The development of the Single-Strand Conformation Polymorphism (SSCP) technique to assess sequence level variation within the Major Histocompatibility Complex (MHC) *DRB1* gene in four South African buffalo (*Syncerus caffer*) populations.

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"Appreciation is a wonderful thing: It makes what is excellent in others belong to us as well."

Voltaire

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Abstract:

This thesis reports the development of Single-Strand Conformation Polymorphism (SSCP) technique to assess sequence level variation within the Major Histocompatibility Complex (MHC) *DRB1* gene in four South African buffalo populations. MHC gene products are involved in the immune response, and so variation within these genes provides information on the immunological fitness of the population under study. The aims of this study were: (i) to develop the SSCP technique; (ii) to investigate the level of genetic variation at the peptide binding region (PBR) of the *DRB1* gene in four South African buffalo populations. (iii) This data was then compared to data generated previously in a study on the same populations using microsatellite DNA, (iv) the statistical comparisons were used to assess the appropriateness of SSCP data for population genetic analysis.

Levels of heterozygosity, allelic diversity and population differentiation were quantified using MHC *DRB1* gene. The amplified region (Exon 2 of the *DRB1* gene) showed high levels of variability, with 77 alleles found in the 84 individuals examined using SSCP analyses. In addition, a smaller sub-set of individuals were analysed by RFLP. RFLP found 13 genotypes in 31 individuals.

Comparisons to a microsatellite study found a similar trend with regard to F_{ST} and heterozygosity, although the microsatellite study reported higher F_{ST} values these differences were not statistically significant. Slight levels of population structuring were determined; this differentiation is proposed to be a result of genetic drift.

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Abbreviations:

α	alpha
A	adenine
ANP	Addo National Park
β	beta
bp	base pair
C	Cytosine
°C	degrees Celsius
dH_2O	distilled water
cfu	colony forming units
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EDTA	disodium ethylenediaminetetraacetate dihydrate
EtBr	ethidium bromide
Fig.	Figure
FORS-D	folding of randomised sequence difference
γ	gamma
g	gram
G	guanine
HWE	Hardy-Weinberg Equilibrium
H_o	Heterozygosity (observed)
H_{oc}	corrected H_o values
H_e	Heterozygosity (expected under HWE)
HLA	human leucocyte antigen

IPTG	isopropyl β-D-thiogalactopyranoside
Kb	kilobase, kilobasepair
KNP	Kruger National Park
λ	lambda
L	liter
MHC	major histocompatibility complex
M	molar
mg	milligram (10^{-3}g)
ml	milliliter (10^{-3}L)
m*M*	millimolar (10^{-6}*M*)
μg	microgram (10^{-6}g)
μl	microliter (10^{-6}L)
μ*M*	micromolar (10^{-6}*M*)
min	minutes
mt	mitochondrial
NaOH	sodium hydroxide
Ne	effective population size
ηg	nanogram (10^{-9}g)
PAGE	polyacrylamide gel electrophoresis
PBR	peptide binding region
PCR	polymerase chain reaction
ρmol	picomole (10^{-12}mole)
RAPD	random amplified polymorphic DNA
RE	restriction enzyme
RFLP	restriction fragment length polymorphism

sec	seconds
SSCP	single strand conformation polymorphism
StL	St Lucia
T	thymine
TE	Tris-EDTA buffer
MHC	major histocompatibility complex
TBE	tris-borate-EDTA buffer
UHC	Umfolozi-Hluhluwe Complex
u	unit
UV	ultra violet
V	volts
v/v	volume per volume
w/v	weight per volume
X-Gal	5bromo-4-chloro-3-indolyl-β-D-galactopyranoside

One-Letter Amino Acid Abbreviations

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

"We do not understand when the buffalo are all slaughtered, the wild horses are tamed, the secret corners of the forest heavy with scent of many men, and the view of the ripe hills blotted by talking wires. Where is the thicket? Gone. Where is the eagle? Gone. The end of living and the beginning of survival."

Seattle (c. 1784 – 1866) Native American Chief

Chapter 1: Introduction

"Adapt or perish, now as ever, is nature's inexorable imperative"

H. G. Wells

1.1 Natural history of African buffalo (Syncerus caffer)

The *Bovini* sub-family originated during the lower Pliocene, in Asia, as an offshoot of the nilgai (*Baselaphus tragocamelus*), the largest of the Asian antelope **(1)**. The most recently evolved of the ruminants, African buffalo (*Syncerus caffer*), the only wild member of the Bovini tribe south of the Sahara were once widely distributed in large herds throughout sub-Saharan Africa **(2).** Hunting and habitat loss led to a reduction in buffalo numbers at the end of the 19th century; however, a foot-and-mouth disease outbreak and the morbillivirus rinderpest pandemic of 1894 and 1896 respectively resulted in a loss of approximately 95% of the original population **(3)**. The remaining South African buffalo are confined to the game reserves: Kruger National Park (KNP), Umfolozi-Hluhluwe Complex (UHC) and Addo National Park (ANP) (Fig. 1.1). These remnant populations have been isolated from each other for 100 years as a result of disease management legislation **(3)**.

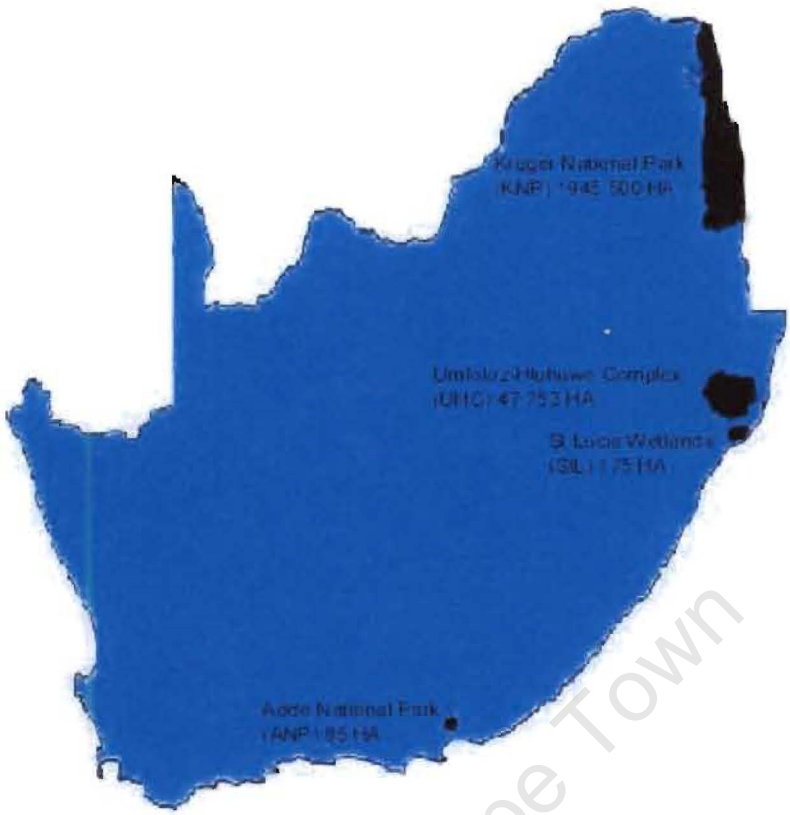


Figure 1.1: Map of the three remnant populations and a seeded population (StL) seeded from UHC in 1977.

Buffalo are known reservoirs of diseases such as foot-and-mouth disease, corridor disease, bovine tuberculosis and brucellosis. Foot-and-mouth disease confined areas contain 89% of South African buffalo while 95% reside in corridor disease and tuberculosis confined areas **(4)**. These diseases would negatively affect the South African livestock industry should transmission occur. As a result, legislation prevents the movement of buffalo from foot-and-mouth, tuberculosis and corridor disease controlled areas **(4)**. Consequently, there is a significant demand for buffalo from the few disease-free herds to stock game ranches. The only disease free, remnant buffalo population is the population in the ANP. Presently individuals from various zoological gardens or the ANP, with a very small population of only 45 (most recent census figures), founded all of the South African buffalo in disease free areas **(4)**.

African buffalo are the only representatives of the sub-family *bovini* that still occur in substantial numbers in the wild **(5)**. Buffalo are important in terms of biomass, as they are the most dominant species in their natural habitat, ecologically buffalo prefer grass lands with plenty of water and some dense cover, but may occur in a variety of habitats **(6)**.

Because there has been no gene flow between the remnant South African populations for approximately 15 generations **(2)**, the African buffalo is an ideal model organism to study the long-term effects of habitat fragmentation on population genetic diversity. These extant buffalo populations have been subjected to human disturbances, cycles of local extinctions and recolonisations that could lead to a loss of genetic variability, manifesting itself in the form of low levels of molecular diversity **(5, 6)**. Decreased genetic variability within a population is thought to be the result of inbreeding and random genetic drift. This loss of genetic variability is manifested as a decrease in observed heterozygosities **(7, 8)**. Heterozygosity is the occurrence of two distinguishable alleles at a genetic locus **(9)**. We would expect genetic drift to affect a loss in genetic diversity proportional to the population size (Fig. 1.2). The genetic variation seen in this study may be historical; as there have been approximately 11 generations since fragmentation.

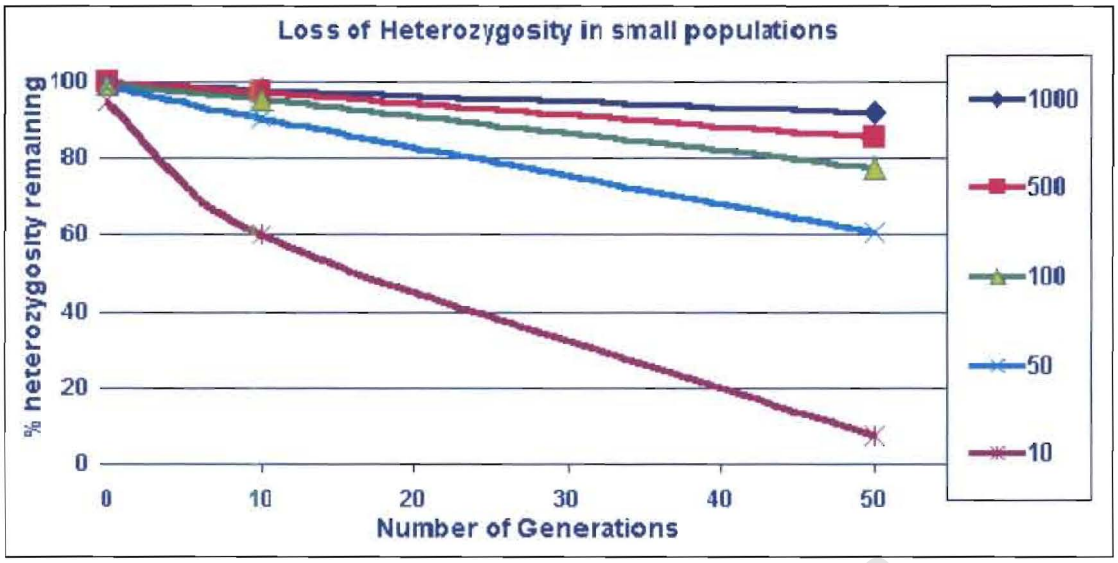


Figure 1.2: Hypothetical graph representing a calculated loss of heterozygosity in various sized small populations over time. This graph was produced by applying equations provided in **(77)**.

Africa buffalo are water-dependent roughage feeders. They can survive on most major vegetation types, provided there is a permanent water source **(2)**. Without an efficient moisture conservation mechanism, buffalo must drink regularly **(1)**. Consequently most population bottlenecks in the Addo National Park (ANP) have occurred during periods of drought (discussed in the next section).

Buffalos are highly gregarious, living in large mixed herds with male dominance hierarchy **(2)**. These large herds are often composed of small units, which occur in separate home ranges **(1)**. While there appears to be minimal contact between units, officials at the Kruger National Park noted that after drought units often reform with different members.

1.1.2 Demographics of study populations

The Addo National Park (ANP) population size has fluctuated greatly in relation to annual rainfall. The 1980-81 drought caused drastic declines, and the 1984 drought reduced the census numbers from 157 in 1983 to 42 in 1985 (a 73%

reduction in numbers). The numbers remained below 100, and at time of sampling in 1994, the census count was 66 **(Appendix F)**.

In contrast the Umfolozi-Hluhluwe Complex (UHC) population increased steadily in numbers from the earliest census in 1929 of 76 animals to 8400 animals at time of sampling in 1993. During the 1960's the Umfolozi National Park and the Hluhluwe National Park were combined to form the UHC, this opening of the parks borders partly accounts for the dramatic increase in population size during this time. The population size was maintained within carrying capacity limits by annual removals of animals from 1980 **(Appendix F)**.

The St Lucia (StL) population was founded with 23 animals from the UHC during 1977, but 4 animals died that year. Effectively this population was founded by 19 individuals. This population, whilst maintained by an annual cull' increased over the years to a size of 180 individuals at time of sampling in 1992 **(Appendix F)**.

In the Kruger National Park, rivers divide the herds for most of the year. During dry periods, these rivers get low enough to cross, as evidenced by the crossing of buffalo tuberculosis across the Sabie River. This freedom of movement under these circumstances may also improve genetic diversity **(Appendix F)**.

1.2 The Major Histocompatibility Complex class II:

The Major Histocompatibility Complex (MHC) was discovered at the beginning of the 20th century when scientists proposed that transplant rejection was caused by the host immune response to antigens displayed on the donor cells **(11)**. It was determined that the gene products implicated in the rejection process were located together in a specific region of the genome **(12)**, later named the Major Histocompatibility Complex (MHC) **(11)**. Further characterisation revealed that the MHC is composed of several hundred genes and a relationship between these loci and disease resistance was observed and investigated. This research led to the discovery that cell surface proteins of the MHC were involved in immunological recognition through antigen presentation and cell-cell interactions **(13)**. Indeed the molecules of the MHC are responsible for the greatest allogenic reaction in all species studied to date **(11)**. The MHC genes have an important immunological role in all vertebrates **(22)**. MHC antigens are co-dominant (11), suggesting that heterozygotes would express twice as many MHC genes as homozygotes.

Although developments in the medical field have led to a wealth of data on the MHC in relation to disease resistance **(14, 15)**, population genetic studies **(16-20)** are a recent extension in the development of MHC techniques. Population genetic studies utilise the highly variable Peptide Binding Region (PBR), which contains a greater number of non-synonymous to synonymous nucleotide substitutions than in the more conserved non-peptide binding regions of the molecule **(21)**.

Much of conservation genetics research is based on the implicit assumption that molecular markers studied should be selectively neutral **(22, 23, 24)**. However, MHC genes provide an opportunity to study a genetic marker under balancing selection or overdominance, where heterozygotes have an immunological advantage over either homozygote **(25)**. MHC markers are important when investigating the adaptive evolution of populations since the differential selective effects of each genetic variant impact directly on the fitness of individuals within the population **(22)**.

MHC molecules are cell surface glyco-proteins (Fig. 1.3) that are involved in cell-cell interactions and the presentation of antigenic peptides to the helper T lymphocytes in order to initiate an immune response **(26)**. The MHC is one of the most gene dense regions in any vertebrate genome, and encodes some of the most polymorphic and functional proteins **(27)**. Two hundred and twenty four gene loci were identified when the human MHC was sequenced **(27)**; it was determined that 128 of these genes are expressed **(28)**. Some sequences were estimated to predate the emergence of the immune system by 300 million years **(27)**.

While the present study focused on MHC class II, some mention needs to be made of MHC class I. MHC class I molecules consist of the class I gene product bound to β_2 microglobulin. Class I genes mutate more rapidly than class II genes **(29)**, making them difficult to use in a population genetics context as the class I alleles are so short lived that relationships among populations are obscured. MHC class I molecules are outside the scope o this study and will not be elaborated on further.

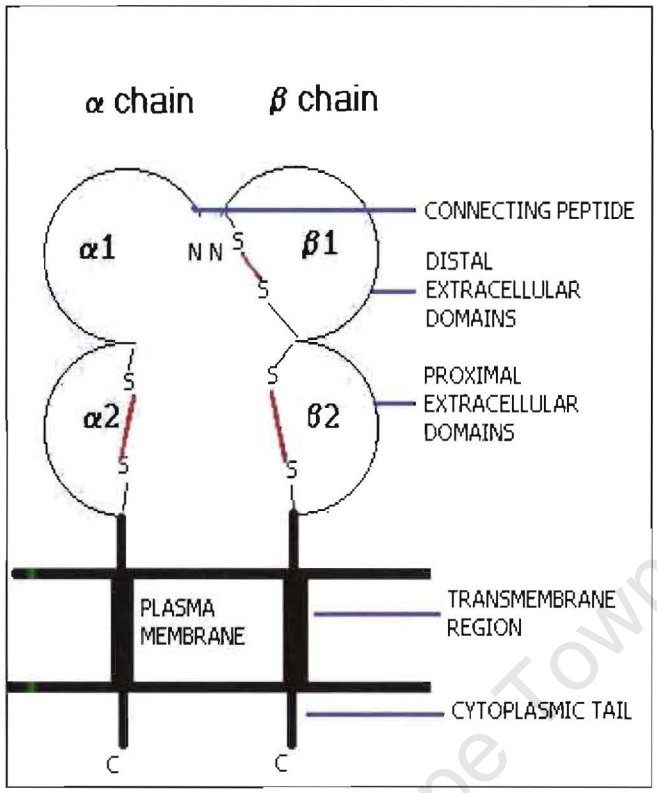


Figure 1.3: The secondary structure of the class II MHC molecule **(30)**.

The class II MHC molecule antigen-binding site consists of two α-helices and an eight-strand β-sheet **(26)**, equally representing the α1 and β1 regions **(26)**. In the particular case of the DR genes only the β genes are polymorphic **(31, 32)**.

Provided the peptide structure is complementary to the MHC binding cleft **(26)**, class I and classII histocompatibility molecules bind antigenic peptides of 8-10 and 12-24 amino acid residues respectively **(33)**.

1.2.1 Function of the major histocompatibility complex

Approximately 40% of the MHC genes encode proteins involved in the immune response, the remaining 60% encodes genes of unknown function, pseudogenes and chaperones **(27)**.

The Immune Response:

MHC class II antigen presentation (illustrated in Fig. 1.4) is characterised by the trafficking of MHC class II molecules through an endocytic pathway where they are loaded with exogenous antigenic peptides for presentation to T-helper cells.

