	124	287	287	115	115	144	128	314	314	209	201	207	203	295	295	235	235
GSD019	South Africa	Shw/Sp	132	132	182	162	133	129	180	180	101	101	94	94	0	0	287	287	125	117	140	128	318	314	205	201	211	207	299	295	239	223
GSD020	South Africa	Show	132	132	170	166	133	133	188	188	101	101	92	90	138	138	287	287	115	115	140	128	318	314	205	205	211	207	295	295	235	227
GSD021	Germany	Sport	132	132	182	166	157	133	188	180	101	101	94	92	138	122	287	287	125	115	144	140	318	314	209	201	207	203	299	295	235	223
GSD022	Germany	Sport	132	132	178	170	133	129	188	180	103	103	92	88	138	138	293	285	125	115	140	128	326	314	209	201	243	243	295	295	239	223
GSD023	Germany	Sport	140	132	178	170	133	129	188	186	101	101	96	90	0	0	287	287	125	125	140	140	314	314	209	205	243	199	299	295	247	239
GSD024	Germany	Show	132	132	170	166	157	133	180	180	101	93	90	90	138	138	293	287	125	115	144	140	318	314	205	201	235	195	295	291	235	223
GSD025	Germany	Show	132	132	170	170	141	137	188	180	107	101	94	86	124	124	293	287	125	125	140	128	322	318	205	201	199	195	295	295	239	227
GSD026	Germany	Show	132	132	182	170	133	133	180	180	103	101	90	86	138	138	287	287	125	115	144	140	326	318	205	201	199	195	295	291	235	223
GSD027	South Africa	Show	132	132	170	170	141	137	180	180	99	99	96	90	138	130	293	287	115	115	144	140	314	314	209	205	207	207	299	291	239	231
GSD028	South Africa	Show	132	132	182	170	141	137	188	180	103	101	94	90	128	122	287	287	117	115	144	128	322	314	209	209	207	207	299	299	243	235
GSD029	South Africa	Show	132	132	182	170	141	133	180	180	101	93	90	90	138	138	293	287	125	115	128	128	326	314	209	209	207	207	295	295	239	223
GSD030	Germany	Sport	132	132	186	178	141	133	188	180	107	97	94	90	142	138	287	287	117	115	144	128	354	314	209	201	207	207	295	295	243	235
GSD031	South Africa	Show	132	132	186	166	161	133	188	188	101	101	96	90	138	138	287	287	117	115	144	128	326	314	209	201	203	195	295	295	235	235

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Sample	Origin	Type	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
GSD032	Germany	Sport	132	102	194	170	161	137	200	188	101	99	96	90	138	138	287	287	117	117	128	128	322	314	213	205	211	207	289	295	247	223
GSD033	South Africa	Shw/Sp	132	132	182	170	141	137	188	180	101	99	94	90	138	138	287	287	125	117	144	140	314	314	209	205	203	199	295	295	223	223
GSD034	Germany	Sport	132	132	182	170	137	133	188	188	99	93	96	94	138	130	293	285	125	125	140	128	326	314	209	197	207	207	295	287	227	223
GSD035	Germany	Sport	140	132	186	182	157	141	188	188	101	101	96	96	138	138	293	287	115	115	144	140	314	314	209	209	247	207	295	295	243	231
GSD036	South Africa	Show	132	132	186	166	141	133	180	180	101	99	96	90	138	124	0	0	125	117	144	128	314	314	209	205	243	207	299	299	239	223
GSD037	South Africa	Show	132	132	178	170	133	133	188	188	101	101	98	86	138	138	0	0	125	115	144	140	326	318	209	209	199	195	295	295	239	235
GSD038	Germany	Sport	132	132	182	170	157	133	188	180	103	101	98	90	138	124	287	287	117	115	140	128	314	314	205	205	211	207	295	291	223	219
GSD039	Germany	Sport	132	110	182	162	141	137	180	180	99	79	98	90	138	130	293	287	125	115	140	128	314	314	209	205	247	207	295	295	235	223
GSD040	South Africa	Show	132	132	178	170	141	137	188	180	103	101	94	94	138	138	287	287	115	115	144	140	330	314	209	201	199	195	299	291	227	223
GSD041	Germany	Show	132	132	182	170	137	133	180	180	101	101	90	90	0	0	287	287	117	117	144	144	318	314	209	205	243	195	295	291	235	223
GSD042	South Africa	Show	132	132	170	170	157	141	188	188	101	93	98	86	124	124	295	287	115	115	144	140	314	314	209	209	195	195	299	291	235	235
GSD043	South Africa	Show	132	132	166	166	137	133	180	180	101	101	98	90	130	130	293	287	125	115	140	128	314	314	213	201	247	195	295	291	235	235
GSD044	Germany	Show	132	132	186	170	133	133	180	180	103	101	94	90	0	0	287	285	125	115	144	144	314	314	209	205	207	207	299	299	235	227
GSD045	South Africa	Show	132	132	182	166	141	141	188	186	101	101	90	90	138	124	0	0	0	0	0	0	314	314	0	0	0	0	0	0	0	0
GSD046	Germany	Show	132	132	182	170	133	133	188	180	101	93	94	90	138	138	287	287	115	115	144	140	314	314	209	209	195	195	295	295	235	235
GSD047	South Africa	Show	132	132	170	166	157	141	188	180	101	93	90	86	138	124	293	287	115	115	144	140	326	314	209	205	247	195	295	291	239	223
GSD048	South Africa	KUSA	132	132	182	170	141	137	188	188	101	101	90	90	138	138	293	287	125	117	128	128	314	314	209	205	195	195	295	295	235	223
GSD049	South Africa	Show	132	132	182	170	133	133	188	180	101	101	96	94	138	138	287	287	115	115	144	128	314	314	205	201	243	211	291	291	239	223
GSD050	South Africa	Show	132	132	186	182	141	133	188	180	103	103	90	86	0	0	287	287	115	115	140	128	318	318	209	205	207	195	295	291	235	235
GSD051	South Africa	KUSA	136	132	170	166	141	133	188	180	101	99	96	94	0	0	287	287	125	115	140	128	318	314	209	205	223	207	299	295	235	223
GSD052	South Africa	Show	132	132	182	166	133	133	188	180	101	101	94	90	122	122	293	287	115	115	140	128	314	314	209	201	207	195	295	295	235	223
GSD053	South Africa	Show	132	132	170	170	157	145	188	180	103	101	94	86	124	124	287	287	115	115	128	128	314	314	209	201	203	195	295	291	243	235
GSD054	South Africa	Show	132	132	170	166	157	141	180	180	103	101	90	90	138	124	297	287	125	117	144	140	314	314	205	201	243	207	299	295	239	227
GSD055	South Africa	Show	132	132	170	166	157	157	188	180	101	101	98	90	122	122	287	285	117	115	144	128	322	314	209	201	199	195	299	295	235	227
GSD056	South Africa	Show	132	132	170	166	157	141	188	180	103	101	96	86	124	124	287	287	117	115	140	128	314	314	209	209	203	195	289	291	243	235
GSD057	South Africa	Show	132	132	170	166	137	137	188	180	101	93	94	90	138	124	287	287	125	115	140	128	318	314	209	201	203	199	291	291	239	223
GSD058	South Africa	Show	132	132	182	170	133	133	188	180	101	101	98	94	138	138	293	287	125	115	128	128	318	314	209	205	199	195	295	291	239	223
GSD059	South Africa	Show	140	136	182	170	141	133	188	180	101	101	98	90	138	138	287	287	125	115	144	140	318	318	201	201	195	195	289	295	239	223
GSD060	South Africa	Show	132	132	186	178	157	133	188	180	103	93	94	86	138	124	287	287	125	115	144	140	318	314	209	209	203	195	295	295	235	223
GSD061	South Africa	Show	132	132	182	182	157	157	188	180	103	101	96	96	0	0	287	285	125	115	144	128	326	318	209	209	195	195	295	295	235	235
GSD062	South Africa	Show	132	132	166	166	157	133	188	186	103	101	96	96	138	138	293	287	125	115	144	132	326	314	209	201	195	191	295	295	235	235
GSD063	South Africa	Show	132	132	170	166	157	133	188	186	103	103	96	90	138	124	287	287	125	115	132	128	314	310	209	201	195	195	299	295	235	223

