# Detection of Genetic Substructure and Diversity in the Endemic South African Antelope Species, *Damaliscus pygargus*

by

Joelle Marie van der Walt

B.S. (Kutztown University, U.S.A) 1991
M.Sc. (Hood College, U.S.A.) 1996

Ph.D. THESIS

DOCTORATE OF PHILOSOPHY

in

Molecular Evolution

in the

DEPARTMENT OF MICROBIOLOGY

University of Pretoria

February 2002

## COPYRIGHT WAIVER

# DEDICATION

to Nico

## ACKNOWLEDMENTS

I wish to express my gratitude to my promoter, Prof. L. H. Nel for his encouragement and guidance that has sustained me throughout this study. His willingness to tackle a new avenue in molecular evolution and his strong interest to the conservation of species has made this research possible. I would also like to thank Prof. Nel for sharing his love of both photography and science with his students. I am also grateful to the long distance support of my co-promoter, Dr. Rus Hoelzel who has led me through the intricacies of population genetics for over eight years. I also appreciate his quick replies and useful comments to my frantic questions through electronic mail. I was inspired to pursue scientific research by Dr. William Towne over a decade ago and I am also indebted to him for his continual support over the years.

Deep gratitude is extended to my two families. My mother and father in America have always heartened me to pursue my goals even when it meant moving thousands of kilometers away from them. I wish to thank my twin sister for visiting me in South Africa and her daily e-mail messages, which kept me going during the blue days. I am also grateful to my South African family, the van der Walts, who have expressed unlimited love and generosity to me from the very beginning.

I also wish to thank both my American and South African friends for being a great support during this project. I especially like to thank the Gecko Posse for showing me friendship and adventure in South Africa. Special thanks to my dogs, Ruby and Sammy, for keeping me company during the hours I spent typing at home.

And lastly, I am forever grateful to my husband, Nico, for his understanding, love, patience, and support during this project.

## TABLE OF CONTENTS

**Statement of Use and Copyright** ii

**Dedication** iii

**Acknowledgments** iv

**Table of Contents** v

**List of Tables** vii

**List of Figures** viii

**List of Abbreviations** ix

**Abstract** 1

**Chapter 1: Introduction and Literature Review** 2

1. Natural History of *Damaliscus pygargus* (Pallas, 1766) 2
   - Evolution of *Damaliscus* (Artiodactyla: Bovidae) 2
   - Taxonomy and Subspecies Classification 4
   - Description of Physical and Behavioral Traits 7
   - Early Genetic Studies 10
   - Bontebok and Blesbok Conservation 11

2. Conservation of Genetic Diversity 15
   - Conservation Genetics 15
   - Inbreeding Depression 16
   - Hereditary Disorders 16
   - Fitness traits 17
   - Fluctuating Asymmetry 18
   - Predisposition to Disease 18
   - Genetic Introgression 19

3. Evolutionary Units for Conservation 20

4. Measurement of Genetic Diversity and Substructure 22

5. Aims of Study 22
   - Hypothesis- Objectives

**Chapter 2: Control Region Variation** 23

Introduction 23
Materials and Methods 27
- Sample Collection 27
- PCR Conditions 27
- SSCP Analysis 29
- Purification of Gene Fragments and Sequencing Procedure 29
- Data Analysis 30

Results 31
Discussion 44

**Chapter 3: Microsatellite Diversity** 49
Introduction 49
Materials and Methods 53
Bovine and Ovine Primers 53
PCR Conditions 53
GeneScan Electrophoresis and Analysis 55
Descriptive Statistical Analysis 56
Clustering Analysis 57
Class Assignment of Individuals 58
Loss of Genetic Diversity 59
Results 59
Discussion 76

**Chapter 4: MHC-DRB Evolution** 84
Introduction 84
Materials and Methods 90
Samples and Collection Sites 90
PCR-SSCP of the DRB Locus 90
Cloning and Sequencing 92
Statistical and Phylogenetic Analyses 92
Genbank Sequences 93
Results 94
Discussion 107

**Chapter 5: Conclusions** 113
Synthesis of Genetic Findings 113
Evolutionary Units and Conservation 113
Inbreeding Depression 114
Fitness Traits 115
Hybrid Detection 117
Management Recommendations 119
Biodiversity in South Africa 119

**Summary** 121

**Chapter 6: Literature Cited** 123

## LIST OF TABLES

1. Nomenclature of *D. pygargus* 8
2. a. Control region SSCP haplotypes of bontebok samples 32
2. b. Control region SSCP haplotypes of blesbok samples 33
2. c. Control region SSCP haplotypes of test group samples 34
3. Control region sequences for bontebok and blesbok 36
4. Alignment of control region sequences 38
5. Genetic distance matrix of control region sequences 39
6. Microsatellite primer list 54
7.a. Multi-locus composite genotypes for blesbok 61
7.b. Multi-locus composite genotypes for bontebok 62
7.c. Multi-locus composite genotypes for test group 63
8. Microsatellite allelic states 66
9. Microsatellite diversity indices 68
10. GeneClass results for reference class and test group 74
11.a. Bottleneck test simulations for blesbok 76
11.b. Bottleneck test simulations for bontebok 77
12. Heterozygosity values for antelope species 80
13.a. DRB genotypes of blesbok 96
13.b. DRB genotypes of bontebok 97
14. DRB frequencies found within in each subspecies 98
15. Allelic diversity values 99
16. Genetic matrix of DRB amino acid sequences 101
17. Nonsynonymous versus synonymous differences 103
18. Rate of substitution of cattle and other ruminants 104
19. Sequence parameters for phylogenetic analysis 105

## LIST OF FIGURES

1. Phylogeny of Alcelaphini 3
2. Geologic timeline of bovid evolution 5
3. Distribution of *D. pygargus* 6
4. Phenotypic differences between bontebok and blesbok 9
5. Historical distribution of bontebok 12
6. Illustration of the mammalian control region 25
7. Population numbers within provinces 28
8. SSCP analysis 35
9. Distribution of control region haplotypes 37
10. Minimum evolution phylogeny of control region sequences 40
11. Control region diversity across antelope species 41
12. Mismatch distribution of control region sequences 42
13.a. Allele frequency distribution 64
13.b. Allele frequency distribution 65
14. UPGMA-Ps phylogenetic tree 69
15. NJ-PS phylogenetic tree 70
16. UPGMA-Kf phylogenetic tree 71
17. NJ-Kf phylogenetic tree 72
18. Test animal tree (UPGMA-Ps) 73
19. Distribution of microsatellite alleles for all loci 75
20. Major histocompatibility complex 85
21. Three-dimensional DRB 86
22. Sample distribution 91
23. SSCP of DRB alleles 95
24. Amino acid alignment of DRB sequences 100
25. Minimum evolution tree of DRB sequences 106
26. Trans-species mode of evolution 108

# LIST OF ABBREVIAIONS

| | |
|---|---|
| BOLA | Bovine Leucocyte Antigen |
| dS | snynonymous |
| dN | nonsynonymous |
| ESU | Evolutionary significant unit |
| Kf | Kinship coefficient |
| LIS | Low ionic saccharose |
| MU | Management unit |
| MYA | Million years ago |
| NJ | Neighbor joining |
| PBR | Peptide binding region |
| Ps | Proportion of allele shared |
| SSCP | Single strand conformational polymorphism |
| TBE | Tris Borate EDTA |
| UPGMA | Unweighted pair group mean algorithm |
| UV | Ultraviolet |

## ABSTRACT

The antelope (*Damaliscus pygargus*) is an endemic species in South Africa belonging to the contemporary antelope tribe Alcelaphini. The species is subdivided into two subspecies based on phenotypic differences and historic geographic isolation. This study has revealed strong molecular evidence of genetic structuring between (*D. p. pygargus*, bontebok) and (*D. p. phillipsi*, blesbok) based on neutral and coding gene markers. Examination of the control region demonstrated extreme lack of diversity within bontebok revealing only one unique matriline, which was not found within blesbok. Microsatellite analyses confirmed the mitochondrial data and showed strong genetic partitioning and differences in genetic diversity for each subspecies. Allelic diversity of the major histocompatibility class II gene DRB was investigated and found to be extremely polymorphic in numbers of alleles, number of amino acid site changes, and genetic distance. I suggest that the evolution of DRB diversity is governed by over-dominant selection and/or heterosis as evidenced by the high number of non-synonymous changes found within the peptide binding region as well as the high number of heterozygous animals. The results of this study reflect past demographic events experienced by each subspecies. Erosion of molecular genetic variation in bontebok is most likely due severe reduction in population size caused by over-hunting and stochastic events. Blesbok antelope display moderate levels of genetic diversity, which was expected given their relatively stable demographic history. Based on the genetic findings from this study, I recommend the retention of the subspecies designation and that each group be managed separately. Furthermore, I suggest careful management of the remaining bontebok herds in order to conserve the unique genetic diversity of these rare antelope for future evolutionary change.

# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

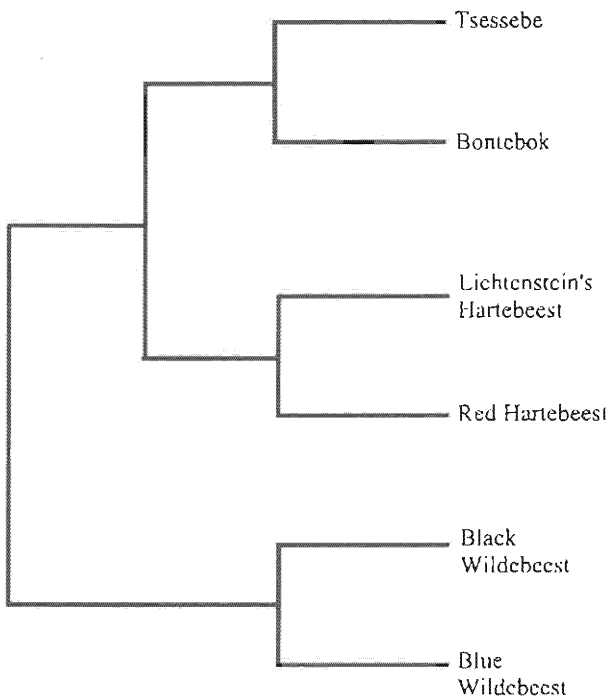
# Chapter 1: Natural History of *Damaliscus pygargus* (Pallas, 1767)

## *Evolution of Damaliscus (Artiodactyla: Bovidae)*

The family Bovidae is characterized by explosive radiation events that took place approximately 18-20 million years ago (MYA) according to fossil record (Vrba 1995). During the evolution of the bovids, over 137 extant species have diverged, making this family one of the most specious groups of mammals (Grubb 1993). Classification methods using morphology alone have divided Bovidae into six subfamilies and 12 different tribes (Ansell 1972, Gentry 1992). Global change during the Late Miocene and Early Pliocene created warmer temperatures and ample rainfall for savanna vegetation to dominate the African landscape (Cerling et al. 1992). This change sparked the major bovid radiation events within the subfamily Bovinae, including the emergence of the tribe Alcelaphini (Vrba 1979).