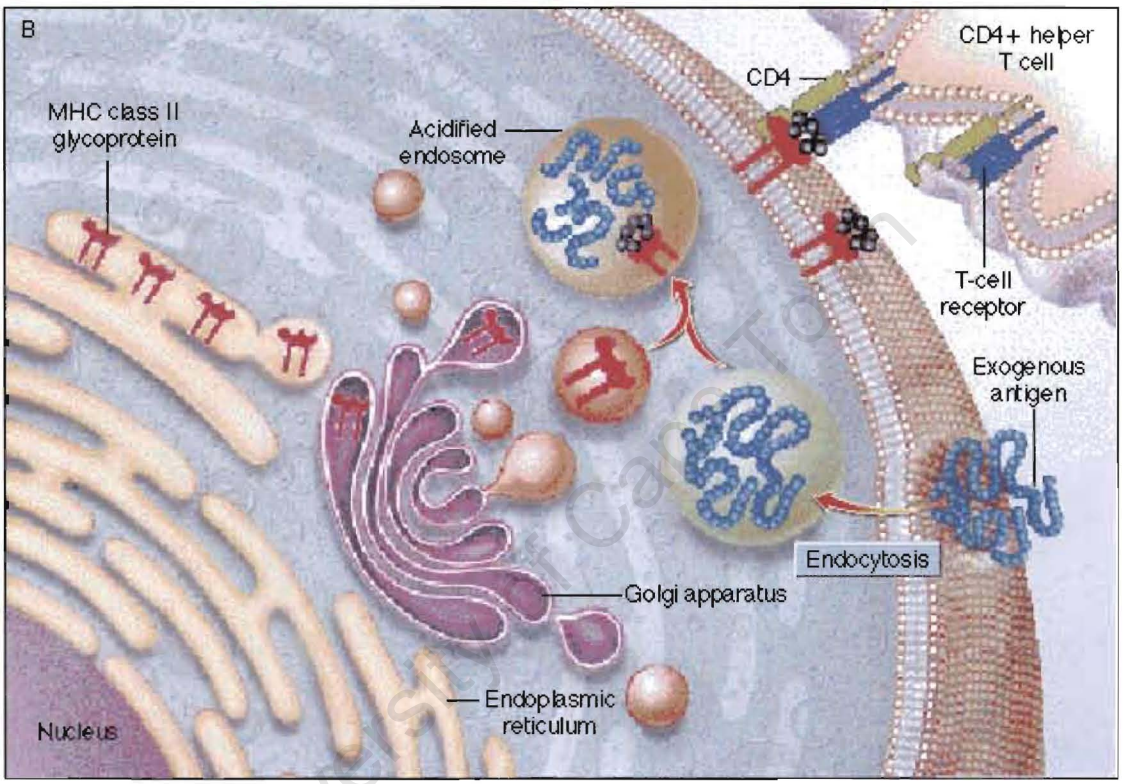


Figure 1.4: Antigen presentation to CD4+ T-helper cells through the MHC II pathway **(34)**.

Helper T cells react only to antigenic peptides presented by 'self" MHC class II molecules. For this reason 'non-self' MHC molecules (tissue transplant cases) initiate an immune response against the tissue carrying them.

At a population level the diversity of the MHC genes means that the recognition systems of individuals within that population are all different. Consequently, the "Perfect Pathogen" cannot evolve an evasive mechanism, capable of evading the

immune systems of all of the individuals in the population, and so cannot spread throughout the entire population **(11)**.

Mate Selection and Kin Recognition

In behavioural recognition systems, one can develop a theory whereby MHC may be responsible for kin recognition and consequently mate selection. Through its immunological function MHC controls microbial flora in the body, and this may produce distinctive odours **(35)**. For example: rats raised in a sterile environment produce no MHC specific odours **(36)**. The ability to distinguish genetically different or similar individuals can be applied by the animal to avoid mating with close relatives, and to favour close relatives with regard to altruistic and co-operative behaviour **(35)**. Studies have shown that mice, rats and human females prefer to mate with males of a different MHC type **(37, 38, 39)**. This observation provides support for the following hypotheses; (i) females choose to enhance their offspring's immunocompetence by mating with a male with complementary MHC alleles she could increase the heterozygosity of her offspring and provide a "moving target" for co-evolving parasites (rare allele advantage). (ii) MHC based mate selection could function as an inbreeding avoidance mechanism. This final hypothesis is supported by observations of kin recognition based on MHC types **(40)**. Landry *et al.* 2001 **(41)** found that the mate choice of Atlantic salmon (*Salmo salar*) aimed to increase heterozygosity of offspring specifically at the peptide-binding region (PBR) of the MHC class II β gene they investigated.

Female mice have been shown to mate with males of different MHC type to their own familial MHC type. In a review, by D. Penn and W. Potts **(40)**, the authors highlighted the experiments in congenic mice strains whereby both male and

female mice select mates of dissimilar MHC types. However, pups raised by cross-fostered (MHC dissimilar) parents chose mates that were dissimilar to their foster parents MHC **(40)**. Human studies by Wedekind in 1995 and 1997 showed a preference of human females for MHC dissimilar males **(42)**. Women were asked to rate the odour of six t-shirts worn for a number of days by three MHC similar and three dissimilar men. Wedekind showed that human females preferred the odour of MHC dissimilar males; however, this preference was reversed in women taking oral contraceptives **(42)**. Ober *et al.* 1997 **(37)** studied a population of Hutterites a small, genetically isolated religious group in North America approximately 400 of whom migrated from Europe in the 1870's because of religious persecution. They found that the Hutterites exhibited MHC-dependent mate preferences; couples were more likely to be MHC dissimilar than by chance. Hutterites have a limited number of five-locus HLA haplotypes (HLA-A, -B, -C, -DR, and –DQ). The authors determined fewer than expected HLA-haplotype matches among spouses based on both computer simulations (using known haplotype data for 411 Hutterite couples) and calculations made from genotype frequencies of the sex and lineage from which the spouse was selected.

1.2.2 Evolution of the Major Histocompatibility Complex

The MHC genes of vertebrates are a multi-gene family with some highly polymorphic loci, acted on by natural selection **(43)**.

Some MHC haplotypes have proven long-lived **(21)**. Hughes *et al.* **(46)** report that humans and chimpanzees share polymorphisms at all shared polymorphic sites in DRβ, and at DQβ, 75% of shared polymorphic site have shared polymorphisms. Human and bovine share 69% polymorphisms at such sites in

DRβ, while human and mouse share 65%. MHC genes have a greater rate of non-synonymous to synonymous nucleotide substitutions in the peptide-binding region than in the more conserved non-peptide binding regions of the molecule. Some MHC polymorphisms have been maintained for millions of years **(21)**. Recently Forsdyke noted that fold pressure decreases in regions under positive "Darwinian" selection. Fold pressure was defined ay Forsdyke as: "The evolutionary pressure on base order which promotes the potential to extrude single strand stem-loops from super coiled duplex DNA". This fold pressure can be measured, and used as supporting evidence for positive selection **(43)**. This fold pressure was measured in mouse MHC class II genes and exon 2 had the lowest 'folding of randomised sequence difference' FORS-D value (table 1.1). Within exon 2 regions involved in peptide or T cell interaction had the lowest FORS-D values **(43)**.

Table 1.1: Average FORS-D values of peptide binding exons compared with non-peptide binding exons **(43)**.

Species	MHC gene	Degree of polymorphism	Peptide binding Exon 2	Non-peptide binding Exon 3
Human	HLA-DRA	Negligible	120±1.30	3.00±0.94
Human	HLA-*DRB4*	High	-2.25±1.13	2.21±1.13
Mouse	H-2Aβ	High	-6.23±0.68	6.64±0.78
Mouse	H-2Aβ2	Negligible	0.96±0.99	2.99±1.46
Mouse	H-2Eα	Low	-0.08±1.20	2.63±1.36

Since MHC diversity plays an important role in the health and reproductive success of vertebrate populations, it is necessary to understand the mechanism by which MHC polymorphisms are maintained **(45)**. Despite a range of research in this field, the mechanisms that maintain the diversity at the MHC loci are controversial.

There are three major explanations for elevated levels of MHC diversity: 1) overdominant selection (heterozygote advantage) Hughes *et al.* **(21)** 2) Pathogen driven selection Hill *et al.* **(14)** and Klein and O'Huigin **(33)** 3) reproductive mechanisms including MHC based dissassortative mating Potts *et al.* **(48)**. These theories are discussed below.

Hughes suggested that overdominant selection is the form of balancing selection acting on the MHC genes **(21)**. Overdominance is also referred to as heterozygote advantage whereby a heterozygote genotype confers a greater advantage to the individual than either homozygote. In contrast, Potts advocates that both pathogen related selection, and reproductive mechanisms, could maintain MHC polymorphisms **(45, 48)**.

A consequence of MHC polymorphism is illustrated by the example of the South African cheetah (*Acinoryx jubatus jubatus*) population. All cheetahs from this population had identical MHC types determined by a lack of rejection to a skin graft, 14 reciprocal skin grafts between unrelated cheetahs were accepted. The consequence of such genetic uniformity was demonstrated in an Oregon breeding colony in 1983. When a feline specific virus, feline infectious peritonitis was accidentally introduced, approximately 50% of the colony died. However, resident lions in the same area, exposed to the same virus, were unaffected. Similarly, domestic kittens of the area were also unaffected **(44)**. Additionally Hill *et al.* 1991 **(14)** found a human MHC haplotype that provides protection against severe malaria in West Africa. This haplotype is common in Gabon, where the study took place, but rare in other racial groups. This data is consistent with the hypothesis of pathogen driven selection at the MHC.

Additional research supporting the occurrence of natural selection at MHC loci is documented by Hill *et al.* **(14)**. Hill suggested that the selection pressure was likely to fluctuate due to the occurrence of epidemics, advocating pathogen driven selection, but not overdominant selection. Hill's study showed that homozygotes were at lower risk of developing severe malaria than heterozygotes at the *DRB1**1302 haplotype. This observation supports the frequency-dependent selection theory (advantage of rare alleles) as opposed to overdominant selection **(14)**.

Klein and O'Huigin supported the co-evolution theory, proposing a pathogen-based model for the maintenance of polymorphism. This theory suggests that co-evolving parasites are the major influence on MHC polymorphism within a vertebrate host, and that 'new' parasites (those which have switched host as recently as 10 000 years ago) have little effect on polymorphism, no matter how virulent **(33)**. The co-evolution theory also encapsulates both negative frequency-dependent selection and overdominant selection. Thus, as pathogens evolve new methods to evade the host immune system, both heterozygotes and rare alleles could become advantageous to the host **(48)**.

The cheetah example (mentioned previously) indicates the importance of inbreeding avoidance for MHC diversity, supporting the use of reproductive mechanisms as a means to maintain MHC diversity. However, mate selection and selective fertilisation or abortion, have not been proved as universal features of the MHC. Since, the biological function of the MHC is the presentation of antigenic peptides to the immune system across all vertebrate taxa, pathogen-based selection could be responsible for maintaining MHC diversity. However, present evidence of pathogen-based selection is largely circumstantial. Although

inbreeding avoidance has not been demonstrated universally, empirical evidence suggests mate selection occurs in mice and humans, and reproductive control mechanisms are employed in rats, mice and humans **(48)**.

1.3 Conservation genetics

Conservation biologists have taken advantage of developments in molecular biology in order to quantify both intra- and inter-population genetic variation. One of the most informative techniques is that of the analysis of microsatellite loci, as illustrated in O'Ryan *et al* 1998 **(3)**. This investigation of the genetic diversity of African buffalo found a relationship between gene diversity and population size, and evidence of population structuring as a result of population fragmentation and genetic drift. Madsen *et al*. 2000 **(49)**, in a study of sand lizards (*Lacerta* agilis) and adders (*Vipera* berus), tested the assumption that variation at microsatellite loci will be reflected in levels of variation at other loci – and provided evidence that microsatellite heterozygosity was not correlated with relative population size. However, these authors, determined that the MHC loci exhibited a significant correlation with population size.

The application of molecular biology in the field of wildlife conservation is increasing. International legislation incorporates genetic diversity as part of its conservation strategies and many conservation programs now use molecular markers to assess the genetic diversity of threatened and endangered species **(50)**.

Mutation, selection, genetic drift and recombination all act on DNA, resulting in variation. Analysis of this variation may provide information at many different levels. Comparative studies within genomic regions can infer time of separation (e.g. when a common ancestor became two sister species) **(51)**. Additionally, regions of DNA with higher mutation rates may be analysed to deduce relationships between individuals or subpopulations **(52)**. Genetic variation can

be compared with geographic distributions to gain information on gene flow and colonisation events. In addition allele distribution and genetic structuring may be used to estimate population size and identify subdivisions **(50)**.

The Use of Molecular Markers in Conservation Genetics

Molecular markers have been identified as being characterised by differential mutation rates and patterns. Specific characteristics of these markers are required to address each of the instances mentioned above. Population genetic studies must begin with the choice of an appropriate molecular marker. This choice must take into account the sensitivity of the marker in relation to the question posed. Assessment at the individual level, such as parentage and relatedness, could best be analysed using genotypic information. Genotypes are disrupted by recombination, and therefore only confer information at an individual level **(23)**. Gene flow and population subdivision might best be assessed at a genic level. Allele and haplotype frequencies are affected by genetic drift; founder effects, gene flow and selection, and so contain population level information. Single copy nuclear markers and mitochondrial markers are used for investigating both among species and, within and between population variation. These markers evolve in relation to mutation rate, selection and changes in effective population size and so are often used for assessing population history **(23)**.

Genetic variation at a molecular level has been observed using allozyme analyses, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), microsatellite analysis and DNA sequencing. Each technique differs with regard to sensitivity, cost, execution time and amount of DNA required **(53)**.

Allozyme analyses underestimate variation because they measure variation of the gene product. Variation at the protein level does not reflect third codon substitutions or any substitution, which does not produce an electrophoretic amino acid substitution thereby underestimating variation **(53)**.

RAPD's is a fairly inexpensive technique, which amplifies arbitrary regions of genomes and reveal more variability than allozymes. However, there are drawbacks related to the use of arbitrary primers, and repeatability problems as reviewed by Black 1993 **(54)**. Black suggests that most polymorphisms detected by this method segregate as dominant markers, rendering the information problematic if working under the assumptions of the Neutrality theory. Empirical observations suggest that RAPD's will have limited application in molecular systematics above the intraspecific level **(54)**.

Mitochondria are maternally inherited molecules, which do not undergo recombination. The mitochondrial genome is found in multiple haploid copes within the mitochondria. Portions of the mitochondrial genome mutate at a high enough rate to be informative at both the within and between level population variation **(2)**. The most variable region is the control region, characterised by sequence and length variation. Because of the uniparental nature of the inheritance, use of the mitochondrial molecule in population genetics is useful as it reduces the effective population size (N_e) by one-fourth that of nuclear genes. The reduction in N_e effectively increases detection of the effects of genetic drift among populations resulting in greater detection of genetic differentiation **(53)**. However, the maternal inheritance aspect of mtDNA leads to a female-biased description of population structure **(2)**.

Another type of marker utilized in population genetics is microsatellite loci. Microsatellites, tandem arrays of short repeats (e.g. CA-repeats), are highly polymorphic and numerous within the genome. Microsatellites mutate through numbers of repeats as opposed to sequence variation, these markers are easily screened by Polymerase Chain Reaction (PCR) and Polyacrylamide Gel Electrophoresis (PAGE) size determination **(55)**. The mutation rate of microsatellite markers, with respect to number of repeats, is approximately 10^{-2} per generation **(2)**.

Choosing a molecular marker

Selection parameters of the marker of choice also relate to the level of variation seen at that marker. Microsatellite markers provide better insight at a population level than mitochondrial DNA, but mitochondrial DNA is a better marker for between population comparisons and phylogenetic studies **(24)**. Most nuclear markers are selectively neutral, but MHC genes are acted on by a selective pressure. This selection pressure allows assumptions to be made regarding the immunological fitness of the population under investigation.

Factors influencing genetic structure and variability

Genetic structure is influenced by migration, generation time, breeding structure and metapopulation structure. Social structured animal populations respond differently to environmental stresses compared to panmictic breeding animal populations. This is because mating systems in these populations involve high levels of non-random mating.

The size of a population, breeding structure, historical bottlenecks and the severity of those bottlenecks may influence genetic structure and variability. For

example, northern elephant seal (*Mirounga angustirostris*) numbers, due to the pressures of hunting, were reduced to 20-100 individuals at the end of the 19th century. The present population consists of 175,000 individuals. This population lacks the genetic variation of the pre-bottleneck elephant seals. In a comparison of 111 contemporary seals with five pre-bottleneck seals (ancient DNA extracted from bone), Weber *et al.* **(56)** found two mtDNA genotypes among the contemporary population and four mtDNA genotypes among the pre-bottleneck seals when using the D-Loop of mitochondrial DNA.

The major histocompatibility complex and conservation genetics

Investigation into the causes of transplant rejection led to the discovery of the MHC at the beginning of the 20th century **(7)**. Research into the MHC is a diverse field ranging from immunology and genetics to animal behaviour and evolutionary studies. Its medical importance has ensured that the MHC has been well characterised both immunologically and genetically in mice and humans, however, research into animal behaviour and conservation biology are developing fields.

Hughes **(57)** ideas about the use of MHC in population and conservation genetics created controversy in 1991, when he suggested that MHC should be used as the only molecular marker in genetic studies of captive populations.. Hughes statement that maintaining MHC diversity to the detriment of other genetic markers, as most genetic loci are selectively neutral **(57)**, led to great debate amongst conservation biologists. Many scientists disagreed with Hughes' statement that a loss of diversity at selectively neutral markers should not cause concern. Variation throughout the genome would allow rapid responses to environmental changes. In response to Hughes, Vrijenhoek and Leberg (1991)

(58) reported that captive breeding programs, which managed populations in order to increase MHC heterozygosity to the detriment of other loci, may improve the health of the population but decrease the ability of the population to adapt to physical challenges in an evolutionary sense **(58)**. Due to the controversy surrounding the use of MHC in captive breeding programs MHC has not been readily used in conservation biology until recently. The importance of MHC in the immune response and inbreeding avoidance, as well as the high levels of polymorphism within MHC genes, makes this genomic region an ideal target for conservation biologists to study the biological fitness of endangered species. MHC diversity is contained mainly within the peptide-binding region (PBR) of the MHC molecules, and the surrounding region of the molecule is highly conserved. This allows universal primers to be designed easily **(59)**, thus reducing time spent isolating markers, cloning, sequencing and designing primers.

Maintenance of MHC polymorphisms may be a dual mechanism whereby mate selection and pathogen-driven selection complement one another. Certain vertebrate species may favour one mechanism over the other, but throughout the vertebrate taxa both mechanisms would be represented.

1.4 Single Stranded Conformation Polymorphism (SSCP)

Single stranded conformation polymorphism (SSCP), a mobility shift analysis of single stranded DNA was first described by Orita *et al* 1989 **(60)**. This technique utilizes the unique sequence specific conformations of single stranded DNA as a basis for rapid screening sequence specific polymorphisms. Once optimised SSCP confers the advantage of being an inexpensive technique which screens for sequence variation in a large sample set without the need for direct sequencing of all the samples.

Initial sequence screening studies were performed using restriction fragment length polymorphism (RFLP) analysis. RFLP is limited because it can only detect variation at specific restriction enzyme sites **(60)**. A more efficient method, developed by Noll and Collins employing denaturing gradient gel electrophoresis (DGGE), employed mobility shift analysis to detect point mutations. SSCP developed from this under the assumption that the mobility shift in DGGE was due to conformational changes of single stranded DNA as the duplex DNA denatured **(60)**.

An SSCP experiment begins with the amplification of source DNA. DNA fragments must be within a size range acceptable for highest SSCP efficiency (100 - 400bp) Kukita *et al.* 1997 **(61)** claim SSCP sensitivity to be 80% for fragments smaller than 300bp. Amplified DNA is then denatured in the presence of NaOH and formamide at 95°C for five minutes. The denatured product can subsequently be electrophoresed on a non-denaturing or mildly denaturing polyacrylamide gel. Gel shifts may be visualised by whichever visualisation

methods are in use in the laboratory. SSCP does, however, require extensive optimisation. Both gel and electrophoretic conditions must be fully optimised.