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Sample	Origin	Type	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
GSD064	South Africa	Show	132	102	170	170	137	133	188	180	103	101	98	90	138	138	293	287	115	115	140	140	322	314	209	205	199	199	295	291	243	223
GSD065	Germany	Show	132	102	170	170	137	133	188	180	101	101	90	90	124	124	287	287	125	125	0	0	314	314	0	0	207	203	295	295	239	235
GSD066	South Africa	Show	132	132	186	166	133	133	188	188	101	93	90	90	138	138	287	287	125	115	144	128	314	314	213	201	203	203	295	295	239	235
GSD067	Germany	Show	132	132	170	166	137	133	180	180	101	93	96	90	124	124	287	287	125	117	144	140	314	314	209	205	195	195	291	287	235	227
GSD068	South Africa	Show	132	132	170	166	137	133	188	188	101	101	94	94	138	138	287	287	125	115	140	140	318	314	209	205	207	203	295	295	235	223
GSD069	South Africa	Show	132	94	170	170	137	137	188	188	101	93	94	94	0	0	287	287	125	115	0	0	314	314	213	209	227	203	295	291	243	239
GSD070	South Africa	Shw/Sp	132	132	186	170	137	133	180	180	101	97	94	86	124	124	293	287	117	115	144	128	314	314	209	205	211	207	299	299	235	223
GSD071	Germany	Sport	132	132	186	170	161	137	188	188	93	93	94	90	138	138	293	287	125	117	128	128	318	314	209	197	207	203	291	287	235	227
GSD072	South Africa	Show	136	132	162	170	141	133	188	188	101	101	96	90	138	138	287	287	125	125	144	140	318	318	201	201	207	195	295	295	235	223
GSD073	South Africa	Show	132	106	166	166	157	133	188	180	103	93	96	94	138	138	287	287	125	117	140	140	326	314	209	201	195	195	295	295	239	235
GSD074	South Africa	Show	132	132	162	166	133	133	188	180	103	101	94	92	138	138	287	287	125	125	140	140	326	314	209	209	199	195	295	291	235	227
GSD075	South Africa	KUSA	132	132	182	170	161	137	188	180	101	101	94	94	124	124	287	287	115	115	140	128	318	318	209	209	243	239	295	291	243	239
GSD076	South Africa	Shw/Sp	132	132	170	166	137	137	188	188	101	101	94	94	130	130	287	287	125	115	144	128	318	318	209	201	243	207	295	291	243	227
GSD077	Germany	Sport	106	94	182	170	137	133	188	188	101	93	90	90	124	124	287	287	125	115	140	128	318	314	201	197	207	203	299	295	235	223
GSD078	South Africa	KUSA	132	106	182	170	157	141	180	180	107	101	96	86	138	138	287	287	125	115	144	128	318	318	209	209	203	199	299	295	223	223
GSD079	Germany	Sport	132	132	186	186	137	133	188	188	103	101	90	90	138	138	287	287	125	117	128	128	326	326	209	209	207	203	295	291	235	227
GSD080	South Africa	Show	132	132	170	166	157	157	188	180	103	101	94	90	130	122	287	287	125	115	144	140	314	314	205	201	243	207	295	295	227	227
GSD081	South Africa	Show	132	132	186	170	137	137	188	188	101	93	96	90	138	138	287	287	125	115	128	128	326	318	209	205	251	207	299	295	227	223
GSD082	South Africa	Show	132	132	182	166	141	133	188	180	101	93	94	86	138	138	287	287	125	125	144	128	318	314	209	209	243	199	295	295	239	223
GSD083	Germany	Show	132	94	182	166	141	133	188	180	101	101	96	96	138	138	287	287	117	117	128	128	318	314	209	209	207	203	295	295	239	239
GSD084	South Africa	Show	132	132	182	170	157	157	188	180	101	101	98	94	138	138	293	287	115	115	144	140	326	314	209	205	247	195	299	291	243	227
GSD085	South Africa	Show	132	132	186	170	141	133	180	180	101	101	94	90	124	124	287	287	115	115	148	140	322	314	209	209	207	207	295	295	235	223
GSD086	South Africa	Show	136	132	174	170	157	129	188	180	103	101	98	90	122	122	287	287	115	115	144	128	318	318	213	209	243	239	295	295	235	223
GSD087	South Africa	Show	132	132	186	170	157	157	188	180	103	101	90	90	142	142	297	287	125	115	144	140	318	306	209	185	195	195	295	287	235	227
GSD088	South Africa	Show	132	132	170	170	157	157	188	180	101	93	94	90	138	138	287	287	125	115	144	144	318	314	205	201	247	243	295	287	235	227
GSD089	South Africa	Show	140	132	182	166	141	137	188	180	101	101	90	86	130	130	287	287	125	125	144	128	318	314	209	205	243	207	295	295	235	223
GSD090	South Africa	Show	132	132	186	170	157	133	188	180	101	93	90	90	138	138	287	287	125	117	144	140	326	314	205	201	247	207	295	287	235	227
GSD091	South Africa	Show	132	132	170	170	133	133	188	188	101	101	94	94	138	128	291	287	125	115	144	128	318	318	209	201	247	207	295	295	243	223
GSD092	South Africa	Show	132	106	186	166	133	133	188	180	101	101	94	94	138	138	295	287	125	125	128	128	318	314	209	205	203	195	299	295	227	227
GSD093	South Africa	Show	148	132	186	186	161	137	208	192	101	101	98	90	126	124	293	287	125	117	144	140	322	318	205	205	219	203	295	287	223	223
GSD094	South Africa	Show	148	132	182	174	161	137	208	192	101	101	96	90	126	124	293	287	125	117	144	140	322	318	205	205	219	203	295	287	243	223
GSD095	South Africa	Shw/Sp	132	132	170	170	153	129	188	180	101	101	94	90	130	130	287	285	125	115	144	144	314	314	205	197	207	203	295	295	235	235

