CHAPTER 2

MATERIALS & METHODS

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2.1. POPULATIONS SAMPLED

Blood samples were taken from four different groups of dogs for this study:

Figure 2: Map showing sampling and/or population locations for the various dog breeds used in this study.

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(a) The Africanis population

In 1995, 86 blood samples were collected from the Africanis breed near Nkandla (28º40'S, 31º10'E), in the north-eastern parts of KwaZulu Natal. These dogs live in and around rural villages and kraals in this area (e.g. Figure 3). Johan Gallant, author and film producer on canine matters, has been studying these animals for several years, and believe that the animals found in this area is a very good example of just such an endemic, southern African dog breed (Gallant, pers comm; in press).

Figure 3: An example of the endemic breed sampled in Nkandla, KwaZulu Natal (South Africa).

(b) The SPCA group

The second group of dogs comprised of several miscellaneous breeds, including Maltese Poodles, Jack Russels, German Shepherds to Rottweilers and Staffordture Terriers. Ninety-two blood samples were obtained mainly from the Boksburg-area, Gauteng. The blood samples were taken from dogs in the squatter camps in the area, where owners allowed the sampling, in exchange for a quick medical check-up, deworming of the animals, sometimes vaccinations and/or treatments and dips for mange and other skin diseases (Figure 4). Animals confiscated by the Society for the Prevention of Cruelty to Animals (SPCA) were also sampled at the premises of the Society.

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Figure 4: Dr Nico Minnaar (left), with a representative from the Boksburg SPCA (middle), examining a sub-adult dog from the local squatter camp.

Some blood samples were also obtained at the Booysens SPCA, Gauteng. These samples were taken at the premises, though, from confiscated or homeless animals kept at the Society, and not necessarily from surrounding squatter camps.

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(c) The Saluqi dogs

Seventeen blood samples from an Arabian indigenous dog breed, the Saluqis were included. These dogs were introduced to the West (predominantly into England) from Egypt, Israel, Jordan, Syria, Iraq and Iran. The founding population was somewhere in the region of 25 individuals, and with several leaving no descendants, only about 16 continue to appear in modern pedigrees. The foundation stock studbooks were closed in 1927 in England and 1928 in the United States of America. "Pure breeding" (inbreeding) commenced from then onwards. However, England permitted continued importations from the countries of origin, if the dogs could be certified purebreds by three dog show judges. Several additional dogs entered the gene pool this way, and more continue to do so (Yocham, unpublished report).

The American Kennel Club (AKC) studbook was closed in 1928 and only Saluqis with four generation (issued by an AKC recognised kennel club) pedigrees are accepted into it. There was an exception made after World War II and two Saluqis brought in from Saudi Arabia (however the male was clearly from northern origin) were permitted into the studbook. The fourth generation of their offspring was registered. However, it is rumoured that several popular bloodlines have false pedigrees due to the use of unregistered Saudi Arabian imports (Yocham, unpublished report).

Apart from four imports since 1928 and the few rumoured false pedigrees, the American foundation stock came from England, not the Middle East; hence English, American and virtually all Western Saluqis share a very narrow gene pool. A few dogs were exported from England to the USA; dogs that had been imported into England from the Middle East (Yocham, unpublished report).

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It is important to specify that the blood samples for this study were obtained from desert-bred Saluqis, as they still prevail in Bedouin camps in the Middle East, and should not be confused with the modern, pure-bred show Saluqi.

(d) The Jericho group

The last group of dogs was sampled in February 1997 in the North West Province (South Africa) and represent an amalgamation of street dogs. Sixteen blood samples were collected during a community project in Jericho (25º17'S, 27º47'E), about 60 km north of Brits (figure 2) near the Borakalalo Game Reserve. Veterinarians and

interested parties from Onderstepoort, Medunsa and the Agricultural Research Council (Animal Improvement Institute) visited, on average, two villages or resource limited communities per day. This was part of a "rapid appraisal" programme. Blood, faeces and hair samples from non-descriptive dog breeds were collected in exchange for veterinary expertise concerning vaccinations, deworming, medical examinations and treatments for various diseases.

2.2. BLOOD SAMPLING AND STORAGE

Blood samples were obtained from the cephalic vein in the front leg, after disinfecting the area. Approximately 3 – 5 ml blood was taken per animal and kept in vacutainer tubes containing either ethylenediamine-tetracetate (EDTA) or heparin (Juneja *et al.*, 1987) as anticoagulants. The vacutainer test tubes were immediately kept on crushed ice for transportation to the laboratory, where they were then stored below freezing temperature (-20°C). Some of the thawed blood samples were later centrifuged for roughly 5 – 10 minutes at 8 000 revolutions per minute, to separate red cells and plasma. The plasma was drawn from the red cells and transferred to alternative test tubes. These separated samples, as well as original whole blood samples, were stored frozen again at -20°C to await electrophoresis.