The Alcelaphini tribe arose in South Africa and spread throughout the African continent (Vrba 1995). This tribe comprises the following extant species: tsessebe (*Damaliscus lunatus*), hirola (*Beatragus hunteri*), red hartebeest (*Alcelaphus buselaphus*), Lichtenstein's hartebeest (*Alcelaphus lichtensteini*), black wildebeest (*Connochaetes gnou*), blue wildebeest (*Connochaetes taurinus*) and the blesbok/bontebok (*Damaliscus pygargus*). Genera of Alcelaphini have been regarded as being quite exceptional by way of sharing distinctive characteristics such as: horned females, specialized limb characteristics, extensive internal sinuses, short braincase and evolutionary advanced teeth (Gentry 1992). The evolutionary relationship of the tribe members supports a basal placement of *Connochaetes* to the sister taxa of *Damaliscus* and *Alcelaphus* (Matthee and Robinson 1999a) (Figure 1).

Figure 1. Evolution of the tribe Alcelaphini adapted from Matthee and Robinson 1999. The phylogeny was reconstructed using cytochrome b sequences by three methods of analysis (maximum parsiomny, minimum evolution and maximum likelihood). All nodes are well supported by each method (Boostrap value range 75 - 100%)



The fossil evidence places the first appearance of Alcelaphini at approximately 5 MYA (Vrba 1995) while mitochondrial DNA data supports an earlier emergence at 10 MYA (Hassainin and Douzery 1999) (Figure 2). The present alcelaphini species are contemporaneous and have arisen between 0.5 – 1.5 MYA according to the fossil record (Vrba 1979). Mitochondrial data supports the fossil dates for the most part, but has revealed extinction of some lineages of species (Arctander et al. 1999). Within this epoch, bovids underwent another macroevolution due to the shifts in temperature and rainfall throughout glacial and inter-glacial periods (Vrba 1979). Many warm regions of Africa with sufficient rainfall became refuges for bovids. These ecological fluctuations shaped the speciation events of some bovid species and lineages while at the same time caused the extinction of others.

***Taxonomy and Subspecies Classification***

Fossil evidence has revealed that *D. pygargus* had a continuous distribution covering the southwest cape region in South Africa to the southern area of Zimbabwe. However, over geologic time the species was split into two groups by climatic and habitat changes and have remained allopatric (Skinner and Smithers 1990) (Figure 3). This isolation has allowed for morphological and behavioral differences to arise in each group. Subspecies recognition of the bontebok (*D. p. pygargus*) and the blesbok (*D. p. phillipsi*) was based upon these differing characteristics. The common names given to each subspecies by the early settlers have become entrenched over 300 years and have been retained to represent each subspecies.

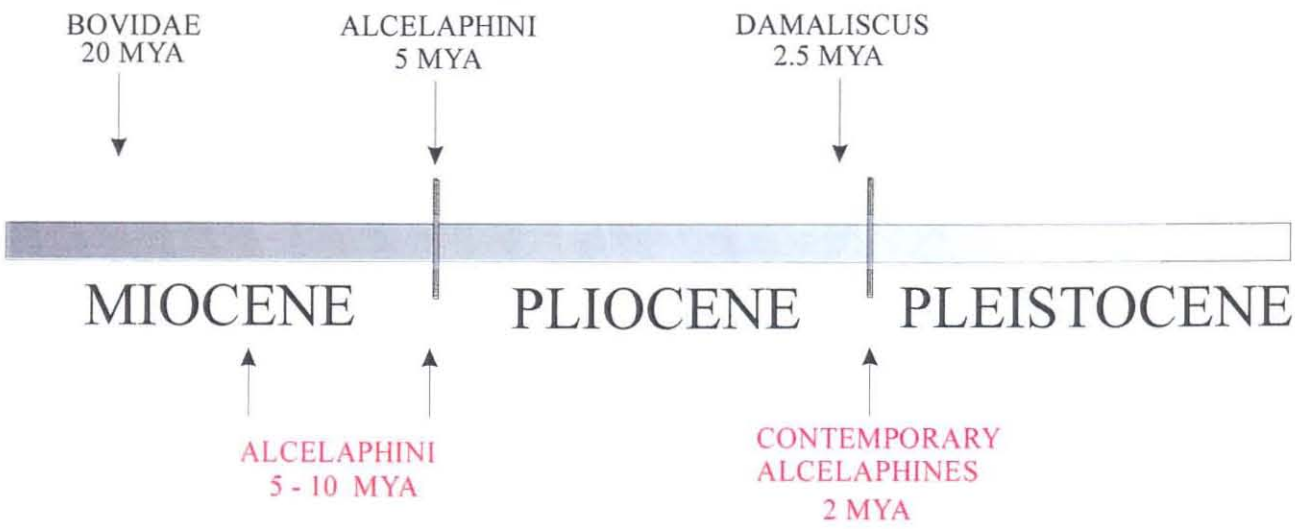



**Figure 2.** Geologic timescale of the major radiations of Bovidae leading to the emergence of *Damaliscus*. The fossil record dates are illustrated above the time line and are featured in black text. Molecular data place the emergence of Alcelaphini within the late Miocene - early Pliocene and the radiation of all contemporaneous alcelaphines during the Pleistocene.


**Figure 3.** Historical distribution of *Damaliscus pygargus*.

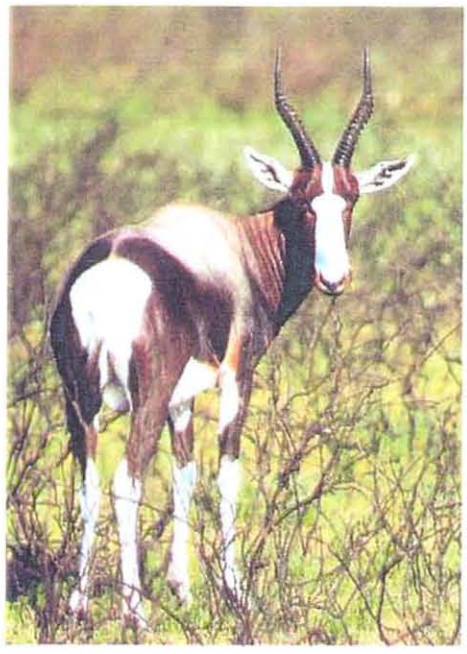
The scientific names of the bontebok and blesbok have changed several times over the last few hundred years due to the confusion over nomenclature codes (Table 1). In 1766, Pallas described a bontebok specimen as *Antilope dorcas* in his Miscellanea Zoologica. Later he realized that he had reserved that name for another species and subsequently changed the species name to *pygargus* in 1767 in his revised Spicilegia Zoologica. The classification became even more confusing when the genus name was reviewed by Scatler and Thomas (1894-1900) and later revised by Harper (1940). The classification was changed to the combination of *Damaliscus* and *dorcas*. Finally, the 1985 Code of Zoological Nomenclature (Article 59b) stated that a secondary homonym was invalid if changed before 1961. This code invalidates the *dorcas* homonym and supports the use of *pygargus* as species name (Rookmaaker 1991). Many researchers still continue to use the incorrect scientific name (*D. dorcas*) for the bontebok and blesbok antelope. The taxonomic description of this antelope species should further on be referred to as *D. pygargus* (Pallas, 1767).

***Description of Physical and Behavioral Traits***

Blesbok and bontebok are the smallest antelope of the Alcelaphini tribe (Estes 1991). The antelope were classified as separate subspecies based upon morphological differences and geographic isolation. Historically, bontebok were restricted to the coastal plains of the Western Cape region while blesbok roamed the grasslands of the Eastern Cape, Free State, Gauteng and Mpumalanga (former Transvaal). The subspecies have distinct color characteristics including coat color, body markings and facial blaze (Bigalke 1955) (Figure 4). The markings of the bontebok are quite striking having a dark,

**Table 1.** Nomenclature of *D. pygargus*

| | **Taxonomy** |
|---|---|
| Family | Bovidae |
| Subfamily | Hippotraginae |
| Tribe | Alcelaphini |
| Genus/Species | *Antilope dorcas (Pallas 1766)* |
| Species (revised) | *A. pygargus (Pallas 1767)* |
| Genus/Species (reviewed) | *A. pygargus* (Sclater & Thomas, 1894) |
| Genus/Species (revised) | *Damaliscus dorcas* (Harper 1940) |
| Bontebok subspecies | *D. d. dorcas* (Pallas, 1767) |
| Blesbok subspecies | *D. d. phillipsi* (Harper, 1940) |
| **Reclassification** | (Rookmaaker, 1991) |
| Genus/Species | ***Damaliscus pygargus* (Pallas, 1767)** |
| Bontebok subspecies | ***D. p. pygargus*** |
| Blesbok subspecies | ***D. p. phillipsi*** |



(J. van der Walt 1998)

(D.Lynch 1998)

**Figure 4.** The blesbok (D.p. phillipsi) is shown on the right in its grassland habitat while the bontebok (D.p. pygargus) is figured in renoster shrubland on the left. The horn and body color of blesbok are normally darker than that of bontebok. Each subspecies is distinguished by a white face blaze which is continuous in bontebok and disrupted in the blesbok by a horizontal brown band. The white rump patch is characteristic of bontebok.

purple sheen to the coat, large white rump patch surrounding the tail, and white lower legs and belly. Bontebok have a white facial blaze, which runs continuously from the base of the horns to the nose. The color of the blesbok is lighter brown with a light tan rump patch and tawny legs and belly. Blesbok are also distinguished by a white facial blaze that is divided by a light brown band between the eyes.

The social structure of the bontebok consists of small nursery herds (females and calves), territorial males and large herds of bachelor males throughout all seasons. The bachelor and nursery herds rove between the park boundaries in search of short grass while reproductive males defend territories and display to mature females (David 1973). The social structure of the blesbok differs somewhat from that of the bontebok Reproductive males strongly defend territories during the breeding season, like the blesbok: however, all territories are given up after the rut in the winter season (June – August). Large aggregations of antelope of every age and sex are formed to increase individual likelihood of grazing during the dry months. All herds re-group in November after calving begins and territorial males re-establish territories for the next breeding season (Lynch 1971).

***Early Genetic Studies***

Although ecological and behavioral data exists for *D. pygargus*, little genetic data is available for this antelope. An early genetic study of blesbok using protein markers (transferrin, hemoglobin, amylase, albumin and carbonic anhydrase) revealed no variation for these blood factors (Osterhoff et al. 1972). The same study did, however, report slight variations in blood serum of blesbok using antisera to goat red cell factors. A second study screened the mitochondrial genome for variation using restriction fragment length

polymorphism (Essop et al. 1991). The structure and mode of mitochondrial DNA (mtDNA) evolution provides a finer measure of genetic variation than protein markers below the species level (Avise 1994). The restriction maps revealed genetic difference between the bontebok and blesbok and a calculated sequence divergence of 0.47% (Essop et al. 1991). This study also estimated an approximate time of divergence between blesbok and bontebok at 250,000 years based upon the mean rate of mtDNA divergence of 2% per million years (Wilson et al. 1985). Lastly, G and C banded karyotypes (2n = 38) of both subspecies displayed complete homology (Kumamoto et al. 1996).