SSCP revolutionised sequence screening in medical laboratories and in 1995 Murray *et al.* 1995 utilised the technique for sequence screening MHC of a population of beluga whales (*Delphinapterus leucas*) **(62)**.

1.5 Research objectives

The aims of this study are to :

(i) Optimise the SSCP technique with regard to PCR product denaturation, polyacrylamide gel composition, electrophoretic conditions and visualisation methods.

(ii) Use the optimised SSCP technique to investigate the level of genetic variation within the four South African buffalo populations (KNP, ANP, UHC and StL) at the PBR of the MHC *DRB1* locus.

(iii) Perform statistical analysis comparing MHC *DRB1* data with previous microsatellite data on the same samples, in order to assess the value of MHC genes as markers of overall genetic fitness. Additionally the genetic status of South African buffalo as a whole will be evaluated.

(iv) Assess the appropriateness of the SSCP technique to obtain data for population genetic analysis.

Chapter 2: Materials and Methods

"In all things in nature there is something of the marvellous"

Aristotle

2.1 Sample Information:

Samples consisting of ear nicks (or muscle in the case of the Kruger National Park (KNP)) were collected from 105 individuals from all three remnant populations (Kruger National Park (KNP), Umfolozi-Hluhluwe Complex (UHC) and the Addo National Park (ANP)) and one seeded population (St Lucia wetlands (StL) seeded from UHC).

Table 2.1: Summary of populations sampled.

Locality	Year established	Size (HA)	Total population	DNA source	Year sampled	No. Samples	No. Samples analysed
KNP	1898	1 945 500	35 000	Muscle	1992-93	34	30
UHC	1897	47 753	8 400	Blood	1993	38	31
StL	1977*	175	175	Blood	1992	23	13
ANP	1931	85	66	Blood	1994	10	10

* Year buffalo were introduced

2.2 DNA extraction

DNA samples from South African buffalo of the Kruger National Park (KNP), Umfolozi-Hluhluwe Complex (UHC), St Lucia Wetlands (StL) and Addo National Park (ANP) were received from Dr. C. O'Ryan **(3)**. DNA had been extracted using a standard phenol-chloroform technique described in Sambrook *et al.* **(65)**.

2.3 Selection of primers

Although the PBR is highly polymorphic, the region surrounding it is highly conserved, making the use of universal MHC primers possible. See table 2.2 for a summary of primers used.

Table 2.2: Summary of primers used.

Primer name:	Primer Sequence:	Reference:
DQB1	5′-ctg gta gtt gtg tct gca cac-3′	**62**
DQB2	5′-cat gtg cta ctt cac caa cgg-3′	**62**
HLO30	5′-gag cga gtg tca ttt ctt ca-3′	**6**
HLO32	5′-tgt ctg cag tac gtg tcc a-3′	**6**
DRB1F	5′-gtg tct gca gta cgt gtc c-3′	Designed in this study
DRB1R	5′-gag cga gtg tca ttt ctc c-3′	Designed in this study

The second exons, containing part of the (PBR), of two MHC genes, *DQB* and *DRB1*, were amplified. Primers: DQB1 and DQB2 (developed in the Beluga whale, *Delphinapterus leucas*) **(62)** were used to amplify *DQB*. HLO30 and HLO32 primers developed in cattle, *Bos taurus*, **(6)** were used initially to amplify *DRB1*.

DQB provided irreproducible results and so data was generated using the *DRB1* gene. Initial amplifications of the *DRB1* gene with the HLO30/HLO32 primer set were used to generate sequence data in order to verify initial SSCP results. This sequence data was subsequently used to design the primers used throughout this study, DRB1F and DRB1R. Further analysis was performed on *DRB1* PCR products. These primers were developed using DNAman **(63)**.

Primers were custom synthesized at the DNA Laboratory, Department of Molecular and Cell Biology, University of Cape Town.

2.4 PCR: Amplification of target region

The polymerase chain reaction (PCR) is a primer-mediated enzymatic DNA amplification, was invented by Kary Mullis in 1987. The PCR process requires a repetitive series of three steps: 1) denaturation of the double stranded DNA (94-96°C). 2) Annealing of the primers to the single-strand template (temperature dependent on the T_m or melting temperature of the primer: template hybrid). 3) Extension of the primers (72°C) via *Taq* polymerase activity to produce copies that can be used as template in subsequent cycles.

Ten to 50 ng of genomic DNA was amplified in 0.2ml thin walled Eppendorf tubes. The PCR reaction mixture containing the following: 1.0μ*M* of each primer, 0.8m*M* dNTP mix 0.375u BIOTAQ™ DNA Polymerase (Bioline UK Ltd) and 1X corresponding reaction buffer. PCR was carried out in a final volume of 25μl. The PCR conditions for *Syncerus* leukocyte antigen *DQB* (SyLA-*DQB*) DQB1/DQB2 gene included 2m*M* $MgCl_2$. Thermal cycling was carried out on a Hybaid PCR Sprint (Hybaid, UK) as previously described **(6)**. A modified annealing temperature of 57°C was used. SyLA-*DRB1* HLO30/HLO32 was amplified under similar conditions to SyLA-DQB. The following modifications were used: 1m*M* $MgCl_2$ was used in the reaction mix. Thermal cycling was carried out on a Hybaid Thermal Reactor (Hybaid, UK). Cycling consisted of an initial denaturation step for 4 min at 94°C followed by 35 cycles of 1 min at 94°C, 45sec at 62°C and 1 min 15sec at 72°C. A final extension step of 5 min at 72°C completed the reaction cycle. SyLA-*DRB1* DRB1F/DRB1R was carried out using 1X*taq* polymerase buffer (Southern Cross Biotechnology, Cape Town), 1mM $MgCl_2$ and 1.25u *Taq* polymerase (Southern Cross Biotechnology, Cape Town). PCR was carried out

with an initial denaturation of 95°C for 5 min, followed by 30 cycles of 94°C for 30sec, 60°C for 20sec, and 72°C for 20sec, a final elongation step of 72°C for 5 min completed the reaction. Reactions were performed on a Gene Amp PCR System 9700 (Perkin Elmer Applied Biosystems, USA). A negative control was included in all amplification experiments, and comprised of a sample that contained all of the above components except for the template DNA. The size of the PCR products was determined by 2% (w/v) agarose gel electrophoresis before continuing with SSCP and RFLP analyses.

2.4.1 PCR Optimisation

In order to optimise PCR a series of titrations for $MgCl_2$, primer and DNA concentration were carried out as well as a range of melting temperature experiments. These optimisation experiments took place over approximately 40 PCR's and were visualised by ethidium bromide staining of 2% (w/v) agarose gel. Further optimisation was required under SSCP conditions.

2.4.2 PCR purification

Ambiguous SSCP banding patterns necessitated the purification of PCR products prior to SSCP analysis. This purification was carried out using standard plugged tips.

PCR products were electrophoresed on a 2% (w/v) agarose gel, confirming the product size and purity. Bands were excised. TE was washed through plugged tips by centrifugation at 13 000 rpm for one min. Plugged tips were transferred to a clean 1.5ml eppendorf tubes and the excised agarose gel slices placed on the filter. This was then centrifuged at 13 000 rpm for 10 min. Ultimately the solution in the eppendorf tub is sufficiently pure to perform SSCP experiments. Initial tests comparing this method with a commercially available purification kit showed that the plugged tip method produced marginally better results.

2.5 Single Stranded Conformation Polymorphism:

2.5.1 Optimisation

The SSCP protocol required extensive optimisation with regard to denaturation conditions, gel components (polyacrylamide concentration, acrylamide: bis-acrylamide ratio, gel additives such as glycerol and urea), electrophoretic conditions and visualisation methods.

Polyacrylamide gels used ranged from 5-12% neutral polyacrylamide gels **(60, 62, 64)** with 0%, 5% or 10% glycerol. These gels were electrophoresed at room temperature or 4°C at 200V overnight.

The SSCP profile was visualised in one of three ways: silver staining **(Appendix A)**, ethidium bromide (EtBr) staining **(Appendix A)**, or γ32P end labelling. Ethidium bromide staining followed the protocol of Hongyo *et al.* 1993 **(64)**, with the following modifications: 30μl of PCR product was mixed with 15μl of a denaturing solution **(60)**. After heat denaturation, 25μl was loaded onto a 12% non-denaturing polyacrylamide gel and electrophoresed for 2h at 300V at 4°C. Primer end labelling method was performed according to O'Ryan *et al.* **(3)**. Either one or both primers were radioactively end-labelled prior to thermal cycling under standard conditions as mentioned above. Silver staining proved to be the optimal visualisation method.

2.5.2 Parameters used

After amplification, 10μl of PCR product was combined with 10μl of SSCP loading dye containing 10mM NaOH, 1mM EDTA, 0.01% bromophenol blue, 0.01%

xylene cyanol and 80% formamide (http://europium.csc.mrc.ac.uk/usr/database.dir/methods.dir/pcrpract.htm) **(Appendix A)**. Samples were heat-denatured at 95°C for 5min and immediately cooled on ice for 2min; 15µl was loaded onto a 10% "Mildly Denaturing" SSCP polyacrylamide (10% MD-SSCP) gel (a polyacrylamide gel containing 2.66*M* Urea, 0.5XTBE, 5% glycerol, 10% (39:1) Polyacrylamide, 0.1% AMPS, 0.001X TEMED) **(Appendix A)**. This gel had been glued to the plate with γ-Methacryloxypropyl trimethoxysilane (Sigma) used as per manufacturers instructions. Control samples, which were co-electrophoresed, included a non-denatured sample as control for the electrophoretic migration of the double stranded DNA and λPst or λEcoR*V* as molecular size markers **(Appendix A)**. Samples were electrophoresed overnight at 200V at 4°C. The SSCP profile was visualised by silver staining. After disassembling the plates the plate with the gel adhering to it is first washed with distilled water (dH_2O) and then agitated in the first silver staining solution of 0.1% (w/v) $AgNO_3$ for 10 min and subsequently washed with dH_2O. The gel is then immersed in the second solution of 1.5% (w/v) NaOH, 0.01% (w/v) $NaBH_4$ and 0.4% (v/v) formaldehyde **(Appendix A)**.

2.5.3 SSCP gel scoring method

SSCP gels were scored by a numerical system whereby each unique banding pattern was given a numerical value. Heterozygote-banding patterns were matched against homozygote-banding patterns as shown in fig 2.1. These numerical values were then converted to a di-alleic scoring system (e.g. 0101 for a homozygote and 0102 for a heterozygote) Fig 2.1. This data conversion ensures interpretation by statistical packages.

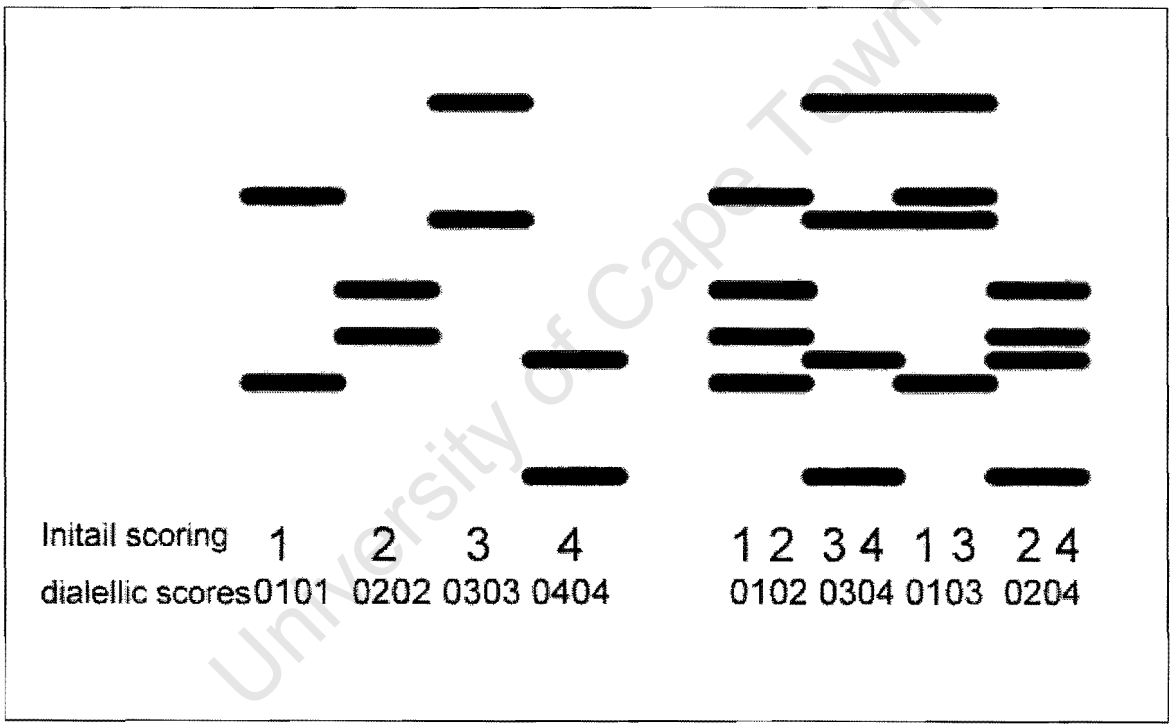


Figure 2.1: An example of SSCP banding patterns and there representative scores. Heterozygotes are compared with homozygotes in order to assign representative score for use in statistical packages.

2.6 Restriction Fragment Length Polymorphism:

Ten microlitres of PCR product was digested to completion at 37°C with 10U of *RsaI*, *HhaI* or *HaeIII* in a final volume of 50µl. Restriction fragments were resolved on 4% agarose gels, and stained with either EtBr at 0.5µg/ml or GelStar® Nucleic Acid Gel Stain (BioWhittaker Molecular Applications, USA) which was used according to manufacturer's specifications.

The recognition sequences for the above mentioned enzymes are *RsaI*: GG^{Y}CC, *HhaI*: GCG^{Y}C and *HaeIII*: GT^{Y}AC.

Restriction Fragment Length Polymorphism (RFLP) was carried out as a preliminary study to establish sequence variability while developing the SSCP technique. Subsequent comparisons are based on the same sample set.

2.7 Automated sequencing:

Validation of preliminary SSCP results

Initial PCR products amplified using HLO30/HLO32 primer set **(6)** was sub-cloned and sequenced to validate preliminary SSCP results and to provide sequence data for the design of buffalo specific primers.

The second exon of *DRB1* (HLO30/HLO32) was amplified and purified using a QIAquick™ PCR purification kit (Qiagen, Germany). The amount of purified product was quantitated and ligated using the pGEM®-T Easy Vector System I (Promega, USA) as per the manufacturer's instructions. Competent XL1-Blue *Escherichia coli* cells were transformed using heat shock **(65)** to an efficiency of 3.4 X 10^7cfu/μg and 200μl of each transformation culture was plated onto Luria Agar containing 100μg/ml ampicillin, 50mg/ml X Gal and 100μ*M* IPTG **(Appendix A)**. The plates were incubated at 37°C overnight and blue/white selection was used to identify positive transformants. To confirm the presence of an insert, three positive colonies were picked with a sterile toothpick and used directly as template in a standard *DRB1* (HLO30/HLO32) PCR reaction. The same three positives were then also used to innoculate a 5ml liquid culture of Luria Broth containing 100μg/ml Ampicillin. Plasmid purification of the 5ml cultures was carried out using a QIAprep® Spin Miniprep Kit (Qiagen, Germany) and 2μl of the purified plasmid was then used as a template for a standard PCR to check for the presence of an insert. Subsequently, two clones were selected for three individuals: one putative homozygote and two putative heterozygotes as determined by preliminary SSCP data. The plasmids isolated from these clones were cycle sequenced (Sequencing Service, Department of Molecular and Cell

Biology, University of Cape Town) on a GeneAmp PCR System 9700 (Perkin Elmer Applied Biosystems, USA) and the products run on an ALFexpress DNA Automated Sequencer (AEC Amersham, SA) using the chain termination protocol of Sanger *et al.* 1977 **(66)**.

Validation of SSCP gel scoring method

Direct sequencing of PCR products (DRB1F/DRB1R) from the ANP population was used to confirm SSCP gel scoring.

PCR products were electrophoresed through a 2% w/v agarose gel **(Appendix A)**, the distinct bands excised and gel extracted using a QIAquick™ gel extraction kit (Qiagen, Germany). Purified products were then cycle sequenced using DYEnamic ET Dye terminator Cycle sequencing Kit for MegaBACE on a GeneAmp PCR System 9700 (Perkin Elmer Applied Biosystems, USA) as above using either (DRB1F/DRB1R) primer. These sequencing reactions were electrophoresed through LPA long-read gel matrix on the MegaBACE 500 Automated Capillary DNA Sequencing System (Molecular Dynamics part of Amersham Pharmacia Biotech (Amersham Biosciences, USA). Sequences were analysed with MegaBACE 500 Sequence Analyser v2.4. All sequencing was performed at the DNA Sequencing Facility, Department of Molecular and Cell Biology, University of Cape Town.

2.8 Population genetic analysis

SSCP is a qualitative sequence screening technique, but for the purposes of comparing the SSCP data with microsatellite data the banding patterns were scored in a similar fashion to microsatellite gels and analysed using the same population genetics programs. GenePop (see **Appendix B** for input file format) **(67)**, FSTAT (see **Appendix D** for input file format) **(68)**, Arlequin (see **Appendix E** for input file format) **(69)**, and GenAlEx (see **Appendix C** for input file format) **(70)** were used for data analysis. GenePop **(67)** was used to estimate Hardy-Weinberg proportions. FSTAT **(68)** was used to calculate F-statistics. Arlequin **(69)** was used for Analysis of Molecular Variance (AMOVA). GenAlEx **(70)** was used for population assignment.

2.8.1 Sequence alignment

DNAman **(63)** was used to align sequences and construct homology trees, as well as translating to amino acid sequence for the purposes of alignments and synonymous: non-synonymous change calculations.

2.8.2 Genetic Variability Measures

SSCP gels were scored according to haplotype patterns; homozygotes were given an arbitrary score representative of the banding position on the gel. Heterozygotes were subsequently scored as double homozygotes, hence heterozygotes without corresponding homozygote patterns within the population were discarded, as they could not be accurately scored.

Genetic variation within the South African buffalo populations sampled was quantified using the mean number of alleles per locus (A), observed

heterozygosity (H_o) and expected heterozygosities (H_e) under Hardy-Weinberg equilibrium **(71)**. All three values were calculated for *DRB1* and previously reported microsatellite loci **(3)** for each population, and averaged over all loci using GenePop **(67)**.

Hardy-Weinberg Equilibrium

GenePop **(67)** was used to calculate observed heterozygosity (H_o) and expected heterozygosity (H_e). Deviations from Hardy-Weinberg expectations **(Equation 1)** were determined by chi-squared analysis **(Equation 2)** **(72)**. The inbreeding estimator of Weir and Cockerham 1984 **(71)**, F_{IS} **(Equation 3)** is a measure of allele frequency in the individual ($_I$) relative to the sub-population ($_S$). F_{IS} was calculated for each population at both the *DRB1* locus and the previously reported microsatellite loci **(2)**. Hardy-Weinberg exact probabilities were estimated using the Markov chain method (Guo and Thompson 1992) **(74)**.

$$P^2 + 2pq + q^2 = 1 \qquad \text{Equation 1}$$

$$X^2 = \Sigma (obs - exp)^2 / exp \qquad \text{Equation 2}$$

$$F_{IS} = (H_s - H_i)/H_s \qquad \text{Equation 3}$$

2.8.3 Estimates of Population Differentiation

Levels of population differentiation were calculated using F-statistics from the programs FSTAT **(68)**.