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Sample	Origin	Type	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
GSD096	Germany	Sport	132	132	186	186	137	137	188	188	103	101	94	92	138	138	287	287	125	115	144	128	318	314	209	209	207	203	295	287	235	223
GSD097	South Africa	Show	132	132	170	170	153	137	188	180	101	101	94	90	138	124	297	287	117	115	140	128	318	314	209	201	207	203	299	295	235	223
GSD098	South Africa	Show	140	132	170	170	153	133	188	188	101	101	90	90	138	138	287	287	125	115	144	144	0	0	209	205	199	195	0	0	0	0
GSD099	South Africa	Shw/Sp	132	132	186	170	137	133	188	188	103	101	98	94	138	138	293	285	115	115	128	128	314	314	209	209	207	207	295	295	235	223
GSD100	Germany	Sport	132	132	178	170	137	133	188	188	103	101	90	90	130	130	287	287	125	117	144	128	322	314	205	201	247	191	291	291	223	223
GSD101	South Africa	Show	132	132	182	170	133	133	180	180	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Sample	Type	Coat	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
DH01	Standard	Short	140	102	170	162	137	137	208	204	113	93	94	86	130	130	285	285	125	115	140	128	314	314	197	197	195	191	311	295	239	235
DH02	Standard	Short	140	102	178	170	137	137	208	196	93	93	94	94	130	130	285	285	125	115	128	128	318	318	201	197	191	191	307	307	239	235
DH03	Miniature	Short	144	132	170	162	137	137	196	184	111	105	98	90	130	130	285	285	117	115	128	128	322	322	205	197	223	195	339	307	235	231
DH04	Miniature	Short	140	136	162	150	137	137	200	192	109	99	92	92	124	124	289	289	129	115	140	136	322	314	217	201	219	191	307	287	231	227
DH05	Miniature	Long	136	110	150	150	145	137	192	188	105	105	96	92	142	124	287	287	125	125	140	128	322	314	209	205	243	203	287	287	235	227
DH06	Miniature	Long	110	110	162	162	137	133	184	184	103	103	98	94	124	124	287	287	125	125	144	124	322	318	213	209	195	179	287	283	235	227
DH07	Standard	Short	136	102	162	150	137	137	196	184	99	99	94	94	130	124	291	291	129	115	140	132	322	318	209	197	219	195	307	303	239	239
DH08	Miniature	Short	140	136	170	162	157	157	208	208	91	91	92	92	130	126	285	285	123	115	128	128	314	314	201	201	195	191	295	295	235	231
DH09	Miniature	Long	106	106	170	170	137	133	192	188	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH10	Miniature	Long	110	110	162	150	157	137	192	192	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH11	Miniature	Long	110	110	170	170	133	133	188	184	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH12	Miniature	Short	140	132	162	150	137	137	204	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH13	Miniature	Short	136	136	170	162	137	137	204	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH14	Miniature	Short	144	136	162	162	153	137	204	184	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH15	Miniature	Short	144	140	178	162	137	137	192	184	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH16	Miniature	Short	144	144	162	162	133	133	204	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH17	Standard	Short	140	98	174	170	153	137	200	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH18	Standard	Short	140	140	170	162	137	137	204	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH20	Standard	Short	140	140	178	170	137	137	196	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH21	Standard	Short	98	98	162	150	137	137	200	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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Sample	Type	Coat	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
DH22	Standard	Short	140	140	178	170	137	133	204	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH23	Miniature	Short	144	136	170	162	137	137	180	180	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH24	Miniature	Short	136	132	170	162	137	137	192	192	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH25	Miniature	Short	136	136	162	150	137	133	200	180	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH26	Miniature	Short	140	132	170	162	153	137	192	184	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH27	Miniature	Short	136	136	162	162	141	137	204	192	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Sample	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
SBT01	132	132	174	170	141	137	200	192	97	91	96	92	126	126	291	287	119	117	140	136	318	314	217	205	203	199	319	279	231	227
SBT02	132	132	162	162	165	141	196	188	101	99	98	92	124	124	291	287	117	117	140	128	322	310	217	213	251	199	291	279	235	227
SBT03	140	98	162	162	165	141	204	200	101	91	92	90	134	124	0	0	123	117	0	0	0	0	0	0	0	0	0	0	0	0
SBT04	132	132	178	162	157	157	196	192	97	91	96	90	140	126	291	287	119	117	140	140	306	274	209	209	247	191	311	279	239	227
SBT05	132	132	162	162	157	137	200	180	97	91	96	92	134	126	291	287	123	119	144	140	318	310	209	209	251	251	311	311	231	227
SBT06	132	132	170	166	141	137	208	208	97	91	96	96	134	130	287	287	123	117	144	140	326	322	213	213	207	183	311	279	231	231
SBT07	132	132	182	166	141	137	200	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SBT08	132	132	174	166	157	137	200	192	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SBT09	132	132	162	162	137	137	192	188	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SBT10	132	132	170	166	157	137	196	192	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SBT11	132	132	182	166	141	133	200	188	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SBT12	132	132	162	162	157	141	208	188	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SBT13	132	132	174	166	141	137	208	208	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SBT14	132	132	166	162	157	141	204	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SBT15	140	132	186	162	157	141	216	208	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SBT20	140	132	162	162	157	137	192	180	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SBT21	144	132	178	174	157	141	204	192	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SBT23	132	132	174	170	141	137	200	192	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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Sample	Breed	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
CB01	Dobermann	140	140	166	162	137	133	192	192	97	95	102	96	134	124	287	285	119	117	140	140	346	306	209	205	231	195	283	279	231	223
CB02	Weimaraner	136	132	186	182	149	137	204	200	101	97	96	96	138	124	287	285	123	115	144	128	338	322	201	193	195	195	347	295	235	235
CB03	Scottish Terrier	132	132	162	162	145	133	208	200	97	93	96	90	132	124	291	289	117	117	132	132	322	322	217	205	191	191	291	283	235	235
CB04	Labrador Retriever	136	132	186	186	153	137	200	196	105	101	96	94	130	130	287	283	123	123	144	144	318	318	205	197	243	243	303	299	243	235
CB05	Miniature Schnauzer	0	0	0	0	137	137	204	196	101	101	102	102	140	140	287	283	123	119	144	144	0	0	209	201	195	195	291	271	227	227
CB06	Standard Schnauzer	136	136	186	182	141	141	196	188	107	103	90	90	140	140	287	287	123	123	140	136	338	322	209	205	223	207	323	323	239	215
CB07	Goldern Retriever	136	136	194	174	133	133	208	192	101	97	100	94	124	124	291	287	117	117	144	140	342	338	213	205	203	199	287	283	231	231
CB08	Whippet	136	110	186	178	137	133	192	188	105	97	96	90	134	132	285	285	117	115	140	140	330	322	205	197	207	195	287	287	243	215
CB09	Greyhound	132	102	186	178	153	145	204	188	99	97	90	90	134	130	289	289	125	115	132	128	306	306	213	201	203	195	299	299	231	223
CB10	Great Dane	132	132	158	158	141	129	188	188	107	79	90	90	138	126	289	287	115	115	144	140	342	318	209	205	223	203	299	295	223	223
CB11	Labrador Retriever	132	132	182	158	157	133	204	196	105	105	96	90	140	130	283	283	123	117	144	144	318	318	205	205	251	251	303	303	239	235
CB12	Bernese Mountain Dog	132	132	182	182	133	133	196	180	97	97	100	96	140	124	289	289	123	123	132	128	342	314	205	205	215	183	331	327	239	239
CB13	Jack Russell Terrier	110	110	182	182	141	137	212	188	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB14	Border Collie	102	102	170	166	141	137	204	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB15	Standard Schnauzer	132	132	158	158	141	141	200	188	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB16	Wire-haired Fox Terrier	136	136	182	166	141	137	208	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB17	Beagle	140	102	174	166	157	137	204	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB18	Miniature Schnauzer	98	98	0	0	137	137	204	192	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB19	Boxer	136	132	0	0	153	137	192	188	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB20	Mastiff	132	132	158	158	141	141	196	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB21	Mastiff	132	102	182	158	137	137	196	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB22	Belgian Tervueren	148	98	186	174	145	145	196	192	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB23	Bulldog	132	102	0	0	141	133	196	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB24	Standard Poodle	144	102	186	186	137	133	208	208	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB25	Boxer	136	132	186	162	137	137	192	192	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB26	Giant Schnauzer	136	132	186	182	137	133	200	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB27	Bouvier des Flandres	132	132	178	178	137	133	196	192	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB28	Cocker Spaniel	140	140	190	162	157	129	192	188	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB29	Airedale	102	102	182	170	137	137	204	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB30	Rhodesian Ridgeback	136	132	170	170	137	137	200	188	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB31	Scottish Terrier	136	132	162	162	145	133	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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Sample	Breed	DTRCN1		FH2137		FH2140		FH2328		AHT121		INRA21		AHTh171		AHTk253		CXX279		FH2001		FH2164		FH2611		FH2247		FH2289		PEZ08	
CB33	Weimaraner	136	132	178	162	149	137	196	188	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB34	Rough Collie	136	132	178	166	145	133	208	204	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB35	Siberian Husky	0	0	170	166	141	137	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB36	Short-haired Pointer	140	140	162	162	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB37	Elkhound	144	140	166	158	133	129	200	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CB38	Irish Setter	136	136	162	162	153	137	200	196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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Appendix II

Supplementary Results

Graphic representations of the allele frequency and distribution of each of the 15 microsatellite markers for the German Shepherd Dog (GSD), Dachshund (DH), Staffordshire Bull Terrier (SBT), composite breed group (CB), and outbred dogs (OBD).