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2.3. ELECTROPHORESIS – HORIZONTAL STARCH GEL AND PAGE

2.3.1. Biochemical techniques

The blood samples were analysed using two methods of one-dimensional zone electrophoresis, namely starch- and polyacrylamide gel-electrophoresis (PAGE). Several similar genetic studies were also based on these techniques. Already in the early days, Braend (1967), Stevens and Townsley (1970) and Day *et al.* (1971) reported protein polymorphisms in canine serum as mentioned previously. Also, Bauer and Shorr (1969), Braend and Austad (1973) and Meera Khan *et al.* (1973) described genetic polymorphisms of some enzymes in canine erythrocytes. It is now known that the shape, size and ionic charge of the protein molecules (Johnson, 1979), the composition and pH of the buffer system (Smithies, 1959) and the percentage, pH and composition of the gel (Ferreira *et al.*, 1984) are variables affecting the separation and resolution qualities of the electrophoresis technique.

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Two-dimensional polyacrylamide electrophoresis was also attempted, but no interpretable results were obtained, due to poor resolution of the gel banding patterns.

The starch gels were prepared as 12% gels, following procedures by Hillis *et al.* (1996). The protocols for horizontal starch gel electrophoresis as described by Grant (1989) were used. After preliminary experiments, using various buffers to determine optimal electrophoretic conditions, four systems were identified and applied: *(1)* **MF** – a continuous Tris, boric acid and EDTA buffer with pH 8.6 (Markert and Faulhaber, 1965); *(2)* **TC** – a continuous Tris and citric acid buffer with pH 6.9 (Whitt, 1970); *(3)*

A – a continuous Tris, EDTA and borate buffer with pH 8.6 (Goncharenko *et al.*, 1992); and *(4)* **RW** – a discontinuous Tris, citric acid (gel pH 8.7), lithium hydroxide and boric acid (electrode pH 8.0) buffer (Ridgeway *et al.*, 1970).

Systems using polyacrylamide gel-electrophoresis (PAGE) for analysis were prepared as described by Gahne *et al.* (1977). This procedure was carried out at the Animal Improvement Institute, Pretoria, at a laboratory subject to annual international comparison tests to ensure that an excellent standard is maintained (Figure 5). The buffers used, for optimal resolution and clarity, for each protein loci is indicated in Table 1 (see Chapter 3 - *Results*).

Figure 5: Laboratories of the Animal Improvement Institute at the Agricultural Research Council (ARC), Pretoria (South Africa).

Continuous starch gels with concentration of 13.8% were used for analysis of hemoglobin consisting of 60.5g Tris, 6.0g EDTA and 4.6g boric acid per litre of distilled water (pH 8.9). These gels were run on 50 volts until the visible banding patterns that could be clearly observed, had migrated approximately 3 cm into the gel.

2.3.2. Histochemical techniques

Following electrophoresis, the starch gels were sliced into four slabs and each slab was assayed for a different protein. Standard recipes for the specific stains were used (Harris and Hopkinson, 1976; Hillis *et al.*, 1996). Two methods were used for staining of the gels. The first method was performed by soaking the gel in a solution containing the staining components, followed by destaining the gel in a different solution which was devoid of the staining agent (Grant, 1989). PAGE and some starch gels were stained in this fashion using a general protein (PT) stain from recipes of Harris and Hopkinson (1976). The second technique entailed using a 2% agar overlay in which the components for staining were mixed. The latter method is used for detection of the majority of protein systems in starch gels (Evans, 1987). The stained gels were photographed for quantifying the bands and as reference, once the staining was completed.

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Alleles at each locus were designated alphabetically by the electrophoretic mobilities of the gene products they encode, relative to the mobility of the most common allelic product at the locus. The locus nomenclature in Van der Bank *et al.* (1992) was followed.

2.4. GEL INTERPRETATION AND DATA ANALYSIS

2.4.1. Nomenclature

The genotype at the gene locus coding for the enzyme can be inferred for each individual in the sample from the number and positions of the stained spots on the gels (Ayala, 1982). The electrophoretically detectable variants, which can be assumed to differ genetically from each other, may be referred to as alleles (Gutiérrez *et al.*, 1983).

The banding patterns were interpreted and the loci numbered from the cathode to the anode, e.g. the zones of activity closest to the origin (the most cathodal end of the gel) were designated as the first locus, with the more anodal loci indicated by progressively higher numbers. The alleles in each zone (or locus) are also labelled from the origin, according to the relative mobilities of the gene products that they encode. They are labelled alphabetically from the cathode and proceeding towards the anodal end or buffer front.