***Bontebok and Blesbok Conservation***

The former distribution of the bontebok covered the coastal sandveld of the south-western Cape region from the settlements of Caledon and Mossel Bay (van der Merwe 1968). An array of geographic barriers, including the Bot River in the west, the Langeberg and Zonderend mountain ranges in the north and the Atlantic and Indian oceans of the south restricted movement of bontebok from the narrow confines of this region (Figure 5). Human settlement within the western Cape began in 1652 and occupied the most fertile land for agriculture. This encroachment forced the bontebok herds into the less suitable grasslands for grazing. Large herds of bontebok and other antelope were still sighted by the early Cape naturalists in the late 1600's and 1700's, then drastically declined by the early 1800's (Skead 1980). Intensive human encroachment and strong hunting pressure attributed to the near extinction of the endemic South African bontebok.

Fortunately, several landowners within the area had the insight and initiative to protect herds of bontebok on their farms, which in turn, led to the recovery of this

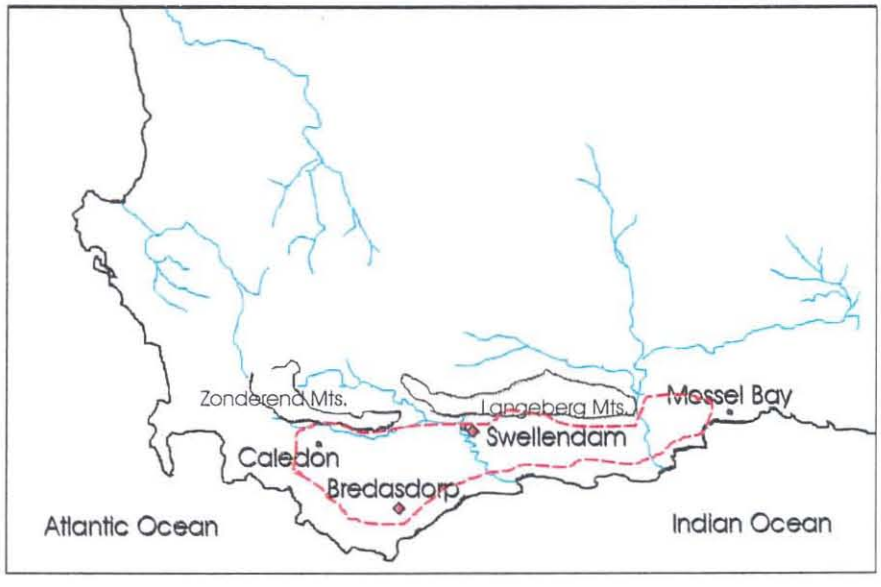



**Figure 5.** The historic distribution of bontebok is shown by the red outline. Due to the drastic reduction in population size, the first Bontebok National Park (BNP) was proclaimed in Bredasdorp in 1931. Surviving animals from the original herd were translocated in 1960 to the present BNP located in Swellendam.

antelope. In 1837, a small herd of 27 bontebok was protected on a farm owned by the van der Byl family within the Bredasdorp area. The herd number increased slowly at two animals per year but tended to decrease during seasons of drought. By the early 1900's, the total number of protected bontebok increased to approximately 250 individuals on the combined area of the van der Byl and Albertyn farms (Skead 1980).

In 1931, the first Bontebok National Park was proclaimed in Bredasdorp in order to protect the remaining bontebok from further hunting pressure. The park was stocked with 17 bontebok from the van der Byl and Albertyn farms (N. Fairall–National Parks Board). Within three decades, this herd increased (n = 180) well beyond the carrying capacity of the small reserve. The only remedy at the time was to graze bontebok on artificial pastures that were deficient in copper (Barnard and van der Walt 1961). The deficiency presented in the forms of swayback and ataxia in the antelope. Animals also suffered from massive worm infestations of conical fluke (*Paramphistomum* sp.), lung worm (*Protostrongylus* sp.), wireworm (*Haemonchus* sp.), brown stomach worm (*Ostertogia* sp.) and bankruptworm (*Tirchostrongulus* sp.) which aggravated the swayback condition. A large mortality (50%) resulted within the herd from the parasitic infection, poor grazing conditions and related syndromes. A decision was made to translocate antelope from the park to a suitable habitat farther north in Swellendam. The new Bontebok National Park was established in 1960 with 61 bontebok that survived the translocation event. The estimated 2,500 - 3,000 bontebok today are derived from this founding population. Bontebok are still considered a rare antelope species and are listed as vulnerable in Appendix II of the International Trade in Endangered Species Red List (World Conservation Union, 1993).

The blesbok antelope were also persecuted by the early settlers for their hides and were reported as having been slaughtered by the thousands after the Great Trek in 1836

(Lynch 1971). The naturalist H. A. Bryden (1893) wrote "The blesbok which not long since scoured the plains of Lower Bechuanaland (Northwest Province SA-Botswana), the Transvaal (Guateng and Mpumalanga) and Free State in the countless thousands, is now seldom seen". The extermination of blesbok populations persisted until 1899 when the trade in skins was brought to an end by the Boer War. After the war, the majority of the open grassland regions were taken over by settlers forcing the remaining populations to exist only on fenced farms. Their distribution was then artificially expanded by translocation events to the Cape region and Natal. By 1962, the population size of the South African blesbok totaled approximately 42,000 (Kettlitz 1967). Blesbok are still a popular game antelope today, however, the hunting is now confined to private lands and national hunting concessions. The population size of blesbok is currently 120,000 (David 1998).

The subspecies had been separated by over 320 km and remained allopatric until recent times. Many herds have been translocated out of their natural ranges to stock private farms and reserves. The blesbok and bontebok antelope readily hybridize given their close evolutionary relationship. Animals resulting from these hybridizations were also translocated throughout South Africa (Allardice and Gaigher 1979). The genetic admixture of the subspecies threatens the genetic purity of the bontebok. Hybrid animals look very similar to blesbok but are larger in body size (N. Fairall per com.). The blesbok are normally darker in color; however, pelage can become very pale in color if copper is deficient in their diet (Penrith et al. 1996). The white markings can also be used to identify subspecies such as the amount of white on the legs and the shape of the facial blaze. Fabricius et al. (1989) used a discriminant function analysis of rump patch measurements to differentiate between the pure subspecies and hybrid animals. They

reported that the hybrid scores fell between the continuum of the pure subspecies. Parks Board officers have used this method to verify purity of bontebok and blesbok herds.

## 2. Conservation of Genetic Diversity

### *Conservation Genetics*

The endangered status of a species largely results from drastic population reduction caused by habitat destruction, over-hunting, disease outbreaks or environmental changes. Massive global population declines of vertebrate species have been noted as early as the 18th century, but still today, the numbers of endangered and extinct species have escalated to a tragic figure. A new scientific discipline-conservation genetics-has surfaced from these catastrophic events. This science is used to conserve biodiversity by applying the theories of molecular evolution and population genetics and the new methods of biotechnology (Avise 1989, Moritz 1994a, O'Brien 1994a, Hedrick 1999). The genetic data created by molecular techniques can provide traces of historical events as well as the present status of the species (O'Brien 1994b). Genetic findings, together with ecological, behavioral, demographic, and clinical data, can be used to design management plans for the conservation of the species.

Genetic diversity has been slated as an important level of biodiversity demanding conservation (McNeely et al. 1990). This recognition aims to preserve the existing genetic variation of the species for future evolutionary change. Having a substantial amount of variation, the species is better able to adapt to new ecological changes and disease challenges (Fisher 1930, Frankham 1996). This evolutionary potential has been largely theoretical until recent studies have provided empirical data that supports the relationship between genetic variation and fitness (Frankham et al. 1999).

*Inbreeding depression*

In natural populations, severe population contractions often lead to inbreeding within a population. Mating between close relatives causes the decline of the value of a trait (Wright 1931) and loss of fitness. Although, inbreeding depression is slowly becoming accepted as an important factor on species survival, the impact is still argued against by some (Caro and Laurenson 1994). Because the physical symptoms of inbreeding depression are difficult to observe in the wild, the threat has often been ignored. However, Crnokrak and Roff (1999) have thoroughly reviewed the ill effects of inbreeding in wild populations. The consequences of inbreeding depression are the following: increased incidence of hereditary disorders, loss of fitness and increased risk of disease susceptibility.

*Hereditary disorders*

The genetic defects are caused by inheritance of deleterious alleles that may have become fixed in the population (Charlesworth & Charlesworth 1987, Lande 1998). This kind of inbreeding depression results from inheritance of lethal alleles at one single locus or a few loci. The amount of lethal alleles found in a population was termed as the genetic load by Crow in 1930. Laikre (1999) summarized current observations of inherited diseases found in captive carnivore species, which include hereditary blindness, cryptorchidism, albinism, skeletal defects, and congenital heart anomalies. Similar conditions of heart defects, cryptorchidism and vertebrate deformity (kinked tail) have been found in the relic population of Florida panther (Roelke et al. 1993).

***Fitness traits***

Inbreeding is also associated with loss of individual fitness and population viability. The traits affected by inbreeding depression are related to reproductive fitness, fecundity, and juvenile survival (Wright 1977). These consequences of inbreeding depression had been documented in humans (Morton et al. 1955) as well as domestic (Wright 1922) and laboratory animals (Bowman and Falconer 1960). But the threat to exotic animals was only made evident in 1979 by a seminal paper published on inbred captive ungulates by Ralls and colleagues. They reported on a high degree of juvenile mortality in captive inbred ungulate species and suggested that the same phenomenon may occur in the wild. Pedigrees of captive exotics have also been used to estimate inbreeding coefficients and the "costs" of inbreeding (juvenile mortality) in each population (Ralls et al. 1988). Again, this work demonstrated the negative effects of inbreeding and highlighted the possibility that it may pose a severe threat to natural populations.

Sperm quality is another reproductive trait that can be negatively affected by inbreeding depression. The morphology, mobility, viability and concentration of sperm are important factors for successful fertilization to occur (Drobnis and Overstreet 1992). These sperm traits, and other characteristics, have been intensely studied in wild felid species that have undergone drastic population reductions. The African cheetah (*Acinonyx jubatus*), the Ngorongoro lion (*Leo leo*), and the Asian lion (*Leo persica*) all demonstrate reduced male reproductive fitness due to poor sperm quality (Wildt et al. 1983,1987). Two studies of captive gazelle species (*Gazella sp.*) confirmed that individual inbreeding coefficients are related to percentage of normal sperm and length of sperm mid-piece (Roldan et al. 1999, Gomendio et al. 2000). These reports provide

strong evidence for the high reproductive costs associated with inbreeding depression in ungulates.

### *Fluctuating Asymmetry*

Fluctuating asymmetry (FA) is defined as the deviation of a morphological character (e.g., fin length, horn width) from normal bilateral symmetry (Van Valen 1962). The degree of asymmetry has been hypothesized to intensify due to genetic factors (inbreeding, hybridization) or severe environmental conditions (pollution, habitat changes). These factors may alter the normal development of the organism and cause an increase in fluctuating asymmetry (Palmer and Strobeck 1986). It has been suggested that levels of FA have been negatively correlated to levels of heterozygosity (Mitton 1993). Moreover, FA has been strongly linked with genetic stress (inbreeding) and ejaculate quality in gazelles (Roldan et al. 1999, Gomendio et al. 2000).