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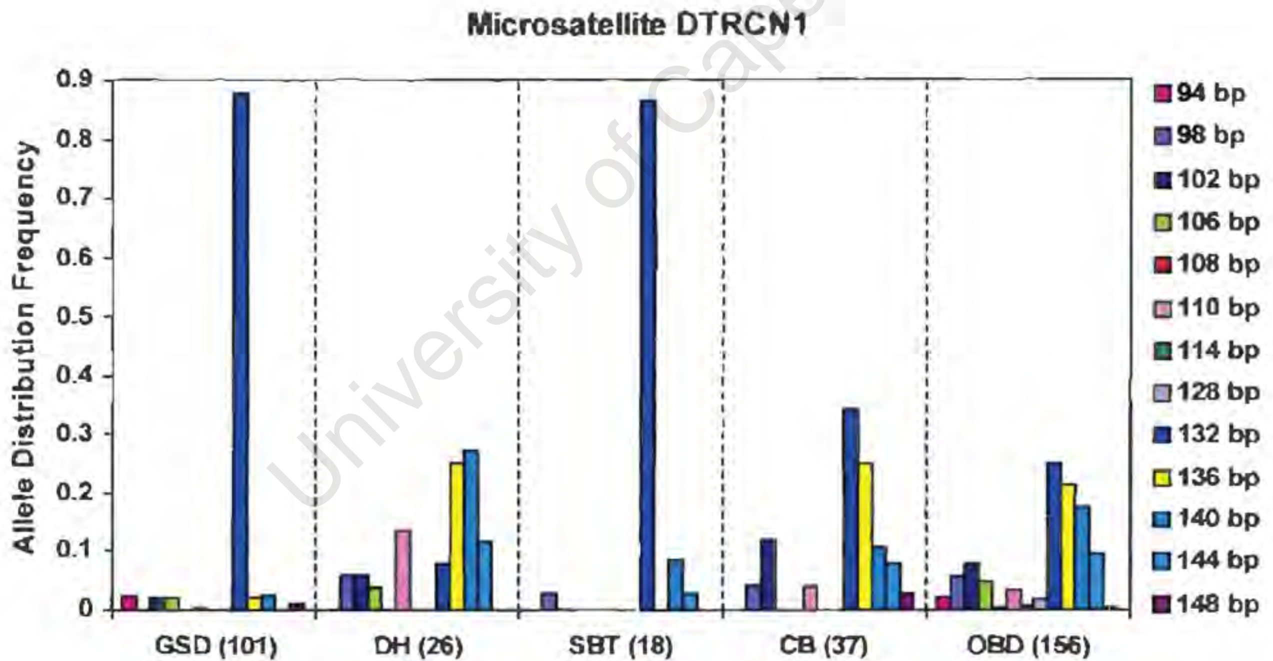
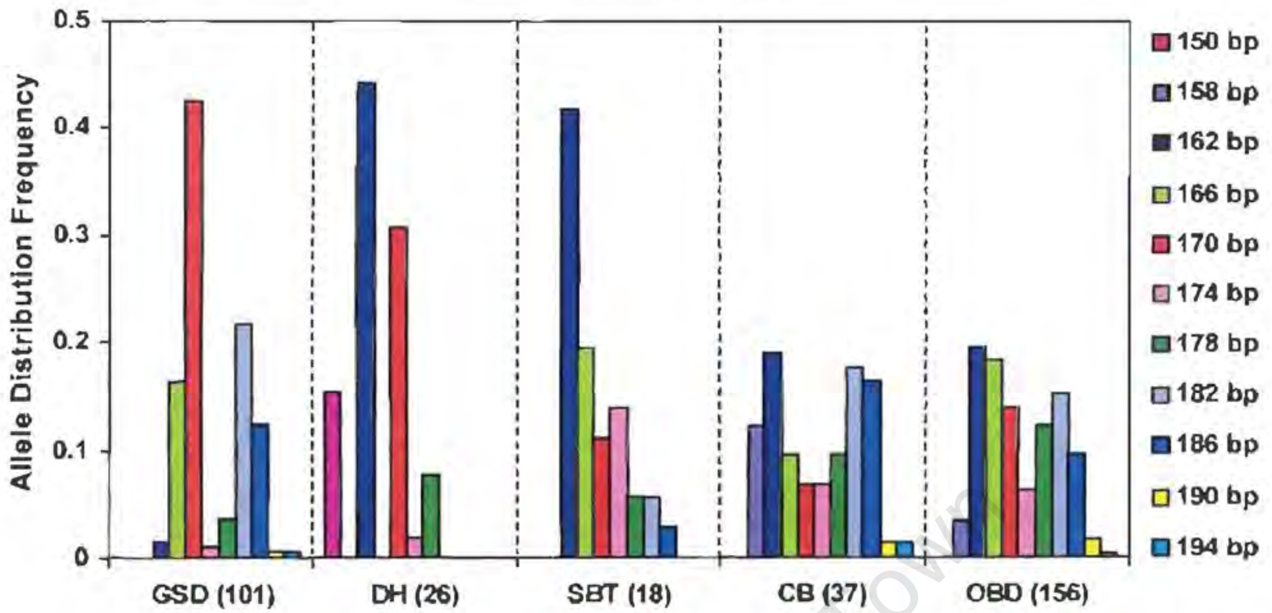
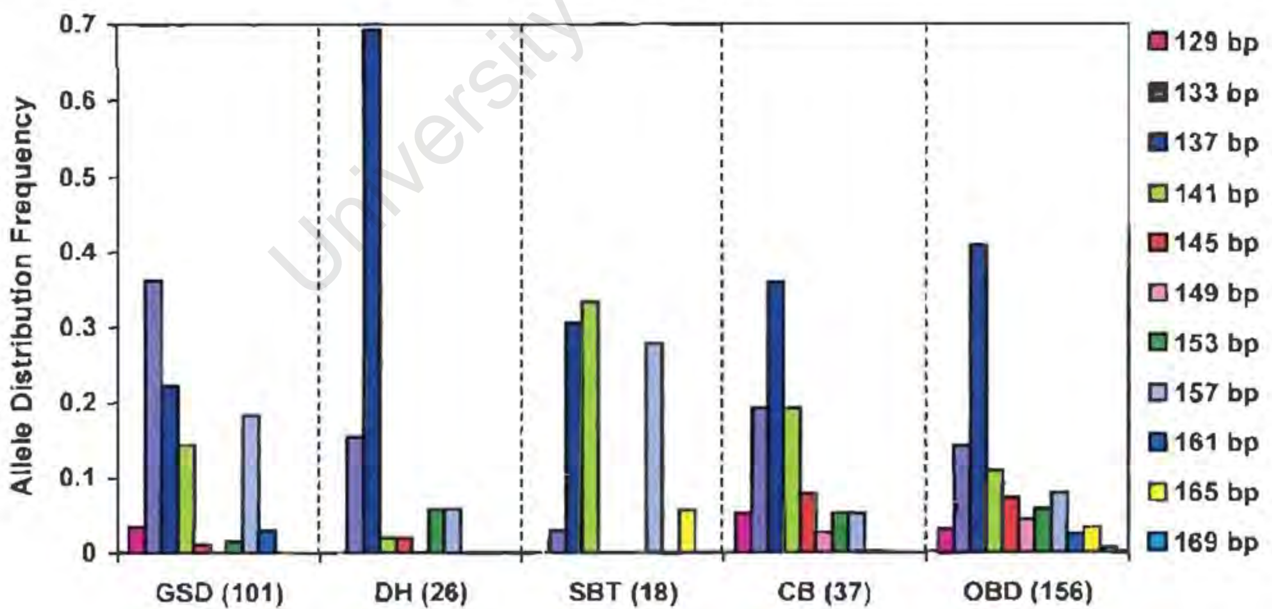
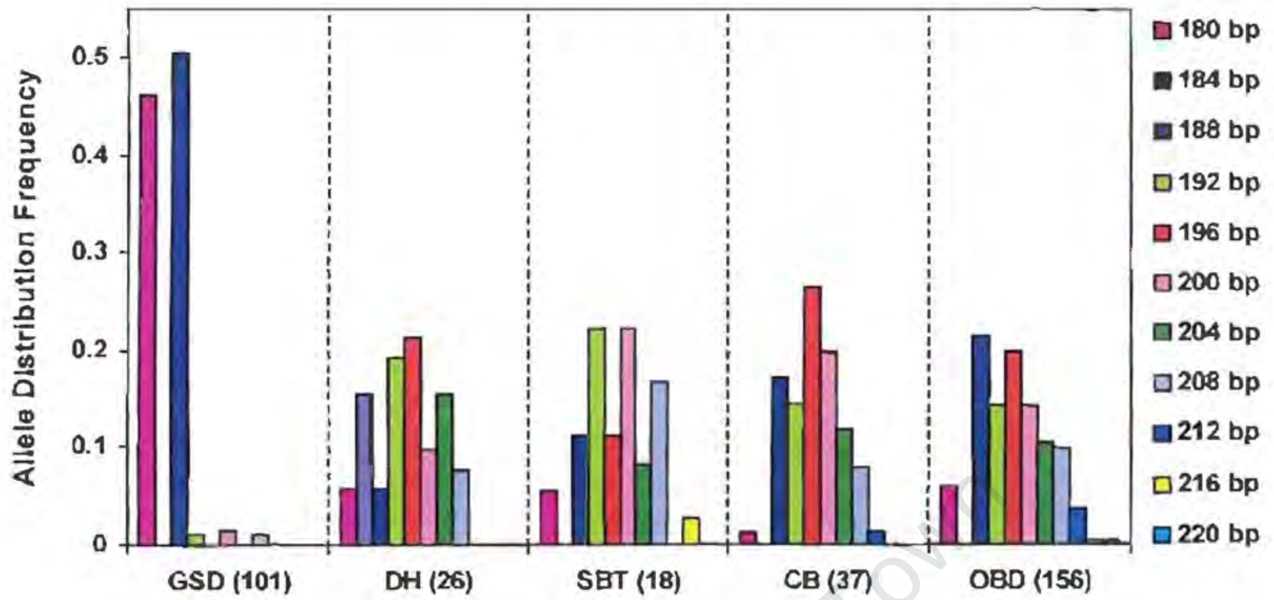



Microsatellite FH2137
Allele Distribution Frequency
0.5
0.4
0.3
0.2
0.1
0
GSD (101)
DH (26)
SBT (18)
CB (37)
OBD (156)
150 bp
158 bp
162 bp
166 bp
170 bp
174 bp
178 bp
182 bp
186 bp
190 bp
194 bp


Microsatellite FH2140
Allele Distribution Frequency
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
GSD (101)
DH (26)
SBT (18)
CB (37)
OBD (156)
129 bp
133 bp
137 bp
141 bp
145 bp
149 bp
153 bp
157 bp
161 bp
165 bp
169 bp



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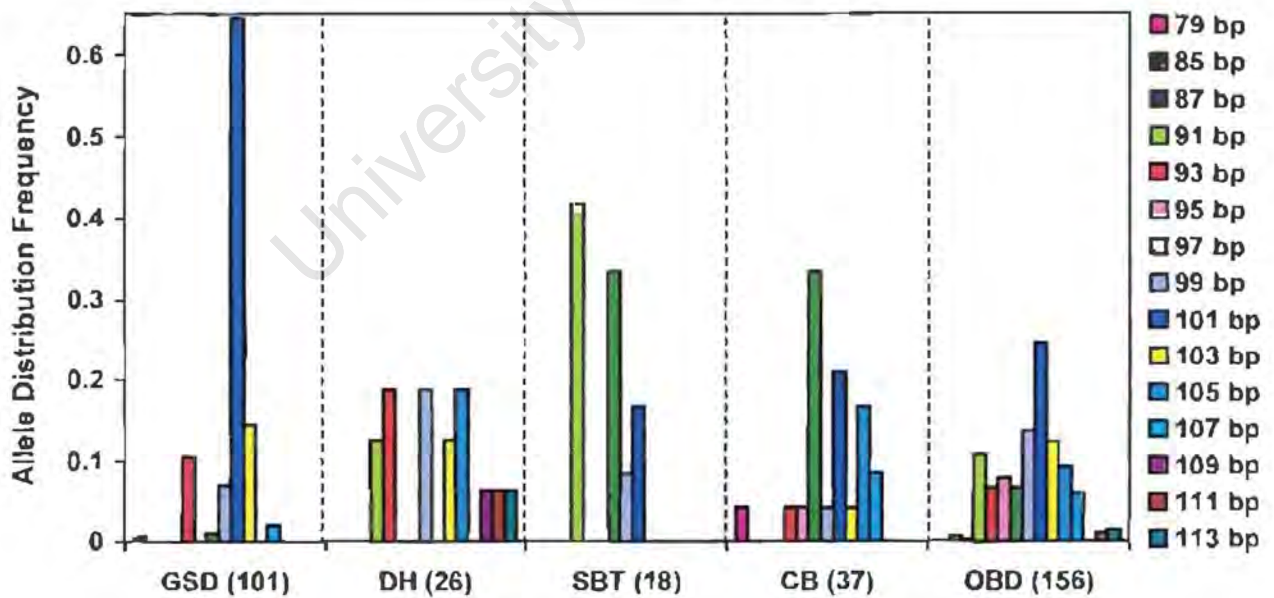