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2.4.2. Statistical Analysis

2.4.2.1. Genetic variation within populations

The BIOSYS-1 program was used to calculate average heterozygosity values (**H**) using the formula of Nei (1978) and Chi-squared (χ^2) values for significant deviations of allele frequencies from expected Hardy-Weinberg equilibrium at each locus. The

expected frequencies were calculated using Levene's (1949) formula for small sample size. The coefficients of heterozygosity deficiency or excess (**d**) for each locus, mean number of alleles per locus (**A**) and percentage of polymorphic loci (**P**) using the 0.95 criterion were calculated. Sample size easily effects the latter measure (Ayala, 1982), hence the necessity for a criterion.

A locus is usually only considered polymorphic when the most common allele has a genotypic frequency of no more than 0.95 (Ayala, 1982), although some authors prefer a criterion of 0.99. Apart from sample size complications, another disadvantage of this polymorphism is that it is an imprecise measure of variation since a slightly polymorphic locus counts as much as a very polymorphic one. Nevertheless, this measure still gives an important aspect of genic variation within populations, as long as a large number of individuals and loci are studied (Nei, 1987). Hartl and Pucek (1994) even argue that the proportion of polymorphic loci may be more biologically significant than **H**, since one very polymorphic locus or a few moderately polymorphic loci give approximately the same values for **H**, whereas the **P** reflects the loss of individual alleles more directly.

A more appropriate measure of genic variation is average heterozygosity (**H**) or gene diversity as it estimates the probability that two alleles taken at random from a population are different (Ayala, 1982; Nei, 1987). It can be defined unambiguously in terms of gene frequencies and therefore does not depend on the arbitrariness of the definition of polymorphism. Although there are several ways in which to measure and estimate average heterozygosity, BIOSYS-1 was used to calculate **H** as the usual estimate, based on Hardy-Weinberg expectations (biased estimate) for this study.

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Degrees of freedom (**DF**) at all polymorphic loci, as well as individual heterozygosity (**h**) values for each population were also calculated, with the latter being calculated as the ration of the number of heterozygous loci for that individual to the total number of loci assayed for that individual.

2.4.2.2. Genetic differentiation between populations

The genetic distances (**D**) between breeds were determined with Nei's (1972, 1978) formulas. Genetic distance is the extent of gene differences (genomic difference) between populations or species that is measured by some numerical quantity, which refers to the number of nucleotide substitutions per nucleotide site or the number of gene substitutions per locus (Nei, 1987). It does, however, historically refer to the gene differences as measured by a function of gene frequencies (Nei, 1987), as was the case for measurement in this study. The computer programme DISPAN (© Tatsua Ota, 1993, Pennsylvania State University, USA) was also used to generate support values for the phylogenetic tree, using neighbour-joining and ‰ bootstrap tests (1 000 replications).

The fixation statistics $\mathbf{F_{IS}}$, $\mathbf{F_{IT}}$ and $\mathbf{F_{ST}}$ (Wright, 1965, 1978; Nei, 1977) were used as an additional measure of differentiation between populations. The BIOSYS-1 programme was used to calculate these Wright's fixation index (**F**) values for polymorphic loci. The F-statistic is also known as the inbreeding coefficient (Ayala, 1982) and gives an indication of the correlation between uniting gametes and thereby helps to determine if populations are from the same gene pool. The $\mathbf{F_{IT}}$ statistic is an indication of the amount of inbreeding in the population as a result of population

subdivision. $\mathbf{F_{IS}}$ is a measure of the degree of allelic fixation in individuals, relative to the subpopulation, and approaches zero in most natural populations where random mating occurs (Nei, 1986). $\mathbf{F_{ST}}$ is a correlation between two gametes drawn at random from each subpopulation and measures the degree of differentiation of subpopulations (Nei, 1987) or can be seen as a mean measure of relatedness of individuals within a population (Swofford and Selander, 1981, 1989).

The statistical analysis of the allozyme data was executed using the computer programs BIOSYS-1 (Swofford and Selander, 1981) and GENEPOP – Version 3.1b (Raymond and Rousset, 1997). BIOSYS-1 performs hierarchical cluster analysis using any of the four most popular algorithms: unweighted pair-group method with arithmetic average (UPGMA), weighted pair-group method with arithmetic average (WPGMA), single linkage (SL) and complete linking (CL) as described by Sneath and Sokal (1973). In this study UPGMA was used since it is the most commonly used method for cluster analysis. GENEPOP-Version 3.1b program is used to determine if significant differences in allele distribution between breeds or groups occur.

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