### *Predisposition to disease*

High genetic diversity is thought to promote greater fitness as well as higher disease resistance in a population (Hughes 1994). The consequences of infectious disease on a host species depend on many factors including "ecological and epidemiological conditions associated with the outbreak" (Murry et al. 1999). Moreover, the severity of the disease may be affected by factors other than inbreeding, such as the nutritional index, pathogen load and stress levels of the animal (Ullrey 1993). The relationship between genetic diversity and disease susceptibility is often difficult to prove in natural populations given the complexity of disease manifestation. However, a recent study has

related individual heterozygosity levels with disease resistance and survival within a wild population of Soay sheep (Coltman et al. 1999).

### *Genetic Introgression*

Hybridization between closely related species or subspecies is one of the natural processes of speciation (O'Brien and Mayr 1991). This natural occurrence may develop from changes in ecology, which unite formally allopatric species ranges. It has been postulated that the red wolf (*Canis rufus*) originated by this process when the grey wolf (*Canis lupus*) and coyote (*Canis latrans*) hybridized during the late Pleistocene (Reich et al. 1999). However, most hybridization events are caused by un-natural or man-induced situations. In these cases, exotic species or subspecies are introduced to an area that is inhabited by native taxa. The subsequent mating events ultimately contaminate the genetic purity of the native species. For endemic species and subspecies, genetic introgressions pose a great risk to their survival as distinct evolutionary lineages. Furthermore, failure to protect these lineages from extinction represents a loss in biodiversity.

Illegal translocations of vertebrate species for economical gain have created numerous conservation problems in southern Africa. Antelope species, in particular, are moved out of their natural range to game farms for trophy hunting. Hybridization events are common between antelope subspecies including the bontebok and blesbok (Allardice and Gaigher 1979), and also the black-faced impala (*Aepyceros melampus petersi*) and common impala (*A. m. melampus*) (Green and Rothstein 1997). The severity of the problem is also evident in the hybridization between species such as black and blue

wildebeest (Fabricius et al. 1988) and the red hartebeest and tsessebe (Robinson et al. 1991).

Hybridization between closely related breeds, races or subspecies may result in two very different manifestations of heterosis. The fitness costs or consequences of the hybrids may vary greatly among species (Charlesworth and Charlesworth 1987). In one case, a negative effect of hybridization may cause the reduction of fitness of the offspring in outbreeding depression. That is, loss of fitness will occur if the two parental groups have evolved local adaptations such as mating behaviors, feeding strategies and disease resistance (Templeton 1987). In this regard, hybridizations may result in unfit progeny that are not successful in the wild (Hatfield and Schluter 1999).

In another scenario, hybridization can result in positive heterosis or "hybrid vigor". This cross-mating between groups is thought to cause the purging of deleterious alleles at homozygous loci and the subsequent increase heterozygosity levels (Crow 1948). Therefore, the effects of inbreeding will be diminished and the progeny will inherit higher fitness (Thornhill 1993).

## 2. Evolutionary Units for Conservation

The Biological Species Concept (BSC) recognizes that the genetic diversity of a species can be subdivided among separated populations into subspecies (Mayr 1963). Intraspecies classification, or taxonomy below the species level, has historically been based upon phenotypic differences and geographic distribution. Morphological characteristics such as pelage, stripe pattern and body size have been used to describe subspecies of mammals. In most cases, geographic distance or physical boundaries

prevent contact and gene flow among subspecies. The temporal and spatial distribution of the subspecies allows for genetic differences to arise (O'Brien and Mayr 1991).

These genetic differences are being utilized to describe evolutionary lineages for conservation (Ryder 1986). This concept emerged out of necessity for genetic diversity to be included in the criteria used for conservation management. An evolutionary significant unit (ESU) was originally defined as any population having a distinct evolutionary history, adaptive differences and reproductive isolation (Ryder 1986, Waples 1991). Through genetic analysis, the taxa that are significantly divergent can be recognized as an ESU and prioritized for conservation. Further refinement of the concept has incorporated reciprocal monophyly for mtDNA sequences and divergence of nuclear gene frequencies to identify units for conservation (Moritz 1994a). Populations that display allele frequency differences are defined as management units (MU). These groups are not phylogenetically distinct, but they do however, show structure by way of frequency distribution that is determined by breeding units within the population (Moritz 1994b).

Since the inception of the ESU concept, biologists have argued that many populations have been labeled as distinct units based solely on genetic data and have failed to include ecological perspectives (Taylor and Dizon 1999, Crandall et al. 2000). They caution the abuse of ESU terminology that limits the definition of conservation units to only phylogenetically distinct taxa. Crandall and colleagues (2000) also highlight that such treatment of taxa may prevent the maintenance of evolutionary potential by restricting gene flow and adaptation.

## 4. Measurement of Genetic Diversity and Substructure

The recent advances in genetic technology have made it possible to address specific questions concerning patterns of genetic diversity found within natural populations (Sunnucks 2000). The highly variable molecular markers described in the following chapters have been found to be highly informative in the examination of the genetic dynamics of populations.

## 5. Aims of Study

***Hypothesis***

*Damaliscus pygargus* diverged into two genetically distinct subspecies through geographic isolation over a long period of time.

***Objectives***

1. Evaluate the extent and character of genetic differentiation between the bontebok and blesbok in order to:
   a. Validate or contest subspecific classification.
   b. Infer past demographic events for each subspecies.
   c. Detect hybridization events between the subspecies by genetic screening methods.

# CHAPTER 2

# CONTROL REGION VARIATION

## Chapter 2: Evolution of *D. pygargus* Revealed by Control Region Analysis

### *Introduction*

Historically, African bovid migrations were governed by the availability of vegetation and water resources (Estes 1991). Many of these migration patterns are still followed by antelope species inhabiting central, eastern and western regions of Africa; however, urban development has disrupted dispersal patterns of bovid species within much of southern Africa. Human encroachment in South Africa has forced *Damaliscus pygargus* (along with numerous mammal species) to exist within islands of protected land without natural migration corridors. Migration of individuals between sympatric populations allows for gene flow and prevents inbreeding between related individuals (Allendorf 1983). Although blesbok and bontebok populations are restricted by fences, genetic exchange is promoted by translocation events throughout South Africa. In this regard, translocation of animals between populations furthers gene flow but at the same time, obscures any genetic substructure that may be present within the species.

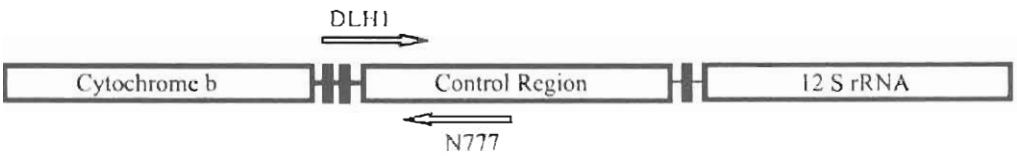
According to neutral theory, alleles in a population are either lost or gained by chance through genetic drift (Kimura 1963). Genetic variation within a species is dependent on the neutral mutation rate of the gene and the effective population size (*Ne*) of the population (Frankham 1996). This diversity may also be divided among its populations in a genetic substructure. The division of haplotypes may arise if distance or geographic barriers have isolated populations over a long period of time. Therefore, genetic diversity reflects phylogeographic separation of discrete populations within the species whereby genetic distances are expected to increase with geographic distance (Avise 1989). The relationships of the isolated populations are reflected by the matrilineal structure of the species (Avise 1994). This partitioning will not occur, however, in a

species that has gene flow between populations and high dispersal rates. In these cases, it is predicted that populations will share many haplotypes and the genetic distances will not increase with physical distance between populations (Wayne et al.1992).

The variable genes of the mitochondrial genome have been used extensively in determining genetic diversity, substructure, introgression and gene flow in mammalian populations (Avis 1994). The high evolution rate of mitochondrial DNA provides polymorphic gene markers for addressing population parameters (Saccone et al. 1991). Vertebrate mitochondrial DNA exists as a circular, supercoiled molecule of approximately 15-20 kilobases. The genes that comprise this molecule include a non-coding region control region and 37 coding genes (Anderson et al. 1982). The mitochondrial genome evolution differs greatly from that of the nuclear genome due to its high rate of evolution, maternal inheritance, and lack of recombination (Brown 1979). The evolution of the mitochondrial genome is 10x faster than that of the nuclear genome. The rapid rate of sequence divergence results from two different mechanisms that include a lack of repair mechanisms for mutations (Wilson 1985) and base mis-pairing from strand slippage (Hoelzel 1993). The mutations of the mitochondrial genome are characterized by large transitional bias.

The control region is the most rapidly evolving gene of the mitochondrial genome and is situated 5' between the $tRNA^{Pro}$ on the light strand and 3'on the side of the $tRNA^{Phe}$ heavy strand (Figure 6). In most vertebrate species, the rate of control region mutation can be 3 to 5 times higher than other mitochondrial genes (Cann et al. 1984, Lopez et al. 1996). The gene length averages about 1 kb for vertebrates and is divided into two hypervariable regions, 5' (I) and 3' (II), a central conserved region and repetitive arrays (Hoelzel 1993). Hypervariable region I has been widely used in population studies to measure genetic variation and substructure (Boyce et al. 1999, Prithiviraj et al. 2000).

Figure 6. Illustration of the mammalian control region. Primers DLH1 and N777 are indicated.



uncover domestication events (Vila et al. 1999), establish genetic introgression (Wayne and Jenks 1991, Rebholz and Harley 1997) and reveal evolutionary relationships (Johnson and O'Brien 1997, Gagneux et al. 1999).

The molecular clock hypothesis applies the assumption that genetic mutations accumulate in a stochastic but steady fashion (Zuckerkandl and Pauling 1962) to estimate the time of divergence between organisms. In this manner, the amount of sequence divergence between taxa would be proportional to the amount of time that had elapsed since sharing a common ancestor. Absolute time is applied to the molecular data by using the fossil record calibrations. The molecular clock calibration for mitochondrial DNA was estimated at 2% per million years in primates (Brown et al. 1982). This clock has been applied universally over 20 years to estimate divergence times in a diverse array of taxa. Current research has revealed that mutation rates differ between genes and lineages (Palumbi 1989, Lopez et al. 1996). Moreover, the neutral theory of mitochondrial gene evolution has recently been challenged due to the discovery of high replacement site changes found in a few of the coding genes including NADH dehydrogenase genes, cytochrome oxidase genes, cytochome b, and ATPase genes (Ballard and Kreitman 1995). Evidence of rate heterogeneity and non-neutrality prohibits the use of a universal clock for all taxa and all genes. New molecular clocks are being calibrated specifically for each gene within a lineage based on fossil dates, calculated mutation rates and genetic distance estimates (Hassanin and Douzery 1999, Kringes et al. 1999, Matthee and Robinson 1999).