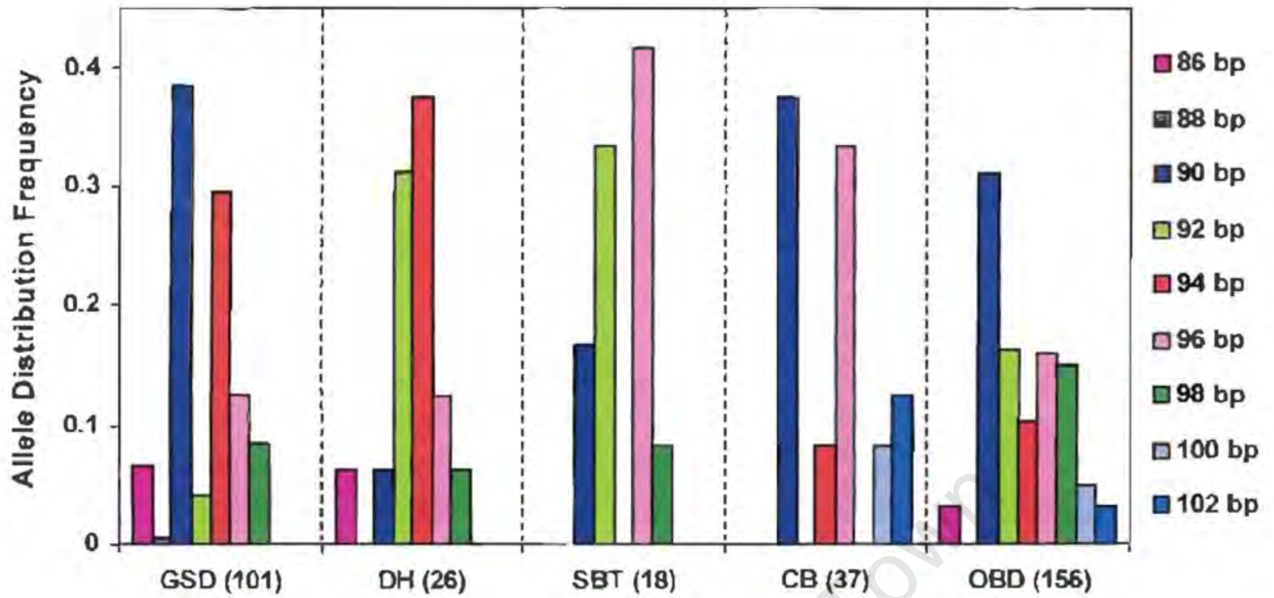

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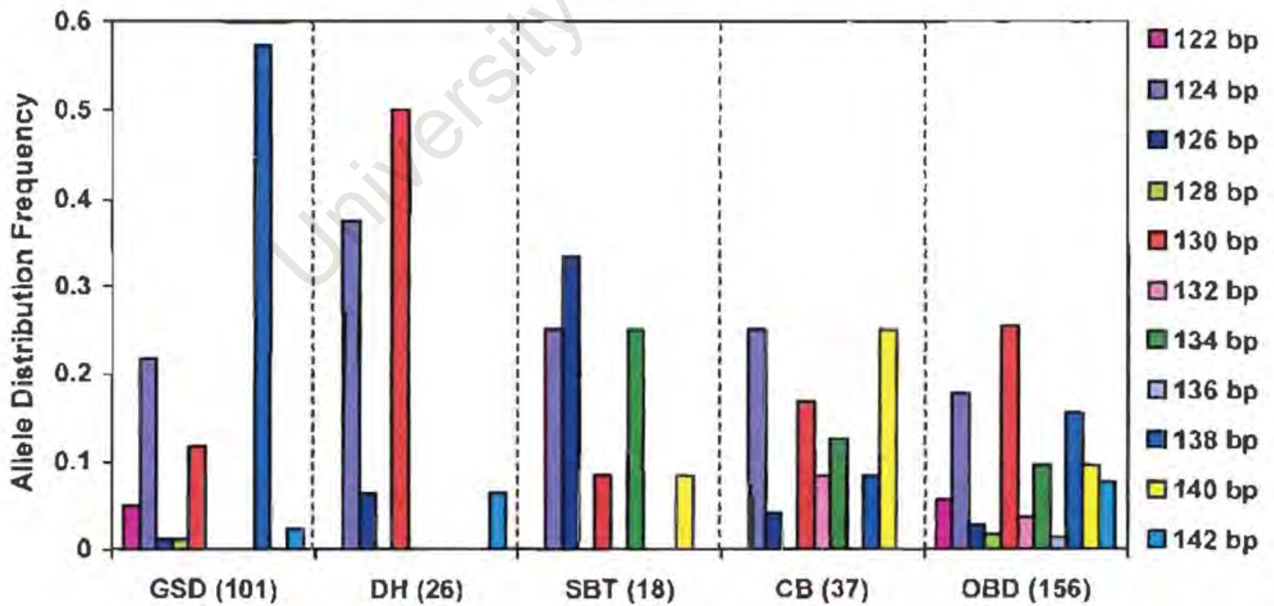