F_{ST} **(Equation 4)** **(75)**, designed as a measure of genetic differentiation, was used in this study for comparison with a previous microsatellite study **(3)**. F_{ST} is a measure of allele frequency variance in a sub-population ($_S$) relative to the total population ($_T$) **(75)**.

$$F_{ST} = (H_t - H_s) / H_t \qquad \text{Equation 4}$$

Analysis of molecular variance (AMOVA) was used to identify the distribution of variation within and among populations. AMOVA was performed using Arlequin **(69)**. The Arlequin **(69)** AMOVA calculation is based on Excoffier *et al.* 1992 **(76)**.

Assignment tests were implemented to strengthen population differentiation analysis by determining whether sampled individuals could be assigned to their respective populations based on locus specific identity. These tests were carried out on all individuals in each population, using GenAlEx **(70)**. GenAlEx calculates the log likelihood assignment values for each sample in the data set. Based on the assignment value for each individual, the most likely genetic origin of the sample is identified. Assignment tests are calculated based on a method described in Cornuet *et al.* 1999 **(77)**.

Chapter 3: Results

"Their understanding
Begins to swell and the approaching tide
Will shortly fill the reasonable shores
That now lie foul and muddy."

William Shakespeare

3.1 PCR Products:

PCR products of the expected size were obtained for *DQB*, *DRB1* (HLO30/HLO32) and (DRB1F/DRB1R) and were 171bp, 286bp, and 225bp respectively. For both genes there were no noticeable size differences among the amplicons for all the buffalo samples tested. *DQB* produced irreproducible SSCP results and thus further analysis of this gene was not pursued. Buffalo specific primers, DRB1F and DRB1R were designed in this study after initial sequencing data had been generated with HLO30/HLO32 and so only *DRB1* (DRB1F/DRB1R) was used for further SSCP analyses.

3.1.1 PCR Optimisation

The titration curves for different $MgCl_2$ concentrations at different primer concentrations is shown below in Fig. 3.1.

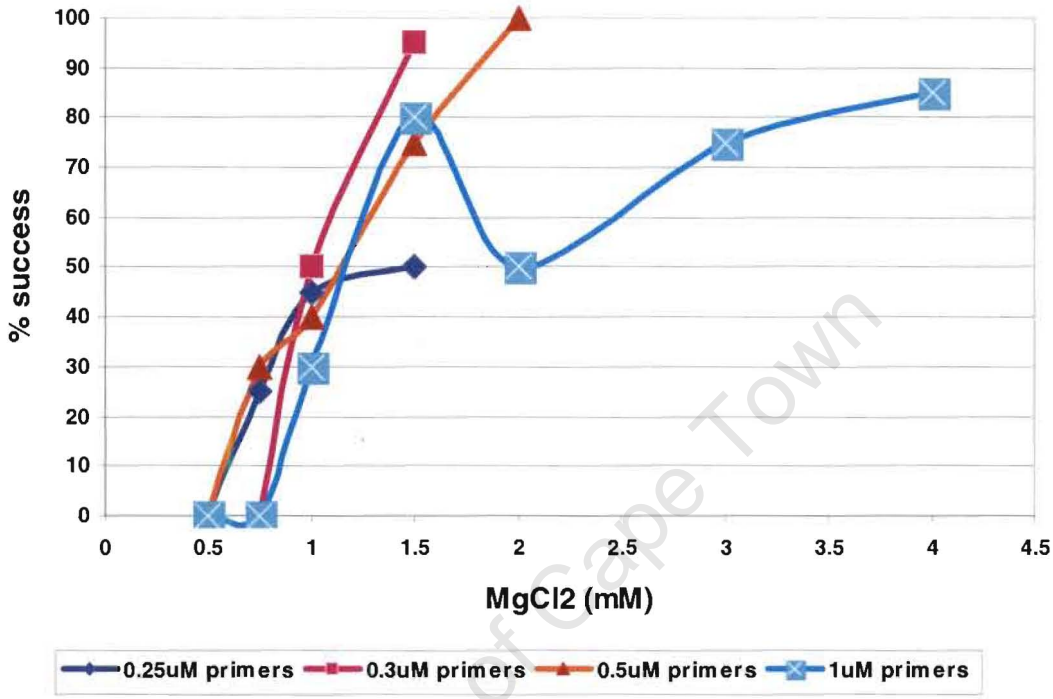


Figure 3.1: The $MgCl_2$ titration curves of the DRB1F/DRB1R primer set. These data points were obtained by agarose gel electrophoresis. 2m*M* $MgCl_2$ and 0.5µ*M* primer concentration were determined to be optimal for agarose gel electrophoresis. PCR success was determined as the presence of a single, clear band of expected size on an ethidium bromide stained agarose gel.

Further optimisation was required under SSCP conditions. The optimal primer concentration was increased to 1µ*M* and further SSCP analysis was performed at this higher concentration.

3.1.2 PCR purification

Initial PCR's were performed using Bioline *Taq* polymerase (Bioline UK Ltd.) resulted in irreproducible SSCP patterns. However, the purification of PCR products extracted from agarose gel slices solved this problem (Fig. 3.2). Two PCR purification methods were compared for purity and yield of PCR product: purification through p1000 plugged tips were compared to GenElute™ columns (Fig. 3.3).

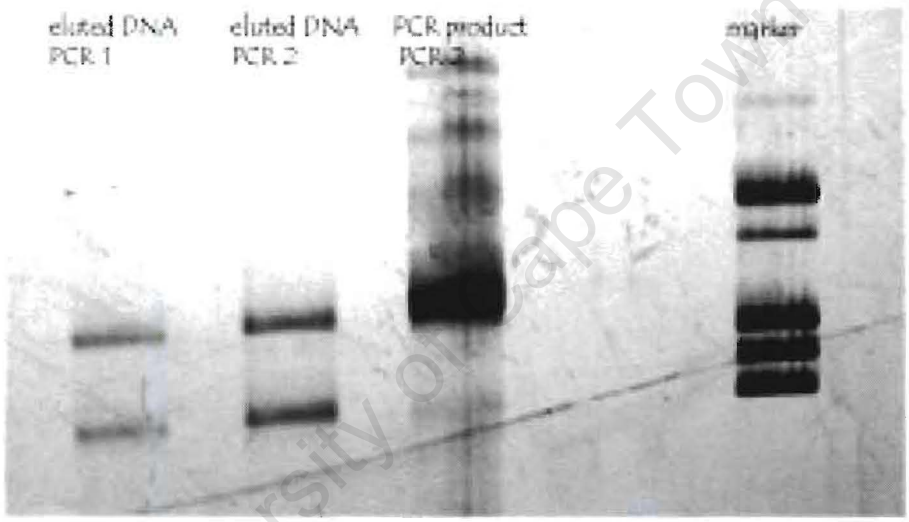


Figure 3.2: Comparison of purified and unpurified PCR products after SSCP. "Eluted DNA" has been purified through a plugged tip prior to SSCP; "PCR product" is unpurified but has undergone the same SSCP process.

Effect of storage on PCR products. PCR 1 and PCR 2 represent the same sample amplified in two reactions. PCR 1 had been stored at 4°C for 7 days prior to SSCP and PCR 2 is a freshly amplified sample.

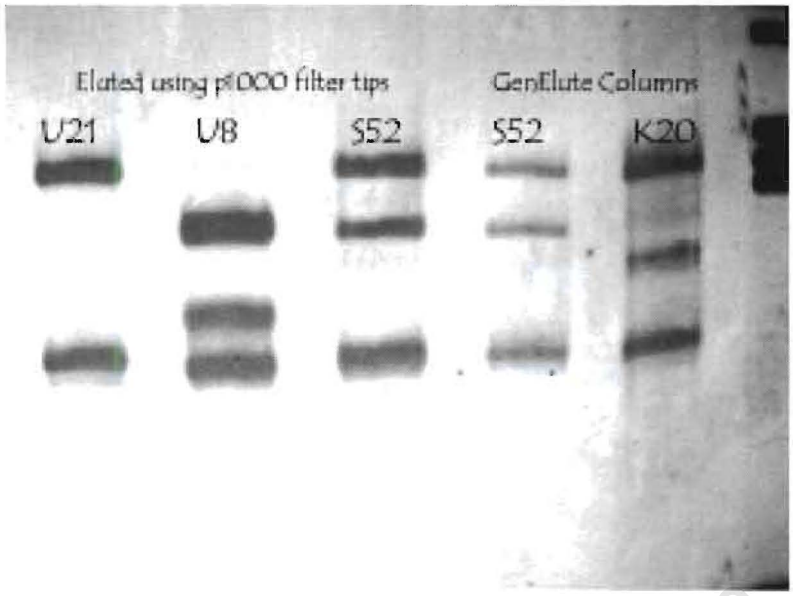


Figure 3.3: Comparison of purification methods using a P1000 filter tip or a GenElute™ column for sample S52. The amplicons purified through filter tips produced a stronger, clearer banding pattern on a silver stained 10% MD-SSCP gel. Sample codes are representative of population specific samples. U21and U8 are UHC samples 21 and 8 respectively, S52 and K20 represent StL sample 52 and KNP sample 20 respectively.

3.2 RFLP

A preliminary RFLP screen was performed to assess the variability of the *DRB1* gene. Three restriction enzymes were used, but only *RsaI* gave reproducible results. High levels of variation were found with 14 gel phenotypes (labelled A to N). The gel is shown in figure 3.4 with a graphical representation below. In spite of numerous attempts to produce a clear photograph, the representation was necessary because the gel photograph is a composite of a number of gels to compare restriction patterns of the entire sample set.

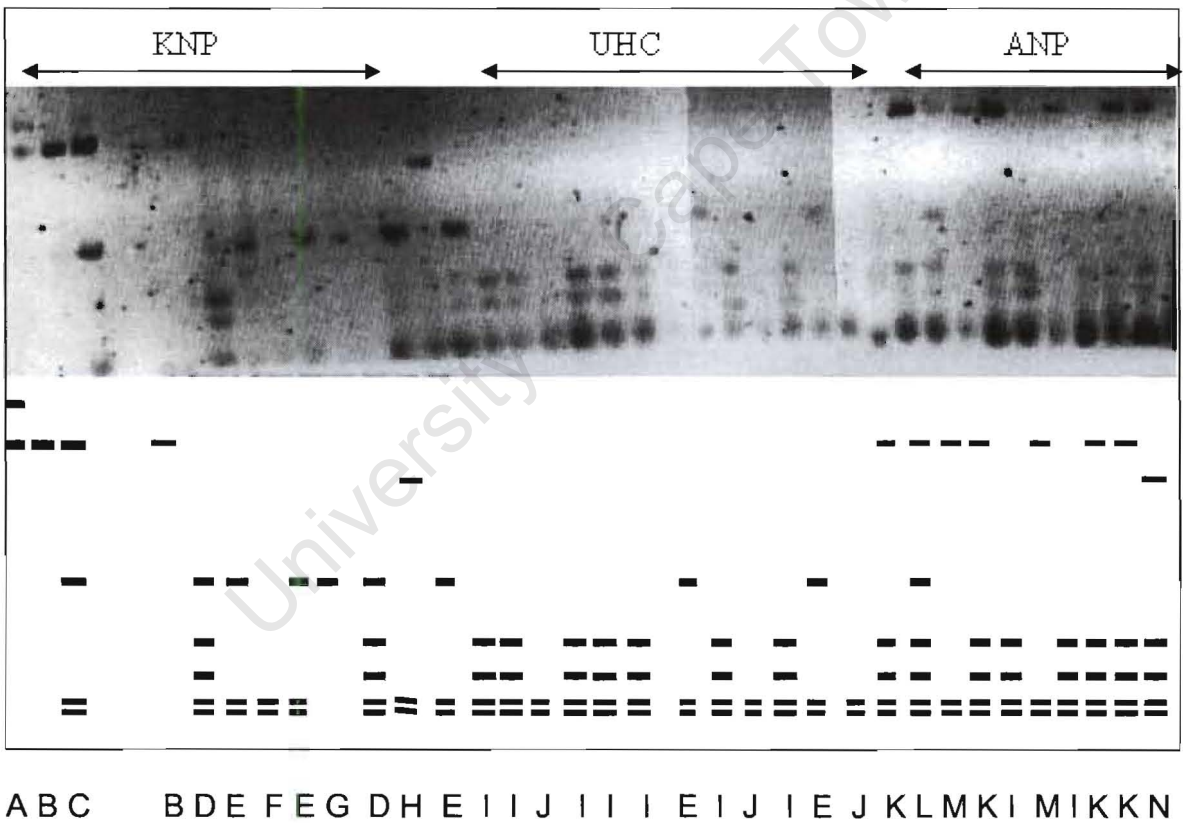


Figure 3.4: RFLP products (*RsaI* digested), electrophoresed on a 4% Agarose gel, visualised with GelStar™. Below is a diagrammatic representation of the gel. (Each banding pattern represents a discrete genotype, reflecting sequence variation at the PBR of the SyLA-*DRB1* locus)

3.3 SSCP

After optimisation of all the variables discussed in the methods (p 45), the optimum conditions in terms of reproducibility and clarity of SSCP patterns were as follows: equal volumes of PCR product and SSCP loading dye were denatured at 95°C for 5 min and cooled on ice for 2 min before being loaded onto a 10% polyacrylamide (39:1) gel containing 2.66*M* urea and 5% glycerol. Samples were electrophoresed overnight at 200V at 4°C. The SSCP profile was visualised by silver staining.

Data from optimised conditions is shown in Fig. 3.5. SSCP reveals very high levels of variation. SSCP gels were scored by a numerical system where each unique banding pattern was given a numerical value. Subsequently heterozygote-banding patterns were matched against homozygote banding patterns. Heterozygotes were then given numerical values similar to di-allelic markers (e.g. 0103); the homozygote scores were also converted to a di-allelic scoring system (e.g. 0101). This data conversion ensures interpretation by statistical packages.

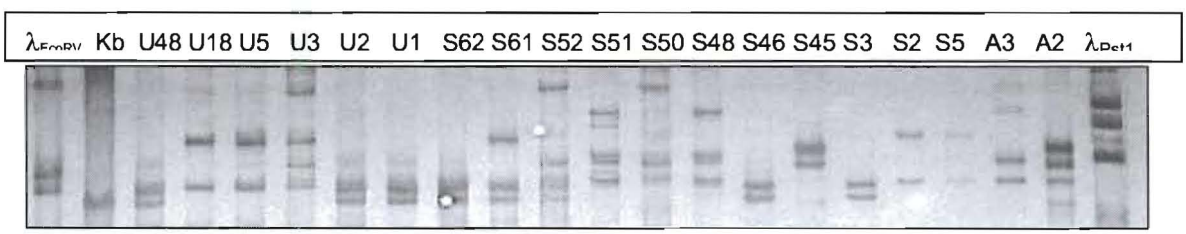


Figure 3.5: SSCP products electrophoresed on a 10% MD-SSCP gel visualised by silver staining. Lane labelling as follows: K = KNP, U=UHC, S=StL, A = ANP

SSCP analysis identified 74 gel phenotypes in 84 individuals. The KNP contained 26 gel phenotypes, whilst ANP contained 9; the UHC and StL contained 33 and 9 gel phenotypes respectively. The 74 gel phenotypes found in the total sample set translated into 77 alleles based on the above scoring method.

3.4 Sequencing:

Validation of preliminary SSCP results

The sequencing results showed that the two heterozygotes (U08 and K13) from UHC and KNP respectively, each produced two unique sequences. The putative homozygote, U21, yielded two sequences that differed by two base pairs. Two transitions in the U21 DNA sequence resulted in two non-synonymous mutations between alleles:(i) leucine (L) changed to a proline (P), both of which are neutral and hydrophobic; (ii) a polar amino acid threonine (T) was converted to a hydrophobic amino acid alanine (A) (Fig. 3.6). The two sequences for individual K13 revealed 35 base pair differences between two alleles, which correlated to 21 amino acid substitutions (Fig. 3.6). Individual U8 has nine nucleic acid mutations, which manifest themselves as seven non-synonymous mutations (Fig. 3.6).

Figure 3.6: Amino acid sequence comparison of the *DRB1* exon 2 for three individuals. These sequences reflect a portion of the PBR for the *DRB1* gene. (Amino acid changes indicated in red are conversions between amino acids of the same group, those changes indicated in blue are conversions between amino acids of different groups)

The above mentioned sequences were used to design buffalo specific *DRB1* primers (DRB1F and DRB1R), which were subsequently used in PCR-SSCP analysis.

Validation of SSCP gel scoring

All individuals in the ANP sample set were cycle sequenced directly from PCR products in order to confirm SSCP data. These sequences were aligned and a homology tree based on sequence comparisons was constructed in DNAman **(63)** (Fig. 3.7).

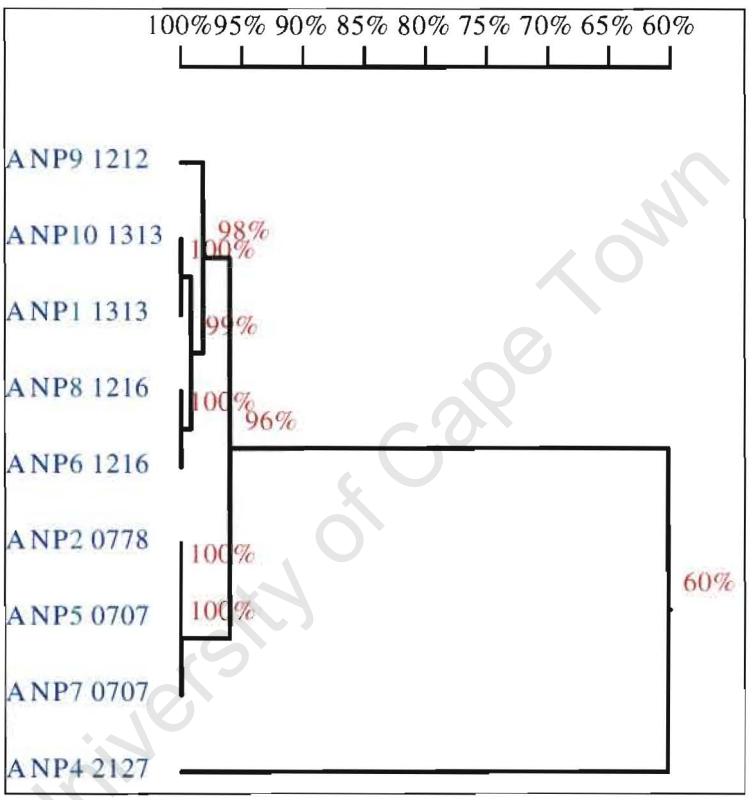


Figure 3.7: A homology tree drawn in DNAman **(63)** from a multiple sequence alignment. Sample names are followed by their GenePop genotype designations.