In this study, I have attempted to evaluate the degree of genetic variation found in the control region for *D. pygargus* in order to infer past demographic events. The haplotypic diversity of this antelope was compared to that of other African antelope species to assess relative haplotypic diversity values. Control region differentiation was

examined with the intention to identify genetic partitioning between subspecies and describe evolutionary units. Finally, the sequence diversity was used to determine an approximate time of divergence since bontebok and blesbok shared a common ancestor.

## MATERIALS AND METHODS

### *Sample Collection*

National Parks Board veterinarians and qualified technicians drew all blood samples on sedated animals. Tissue samples (ear clippings, heart and liver) were preserved in a DMSO/NaCl buffer at room temperature (Amos and Hoelzel 1991). Total genomic DNA was extracted from bontebok and blesbok samples by standard methods (Sambrook et al. 1989). Figure 7 lists the name of each population, the number of individuals sampled and geographic locations

### *PCR conditions*

The 5' hypervariable region I of the control region was amplified for the analysis by using primers situated in the $tRNA^{pro}$ and central conserved block (Hoelzel et al. 1991) (see Figure 6). The ~600 bp fragment was amplified by using primers DL-H1 (5'- ATC CTC TCT CTG CAG CAC ATT TCC- 3') and N777 (5' -TAC ACT GGT CTT GTA AAC C- 3'). PCR reactions were performed using a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer) using the following protocol: 94° C for 3 min denaturation, 30 cycles of 94° C for 1 min, 50° C for 1 min, and 72° C for 1 min, then finishing with a 72° C extension step. Each PCR reaction consisted of 100 ng of template DNA, 50 pmol of each primer, 2.0 mM dNTPs, 10x reaction buffer (50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl) and 1.5 units of *Taq* DNA polymerase (Promega). A total of 125 samples were

**Figure 7.** Population numbers within each South African province.


Sampling locations and number of animals genotyped from each population are as follows: A = West Coast NP (12), B = Overberg Farm (8), C = Bontebok NP (15), D = Heidleberg (2), E = Elandsberg (5), F = Cradock (3), G = De Brug (4), H = Golden Gate (3), I = Bloemhof Farm (3), J = Parys (25). K = Suikerbosrand Reserve (3), L = Sterkfontein Dam (5), M = TDR Farm(6), N = Fairview Farm (5), O = Maria Moroka Reserve (10), P = Rosedale Farm (5), and Q = Wag 'n Bietjie (10)

screened for polymorphism in hypervariable region I of the control region by SSCP analysis. After screening, a subset of the samples was randomly chosen from each population to assess sequence diversity within the control region.

*SSCP Analysis*

PCR products (10 µl) were mixed with a 50 µl volume of a low ionic strength buffer (LIS) and subjected to heat denaturation at 97 C° for 2 minutes. The single strands were formed within the LIS sugar matrix (10% saccharose, 0.01% bromophenol blue, 0.01% xylene cyanol FF) and remained stable at room temperature (Maruya et al. 1996). Single-stranded products were then subjected to electrophoresis (24 mA) through a 10% non-denaturing polyacrylamide gel (1.4% cross-linking) in 1X TBE buffer (Tris Borate EDTA) at room temperature for 24 hours (Glavac and Dean 1993). After electrophoresis, the gel was incubated for 30 minutes with the fluorescent GelStar Nucleic Acid Gel Stain (BioWhittaker). Staining was carried out by pouring a solution composed of 2X GelStar, 10 ml glycerol and 10 ml 1X TBE over the gel surface. Alleles were visualized by UV illumination and images were captured with a Kodak digital camera. Unique SSCP patterns were selected for sequence analysis. Forward and reverse sequences were generated for three to five individuals from each population.

*Purification of Gene Fragments and Sequencing Procedure*

PCR products (~600 bp) were electrophoresed on a 1% agarose gel, isolated, and then purified using the Gene Clean protocol. Purification of gel fragments was carried out by first melting the agarose in 300 µl of 6M NaI solution at 55° C for 10 min. After the agarose and DNA had gone into solution, 10 µl of ionized beads were added to the epindorf tube containing the DNA fraction. The tube was incubated on ice for 10 minutes

with vortexing every 2 minutes. This incubation step allowed for the trapping of DNA to the beads. The complex was subsequently washed three times with a wash solution to rid all excess agarose and NaI. The final step required elution of the bound DNA from the beads. This elution was carried out by adding 15 µl of ddH2O, incubating at 55° C for 5 min, centrifugation at 12,000g for 4 minutes, and transferring of purified DNA to a new storage epindorf tube. Each DNA preparation was subjected to a cycle sequencing reaction using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI-Perkin-Elmer). The labelled PCR fragments were analysed by an ABI 377 automated sequencer.

***Data analysis***

Sequences were edited by Sequence Navigator (ABI:Perkin-Elmer) and aligned by eye. Genetic distances were estimated for pair-wise comparisons using Kimura's 2-parameter model (1980). The distance matrix generated was used to create a neighbor-joining tree (Saitou and Nei 1987). The computer package MEGA was used for phylogenetic and bootstrap analyses (Kumar et al. 1993). Bootstrap resampling (500 iterations) provided statistical support for each node within the distance tree. Arlequin 2.0 software (Schneider et al. 1999) was used to estimate haplotypic and nucleotide diversity as well as population expansion. Estimations of haplotypic diversity (δ) within *D. pygargus* was determined by dividing the number of haplotypes by the sample size (Nei and Tajima 1981). Nucleotide diversity (Nei 1987;10.5) was used to estimate control region variation within each subspecies. The historical demography of blesbok was estimated from the number of differences between pairs of sequences in a mismatch distribution analysis (Rogers and Harpending 1992).

## RESULTS

A total of 125 control region samples, including animals of each subspecies and a test (suspected hybrid) group, were screened by SSCP. Initial screening by SSCP analysis (Table 2) revealed only one pattern for each subspecies (Figure 8). Although SSCP analysis is sensitive enough to resolve 1 bp difference between sequences (Orita 1991), the large size of the control region PCR fragment (~600 bases) prevented identification of slight sequence differences. On closer examination using sequence analysis, five control region haplotypes were found within blesbok samples and only a single bontebok sequence was revealed within bontebok (Table 3). Samples (3 – 5) were sequenced from each population within each subspecies and test group. The distribution of bontebok and blesbok haplotypes is shown in Figure 9. All substitutions found within the 520bp control region fragment were transition changes (Table 4). The bontebok sequence was not found within the blesbok populations examined and differed by an average sequence divergence of 0.044 (Table 5). The sequence divergence among blesbok haplotypes was low ranging from 0.0039 – 0.0117. The evolutionary relationships of the haplotypes are depicted in a neighbor-joining tree in Figure 10. The total nucleotide diversity was $\pi = 0.0226$ (0.0122) while the gene diversity was estimated at 0.738 (0.0446). Haplotypic diversity ($\delta$) was estimated by dividing the number of control region haplotypes by the sample size. This index was compared to that of other antelope species and found to be significantly lower (Figure 11). A mismatch distribution analysis estimated the historical demography of blesbok by the number of differences between pairs of sequences (see Figure 12). The unimodal pattern represents the expansion of the blesbok populations rather than a stable population (Rogers 1995).

The test group of antelope was screened for polymorphism in order to detect possible hybridization events (see Table 2.c.). In the populations tested, most animals

| SAMPLE | POPULATION | PROVINCE | SUBSPECIES | SSCP |
|---|---|---|---|---|
| BN1 | BBNP | WC | Bontebok | A |
| BN2 | BBNP | WC | Bontebok | A |
| BN3 | BBNP | WC | Bontebok | A |
| BN4 | BBNP | WC | Bontebok | A |
| BN5 | BBNP | WC | Bontebok | A |
| BN6 | BBNP | WC | Bontebok | A |
| BB1 | BBNP | WC | Bontebok | A |
| BB2 | BBNP | WC | Bontebok | A |
| BB3 | BBNP | WC | Bontebok | A |
| BB4 | BBNP | WC | Bontebok | A |
| BB5 | BBNP | WC | Bontebok | A |
| BB6 | BBNP | WC | Bontebok | A |
| BB7 | BBNP | WC | Bontebok | A |
| BB8 | BBNP | WC | Bontebok | A |
| BB9 | BBNP | WC | Bontebok | A |
| BB10 | BBNP | WC | Bontebok | A |
| DH1 | De Hoop | WC | Bontebok | A |
| DH2 | De Hoop | WC | Bontebok | A |
| DH3 | De Hoop | WC | Bontebok | A |
| DH4 | De Hoop | WC | Bontebok | A |
| DH6 | De Hoop | WC | Bontebok | A |
| DH7 | De Hoop | WC | Bontebok | A |
| DH8 | De Hoop | WC | Bontebok | A |
| DH9 | De Hoop | WC | Bontebok | A |
| DH10 | De Hoop | WC | Bontebok | A |
| DH11 | De Hoop | WC | Bontebok | A |
| WC1 | WCNP | WC | Bontebok | A |
| WC2 | WCNP | WC | Bontebok | A |
| WC3 | WCNP | WC | Bontebok | A |
| WC4 | WCNP | WC | Bontebok | A |
| WC5 | WCNP | WC | Bontebok | A |
| WC6 | WCNP | WC | Bontebok | A |
| WC7 | WCNP | WC | Bontebok | A |
| WC8 | WCNP | WC | Bontebok | A |
| WC9 | WCNP | WC | Bontebok | A |
| WC10 | WCNP | WC | Bontebok | A |
| WC11 | WCNP | WC | Bontebok | A |
| WC12 | WCNP | WC | Bontebok | A |
| EL3 | Elandsberg | WC | Bontebok | A |
| EL5 | Elandsberg | WC | Bontebok | A |
| EL6 | Elandsberg | WC | Bontebok | A |
| EL7 | Elandsberg | WC | Bontebok | A |
| EL8 | Elandsberg | WC | Bontebok | A |
| JO1 | Overberg | WC | Bontebok | A |
| JO2 | Overberg | WC | Bontebok | A |
| JO3 | Overberg | WC | Bontebok | A |
| JO4 | Overberg | WC | Bontebok | A |
| JO5 | Overberg | WC | Bontebok | A |
| JO6 | Overberg | WC | Bontebok | A |
| JO7 | Overberg | WC | Bontebok | A |
| JO8 | Overberg | WC | Bontebok | A |
| HB1 | Heidelberg | WC | Bontebok | A |