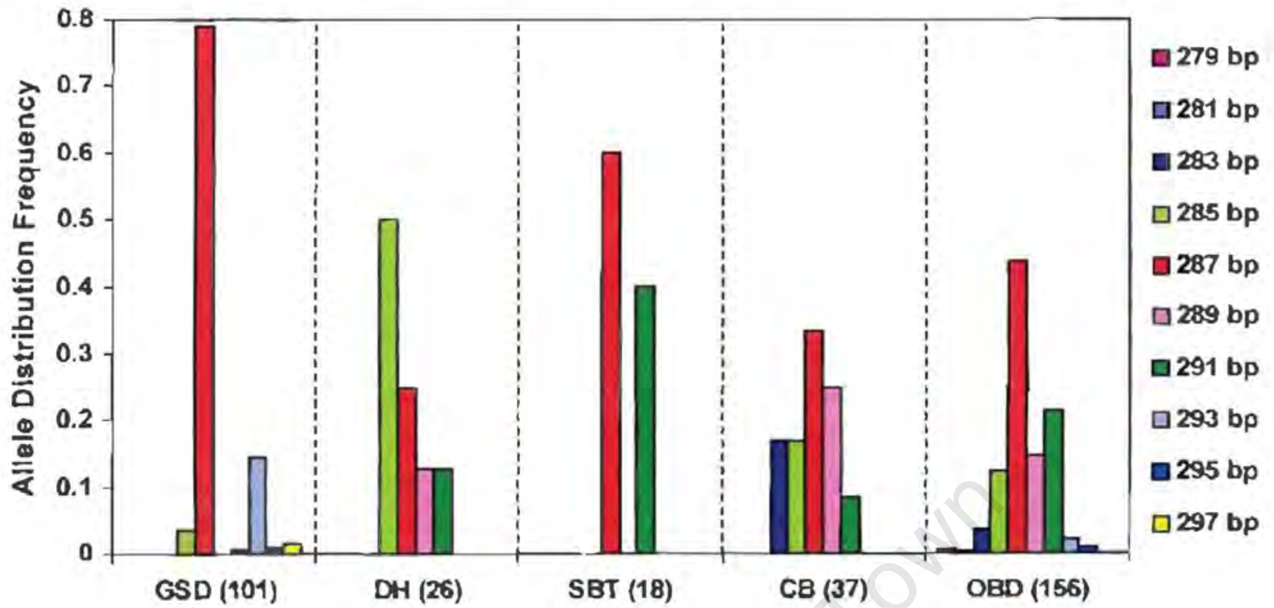

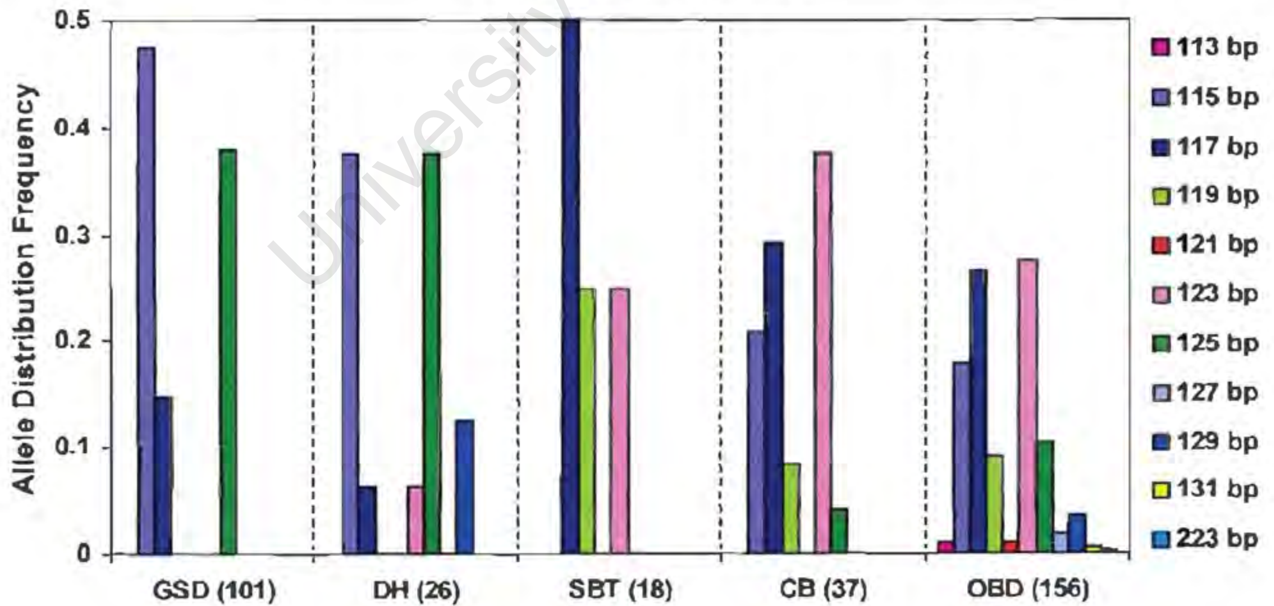

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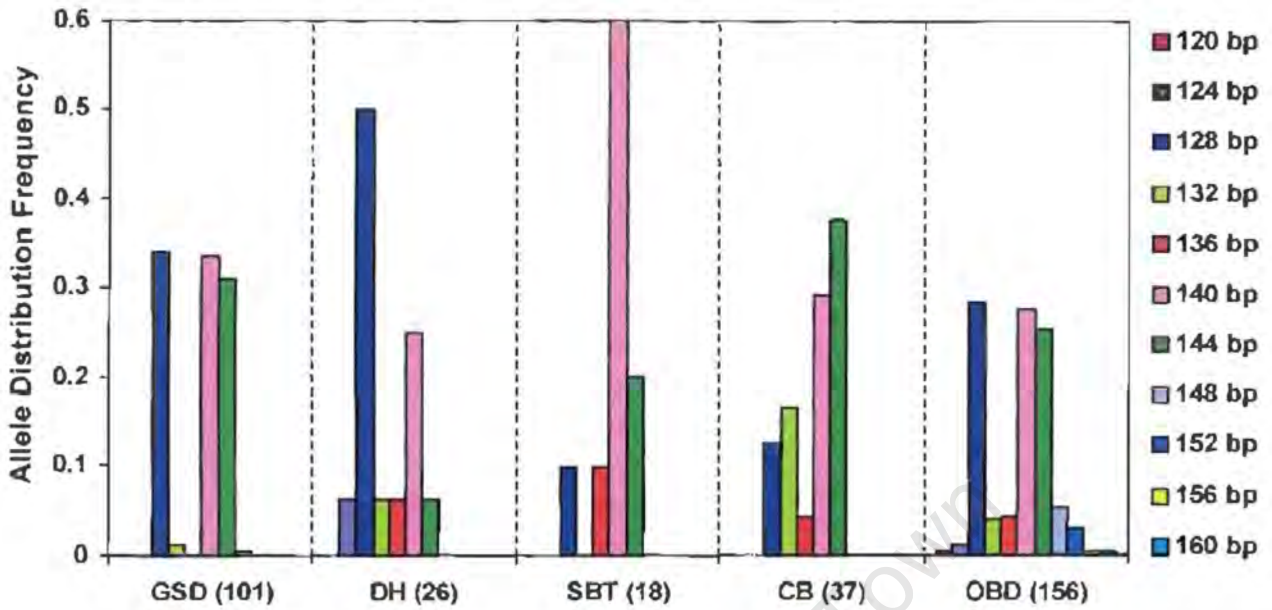

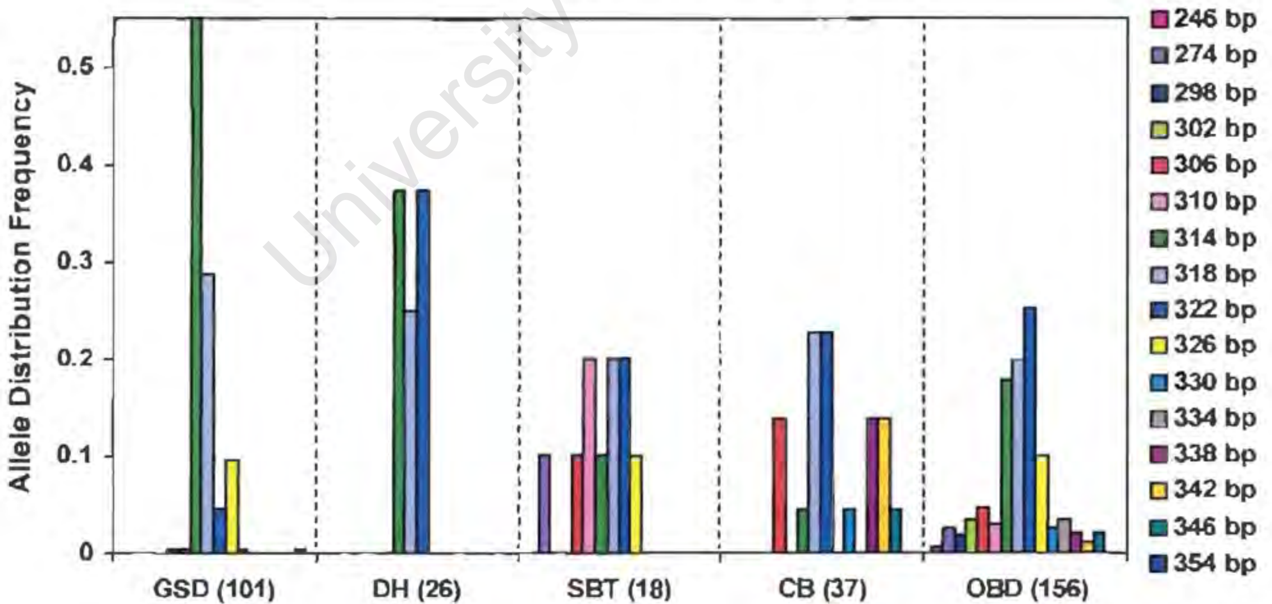

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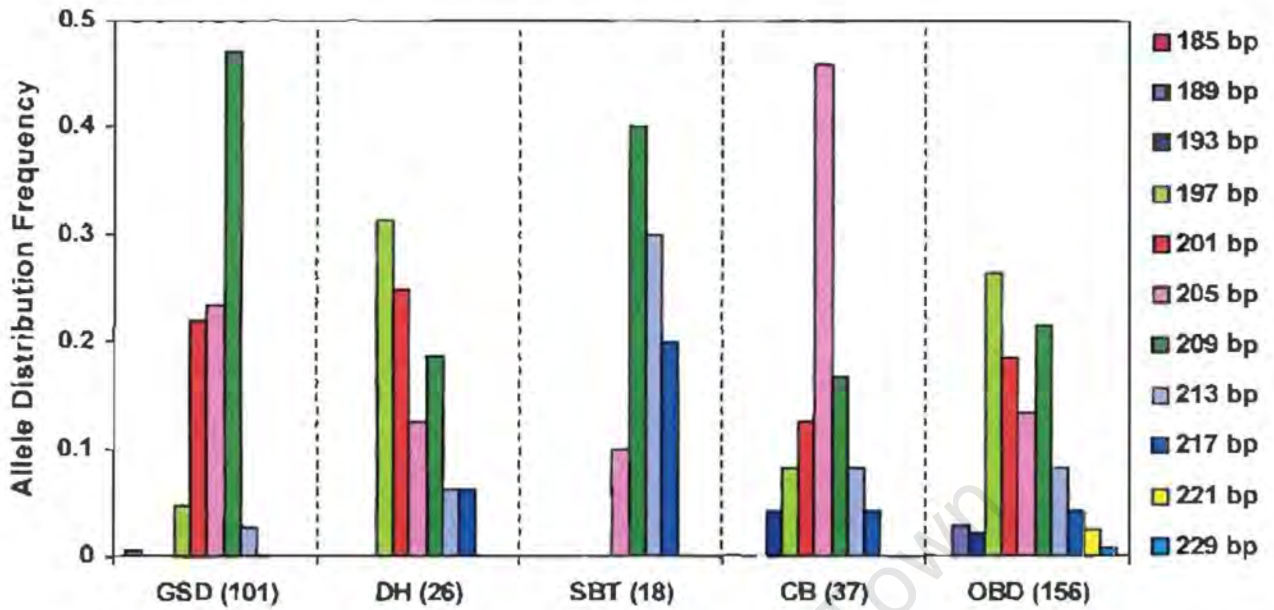