The alleles of the heterozygote ANP2 differ by two base pairs, SSCP analysis could not discern between the two alleles and so ANP2 was initially scored as a homozygote (0707). Sequencing revealed the polymorphism and so ANP2's genotype was changed to (0778). In the remaining ANP individuals the sequencing data confirmed SSCP results. That is homozygotes that were scored as having the same genotype grouped together in the homology tree. For

example ANP1 and ANP10 were both scored 1313 and sequencing results confirmed that they were in fact the same allele. Likewise, the two heterozygotes ANP8 and ANP6 that were scored as the same genotype 1216, were grouped together based on sequence data in the homology tree (Fig. 3.7).

3.5 Population Genetic Analysis

3.5.1 Levels of genetic variability

Allelic variation

Analysis of 84 African buffalo from the four populations using SSCP resulted in 77 alleles observed at the *DRB1* locus (Table 3.1).

Table 3.1: Variation at the *DRB1* gene within South African buffalo populations.

Population	Number of samples	Number of alleles	Number of unique alleles	Number of heterozygotes	Heterozygosity (Ho)
KNP	30	38	32	21	0.70
UHC	31	29	26	14	0.45
StL	13	11	7	6	0.46
ANP	10	8	3	5	0.50
Total	84	77	68	50	0.59

Allelic patterns and frequencies in the four populations are illustrated in Fig. 3.8 below.

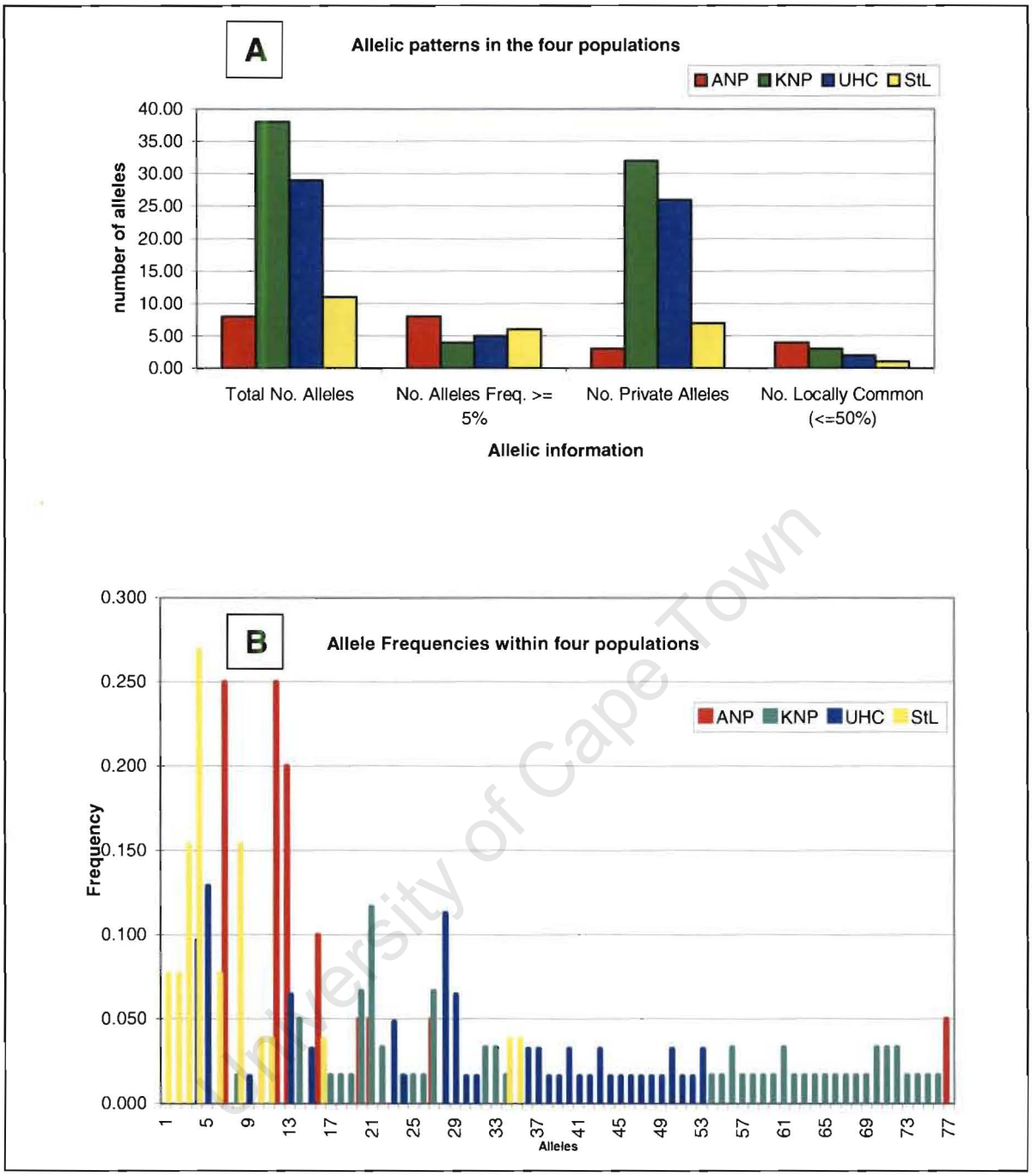


Figure 3.8: Comparison of allelic patterns **A** and allele frequencies **B** as determined by PCR-SSCP analysis, between the four populations.

The SyLA-*DRB1* gene displayed a very high level of allelic diversity, with eight alleles determined for ANP, 38 alleles determined for KNP, 29 alleles in UHC and 11 alleles in StL as summarised in Fig. 3.9. Of these alleles the percentage of unique alleles found in each population was: 37% in ANP, 84% in KNP, 89% in UHC and 63 % in StL as illustrated in Fig. 3.10.

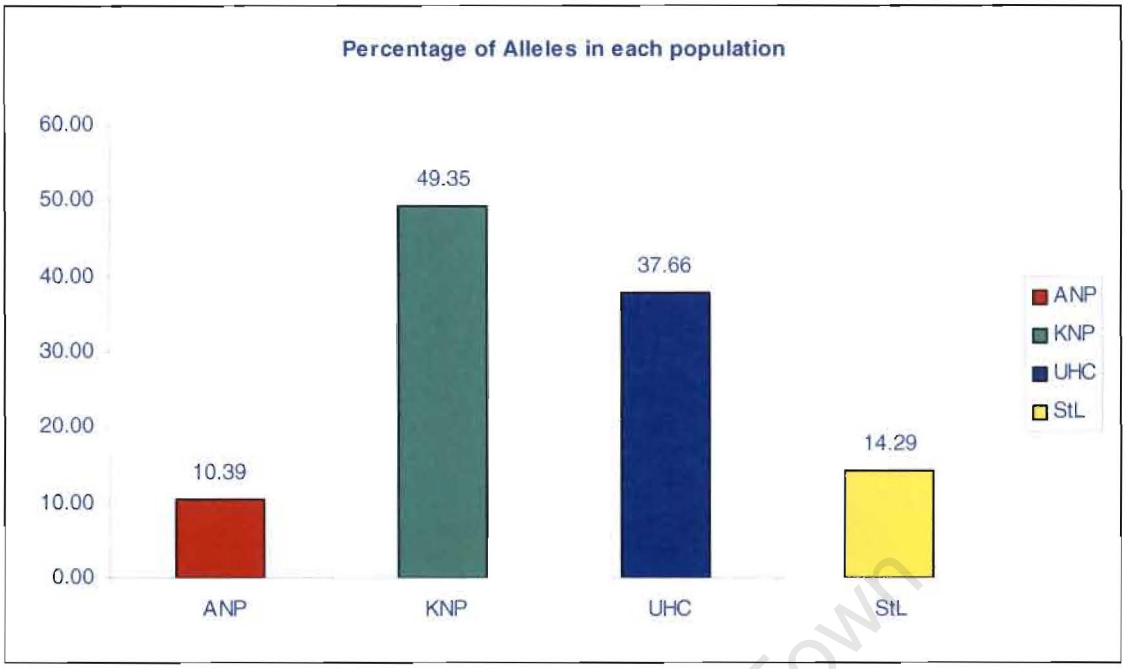


Figure 3.9: The percentage of alleles found in the MHC *DRB1* locus in the four populations investigated.

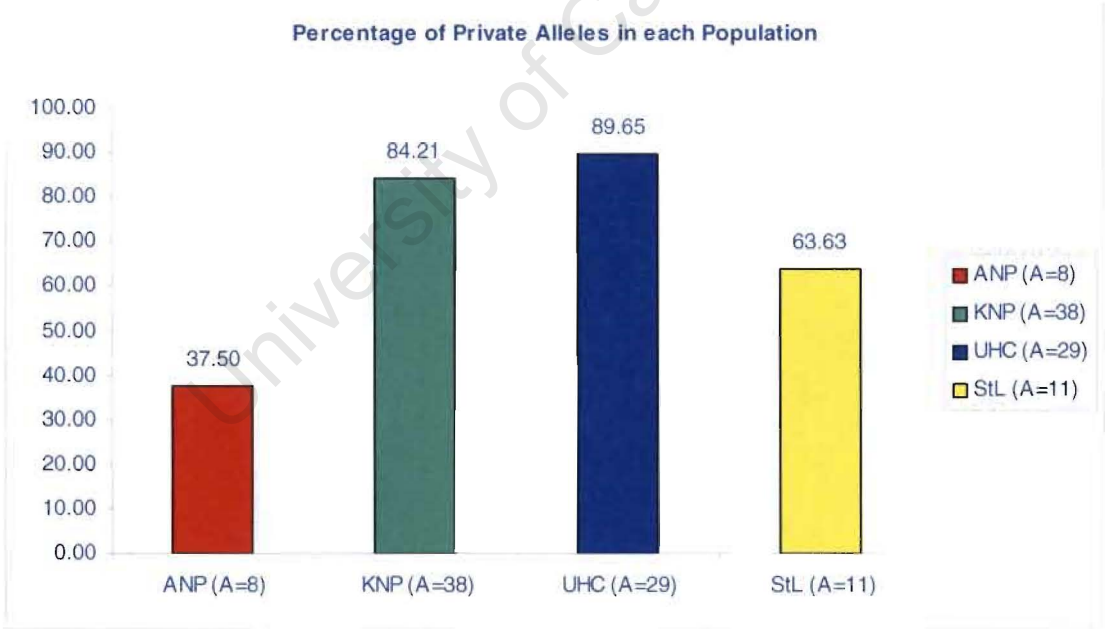


Figure 3.10: The percentage of private alleles at the MHC *DRB1* locus in each population. Total number of alleles is indicated in parenthesis.

The allele frequencies were found to differ markedly between the populations and correlate to population size. KNP (population size: 35 000) supported 38 alleles, 32 of which (84.21%) are unique; UHC (population size: 8 400) had 29 alleles, of

which 26 (89.65%) are unique; StL (population size: 175) has 11 alleles, seven are (63.63%) unique and ANP (population size: 85) had three (37.50%) unique alleles out of eight. The KNP population showed a range of alleles at similar frequencies all above 0.02, with a dominant allele (allele 21) at a frequency of 0.12. UHC and StL, each displayed a dominant allele (allele 5) and (allele 4) respectively, with a frequency of 0.13 and0.27 respectively. ANP has two dominant alleles (alleles 7 and 12), both with a frequency of 0.25.

When using RFLP as an indication of sequence variation, analysis of the same individuals found RFLP to be less sensitive than SSCP at detecting sequence variation. *DRB1* PCR product was digested with *RsaI* and revealed 14 discrete restriction patterns or "genotypes" (A – N) distributed across the three remnant buffalo populations tested (Fig. 3.11).

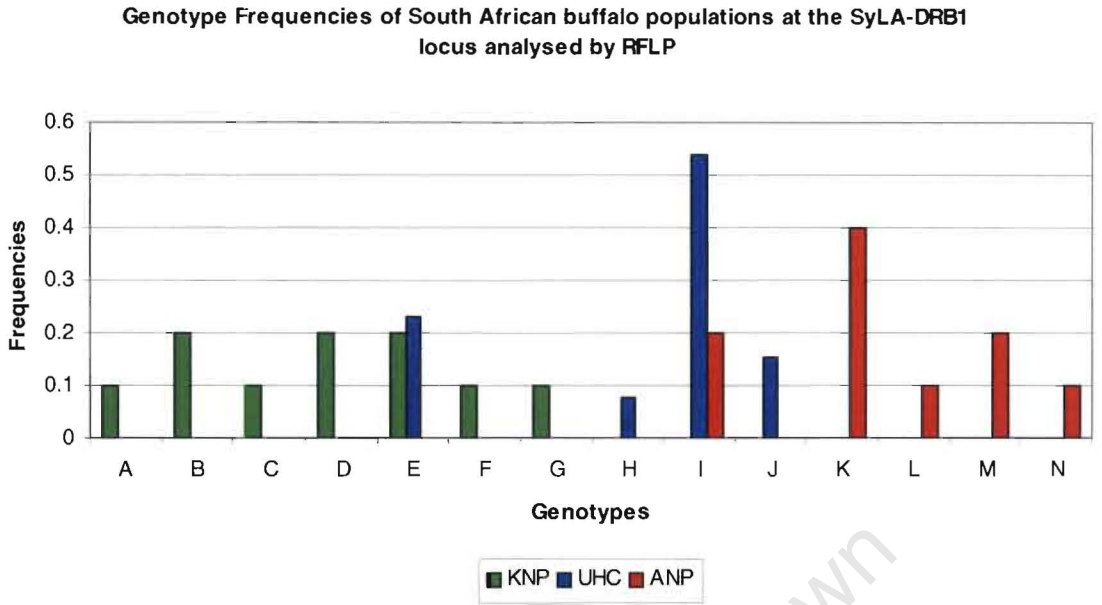


Figure 3.11: A graphical representation of the *DRB1* genotype frequencies as determined by PCR-RFLP analyses.

Two *DRB1* genotypes were found to be shared between the populations: genotype E was found to be shared between KNP and UHC and genotype I was found to be shared between UHC and ANP. As in the case of the SSCP generated allele frequencies, the RFLP genotype frequencies (Fig. 3.11) showed that KNP had many genotypes all at similar, low frequencies, whilst ANP and UHC each had one dominant genotype.

As mentioned previously a total of 84 individuals from four South African buffalo populations (KNP, UHC, StL and ANP) were screened for sequence level polymorphisms by SSCP at the *DRB1* locus. These 84 individuals supported 77 alleles. The microsatellite study conducted by O'Ryan *et* al. **(3)** genotyped 104 individuals in the same four populations at seven polymorphic microsatellite loci, supporting 61 alleles. Measures of gene diversity **(72)** calculated in GenAlEx **(70)** indicated that all loci displayed high levels of genetic diversity as shown in Fig. 3.12.

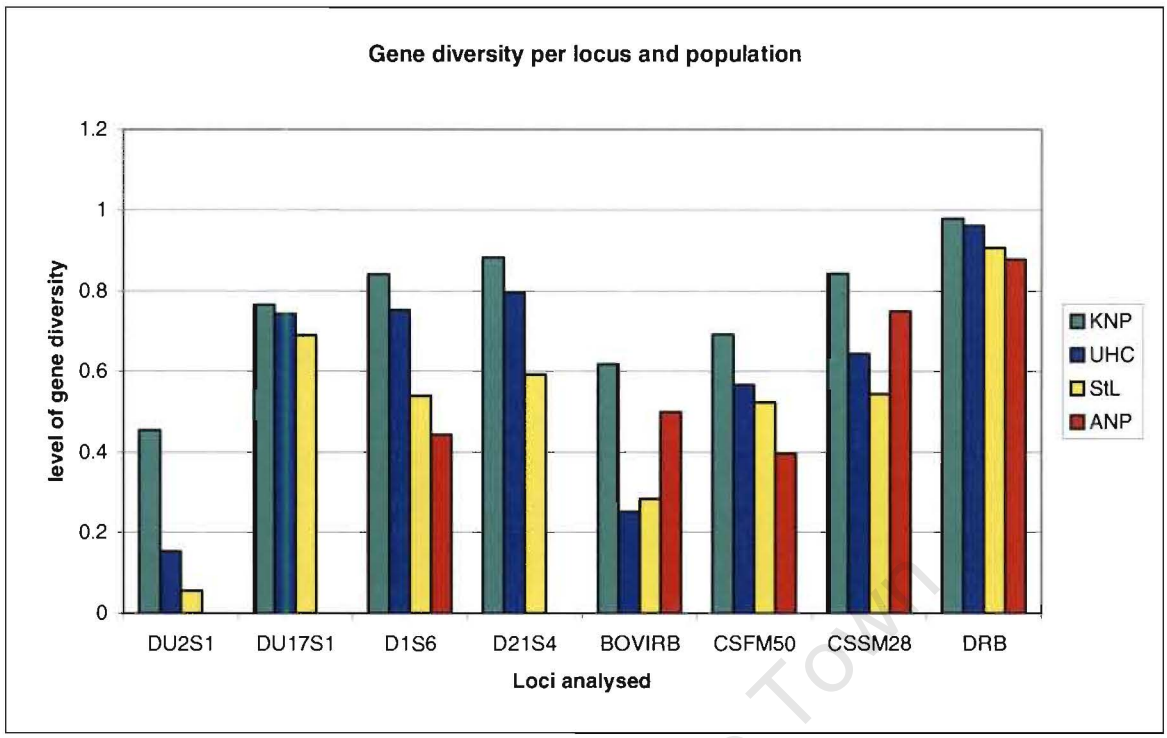


Figure 3.12: A comparison of the levels of gene diversity found at the microsatellite loci **(3)** and *DRB1*. Within the four populations tested.

In comparison with the microsatellite data **(3)**, *DRB1* shows higher levels of gene diversity in the four populations studied (Fig. 3.12).

Heterozygosity

Observed heterozygosity values were corrected (H_{oc}) for SSCP underestimation of heterozygosity. Sequence data from the ANP population indicated that SSCP overestimated homozygosity by 25%. H_o values were corrected for as indicated in Table 3.2. ANP was not corrected for as the entire sample set was sequenced.

High H_{oc} values of 0.78 in the KNP population and 0.59, 0.6 in UHC and StL populations respectively reflect the large number of observed heterozygotes (as summarised in Table 3.2). In contrast, fewer heterozygotes and hence a lower level of H_{oc} of 0.50 was observed in individuals from the ANP population.

When compared to the microsatellite study **(3)**, the MHC study detected similar H_o values (Table 3.2). In both studies the KNP, population displayed the highest levels of H_{oc} (0.78) at *DRB1* and a H_o value of 0.72 for the microsatellite investigation **(3)**. UHC and StL, displayed DRB1 H_{oc} values of 0.59 and 0.60 respectively and microsatellite H_o values of 0.55 and 0.45 respectively. ANP displayed the lowest H_{oc} value of 0.50 in this study and H_o value of 0.49 in the microsatellite study. The Mann-Whitney Test **(77)** was performed to calculate the significance of the observed differences between H_{oc} values at the *DRB1* locus and H_o values at the microsatellite loci, heterozygosity values (H_o and H_{oc}) were assessed at α=0.05, the differences between two data sets were determined to be statistically insignificant with P values between 0.01 and 0.02.

Table 3.2: Comparison of microsatellite and MHC heterozygosity (H) values in the three buffalo populations studied.