**Table 2.a.** Control region SSCP genotypes in bontebok

| SAMPLE | POPULATION | PROVINCE | SUBSPECIES | SSCP |
|---|---|---|---|---|
| JP1 | Parys | GP | Blesbok | B |
| JP2 | Parys | GP | Blesbok | B |
| JP3 | Parys | GP | Blesbok | B |
| JP4 | Parys | GP | Blesbok | B |
| JP5 | Parys | GP | Blesbok | B |
| JP6 | Parys | GP | Blesbok | B |
| JP7 | Parys | GP | Blesbok | B |
| JP8 | Parys | GP | Blesbok | B |
| JP9 | Parys | GP | Blesbok | B |
| JP10 | Parys | GP | Blesbok | B |
| JP11 | Parys | GP | Blesbok | B |
| JP13 | Parys | GP | Blesbok | B |
| JP14 | Parys | GP | Blesbok | B |
| JP15 | Parys | GP | Blesbok | B |
| JP16 | Parys | GP | Blesbok | B |
| JP18 | Parys | GP | Blesbok | B |
| JP19 | Parys | GP | Blesbok | B |
| JP20 | Parys | GP | Blesbok | B |
| JP21 | Parys | GP | Blesbok | B |
| JP22 | Parys | GP | Blesbok | B |
| JP23 | Parys | GP | Blesbok | B |
| JP24 | Parys | GP | Blesbok | B |
| JP25 | Parys | GP | Blesbok | B |
| JP26 | Parys | GP | Blesbok | B |
| JT1 | TDR | GP | Blesbok | B |
| JT2 | TDR | GP | Blesbok | B |
| JT3 | TDR | GP | Blesbok | B |
| JT4 | TDR | GP | Blesbok | B |
| JT5 | TDR | GP | Blesbok | B |
| JT6 | TDR | GP | Blesbok | B |
| SW1 | Swartzkop | FS | Blesbok | B |
| SW2 | Swartzkop | FS | Blesbok | B |
| SR1 | Suikerbosrand | GP | Blesbok | B |
| SR2 | Suikerbosrand | GP | Blesbok | B |
| SR3 | Suikerbosrand | GP | Blesbok | B |
| CR1 | Craddock | EC | Blesbok | B |
| CR2 | Craddock | EC | Blesbok | B |
| CR3 | Craddock | EC | Blesbok | B |
| BL1 | Bloemhof | FS | Blesbok | B |
| BL3 | Bloemhof | FS | Blesbok | B |
| BL4 | Bloemhof | FS | Blesbok | B |
| DB2 | De Brug | FS | Blesbok | B |
| DB3 | De Brug | FS | Blesbok | B |
| DB4 | De Brug | FS | Blesbok | B |
| DB5 | De Brug | FS | Blesbok | B |
| GG29 | GGNP | FS | Blesbok | B |
| GG30 | GGNP | FS | Blesbok | B |
| GG31 | GGNP | FS | Blesbok | B |

**Table 2.b.** Control region SSCP haplotypes for blesbok samples.

| SAMPLE | POPULATION | PROVINCE | TEST | SSCP |
|---|---|---|---|---|
| RD1 | Rosedale | FS | Blesbok | B |
| RD2 | Rosedale | FS | Blesbok | B |
| RD3 | Rosedale | FS | Blesbok | B |
| RD4 | Rosedale | FS | Blesbok | B |
| RD5 | Rosedale | FS | Blesbok | B |
| ST1 | Sterkfontein | FS | Blesbok | B |
| ST2 | Sterkfontein | FS | Blesbok | B |
| ST3 | Sterkfontein | FS | Blesbok | B |
| ST4 | Sterkfontein | FS | Blesbok | B |
| ST5 | Sterkfontein | FS | Blesbok | B |
| Bb1 | Fairview | FS | Bontebok | A |
| *Bb2 | Fairview | FS | Bontebok | B |
| Bb3 | Fairview | FS | Bontebok | A |
| Bb4 | Fairview | FS | Bontebok | A |
| *Bb5 | Fairview | FS | Bontebok | B |
| WB1 | Wag 'n Bietjie | FS | Bontebok | A |
| WB2 | Wag 'n Bietjie | FS | Bontebok | A |
| WB3 | Wag 'n Bietjie | FS | Bontebok | A |
| WB4 | Wag 'n Bietjie | FS | Bontebok | A |
| WB5 | Wag 'n Bietjie | FS | Bontebok | A |
| WB6 | Wag 'n Bietjie | FS | Bontebok | A |
| WB7 | Wag 'n Bietjie | FS | Bontebok | A |
| WB8 | Wag 'n Bietjie | FS | Bontebok | A |
| WB9 | Wag 'n Bietjie | FS | Bontebok | A |
| WB10 | Wag 'n Bietjie | FS | Bontebok | A |
| MM1 | Marie Moroka | NW | Blesbok | B |
| MM2 | Marie Moroka | NW | Blesbok | B |
| MM3 | Marie Moroka | NW | Blesbok | B |
| MM4 | Marie Moroka | NW | Blesbok | B |
| MM5 | Marie Moroka | NW | Blesbok | B |
| MM6 | Marie Moroka | NW | Blesbok | B |
| MM7 | Marie Moroka | NW | Blesbok | B |
| MM8 | Marie Moroka | NW | Blesbok | B |
| MM9 | Marie Moroka | NW | Blesbok | B |
| MM10 | Marie Moroka | NW | Blesbok | B |

**Table 2.c.** Control region SSCP haplotypes for test animals
* Blesbok control region haplotype in hybrid samples

**Figure 8.** SSCP patterns for control region alleles. Blesbok display pattern (B) and bontebok patterns are indicated by (A).

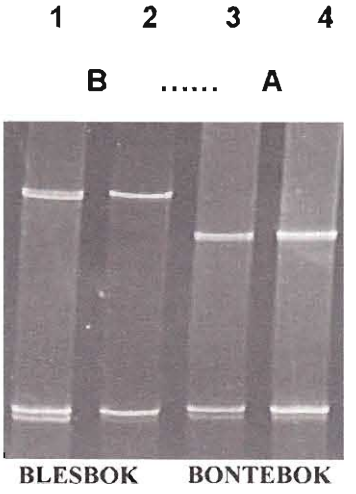


**Table. 3.** Control Region Sequences

| Bles1 | Bles 2 | Bles 3 | Bles 4 | Bles5 | Bont 1 |
|---|---|---|---|---|---|
| BL3 | BL1 | JP4 | JT3 | MM10 | BB2 |
| GG31 | JP3 | JP5 | JT6 | MM11 | BN2 |
| DB4 | JP15 | JP11 | | | BN4 |
| DB5 | BS1 | JP16 | | | BN5 |
| RD2 | | SR2 | | | DH11 |
| JT2 | | | | | EL6 |
| JT4 | | | | | EL10 |
| ST1 | | | | | HB1 |
| ST4 | | | | | HB2 |
| BS5 | | | | | JB1 |
| CR1 | | | | | JB2 |
| CR3 | | | | | JO1 |
| | | | | | JO3 |
| | | | | | JO4 |
| | | | | | JO6 |
| | | | | | KB1 |
| | | | | | WC2 |
| | | | | | WC5 |
| **12** | **4** | **5** | **2** | **2** | **18** |

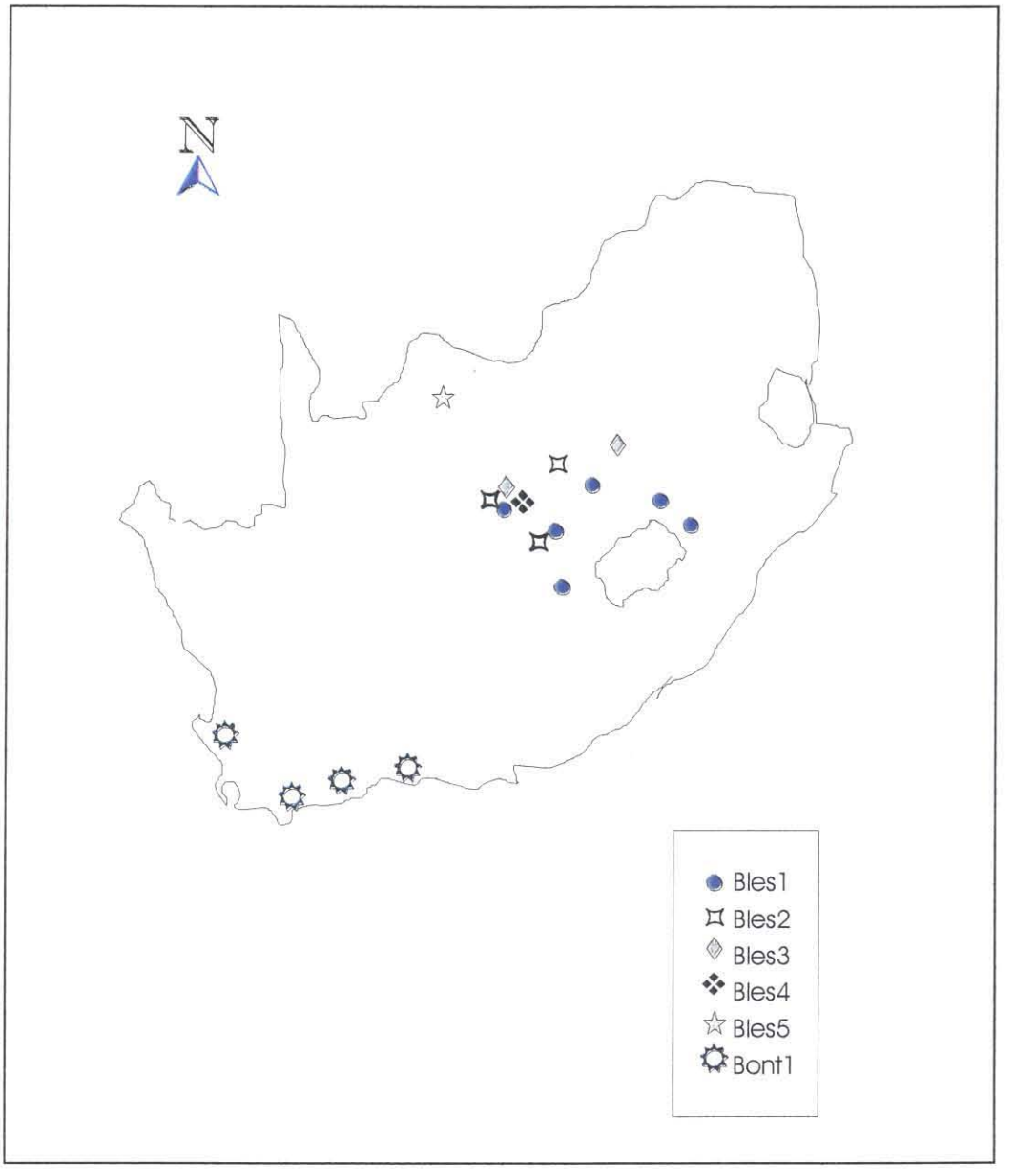



**Figure 9.** Distribution of control region haplotypes.

**Table 4.** Alignment of Control Region sequences.

| | 69 | 90 | 109 | 112 | 142 | 145 | 152 | 154 | 155 | 170 | 178 | 193 | 204 | 244 | 272 | 277 | 298 | 461 | 470 | 480 | 489 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bles1 | C | G | A | A | A | G | C | A | A | G | G | T | T | G | G | T | C | A | A | A | C |
| Bles2 | * | * | * | * | * | * | * | * | G | * | * | * | * | * | * | * | * | * | * | * | * |
| Bles3 | * | * | * | * | * | * | * | * | * | * | * | * | C | * | * | * | * | * | * | * | * |
| Bles4 | * | * | * | * | * | * | * | * | * | * | * | C | * | * | * | * | * | * | * | G | T |
| Bles5 | * | * | * | * | * | A | * | * | * | * | * | C | * | * | * | * | * | * | * | * | * |
| Bont1 | T | A | G | G | G | * | T | G | * | A | A | * | * | A | A | C | T | G | G | * | * |

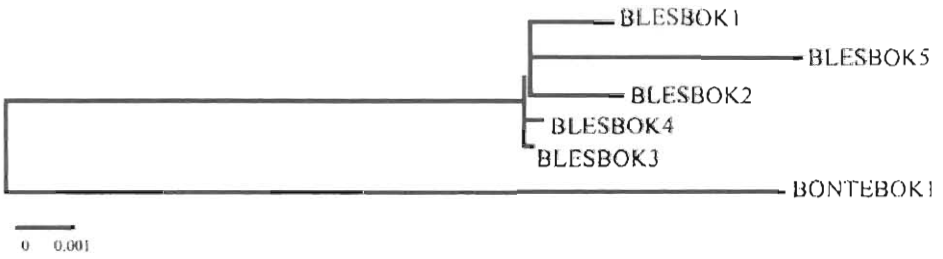
Nucleotide positions of the sequenced fragment were numbered 1 – 520. All mutations are indicated by the nucleotide number in the first row. Sequence similarity is represented by "*".