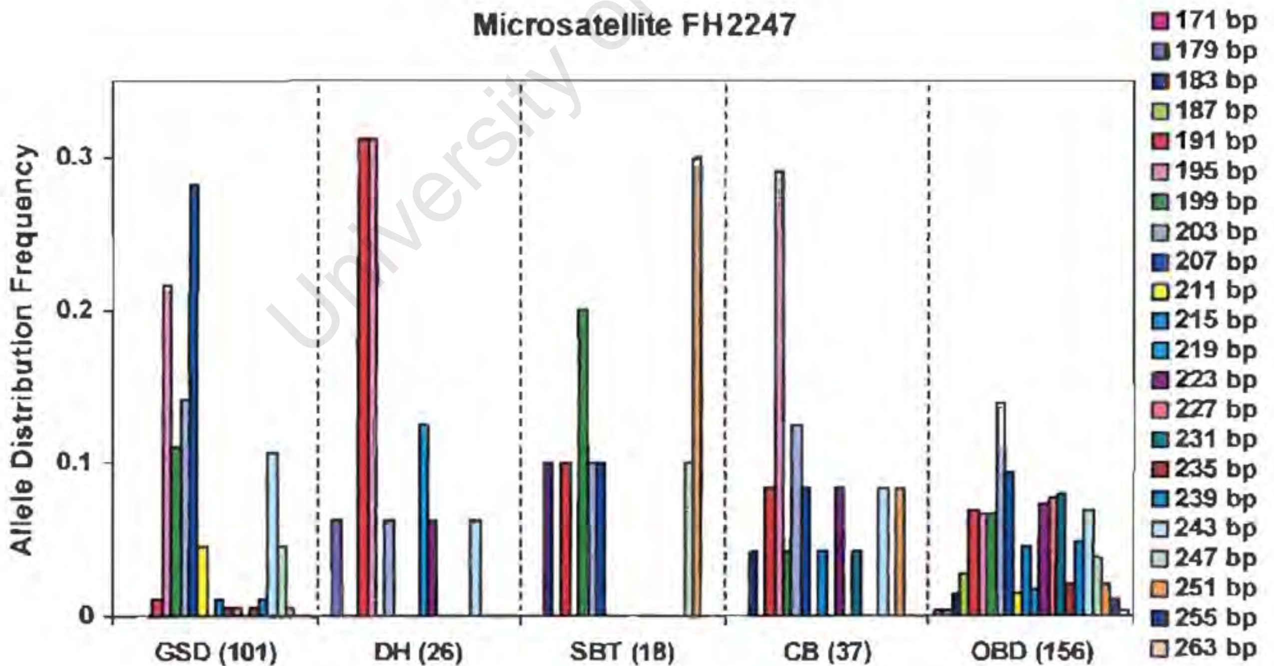

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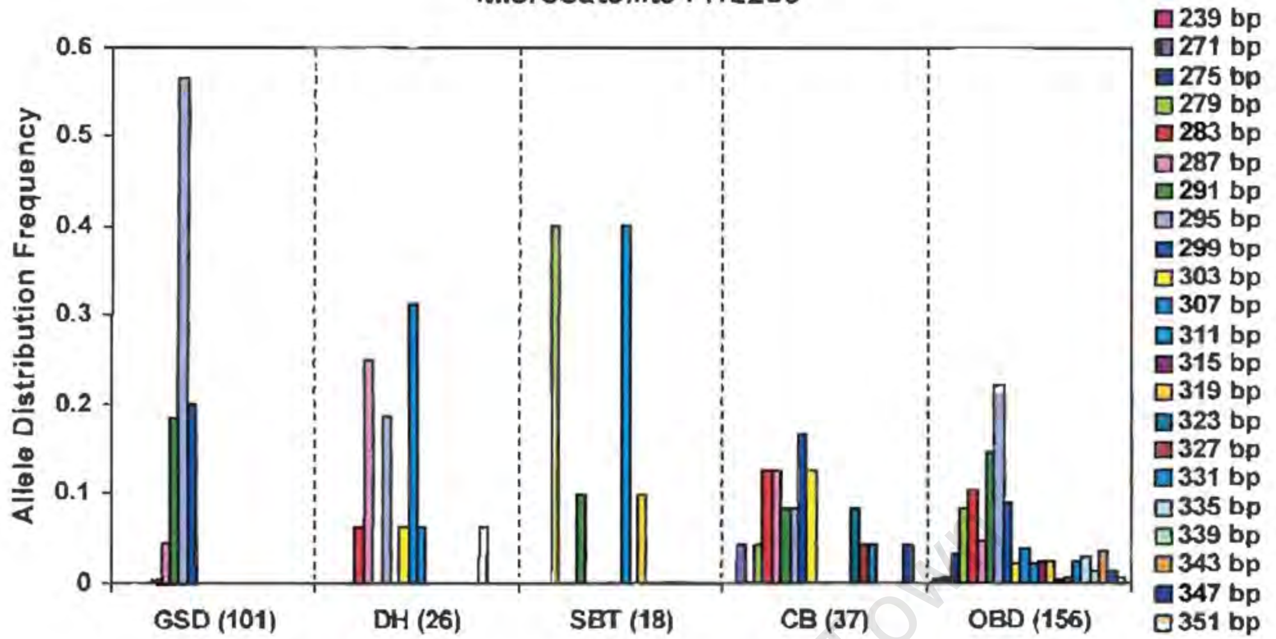

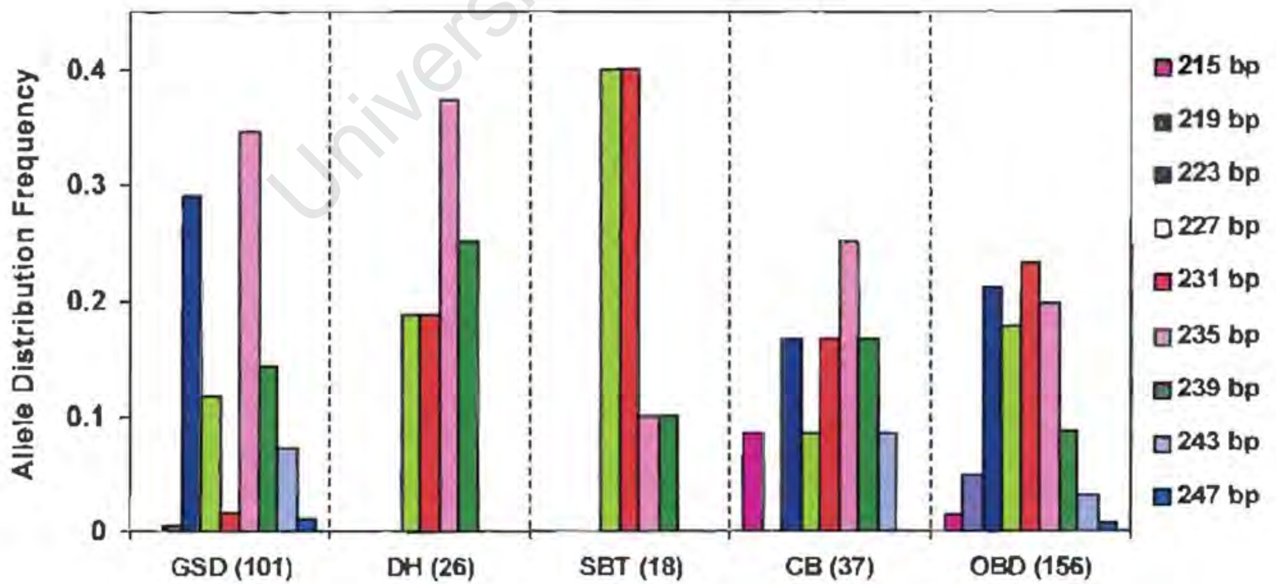

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Tabulated morphological measurements of German Shepherd Dogs representing sport dogs, crossbred show and sport dogs (Shw/Sp), and show dogs. The length of head from nose to sagital crest, height at the shoulder, height at the pelvic bone and body length from chest to pelvis are indicated. The ratio of body length to shoulder height and the ratio of head to body length are calculated from these measurements. The gradient of the slope of the back (topline) is calculated according to the change in the y- and x-axes ($\Delta y/x$).