POPULATION	(H_o) *DRB1*	(H_{oc}) *DRB1* corrected	(H_o) (O'Ryan *et al.*, 1998)
KNP	0.7	0.78	0.72
UHC	0.45	0.59	0.55
StL	0.46	0.6	0.45
ANP	0.5	0.5	0.49

Table 3.3 compares H_o, H_e and associated p-values (α=0.05) for each locus in each population. H_o values tended to be high in the range of 0.06 to 1.00 with a mean H_o value of 0.58. P-values are an indication of whether the deviation of H_o from H_e is statistically significant **(9)**. In comparison with the microsatellite data O'Ryan *et al.* **(3)** MHC *DRB1* is the only locus where p-values indicate that the differences between H_o and H_e are statistically significant. This supports the

results of the Hardy-Weinberg Equilibrium test, which found that the populations are not in Hardy-Weinberg Equilibrium at the MHC *DRB1* locus.

Table 3.3: Observed (H_o) and expected (H_e) heterozygosity values for all microsatellite loci investigated in O'Ryan *et el.* **(3)** and the *DRB1* locus.

	KNP	UHC	StL	ANP
DU2S1				
Number of genotypes	34	30	18	Monomorphic - test not done
H_o	0.41	0.17	0.06	
H_e	0.47	0.19	0.11	
P value	0.79	1	1	
DU17S16				
Number of genotypes	31	31	11	Monomorphic - test not done
H_o	0.71	0.71	0.82	
H_e	0.77	0.74	0.74	
P value	0.28	0.18	0.88	
D1S6				
Number of genotypes	30	31	20	9
H_o	0.77	0.97	0.55	0.56
H_e	0.84	0.77	0.56	0.53
P value	0.64	0	0.67	1
D21S4				
Number of genotypes	25	28	15	Monomorphic - test not done
H_o	0.84	0.79	0.73	
H_e	0.89	0.8	0.6	
P value	0.07	0.74	0.68	
BOVIRBP				
Number of genotypes	26	36	22	2
H_o	0.58	0.28	0.32	1
H_e	0.62	0.32	0.28	0.67
P value	0.33	0.45	1	1
CSFM50				
Number of genotypes	25	21	15	9
H_o	0.68	0.43	0.27	0.22
H_e	0.69	0.61	0.58	0.39
P value	0.66	0.04	0.11	0.35
CSSM28				
Number of genotypes	14	28	16	2
H_o	0.71	0.46	0.25	1
H_e	0.85	0.66	0.53	0.83
P value	0.19	0.01	0.03	1
DRB1				
Number of genotypes	30	31	13	10
H_{oc}	0.78	0.59	0.60	0.5
H_e	0.98	0.97	0.94	0.88
P value	0	0	0	0.27×10^{-3}

Hardy-Weinberg Equilibrium

The four buffalo populations analysed at the *DRB1* locus were not in Hardy-Weinberg equilibrium. This was not unexpected, as Hardy-Weinberg requires a number of conditions to be met before a population can be considered to be in equilibrium. These requirements are an absence of selection, mutation, and migration; random-mating; and constant genotypic proportions from generation to generation **(78)**. These four buffalo populations fail to fulfil the requirement of no selection. There is a positive selective pressure at the PBR of MHC genes, which nullifies the Hardy-Weinberg equilibrium, as it requires neutrality. The statistical tests were performed in order to compare the MHC data with microsatellite data **(3)**. O'Ryan *et al.* **(3)** found a significant departure from Hardy-Weinberg equilibrium caused by a homozygote excess at two loci. After removing these loci from the analysis, there was no longer a significant departure from Hardy-Weinberg equilibrium.

3.4.1 Levels of Population differentiation

F_{ST} comparisons

F_{ST} values are found to increase as allele frequencies diverge **(66)**; therefore, F_{ST} values are a good measure of population differentiation. A pair wise comparison of the buffalo populations using allele frequency data showed low to medium F_{ST} **(9)** values for all population pairs (Table 3.4).

Table 3.4: Pairwise F_{ST} values for the KNP, UHC, StL and ANP buffalo populations. F_{ST} values reported for the MHC study and the Microsatellite study **(3)**.

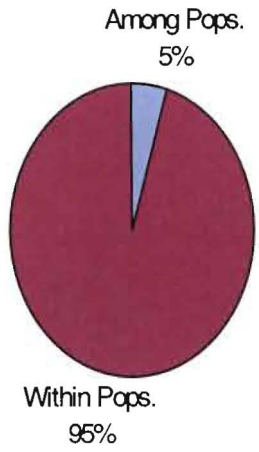
	KNP	UHC	StL	ANP
DRB1				
KNP	-			
UHC	0.03	-		
StL	0.05	0.04	-	
ANP	0.06	0.06	0.1	-
Microsatellite O'Ryan *et al* **(3)**				
KNP	-			
UHC	0.1	-		
StL	0.11	0.06	-	
ANP	0.08	0.26	0.31	-

The highest MHC F_{ST} value of 0.1 was calculated when comparing StL/ANP. The MHC *DRB1* locus indicated lower levels of genetic differentiation between all four populations. The F_{ST} values calculated using microsatellite data are greater than the values calculated for the MHC data, but they follow the same trend. The highest F_{ST} values are observed between StL and ANP, followed by UHC and ANP. The Mann-Whitney Test **(77)** was performed (α=0.05) on the Pair wise F_{ST} values and the differences between these two data sets is statistically insignificant with a P value of between 0.01 and 0.02.

Analysis of molecular variance

Because F_{ST} values indicated low levels of between-population differentiation, the distribution of variance within the data was tested using the AMOVA package in Arlequin **(69)**. AMOVA results are indicated in Fig. 3.13. All three levels of analysis support low between-population F_{ST} values as in all cases within-population variance greatly exceeds among-population variance.

Analysis of Molecular Variance at the MHc DRB1 locus



Analysis of Molecular Variance at the microsatellite loci

Figure 3.13: Analysis of Molecular Variance within and among populations for all data sets.

Assignment Tests

Assignment test likelihood ratio values obtained from GenAlEx **(70)** for *DRB1* are represented graphically in Fig. 3.14 and as an assignment matrix in **(Appendix G)**.

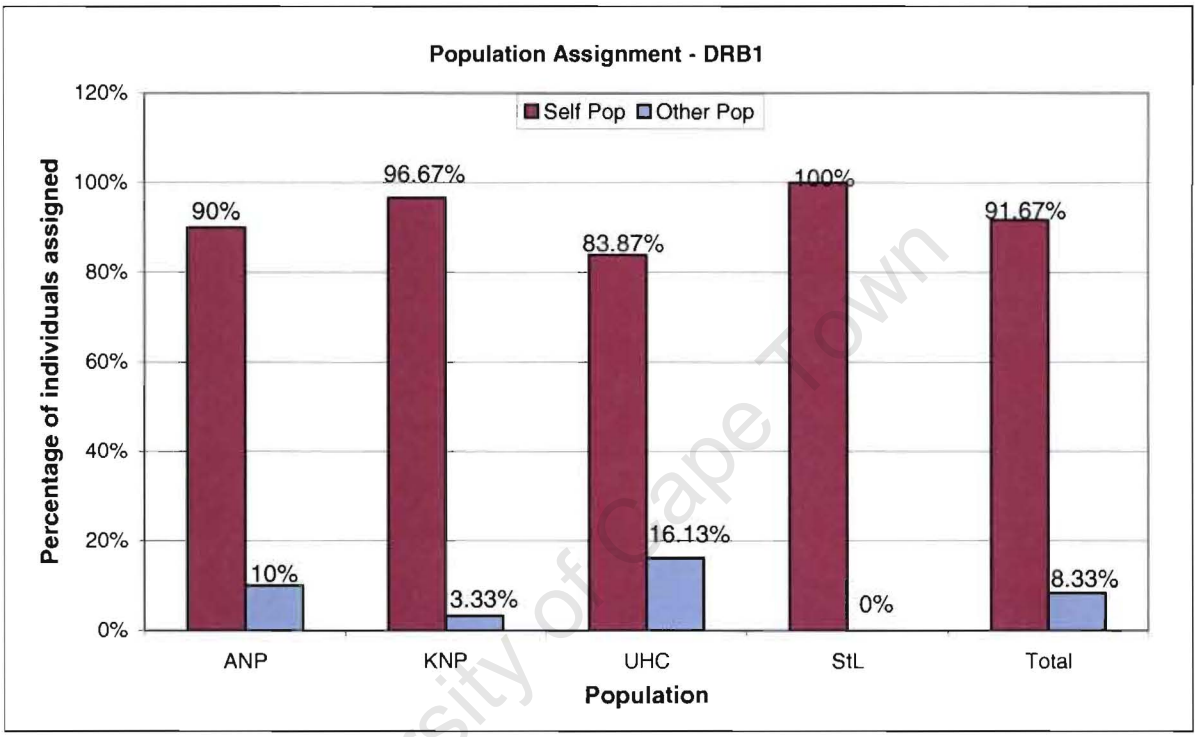


Figure 3.14: Population assignments based on *DRB1* data.

Ten percent (1/10) of individuals from the ANP population were incorrectly assigned to KNP. 3.33% (1/30) of KNP individuals was assigned to StL; and of the 16.13% (5/31) UHC individuals which mis-assigned, 6.45% (2/31) were assigned to ANP and 9.68% (3/31) were assigned to StL. In total 91.67% of individuals were correctly assigned using *DRB1* data. This is consistent with population structuring as indicated by the F_{ST} values and the high proportion of private alleles seen in each population (Fig. 3.10).

Chapter 4: Discussion

"I may not have gone where I intended to go, but I think
I have ended up where I intended to be."

Douglas Adams

African buffalo (*Syncerus caffer*) were once widely distributed across Sub-Saharan Africa **(79)**. Human disturbances led to a severe population crash and resulted in the remaining South African buffalo being confined to three game reserves. These game reserves are: Umfolozi-Hluhluwe Complex (UHC, established in Kwa-Zulu Natal 1897); Kruger National Park (KNP, established in Mpumalanga 1899) and the Addo National Park (ANP, established in the Eastern Cape 1931) **(3)**.

Buffalo carry diseases, which in the event of contact with domestic cattle stocks would negatively affect the South African livestock industry **(4)**. As a result South African legislation prevents the movement of buffalo from disease-controlled areas **(4)**. This places a high demand on the only disease free remnant population, ANP, as a source population to stock various private game farms **(4)**.

Because there has been no gene flow between remnant South African populations, buffalo are ideal for studying the long-term effects of habitat fragmentation on population genetic diversity.

Conservation geneticists identify genetic variation using molecular markers. Genetic variation may be affected by factors such as mutation, selection, genetic drift and recombination. Molecular research can address issues such as: the inter-relatedness of sub-populations and the amount of genetic diversity within a population these factors can then be used to assess levels of population

structuring. International legislative bodies and conservation programs recognise the value of this kind of work **(50)**. Genetic variation is thought to be essential to the long-term adaptability of populations in response to environmental change.

The previous study of South African buffalo populations by O'Ryan *et al.* 1998 **(3)** investigated population level genetic variation using seven microsatellite loci.

We chose to study these populations by investigating sequence level variability at an MHC gene (*DRB1*). *DRB* genes are the most commonly used genes to investigate ruminants, as indicated by vast amounts of literature on MHC-*DRB* variation in various populations **(5, 6, 17, 80, 81, 82)**. A preliminary study of *DQB* proved fruitless and further efforts were concentrated on *DRB1*.

Sequence variation was demonstrated using both SSCP and RFLP techniques. SSCP provided important advantages over conventional sequencing and RFLP for the detection of genetic variation. SSCP can be used to screen large numbers of individuals in a short period of time, saving on both time and expense **(79)**. SSCP can also detect sequence variation across the entire length of sequence, whereas RFLP can only detect variation at discrete endonuclease restriction sites within the sequence **(80)**. In this study, SSCP analysis detected approximately 50% more genotypes than RFLP. However, SSCP is not sensitive enough to detect all point mutations. This study determined SSCP overestimated homozygosity by 25%, this is in agreement with Nataraj *et al.* 1999 **(79)** who indicated that for fragment sizes greater than 200bp SSCP can be expected to detect 80-95% of mutations. Sequencing, however, can detect all the potential alleles in a population. In this study, sequencing data highlighted the rate of synonymous to non-synonymous mutations at the PBR. Mutations that result in

changes at the amino acid level of the PBR result in structural divergence at the protein level. This enables the PBR to interact with different peptides.

Levels of genetic variability

Despite the great reduction in numbers at the end of the 19th century, South African buffalo have retained appreciable levels of diversity within the populations **(2, 3, 4, 5, 6)**. Sampled populations displayed very high levels of heterozygosity and high allelic diversity across all populations.

Assuming an overestimation of homozygotes by 25% as suggested by sequencing data of the ANP population, SSCP heterozygosity values were corrected in the other three populations. When compared to KNP, the largest remnant population, the ANP population has 64% as many heterozygotes, while UHC and StL have 76% and 77% as many heterozygotes respectively at the *DRB1* locus. The results of the microsatellite study **(3)** showed that ANP and UHC heterozygosity values are similar to those previously reported, namely 66% for ANP and 75% for UHC respectively. The heterozygosity value for StL (77% at *DRB1*) was higher than the 61% found by the microsatellite study. However the Mann-Whitney U test **(77)** showed no significant difference between the two studies results.

The *DRB1* locus deviated significantly from Hardy-Weinberg equilibrium (HWE) across all four populations. In contrast, an earlier microsatellite study **(3)** found significant deviation from HWE at two loci because of homozygote excess, but removing these two loci from the analysis there was no longer significant departure from HWE. The reason for differences between these two markers with regard to HWE is: the *DRB1* gene is acted on by balancing selection, which

nullifies HWE; this test was performed as a comparison with microsatellite data **(3)**.

Population Differentiation

F_{ST} data indicated low levels of genetic differentiation between populations at the *DRB1* locus. This was verified by AMOVA, which indicated that 94% of variation found at the *DRB1* locus was within the populations. However, analysis of microsatellite data **(3)** found that although F_{ST} values were higher than at *DRB1*, AMOVA indicated only 1% of variation identified by microsatellite data analysis to be among populations. This lack of differentiation between populations may imply rates of historical gene flow and that 100 years of separation may not be sufficient for the establishment of substantial population structuring. These findings are consistent with a once continuous distribution of the species, as supported by historical records; South African buffalo were once contiguously distributed across sub-Saharan Africa. O'Ryan *et al.* **(3)** reported F_{ST} values for these populations higher than R_{ST} values, and considering the lack of migration in the South African buffalo populations, the small degree of observed population differentiation is likely the result of genetic drift. R_{ST} is an adaptation of F_{ST} specific to microsatellite loci, which assumes a stepwise mutation model **(2)**.

Population Assignment

The high level of correct assignments supports the F_{ST} data, which indicated low but significant levels of population structuring. The high level of correct assignment of individuals to the correct population is also consistent with the high proportion of private alleles seen in each population. Ninety-two percent of individuals were correctly assigned, and if we consider UHC and StL to be one

Natal population, this figure increases to 95%. These assigned values are high when considering F_{ST} and AMOVA calculations indicate low levels of population differentiation. This may be a result of balancing selection at the *DRB1* locus. Balancing selection would drive heterozygote advantage whilst maintaining advantageous ancestral alleles. The maintenance of ancestral alleles and the short period of time since separation could lead to a lack of observable population structure. Balancing selection could maintain high levels of allelic variability, noticeable in AMOVA analysis.

Conclusion

This project successfully addressed the objectives of this study, namely,

i) to develop the SSCP technique to be employed in this study,

ii) to investigate the level of genetic variation within the South African buffalo populations at the MHC *DRB1* locus,

iii) to compare MHC *DRB1* data with previous microsatellite data and,

iv) to assess the appropriateness of SSCP data for population genetic analysis.

SSCP development

The SSCP technique was successfully developed for the analysis of sequence level variation at the MHC *DRB1* PBR in South African buffalo populations. This study reports the experimental conditions to achieve reproducible, SSCP patterns for the DRB locus in buffalo.

Genetic Variation at the MHC DRB1 locus

The total lack of migration in South African buffalo populations has led to a low but significant level of between-population differentiation, indicating that a century of isolation has caused low-level population structuring. Despite being the smallest population, and therefore more susceptible to genetic drift, ANP has not lost significant levels of genetic variation. ANP has retained relatively high levels of heterozygosity when compared to the other remnant populations. In fact, all four populations have retained high levels of genetic variation, in spite of the two disease epidemics at the turn of the previous century, hunting and population fragmentation

High levels of genetic variability at the *DRB1* locus suggest that these populations are immunologically fit despite undergoing such a large bottleneck. If

one assumes levels of heterozygosity are comparable to immunological fitness, then under this assumption, ANP can be considered immunologically fit. This bodes well for South African buffalo. Since ANP is the only disease-free population and is a parent population of many new buffalo populations. However, just because there are appreciable levels heterozygosity at the DRB locus, it cannot be assumed that the same is true at all other loci.

Comparison with microsatellite data

DRB1 data and microsatellite data **(3)** were compared statistically and both data sets indicated similar trends with regard to heterozygosity levels (H_o for microsatellites and H_{oc} for *DRB1*). Pairwise F_{ST} values were considerably lower when calculated for *DRB1* data than microsatellite, but followed the same trend. AMOVA analysis indicated that both *DRB1* data and microsatellite data supported an excess of within-population variation as compared to among-population variation, providing support for the low F_{ST} values calculated for these populations.

Assessment of SSCP data for population genetic analysis

SSCP has not been previously used for this type of gel based data analysis because gel phenotypes are not easily adapted to data input files for population genetics programs. Additionally SSCP underestimates H_o values, and some sequence data must be generated to calculate and correct for the level of underestimation. Sequence comparison within the ANP sample set confirmed the SSCP gel scoring method. Statistical comparisons of *DRB1* data with the previously performed microsatellite study **(3)** showed comparable results. SSCP gel based analysis is therefore appropriate.

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Appendix A: Materials and Reagents

Stock Solutions

40% Polyacrylamide

Acrylamide	39g
Bis-Acrylamide	1g

Add H_2O to 100ml, mix on a magnetic stirrer until fully dissolved, filter and store at 4°C in a dark container.

20% Ammonium Persulphate

Ammonium persulphate	20g
H_2O	10ml

Mix well and store at -20°C in a dark container.

0.1M IPTG

IPTG	1.2g
H_2O	50ml

Filter sterilize (0.2µ*M*)

Store at -20°C

10X TBE

Tris-OH	0.89*M*, pH 8.0
Boric acid	0.89*M*
EDTA	20m*M*, pH 8.0

10X TE

Tris-HCl	100*mM*, pH 8.0
EDTA	10m*M*, pH 8.0

Ethidium Bromide (10mg/ml)

Ethidium bromide	500mg
H_2O	50ml

X-Gal (50mg/ml)

X-Gal	0.1g
N,N'-dimethylformamide	2ml

Store in dark containers at -20°C

Single stranded conformation polymorphism (SSCP)

Stock plate glue solution

Methacryloxypropyl trimethoxysilane (Sigma)	50µl
Absolute ethanol	100ml

Siliconizing agent

Gel Slick™ (FMC)

70% ethanol

Technical ethanol	70ml
H_2O	30ml

10% acetic acid

Acetic acid	10ml
H_2O	90ml

10% MD-SSCP

(Personal communication: Mr T. De Lange, University of Stellenbosch)

Urea	15g
10XTBE	5ml
Glycerol	5ml
H_2O	52.5ml
40% Acrylamide	25.5ml

20% AMPS	500µl
TEMED	100µl

Casting of SSCP Gels

The surfaces of both plates must be thoroughly washed with 100% technical ethanol, 70% technical ethanol and water, and then allowed to dry completely. To the notched plate, siliconizing agent was applied and vigorously rubbed onto the surface to ease the separation of the plate after electrophoresis. To the other plate, a glue mix, containing 90µl 10% acetic acid and 3ml of the plate glue stock, should be thoroughly rubbed into the surface of the plate and allowed to stand for 3 minutes. Excess plate glue was rubbed off with 100% ethanol. Repeat the ethanol wipe to ensure all excess glue is removed because the plates tend to stick together during electrophoresis, due to the diffusion through the gel. The plates may then be assembled with spacers of 1mm thickness and secured with bulldog clips. The gel is poured with the assembled plates held at a slight angle. The assembly is subsequently laid down horizontally and a square-tooth well-forming comb inserted. The gel is then allowed to set for 2 hours.