**Table 5.** Genetic distance matrix of Control Region sequences in blesbok (Bles 1-4) and bontebok (Bont1).

| | **Bles1** | **Bles2** | **Bles3** | **Bles4** | **Bles5** | **Bont1** |
|---|---|---|---|---|---|---|
| **Bles1** | | 0.0039 | 0.0039 | 0.0039 | 0.0055 | 0.0129 |
| **Bles2** | 0.0039 | | 0.0055 | 0.0055 | 0.0068 | 0.0136 |
| **Bles3** | 0.0039 | 0.0078 | | 0.0055 | 0.0068 | 0.0136 |
| **Bles4** | 0.0039 | 0.0078 | 0.0078 | | 0.0039 | 0.0136 |
| **Bles5** | 0.0078 | 0.0117 | 0.0117 | 0.0039 | | 0.0143 |
| **Bont1** | 0.0400 | 0.0442 | 0.0442 | 0.0442 | 0.0484 | |

Distances were estimated using Kimura's -2 parameter method (1980) and are figured in the lower-left side of the matrix. Standard errors are given in the upper-right section.

Figure 10. Minimum evolution phylogeny of control region sequences



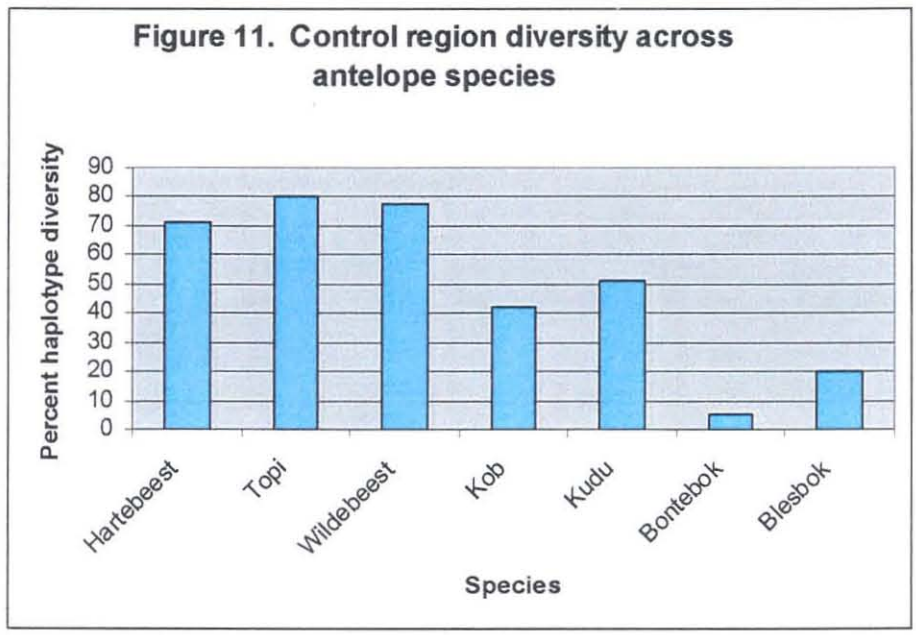


Figure 11. Control region diversity across antelope species
Percent haplotype diversity
90
80
70
60
50
40
30
20
10
0
Hartebeest
Topi
Wildebeest
Kob
Kudu
Bontebok
Blesbok
Species

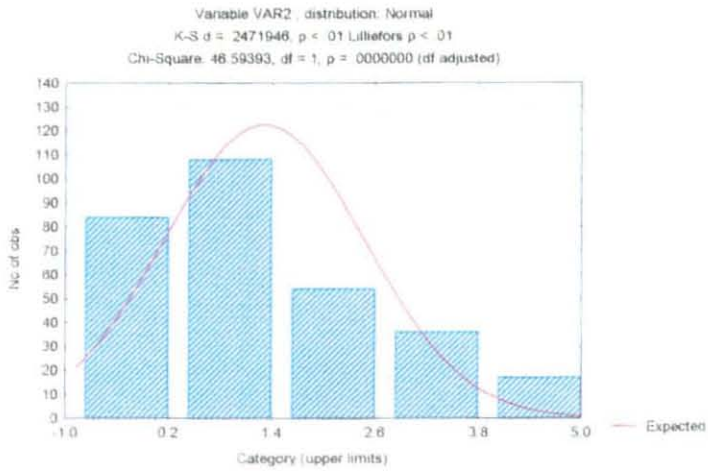



**Figure 12.** Mismatch distribution of control region sequences in blesbok. The histogram reveals an expanding population for this subspecies

displayed a haplotype that was expected for its classification. However, bontebok antelope suspected as being hybrids from the Fairview Farm population contained two B haplotypes and three A haplotypes.

## DISCUSSION

The hypervariable region I of the control region is an extremely polymorphic marker in most mammalian species (Avise 1994). Exceptions have been recorded in species that have undergone bottleneck events. These include the European otter (Cassens et al. 2000) and the northern elephant seal (Hoelzel et al. 1993). Species that exist in matrifocal social structures such as killer whales also demonstrate low genetic variation (Hoelzel et al. 1998). The extreme lack of genetic diversity at the control region locus in the bontebok was expected given their recent population collapse. Genetic variation of mtDNA is rapidly lost during population decline through genetic drift and inbreeding. Mitochondrial diversity only represents ¼ of the *Ne* since it is inherited maternally as a haploid marker while nuclear genes correspond to the paternal, diploid genetic structure of the total *Ne*. A bottleneck event will greatly reduce haplotypic diversity of mitochondrial makers and strongly alter frequencies through genetic drift (Nei 1987).

In comparison, the African buffalo has retained a substantial amount of mtDNA variation (Simonsen et al. 1998) although it had suffered a large population decline throughout the continent due to a rinderpest epidemic in 1889. The historical population size of buffalo must have been extremely large for the large number (n = 87) haplotypes to remain (Simonsen et al. 1998). Genetic theory predicts that erosion of molecular diversity will be less during a short bottleneck event and if population increase is high (Nei et al. 1975). Moreover, species with large population sizes and wide spread distributions are expected to have more variation (Frankham 1996). Given the endemic status of *D. pygargus*, it is possible that its population sizes were historically small and would therefore have less variation than other widespread antelope species.

The low level of control region variation in blesbok was surprising since their population sizes have remained relatively stable, although declined from over-hunting during the late 1800's (Bryden 1886). This paucity of genetic diversity could be explained by an ancient population crash of the species. Southern Africa experienced continual fluctuations of rainfall and temperature during the last glaciation event within the late Pleistocene (Moreau 1962). Evidence of extreme aridity was found within the Free State Province dating back to 40,000 years ago (Clark 1959). *D. pygargus* antelope rely heavily on rainfall to provide short, green shoots for grazing (David 1975, Lynch 1971). Furthermore, the abundance of grass for grazing determines the optimal condition for breeding. The scarcity of food resources could have caused the extinction of blesbok lineages throughout South Africa.

A phylogeographic study of wildebeest, hartebeest and topi by Arctander and colleagues (1999) found high genetic variation in the control region for all species and patterns of range expansion and contraction (see Figure 11). They concluded that wildebeest suffered under the Pleistocene conditions and did not evolve as successfully as the topi and hartebeest. The fluctuations of climate within glacial and inter-glacial periods resulted in "pulses" in the evolution of some species and extinction for others (Vrba 1995). The pattern of mtDNA variation of *D. pygargus* suggests that this species was unsuccessful in withstanding temperature changes and as a result, declined. Fossil evidence also suggests that *D. pygargus* failed to expand their range from south to east and never colonized farther north than Zimbabwe (Vrba 1975). The alcelaphine bovids share similar habitat needs and therefore, would be equally affected by the severe Pleistocene conditions. *D. pygargus* may have been out-competed by the other bovids for food resources within the refugias in South Africa.

Due to the removal of animals and subsequent re-introduction events, it is impossible to define geographic meta-populations of blesbok. It is likely that any unique genetic variation that had evolved in the allopatric populations has been lost through recent admixture particularly within the Free State Province. Similarly, all bontebok populations found within reserves and farms are offspring from the founding population at Bontebok National Park (BNP). Therefore, it is expected that there will not be significant differentiation between bontebok populations.

Female introgression can be detected using mitochondrial haplotypes that are unique (diagnostic) to each subspecies. However, since the mode of mtDNA inheritance is maternal, any male introgression (intra-specific mating) will go undetected. In the best-case scenario, hybridization can be detected with 100% accuracy if all individuals are tested. This scenario is nearly impossible to attain due to the limitations of sample collection and high costs of analysis. Oftentimes, only a fraction of a herd size is sampled: this sampling would only provide information on the matrilines of the subset of animals and a likelihood of the genetic purity of the remaining herd (H. Kloppers, B. Eisenberg, per.com {Dept. of Mathematics, Technicon. Pretoria}). In a sampled population, the probability of hybrid detection within a test group would be dependent on the herd size, samples size and the demographic history of the population.

The average sequence difference (2.8%) of the subspecific haplotypes demonstrates the unique evolutionary lineages of the species. The time of divergence since sharing a common ancestor is calculated at approximately 0.7 to 1.4 million years if the rate of mitochondrial evolution is assumed to be 2 – 4% per million years. This calibration relies on the assumption that mtDNA evolution is constant and mutates in a clock-like fashion (Shields and Wilson 1987). However, this calibration does not take into account the rate differences for each gene and across taxa (Hoelzel and Dover 1991).

Given the inherent faults of this calibration method, molecular clocks are now being recalibrated by regression analysis based on fossil dating. Matthee and Robinson (1999a) estimated transversional sequence divergence values for cytochrome-b in four tribes of Bovidae that included Alcelaphini. They proposed a 0.22% (SD = 0.015%) sequence divergence per million years in bovid species. This molecular clock was applied to the transversional sequence divergence values obtained from cytochrome-b sequences for both subspecies of *D. pygargus* (Hassanin and Douzery 1999, Matthee and Robinson 1999a). The sequence divergence estimate (0.26%) was applied to the calibration formula, which revealed an approximate separation time of 1.2 million years for the subspecies (data not shown). This estimate is concordant with the approximate time divergence based on the control region data thus providing more evidence for separation of bontebok and blesbok within the Pleistocene epoch.