Sample	Type	Nose to Sagital crest (cm)	Shoulder height (cm)	Pelvis height (cm)	Body length (cm)	Ratio Length to Height (%)	Ratio Head to Body (%)	Topline Gradient ($\Delta y/x$)
GSD005	Sport	27.0	66.0	53	79.0	119.70	34.18	0.16
GSD010	Sport	27.0	65.0	56	80.0	123.08	33.75	0.11
GSD011	Sport	27.0	65.0	56	70.0	107.69	38.57	0.13
GSD021	Sport	27.0	64.0	52	70.0	109.38	38.57	0.17
GSD022	Sport	24.0	59.0	57	66.0	111.86	36.36	0.03
GSD023	Sport	24.5	59.0	56	72.0	122.03	34.03	0.04
GSD034	Sport	31.0	65.0	51	81.0	124.62	38.27	0.17
GSD035	Sport	26.0	58.0	49	72.0	124.14	36.11	0.13
GSD071	Sport	26.0	56.0	45	73.5	131.25	35.37	0.15
GSD072	Sport	30.0	65.0	53	79.0	121.54	37.97	0.15
GSD077	Sport	27.0	65.0	57	76.0	116.92	35.53	0.11
GSD079	Sport	27.5	64.0	52	64.5	100.78	42.64	0.19
GSD096	Sport	27.0	59.0	49	69.0	116.95	39.13	0.14
GSD201	Sport	28.0	65.0	58	74.0	113.85	37.84	0.09
GSD414	Sport	26.0	64.0	56	74.0	115.63	35.14	0.12
GSD415	Sport	27.0	65.0	51	73.0	112.31	36.99	0.11
GSD416	Sport	28.0	65.0	55	78.0	120.00	35.90	0.14
GSD417	Sport	26.0	58.0	56	64.0	110.34	40.63	0.11
GSD418	Sport	28.0	65.0	52	71.0	109.23	39.44	0.16
GSD419	Sport	27.0	64.0	54	72.0	112.50	37.50	0.15
Average	**Sport**	**27.1**	**62.8**	**53.4**	**72.9**	**116.19**	**37.2**	**0.13**

Sample	Type	Nose to Sagital crest (cm)	Shoulder height (cm)	Pelvis height (cm)	Body length (cm)	Ratio Length to Height (%)	Ratio Head to Body (%)	Topline Gradient ($\Delta y/x$)
GSD 002	Shw/Sp	27.0	71.0	62.0	71.0	100.00	38.03	0.13
GSD 003	Shw/Sp	28.0	65.0	54.0	69.5	106.92	40.29	0.16
GSD 019	Shw/Sp	30.0	68.0	49.0	73.0	107.35	41.10	0.26
GSD 070	Shw/Sp	30.0	70.0	47.0	79.0	112.86	37.97	0.29
GSD 075	Shw/Sp	25.0	59.0	45.0	66.0	111.86	37.88	0.21
GSD 076	Shw/Sp	29.0	66.0	51.0	72.0	109.09	40.28	0.21
GSD 095	Shw/Sp	27.0	62.0	52.0	72.0	116.13	37.50	0.14
Average	**Shw/Sp**	**28.0**	**65.9**	**51.4**	**71.8**	**109.17**	**39.01**	**0.20**

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Sample	Type	Nose to Sagital crest (cm)	Shoulder height (cm)	Pelvis height (cm)	Body length (cm)	Ratio Length to Height (%)	Ratio Head to Body (%)	Topline Gradient (Δy/x)
GSD001	Show	30.0	72.0	52.0	82.0	113.89	36.59	0.24
GSD008	Show	30.0	68.0	48.0	80.0	117.65	37.50	0.25
GSD020	Show	31.0	68.0	44.0	75.0	110.29	41.33	0.32
GSD040	Show	24.0	58.0	37.0	67.0	115.52	35.82	0.31
GSD042	Show	26.0	64.0	47.0	71.0	110.94	36.62	0.24
GSD043	Show	31.0	68.0	45.0	75.5	111.03	41.06	0.30
GSD044	Show	30.0	70.0	47.0	73.0	104.29	41.10	0.32
GSD048	Show	26.0	58.0	40.0	68.0	117.24	38.24	0.26
GSD049	Show	30.5	70.0	46.5	79.5	113.57	38.36	0.30
GSD050	Show	29.5	67.0	47.0	78.0	116.42	37.82	0.26
GSD051	Show	28.0	65.0	44.0	75.0	115.38	37.33	0.28
GSD052	Show	28.0	62.0	43.0	77.0	124.19	36.36	0.25
GSD053	Show	27.0	58.0	40.0	69.0	118.97	39.13	0.26
GSD055	Show	29.5	68.0	46.0	80.0	117.65	36.88	0.28
GSD056	Show	27.0	60.0	42.0	71.0	118.33	38.03	0.25
GSD057	Show	27.0	62.0	42.0	74.0	119.35	36.49	0.27
GSD058	Show	27.0	62.0	42.0	77.0	124.19	35.06	0.26
GSD059	Show	27.0	62.0	41.0	69.0	111.29	39.13	0.30
GSD060	Show	28.0	65.0	44.0	77.0	118.46	36.36	0.27
GSD061	Show	29.0	67.0	42.0	80.0	119.40	36.25	0.31
GSD062	Show	25.0	62.0	41.0	70.0	112.90	35.71	0.30
GSD063	Show	24.0	56.0	40.0	67.0	119.64	35.82	0.24
GSD064	Show	24.0	55.0	40.0	65.0	118.18	36.92	0.23
GSD065	Show	29.0	68.0	44.0	83.0	122.06	34.94	0.29
GSD066	Show	29.0	70.0	46.0	78.0	111.43	37.18	0.31
GSD067	Show	25.5	60.0	40.0	72.0	120.00	35.42	0.28
GSD069	Show	28.0	64.0	45.0	78.0	121.88	35.90	0.24
GSD080	Show	29.0	66.0	47.0	73.0	110.61	39.73	0.26
GSD098	Show	27.0	62.0	43.0	70.5	113.71	38.30	0.27
GSD413	Show	27.0	61.0	44.0	69.0	113.11	39.13	0.25
Average	**Show**	**27.8**	**64.1**	**43.7**	**74.0**	**115.59**	**37.45**	**0.28**

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Appendix III

Reagents and Solutions

Red Blood Cell Lysis Buffer	
109.5g	Sucrose (B & M Scientific)
10ml	1M Tris-Hydrochloric acid solution pH 7.6 (B & M Scientific)
10ml	Triton X-100 (BDH Laboratory Supplies)
5ml	1M Magnesium Chloride solution (Saarchem)

Make solution up to 1L with dH_2O

Saline EDTA Solution pH 8.0	
9.3g	Ethylenediaminetetra-acetic acid (SMM Chemicals)
58.4g	Sodium chloride (Saarchem-Holpro Analytic)

Adjust pH until 8.0 (Beckman ϕ32pH Meter) with 5M Sodium hydroxide solution (RPE Analyticals) and make solution up to 250ml with dH_2O

1× TE Buffer	
1.211g	Trishydroxymethylaminomethane (Promega)
0.372g	Ethylenediaminetetra-acetic acid (SMM Chemicals)

Make solution up to 1L with dH_2O

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6% Acrylamide Solution

12ml	40% Acrylamide/Bis solution (Bio-Rad Laboratories)
48g	Urea (Riedel-deHaën)
10ml	10× TBE buffer
~42ml	dH_2O

Polymerise with 280µl 20% Ammonium persulphate (BDH), 60µl TEMED (Promega)

10× TBE Running Buffer

108g	Trishydroxymethylaminomethane (Promega)
55g	Boric acid (B&M Scientific)
7.4g	Ethylenediaminetetra-acetic acid (SMM Chemicals)

Make solution up to 1L with dH_2O and autoclave (Lasec)

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Loading Buffer

20g (40%w/v)	Sucrose (B&M Scientific)
0.125g	Bromophenyl Blue (Merck)

Make solution up to 50ml with dH_2O

pH corrected with half a Sodium chloride pellet (Saarchem-Holpro Analytic)

Molecular Weight Marker (A-T Ladder) using the Sequenase Kit (Amersham)

Hot primer

0.6µl sdH_2O

6.67µl -40 (17mer) universal primer

1.0µl 10× PNK buffer (BioLabs)

1.0µl $\gamma^{32}P$ ATP (20µCi/µl) (Amersham)

0.67µl T4 PNK (BioLabs)

37°C for 30min, 90°C for 3min (Hybaid), centrifuge (Beckman) at store at -20°C .

Annealing Reaction

5.2µl sdH_2O

10µl ssM13 DNA

4µl Annealing buffer

4.8µl End-labelled primer

37°C for 10min and 25°C for 10min (Hybaid).

Add to annealed template

5.2µl H_2O

2.0µl MDTT

1.0µl Multi-pol sequenase

Termination Tubes

2µl × 2 tubes ddATP

2µl × 2 tubes ddTTP

Mix by pipetting gently and centrifuge (Beckman). Transfer 8µl to each of the four tubes of termination mix, pipetting gently and incubate at 37°C for 3min, followed by 70°C for 7min. To each tube add 7µl stop solution and 3µl H_2O, collect all reactions in one tube, mix by pipetting gently and centrifuge. Store at -20°C, and denature at 90°C for 3min before use, load 2.5µl per well.

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