Sample preparation and electrophoresis

10µl of the PCR was mixed with 10µl of the SSCP loading dye. The tubes were incubated @ 95°C for 5 minutes to denature the DNA and immediately put on ice. The combs were removed from the gel to form the wells. The gel is now placed into the electrophoresis tank and filled with 0.5X TBE buffer. The wells must be cleaned with a syringe. Initially the Gel is electrophoresed for 75W for 5 minutes and then the wattage is decreased to 9W overnight.

Molecular Size Markers

λEcoRV

Bacteriophage λ genomic DNA (50μg)	100μl
EcoRV (10U/μl)	3μl
Buffer B	20μl
H_2O	77μl

Incubate at 37°C overnight.

Fragment sizes (bp):
5765, 5376, 4613, 3873, 3744, 3595, 3326, 2884, 1921, 1679, 1434, 1403, 1377, 738, 655, 618, 588, 268, 35, 13.

λ Pst

Bacteriophage λ genomic DNA (50μg)	100μl
Pst (10U/μl)	3μl
Buffer H	20μl
H_2O	77μl

Incubate at 37°C overnight.

Fragment sizes (bp):
11497, 5077, 4749, 4507, 2838, 2560, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264, 247, 216, 211, 200, 164, 150, 94, 87, 72.

Loading Dyes

6X loading dye for agarose and polyacrylamide gel electrophoresis

Bromophenol blue	0.25%
Xylene Cyanol	0.25%
Glycerol	15%

Make up to volume with H_2O, mix well and store at 4°C.

SSCP loading dye

NaOH	10 m*M*

EDTA	1m*M*
0Bromophenol Blue	0.01%
Xylene Cyanol	0.01%
Formamide	80%

Mix well and store at 4°C.

Silver Staining

(Personal communication: Ms L. Swartz, University of Cape Town)

Solution 1:

2g $AgNO_3$
2L-distilled water

Solution 2:

30g NaOH
0.2g $NaBH_4$
8ml formaldehyde
2L-distilled water

Solution 3:

15g Na_2CO_3
2L-distilled water

The two plates must be disassembled and the glass plate to which the gel is adhering should be put into a tray for silver staining. Cover gel in 2L of distilled water and gently agitate for 1min. Discard water and add solution 1. Slowly agitate for 10 minutes. Rinse gel with distilled water, shake for 2 minutes. Discard water and add solution 2. Leave solution 2 in the tray for 30 minutes or until the bands are clearly visible. Discard solution 2 and add distilled water, agitate for 1 minute. Replace the water with solution 3 to sharpen the bands. Finally, discard solution 3 and reimmerse the gel in water. The gel can then be

viewed on a light box and photo's taken. Soak the gel in NaOH to remove the gel from the plate. The plates must then be cleaned and stored.

Ethidium bromide staining

10mg/ml ethidium bromide	50µl
10X TBE	100ml
H_2O	900ml

(0.5µg/ml) ethidium bromide solution is used to stain both 4% agarose RFLP gels and Polyacrylamide SSCP gels. Gels were immersed in EtBr solution for 20 min and visualised with long wavelength UV.

Appendix B: Example of a GenePop input file

SyLA-DRB1 Syncerus Caffer African Buffalo
SyLA-DRB1
POP
A1, 1313
A2, 0778
A3, 1220
A4, 2127
A5, 0707
A6, 1216
A7, 0707
A8, 1216
A9, 1212
A10, 1313
Pop
K1, 0835
K2, 1414
K3, 5556
K4, 5757
K5, 5859
K6, 6061
K7, 6262
K8, 2222
K9, 2126
K10, 2121
K11, 6364
K12, 3233
K13, 3233
K14, 2127
K15, 1417
K16, 2127
K17, 2127
K18, 6566
K19, 6768
K20, 6970
K21, 7171
K22, 7272
K23, 7373
K24, 7475
K25, 2020
K26, 1819
K27, 3476
K28, 2024
K29, 2127
K30, 2025
Pop
U1, 0404
U2, 0404
U3, 0509
U4, 4141
U5, 0505
U6, 3940

U7, 0528
U8, 3737
U9, 1313
U10, 3838
U11, 4243
U12, 2323
U13, 2324
U14, 0528
U15, 1313
U16, 1515
U17, 4444
U18, 4546
U19, 4748
U20, 4950
U21, 5151
U22, 5253
U23, 5454
U24, 2828
U25, 2929
U26, 0528
U27, 0528
U28, 2929
U29, 0404
U30, 3031
U31, 0528
Pop
S1, 0303
S2, 0404
S3, 0303
S4, 0836
S5, 1635
S6, 0606
S7, 0404
S8, 0102
S9, 0808
S10, 0102
S11, 0810
S12, 0411
S13, 0404
Pop
W1, 0707
W2, 0707
W3, 0707
W4, 0707
W5, 0707
W6, 0720
W7, 0720
W8, 0720
W9, 0707
W10, 0707
W11, 0707
W12, 0707
W13, 0707
W14, 0707
W15, 0707

W16, 0720
W17, 0707
W18, 0707
□

Appendix C: Example of a GenAlEx input file

1	84	4	10	30	31	13	1
DRB1			ANP	KNP	UHC	StL	
Sample No.	Pop.	Locus 1					
1	ANP1	13	13				
2	ANP2	7	78				
3	ANP3	12	20				
4	ANP4	21	27				
5	ANP5	7	7				
6	ANP6	12	16				
7	ANP7	7	7				
8	ANP8	12	16				
9	ANP9	12	12				
10	ANP10	13	13				
11	KNP1	8	35				
12	KNP2	14	14				
13	KNP3	22	22				
14	KNP4	21	26				
15	KNP5	21	21				
16	KNP6	32	33				
17	KNP7	32	33				
18	KNP8	21	27				
19	KNP9	14	17				
20	KNP10	21	27				
21	KNP11	21	27				
22	KNP12	20	20				
23	KNP13	18	19				
24	KNP14	20	24				
25	KNP15	21	27				
26	KNP16	20	25				
27	KNP17	55	56				
28	KNP18	57	57				
29	KNP19	58	59				
30	KNP20	60	61				
31	KNP21	62	62				
32	KNP22	63	64				
33	KNP23	65	66				
34	KNP24	67	68				
35	KNP25	69	70				
36	KNP26	71	71				
37	KNP27	72	72				
38	KNP28	73	73				
39	KNP29	74	75				
40	KNP30	76	77				
41	UHC1	4	4				
42	UHC2	4	4				
43	UHC3	5	9				
44	UHC4	5	5				
45	UHC5	39	40				

46	UHC6	5	28				
47	UHC7	37	37				
48	UHC8	13	13				
49	UHC9	38	38				
50	UHC10	23	23				
51	UHC11	23	24				
52	UHC12	5	28				
53	UHC13	13	13				
54	UHC14	15	15				
55	UHC15	28	28				
56	UHC16	29	29				
57	UHC17	5	28				
58	UHC18	5	28				
59	UHC19	29	29				
60	UHC20	4	4				
61	UHC21	30	31				
62	UHC22	5	28				
63	UHC23	41	41				
64	UHC24	42	43				
65	UHC25	44	44				
66	UHC26	45	46				
67	UHC27	47	48				
68	UHC28	49	50				
69	UHC29	51	51				
70	UHC30	52	53				
71	UHC31	54	54				
72	StL1	3	3				
73	StL2	4	4				
74	StL3	3	3				
75	StL4	8	36				
76	StL5	16	35				
77	StL6	6	6				
78	StL7	4	4				
79	StL8	1	2				
80	StL9	8	8				
81	StL10	1	2				
82	StL11	8	10				
83	StL12	4	11				
84	StL13	4	4				

Appendix D: Example of an FSTAT input file

```
4 1 78 2
DRB1
  1  1313
  1   778
  1  1220
  1  2127
  1   707
  1  1216
  1   707
  1  1216
  1  1212
  1  1313
  2   835
  2  1414
  2  5556
  2  5757
  2  5859
  2  6061
  2  6262
  2  2222
  2  2126
  2  2121
  2  6364
  2  3233
  2  3233
  2  2127
  2  1417
  2  2127
  2  2127
  2  6566
  2  6768
  2  6970
  2  7171
  2  7272
  2  7373
  2  7475
  2  2020
  2  1819
  2  3476
  2  2024
  2  2127
  2  2025
  3   404
  3   404
  3   509
  3  4141
  3   505
  3  3940
  3   528
  3  3737
  3  1313
```

3 3838
3 4243
3 2323
3 2324
3 528
3 1313
3 1515
3 4444
3 4546
3 4748
3 4950
3 5151
3 5253
3 5454
3 2828
3 2929
3 528
3 528
3 2929
3 404
3 3031
3 528
4 303
4 404
4 303
4 836
4 1635
4 606
4 404
4 102
4 808
4 102
4 810
4 411
4 404

Appendix E: Example of an Arlequin input file

```
[Profile]
    Title="DRB1"
    NbSamples=4
    GenotypicData=1
    GameticPhase=0
    RecessiveData=0
    DataType=STANDARD
    LocusSeparator=WHITESPACE
    CompDistMatrix=1
[Data]
  [[Samples]]   #Data for 1Loci: Locus
1
    SampleName="ANP"
    SampleSize=10
    SampleData=    {
       1    1   13
                                    13
       2    1   7
                                    78
       3    1   12
                                    20
       4    1   21
                                    27
       5    1   7
                                     7
       6    1   12
                                    16
       7    1   7
                                     7
       8    1   12
                                    16
       9    1   12
                                    12
      10    1   13
                                    13
    }
    SampleName="KNP"
    SampleSize=30
    SampleData=    {
      11    1   8
                                    35
      12    1   14
                                    14
      13    1   22
                                    22
      14    1   21
                                    26
      15    1   21
```

16	1	32	21
17	1	32	33
18	1	21	33
19	1	14	27
20	1	21	17
21	1	21	27
22	1	20	27
23	1	18	20
24	1	20	19
25	1	21	24
26	1	20	27
27	1	55	25
28	1	57	56
29	1	58	57
30	1	60	59
31	1	62	61
32	1	63	62
33	1	65	64
34	1	67	66
35	1	69	68
36	1	71	70
37	1	72	71
38	1	73	72
39	1	74	73
40	1	76	75
			77

```
}
SampleName="UHC"
SampleSize=31
SampleData=    {
  41    1   4
                                4
  42    1   4
                                4
  43    1   5
                                9
  44    1   5
                                5
  45    1   39
                               40
  46    1   5
                               28
  47    1   37
                               37
  48    1   13
                               13
  49    1   38
                               38
  50    1   23
                               23
  51    1   23
                               24
  52    1   5
                               28
  53    1   13
                               13
  54    1   15
                               15
  55    1   28
                               28
  56    1   29
                               29
  57    1   5
                               28
  58    1   5
                               28
  59    1   29
                               29
  60    1   4
                                4
  61    1   30
                               31
  62    1   5
                               28
  63    1   41
                               41
  64    1   42
```

```
                                    43
    65   1   44
                                    44
    66   1   45
                                    46
    67   1   47
                                    48
    68   1   49
                                    50
    69   1   51
                                    51
    70   1   52
                                    53
    71   1   54
                                    54
  }
  SampleName="StL"
  SampleSize=13
  SampleData=   {
    72   1   3
                                     3
    73   1   4
                                     4
    74   1   3
                                     3
    75   1   8
                                    36
    76   1   16
                                    35
    77   1   6
                                     6
    78   1   4
                                     4
    79   1   1
                                     2
    80   1   8
                                     8
    81   1   1
                                     2
    82   1   8
                                    10
    83   1   4
                                    11
    84   1   4
                                     4
  }
[[Structure]]
  StructureName="DRB1 Structure"
  NbGroups=1
  IndividualLevel=0
  Group=   {
```

```
        "ANP"
        "KNP"
        "UHC"
        "StL"
}
```

Appendix F: Parks board communications

Nasionale Krugerwildtuin

Privaatsak X402
SKUKUZA 1350
Tel/Faks (01311) 65611

Kruger National Park

Private Bag X402
SKUKUZA 1350
Tel/Fax (01311) 65611

11 April 1995

Colleen O Ryan
Dept of Chem Path
University of Cape Town
OBSERVATORY
7925

Dear Colleen

INFORMATION ON BUFFALO POPULATIONS

Sorry for the delay, but herewith the info you require. I include a graph that shows the growth of the population, the stabilisation by culling and the plunge during the 1992 drought. During 1983 we also suffered a severe drought that added to the plunge. The upper acceptable limit of 35 000 may alter following further research, as it is now evident that drought will regulate the population, but the current policy is that we will maintain the population between those limits.

During dry periods, the buffalo heards break up into smaller units to reduce foraging competition, and the heards re-unite in wetter years. However, it may not be with the same animals as previously, but most likely from the same general area, which may help with increasing genetic diversity. This can be seen in the additional graphs I forwarded. We are convinced that buffalo cross all our major rivers, as we have seen this in our buffalo tuberculosis crossing the Sabie river.

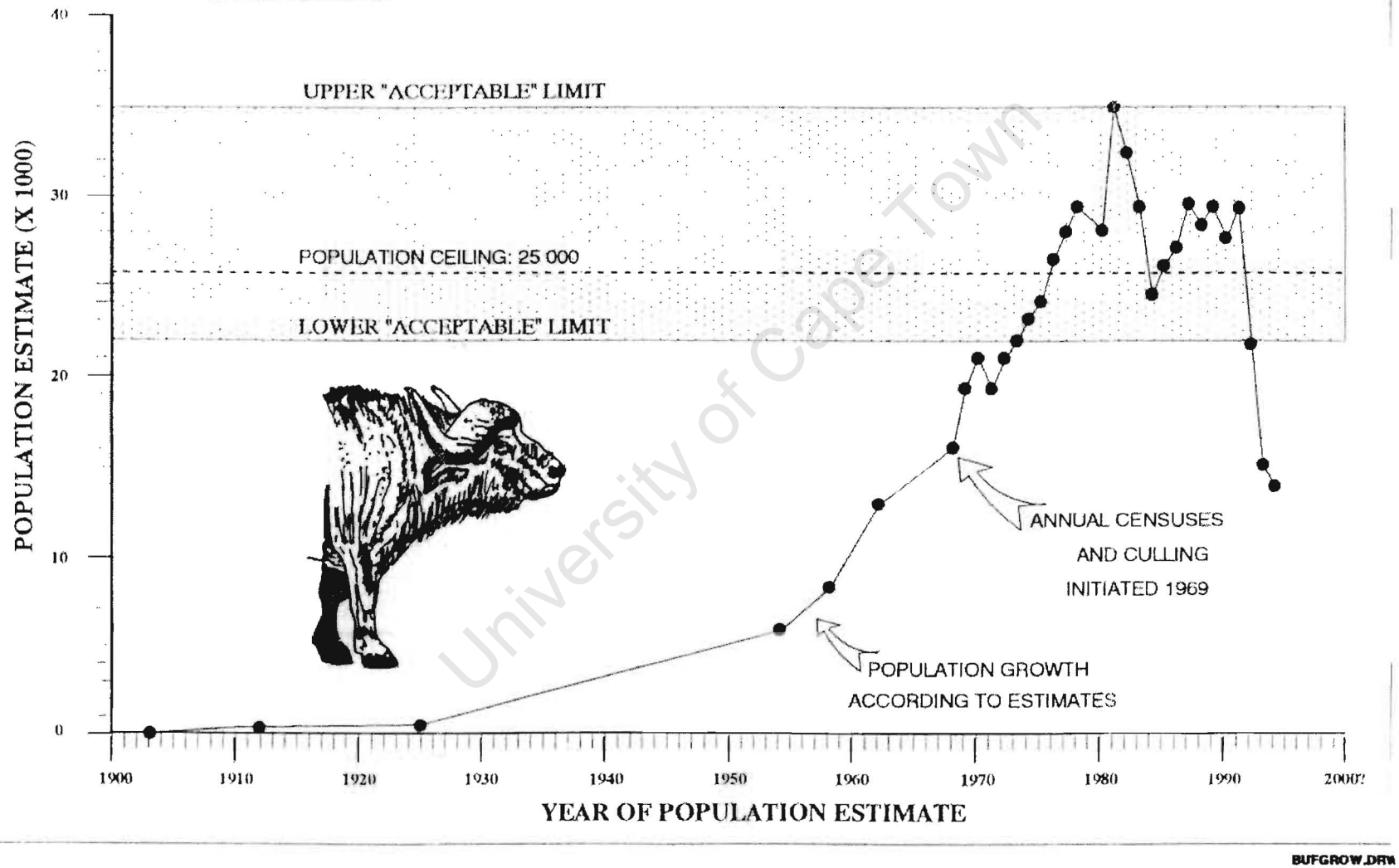
hope this info will help. will be in Skukuza for the rest of the week.

With sincere wishes

Cobus Raath
Manager: Veterinary Services
for Warden

Nasionale Parkeraad Hoofkantoor | Posbus 787, PRETORIA 0001 | Tel. (012) 343-9770
National Parks Board Head Office | P.O. Box 787 PRETORIA 0001 | Tel. (012) 343-9770

FIGURE 5: KNP BUFFALO POPULATION TRENDS: 1903 - 1994



BUFGROW.DRW

Figure 7a: THE RELATIONSHIP BETWEEN RECORDED RAINFALL AND MEAN BUFFALO HERD SIZE THE FOLLOWING YEAR RECORDED DURING ANNUAL AERIAL CENSUSES: 1980 - 1992

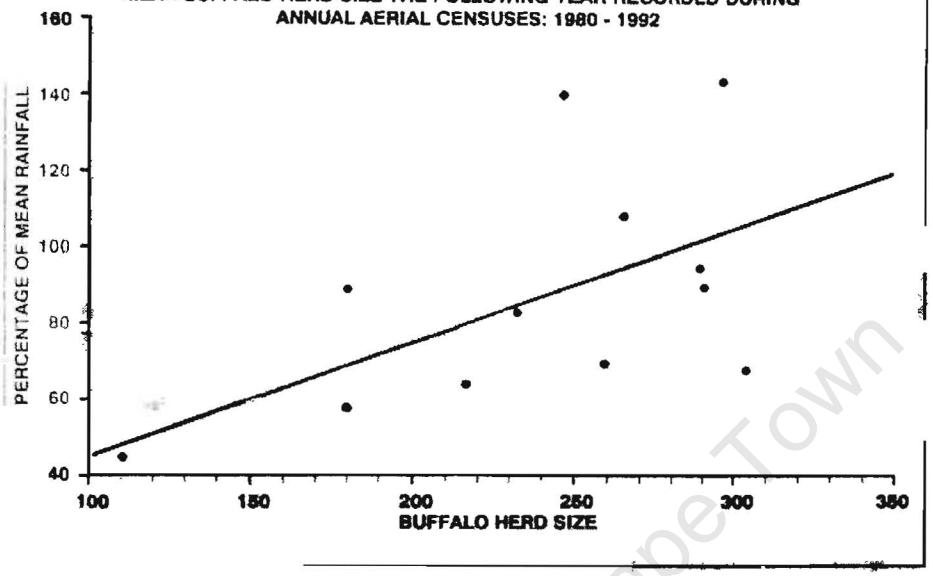
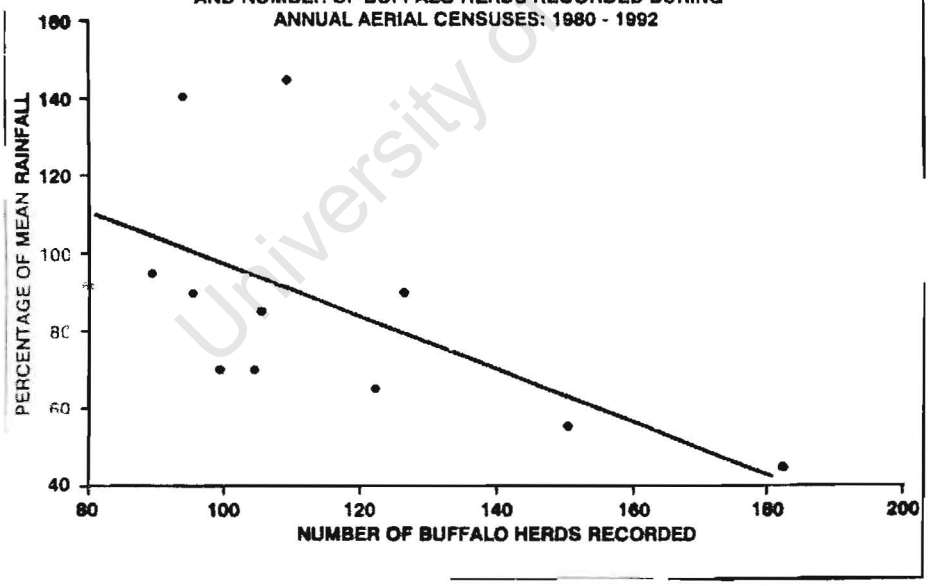


Figure 7b: THE RELATIONSHIP BETWEEN RECORDED RAINFALL AND NUMBER OF BUFFALO HERDS RECORDED DURING ANNUAL AERIAL CENSUSES: 1980 - 1992



From: blackmor@aqua.ccwr.ac.za
Subject: Buf stuf
To: COLLEEN@chempath.uct.ac.za (Colleen o' Ryan
Date: Tue, 24 Oct 95 14:09:01 USAST

Colleen, below are the relevant sections from the report and monitoring
ent. The Age/ Sex stuff will get off the ground this
Regards Andy

ANIMAL POPULATION MANAGEMENT WITHIN THE SOUTHERN REGION OF THE GREATER ST
IA WETLAND PARK - NPB ANNUAL REPORT : 1 April 1994 -

BUFFALO MONITORING
The 1994 buffalo census was delayed by two important management exercises,
mely:

the scheduling of the annual buffalo cull to overlap with the annual census
and
an aerial survey of Chromolaena odorata within the Mfabeni section.

The cull negatively influenced the census as the buffalo population was seen
to be highly fragmented, thus increasing the probabile
cull (Figure 2.1). Despite the fragmented status of the population, only nin
e replications (Table 2.1) were needed to derive a reu
e to derive an index of the buffalo population's performance. In order to dec
rease the number of replicates, manipulation of this
Figure 2.1: 1994 Annual buffalo census. Approximate timing of the cull (() is
indicated. See text for details.0000bservers for theu
s ResultsTable 2.1. General results of the 1994 buffalo survey.Census date 7
/7/9417/8/9418/8/94MeanStandard deviationStandard erro
10 30.60.3300Rhino1010.70.60.3755-jackal2000.71.20.7295Warthog**7610088.017 0
9.84Waterbuck969810499.34.22.41Wildebeest1841713.07.8a
l 10% of the mean number of sighted animals. ** warthog not counted

A comparison can be made between the 1993 and 1994 counts, as the 1993 counts
concentrated solely on buffalo. The inclusion of otht
ion. Given that the aerial census of the buffalo population has now been stan
dardized, comparisons of this nature can be expected When considering the number
of replicates required (Table 2.1), the aerial census appeared to have value in
rerms of counting wart
species of low densities.

Population Estimate
A maximum count of 91 buffalo was achieved during the 1994 total area coverag
e census. This figure is significantly lower than them
ortalities, the buffalo population is estimated (for 1 April 1995) to lie bet
ween 130 and 170 individuals.

Population performance
The 1994 census figure was lower than that determined in 1993 There is,
ver, no evidence to believe the population is decreas
Strategy and Goals

Repeat the aerial census during May / June 1995
Capture historical data into the animal population database.
Initiate the age and sex distribution component of the buffalo monitoring pro
gramme.

Following Be from "Animal population monitoring within the southern region of the Greater St Lucia Wetland Park, Blackmore AC 1998

& BUFFALO MONITORING

INTRODUCTION

A total of 19 buffalo, Syncerus caffer, was introduced, onto the Eastern Shores on the 17 August 1977. This introduction programme

The introduction was to create a small nucleus of breeding buffalo. Once established, the population would be allowed to reach an

The estimate of the buffalo population is obtained by an annual fixed wing census. The age and sex distribution within the popula

and helicopter rhino censes. Limited time and the fragmentation of the herds due to disturbance caused by culling activities, resul

xed-wing.

Table 6-1: Actions taken to manage the buffalo population STRATEGYPOPULATION ESTIMATENo management for estimate less than 100 India

n 150 individuals!!!The carrying capacity was calculated primarily on the basis of available reed habitat within the Eastern Shores

n of threatened herbivores (eg. Lichtenstein's hartebeest and roan antelope) onto the Eastern Shores. In accordance with the Stasow

as installed extending from the lake edge at Sellays Lakes to the beach on the eastern side of the barrier dunes. This fence is to

If buffalo migrate out of the Eastern Shores system, ie northwards into Dzabeni or across the Lake onto the Western Shores, they wi

an for details).

RETURNS SUMMARY
Table 6-1 Summary of the buffalo monitoring exercise

ACTIVITYRESPONSIBILITYTIMINGSDUPLICATE DISTRIBUTION Buffalo MonitoringConserva
tor Eastern Shores ContinuousDDDesign and monitoring g
e and sex distributionOiC MfabeniContinuousENZMortalitiesOiC MfabeniContinuou
sENZPopulation EstimateEcologist Northern ZululandAnnu
ZululandMonthlyNoneAnnual Report as part of the Animal Population Management
ReportEcologist Northern ZululandAnnuallyCCS via CESa
ircraft timeThree observersProductsDistribution and estimate of population si
zetimingAnnually, over period May to JuneTTThe estimas
us is divided into three survey exercises consisting of two days each. Th
hree survey exercises are undertaken during the periods
in the Greater St Lucia Wetland Park, to be overcome. The flight time is book
ed by ENZ and the dates made available to line manage
Management of this species should only be undertaken after the completion of
the antelope census, viz. at the end of August. This o
settle and regroup before the next buffalo census. If these conditions are n
ot adhered to, the census must be abandoned so as to
For each survey, the pilot is to fly east / west transects extending from Sel
leys Lakes southwards. The transects should be approxw
the plane. On observation of buffalo, the pilot (1) makes a mental note of h
is position on the transect, (2) circles the buffalo,w
hich are then counted. The process (3-4) is repeated until consensus on the b
uffalo numbers is achieved. On consensus, the pilot rr
ed on to the buffalo census map (Appendix 8) by the front seat observer. Each
herd is photographed by the front seat observer to d
In addition to the buffalo census, bushbuck, kudu, rhino, warthog, waterbuck,
wildebeest and zebra are counted. As they are encouno
f their population sizes (see also Chapter 7.0 below).

An estimate of the buffalo population is achieved by selecting the maximum nu
mber of buffalo counted during the census. If this fi
and mortalities), then an average of the two is usec An estimate based solel
y on the previous year's counts is used if the census
Age and Sex Distribution Estimate
RequirementsBackground patrollingProductsEstimate of age and sex distribution
within populationPersonnelOiC Mfabeni, rangers, cadea
on the population size as well as sex and age distribution are required. It
is the responsibility of the OiC Mfabeni to ensure thl
o return (Appendix 8) are (1) adult, (2) sub-adult, (3) yearling and (4) new
born numbers (see Appendix 8 for age : shoulder classs
s by ENZ. These data are used annually by ENZ to model medium and long term t
rends of the buffalo population.

TB MONITORING
RequirementsBlood & serum sample, visual observationsProductsTB status of the
Eastern Shores buffalo populationPersonnelNPB Veterit
ected them from coming into contact with animals carrying tuberculosis (TB) b
acilli. Despite this isolation, the buffalo populatioN
PB Veterinarian for TB antibodies. Lungs and intestinal linings of the culled
animals should be inspected for the presence of TB t

Four [illegible] quarantined and were destroyed, and three died on release

Hluhluwe-Umfolozi Park Buffalo

See Brooks & McDonald up to 1982

	Counts	Removals
1983	1618	189
1984	1778	55
1985	1610	1
1986	1939	4
1987	1950	9
1988	2600	9
1989		18
1990		20
1991	6350	
1992		
1993	8400	

Eastern Shores - Buffalo

	Counts	Removals
1982	42	0
1983	42	0
1984	60	0
	75	0
	90	0
	105	0
	130	0
1989	155	1
1990	180	v
1991	170	27
1992	180	18
1993	175	11

(Introduced 23 - 4 deaths; total 19 in 1977)

BOURQUIN

NASIONALE PARKERAAD
NATIONAL PARKS BOARD

FAKSIMILEE
FACSIMILE

Die Parkhoof
Addo Olifant Nasionale Park
Posbus 52
ADDO 6105
Tel: (0426) 400556/7
Faks: (0426) 400196

The Warden
Addo Elephant National Park
PO Box 52
ADDO 6105
Tel: (0426) 400556/7
Fax: (0426) 400196

AAN: TO: U.C.T

AANDAG: ATTENTION: Dr. Colleen O'Ryan.

VAN: FROM: Addo Elephant National Park

DATUM: DATE: 6 Maart 1995

BLADSY: PAGE: of 2

NOTAS: NOTES: Here is the census numbers you are looking for. We've got some more info on the buffalo numbers that we'll post to you. Unfortunately we can't fax it all through.

f results of helicopter census of game animals in Addo Elephant National Pa

able 5. Comparison c
978 - 1994.

pecies	'78	'79	'81	
lephant	92	102	108	
ffalo	247	269	75	
-rhino	9		16	
sh-pig	22			
du	152	203	192	
ysbok	34	35	7	
shbuck	80	81	109	
lker	193	384	392	
trich	99	123	34	
nd	119	138	52	
rtebeest	26	27	27	
ckal	12	10	5	
l-eared	-	-	-	

	Year													
83	'85	'86		'87 Oct	'88	'89 Apr	'89 Dec	'91	'92	'93 Jun	'93 Oct	'94 Mar	'94 Apr	
16	120	127*	133*	135	140	151	162	173	175	185	194	190	193	
7*	42	53	53	43	50	60*	80	60	66	85	75	71	66	
19	16	17	19	18	18	20*	20*	21	29	33	18	28*	34*	
23	11	3	3	2	3	0	0	1	0	6	0	0	0	
93	361	361	276	263	297	308	264	235	284	294	314	259	208	
7	1	8	3	3	6	8	6	0	0	2	0	0	1	
06	123	137	122	109	114	136	95	48	49	75	82	59	41	
89	194	238	105	142	104	226	182	36	62	74	78	51	81	
54	52	52	31	52	27	34	43	28	55	116	67	75	85	
37	49	54	50	49	51	22	25	23	20	31	50	53	56	
24	23	26	37	34	42	35*	20	21	23	35	37	46	38	
13	6	13	9	8	10	9	1	6	11	6	2	4	16	
	1	4	8	0	10	22	0	12	17	20	4	30	12	

evised figures whi ch differ from those given in original census reports
igures include ani als translocated before the census, or in the case of black rhino incorporate
ditional data as we ll.

Appendix G: Assignment Matrix

Results of Population Assignment							
Summary of Population Assignment Outcomes to 'Self' or 'Other' Population							
Pop	Self Pop	Other Pop					
ANP	9	1					
KNP	29	1					
UHC	26	5					
StL	13						
Total	77	7					
Percent	92%	8%					
Assignment Values							
Sample	Pop	ANP	KNP	UHC	StL	Assigned Pop	
1	ANP1	-1.398	-4.000	-2.381	-4.000	1	ANP
2	ANP2	-1.602	-3.699	-3.699	-3.699	1	ANP
3	ANP3	-1.602	-2.875	-3.699	-3.699	1	ANP
4	ANP4	-2.301	-1.808	-3.699	-3.699	2	KNP
5	ANP5	-1.204	-4.000	-4.000	-4.000	1	ANP
6	ANP6	-1.301	-3.699	-3.699	-3.114	1	ANP
7	ANP7	-1.204	-4.000	-4.000	-4.000	1	ANP
8	ANP8	-1.301	-3.699	-3.699	-3.114	1	ANP
9	ANP9	-1.204	-4.000	-4.000	-4.000	1	ANP
10	ANP10	-1.398	-4.000	-2.381	-4.000	1	ANP
11	KNP1	-3.699	-3.255	-3.699	-1.927	4	StL
12	KNP2	-4.000	-2.602	-4.000	-4.000	2	KNP
13	KNP3	-4.000	-2.954	-4.000	-4.000	2	KNP
14	KNP4	-3.000	-2.410	-3.699	-3.699	2	KNP
15	KNP5	-2.602	-1.866	-4.000	-4.000	2	KNP
16	KNP6	-3.699	-2.653	-3.699	-3.699	2	KNP
17	KNP7	-3.699	-2.653	-3.699	-3.699	2	KNP
18	KNP8	-2.301	-1.808	-3.699	-3.699	2	KNP
19	KNP9	-3.699	-2.778	-3.699	-3.699	2	KNP
20	KNP10	-2.301	-1.808	-3.699	-3.699	2	KNP
21	KNP11	-2.301	-1.808	-3.699	-3.699	2	KNP
22	KNP12	-2.602	-2.352	-4.000	-4.000	2	KNP
23	KNP13	-3.699	-3.255	-3.699	-3.699	2	KNP
24	KNP14	-3.000	-2.653	-3.491	-3.699	2	KNP
25	KNP15	-2.301	-1.808	-3.699	-3.699	2	KNP
26	KNP16	-3.000	-2.653	-3.699	-3.699	2	KNP
27	KNP17	-3.699	-3.255	-3.699	-3.699	2	KNP
28	KNP18	-4.000	-2.954	-4.000	-4.000	2	KNP
29	KNP19	-3.699	-3.255	-3.699	-3.699	2	KNP
30	KNP20	-3.699	-3.255	-3.699	-3.699	2	KNP
31	KNP21	-4.000	-2.954	-4.000	-4.000	2	KNP
32	KNP22	-3.699	-3.255	-3.699	-3.699	2	KNP
33	KNP23	-3.699	-3.255	-3.699	-3.699	2	KNP
34	KNP24	-3.699	-3.255	-3.699	-3.699	2	KNP

35	KNP25	-3.699	-3.255	-3.699	-3.699	2	KNP
36	KNP26	-4.000	-2.954	-4.000	-4.000	2	KNP
37	KNP27	-4.000	-2.954	-4.000	-4.000	2	KNP
38	KNP28	-4.000	-2.954	-4.000	-4.000	2	KNP
39	KNP29	-3.699	-3.255	-3.699	-3.699	2	KNP
40	KNP30	-3.699	-3.255	-3.699	-3.699	2	KNP
41	UHC1	-4.000	-4.000	-2.028	-1.140	4	StL
42	UHC2	-4.000	-4.000	-2.028	-1.140	4	StL
43	UHC3	-3.699	-3.699	-2.381	-3.699	3	UHC
44	UHC4	-4.000	-4.000	-1.779	-4.000	3	UHC
45	UHC5	-3.699	-3.699	-3.284	-3.699	3	UHC
46	UHC6	-3.699	-3.699	-1.536	-3.699	3	UHC
47	UHC7	-4.000	-4.000	-2.983	-4.000	3	UHC
48	UHC8	-1.398	-4.000	-2.381	-4.000	1	ANP
49	UHC9	-4.000	-4.000	-2.983	-4.000	3	UHC
50	UHC10	-4.000	-4.000	-2.631	-4.000	3	UHC
51	UHC11	-3.699	-3.477	-2.807	-3.699	3	UHC
52	UHC12	-3.699	-3.699	-1.536	-3.699	3	UHC
53	UHC13	-1.398	-4.000	-2.381	-4.000	1	ANP
54	UHC14	-4.000	-4.000	-2.983	-4.000	3	UHC
55	UHC15	-4.000	-4.000	-1.895	-4.000	3	UHC
56	UHC16	-4.000	-4.000	-2.381	-4.000	3	UHC
57	UHC17	-3.699	-3.699	-1.536	-3.699	3	UHC
58	UHC18	-3.699	-3.699	-1.536	-3.699	3	UHC
59	UHC19	-4.000	-4.000	-2.381	-4.000	3	UHC
60	UHC20	-4.000	-4.000	-2.028	-1.140	4	StL
61	UHC21	-3.699	-3.699	-3.284	-3.699	3	UHC
62	UHC22	-3.699	-3.699	-1.536	-3.699	3	UHC
63	UHC23	-4.000	-4.000	-2.983	-4.000	3	UHC
64	UHC24	-3.699	-3.699	-3.284	-3.699	3	UHC
65	UHC25	-4.000	-4.000	-2.983	-4.000	3	UHC
66	UHC26	-3.699	-3.699	-3.284	-3.699	3	UHC
67	UHC27	-3.699	-3.699	-3.284	-3.699	3	UHC
68	UHC28	-3.699	-3.699	-3.284	-3.699	3	UHC
69	UHC29	-4.000	-4.000	-2.983	-4.000	3	UHC
70	UHC30	-3.699	-3.699	-3.284	-3.699	3	UHC
71	UHC31	-4.000	-4.000	-2.983	-4.000	3	UHC
72	StL1	-4.000	-4.000	-4.000	-1.626	4	StL
73	StL2	-4.000	-4.000	-2.028	-1.140	4	StL
74	StL3	-4.000	-4.000	-4.000	-1.626	4	StL
75	StL4	-3.699	-3.477	-3.699	-1.927	4	StL
76	StL5	-2.699	-3.477	-3.699	-2.529	4	StL
77	StL6	-4.000	-4.000	-4.000	-2.228	4	StL
78	StL7	-4.000	-4.000	-2.028	-1.140	4	StL
79	StL8	-3.699	-3.699	-3.699	-1.927	4	StL
80	StL9	-4.000	-3.556	-4.000	-1.626	4	StL
81	StL10	-3.699	-3.699	-3.699	-1.927	4	StL
82	StL11	-3.699	-3.477	-3.699	-1.927	4	StL
83	StL12	-3.699	-3.699	-2.713	-1.684	4	StL
84	StL13	-4.000	-4.000	-2.028	-1.140	4	StL