Climatic change may have influenced the speciation event of *D. pygargus* as well as affected the expansions and contractions of specific lineages. In order to predict past episodes of population growth or decline, a distribution of control region nucleotide differences was generated between pairs of sequences (Rogers and Harpending 1992). A unimodel distribution pattern was determined indicating past population expansion (Slatkin and Hudson 1991).

The mitochondrial data reviewed here provides evidence for the recent common ancestry and genetic sub-division of *D. pygargus* into two distinct subspecies. The geographic and habitat barriers situated between the south-western cape region and the northern grasslands of South Africa have limited gene flow between these regions. The time since isolation has allowed for the intraspecific mitochondrial variation to arise within *D. pygargus*. Because this species is endemic to South Africa, it is not practical to make comparisons against other widely dispersed African ungulates. However, the

amount of sequence divergence found between bontebok and blesbok is appreciable and can be used with additional gene marker data to verify subspecies classification.

The control region findings revealed a clear pattern of evolutionary history and partitioning *of D. pygargus* at a mitochondrial (single locus) marker. These results were combined with the data from the next three chapters in order to address the question of subspecies classification and to define units for conservation (Chapter 6). In the following chapters, variation at nuclear loci was investigated to provide a finer resolution to the substructure of the species and to predict past demographic events.

# CHAPTER 3

# MICROSATELLITE DIVERSITY

# Chapter 3: Microsatellite Genetic Diversity and Apportionment

## *Introduction*

Classes of simple repetitive DNA, called microsatellites, are the most powerful molecular markers used to answer both evolutionary genetic and behavioral queries in population studies today (Goldstein and Pollock 1998). Microsatellites are highly variable sequences composed of tandem repeat motifs made up of di, tri, tetra, or penta nucleotides (Moore 1991). These sequences are found scattered throughout eukaryotic genomes as pure repeat units, compound (two or more repeat motifs), or interrupted repeat motifs. The inherent properties of microsatellite loci provide for a rich set of data in application to population studies. Firstly, they have an extremely high mutation rate in the order of ($10^{-2}$ –5 x $10^{-6}$) that results in a high number of alleles for a locus found in one population (Dallas et al. 1992, Weber and Wong 1993, Goldstein et al. 1995a). Microsatellites are co-dominant nuclear markers that occur in all chromosomal regions. Multi-locus genotypes are easily scored using PCR and genotyping analyses. Finally, primers developed from one species are usually able to amplify homologous loci in related taxa (Bruford and Wayne 1993). The conservation of the sequences flanking repeat loci allows for amplification of homologous regions among highly divergent species of ruminants (Pepin et al. 1995), felids (Menotti-Raymond and O'Brien 1995), canids (Gottelli et al. 1994) and marine turtles (FitzSimmons et al. 1995).

Genetic studies in a diverse array of species have revealed patterns of evolution of microsatellite loci. Allelic variability at a locus involves changes in the number of repeat motifs (Hamada 1982, Litt and Luty 1989, Tautz 1989, Weber and May 1989). Therefore, it was first assumed that microsatellites mutate by a stepwise mutation model (SMM), whereby an allele is formed by loss or gain of a repeat unit (Ohta and Kimura 1973).

However, additional mutations have been found within microsatellite repeat motifs, creating a departure from this model (Garza et al. 1995, Estoup al 1995). In these cases, alleles mutate by insertion-deletions, or base substitutions under the infinite alleles model (IAM) making the repeat imperfect (Kimura and Crow 1964). The two-phase model (TPM) predicts that mutations tend to be larger jumps between repeat units (Di Rienzo et al. 1994). Research has also revealed a mutational bias in loci of different repeat length. For instance, small repeat loci tend to mutate slower and towards a higher repeat number, while large repeat loci are prone to mutate faster towards a smaller repeat. Mutation may be in a single step (following the strict mutation model) or multiple steps. In many cases alleles are shortened by two repeat units and lengthened by only one. Allele size is constrained by an upper and lower boundary (Garza et al. 1995, Nauta and Weissing 1996). These boundaries restrict variation at a locus. This constraint is either set by the mutational process or by natural selection (Bowcock et al. 1994). No model yet described can explain mutations at all loci.

Microsatellite markers were first successfully used to establish relatedness, measure genetic diversity and assess population substructure (see Bruford and Wayne 1993, Goldstein and Pollock 1997 for review). Microsatellites are now being exploited further for assessing: social structure (Morin et al. 1994), male mating success (Coltman et al. 1999), neonatal fitness (Coltman et al. 1998, Coulson et al. 1998), hybridization (Reich et al. 1999), bottleneck events (Luikart et al. 1998a,b), demographic history (Goldstein et al. 1999) and evolutionary relationships (Grant et al. 1999). Furthermore, the combination of microsatellite analysis and non-invasive sampling has opened up a broader spectrum of inquiry into the behaviors of free-ranging mammals. DNA analysis of dung samples has been used for estimating population size in coyotes (Kohn et al. 1999), tracking movements of brown bear (Taberlet et al. 1997), revealing infanticide

behavior in langur monkeys (Borries et al. 1999), as well as determining dispersal patterns and paternity success in bonobos (Gerloff et al. 1999).

Microsatellite data has been used to investigate hybridization events between taxa of several mammalian species. These markers have been used to detect hybridization between species such as Ethiopian wolf and domestic dog (Gottelli et al. 1994), grey wolf and coyote (Roy et al. 1996), taurine and Zebu cattle (MacHugh 1997), and sika and red deer (Goodman et al. 1999). Microsatellites have also been used to identify introgression below the species level in populations of the great cormorant (Goostrey et al. 1998) and subspecies of tiger (Wentzel et al. 1999). Assignment of individuals into a population is based upon three different methods of classification: the multi-locus genotype, genetic distance estimate and maximum-likelihood score.

A proportion of alleles can be shared between taxa while other alleles can be unique (private) to each taxon. Private alleles or allele frequency differences can be used to detect hybridization in admixture studies (Nason and Ellstrand 1993). In this case, the multi-locus genotype of the individual is used to assign it to as specific category (ie. hybrid or pure taxa). The disadvantage to this method is that the error rate of classifying individuals after the $F_1$ generation will increase even if all loci are diagnostic (Epifanio and Phillip1997).

Phylogenetic methods have been used to group related individuals into categories according to distance estimates calculated from microsatellite data. This approach assumes that individuals from the same population or origin will have similar genotypes and cluster together. Individuals who share common alleles or display small genetic distances are joined together by a tree-joining algorithm (Bowcock et al. 1994, MacHugh et al. 1996, Blott et al. 1999).

Likelihood-based methods use genotypes to assign an individual to a population with the highest likelihood over all other populations (Shriver et al. 1997). Either a frequency method or Baysian approach is employed in the assignment test. The frequency-based method uses the computed allele frequencies in each population and likelihood estimates of each individual multilocus genotype. In effect, the likelihood of a multilocus genotype existing in a population is the product of all likelihoods for each locus (Cornuet et al. 1999). The Baysian approach is similar to the frequency method however; it incorporates a probability distribution of allele frequencies when the distribution is unknown (in the case of rare, or missing alleles).

Although the distance and likelihood methods both utilize observed allele frequencies of each population, the actual assignments are tested by different probability estimates. Simulation models have predicted that in order for an individual to be correctly classified, at least 10 polymorphic loci with heterozygosity values of at least ($H = 0.6$) and $F$st estimates of 0.1 are needed (Cornuet et al. 1999). An accurate estimation of admixture is highly dependent upon the allelic distribution of the loci as well as power of the statistical assignment test. Miller (2000) has recommended that a high number of loci analyzed under a maximum-likelihood method will greatly increase the probability of correct assignment. The major limitation to the multi-locus genotype assignment test is that it only reflects the observed alleles found in the study, and not all possible alleles in the populations. Simulation tests are able to overcome this disadvantage by generating allele frequencies of missing alleles within populations.

Microsatellite analysis allows for a sensitive prediction of demographic expansions or contractions within populations (Goldstein et al. 1999). In this effect, the variances of allele repeat score across microsatellite loci will be reduced in a population under expansion or become inflated under equilibrium. Bottleneck events can be

determined by examination of frequency distributions. The loss of low frequency alleles is indicative of populations that have experienced a large demographic collapse (Nei 1975).

This microsatellite study was carried out in order to explore nuclear diversity within *D. pygargus* and to provide a finer resolution to the examination of genome-wide variation. The allelic distributions of these makers were applied to detect genetic sub-structuring as well as individual classification. Further exploration of microsatellite data was used to infer demographic changes within each subspecies.

## MATERIALS AND METHODS

### *Bovine and Ovine Markers*

A panel of bovine primers was tested for amplification in *D. pygargus* (Table 6). A total of 8 microsatellite loci were chosen for the study based upon reliable amplification and high heterozygosity values measured in cattle (*Bos taurus*) and sheep (*Ovis ovis*). All microsatellite loci were assumed to mutate neutrally with the exception of MDRB3, which is found within intron 2 of the MHC-DRB locus. The mutation of this microsatellite locus is influenced by the evolution of the coding DRB gene in ruminant species such as cattle (Ammer et al. 1992, Ellegren et al. 1993, van Haeringen et al. 1999), bighorn sheep (Patterson 1998) and domestic sheep (Schwaiger et al. 1993).

### *PCR Conditions*

The forward primer for each locus was labeled at the 5' end with a fluorescein tag of the HEX (yellow), TET (green) or 6-FAM (blue) molecule (Perkin-Elmer). The PCR

**Table 6.** Microsatellite primers tested.

| Marker | Source Species | Amplification |
|---|---|---|
| **TGLA142** | Cattle | * |
| **TGLA53** | Cattle | * |
| **DINC** | Cattle | * |
| CSSM18 | Cattle | A, 2 alleles |
| INRA26 | Cattle | A, 2 alleles |
| BM3215 | Cattle | A, 1 alleles |
| **MDRB3** | Cattle | * |
| RBP3 | Cattle | N |
| **MAF46** | Sheep | * |
| **MAF50** | Sheep | * |
| **OarCP26** | Sheep | * |
| **OarCP64** | Sheep | * |
| OarFCB304 | Sheep | N |
| AGLA269 | Cattle | N |
| AGLA218 | Cattle | N |
| TGLA48 | Cattle | A, 2 alleles |
| TGLA263 | Cattle | N |
| TGLA57 | Cattle | A, 2 alleles |
| TGLA73 | Cattle | A, 2 alleles |
| TGLA227 | Cattle | N |
| TGLA126 | Cattle | A, 1 alleles |
| TGLA122 | Cattle | N |
| MGTG4 | Cattle | A, wrong size |
| MGTG7 | Cattle | N |

* = chosen for study, A = amplification of only 1 or 2 alleles

N = no amplification

reaction conditions are as follows: PCR amplification of individual microsatellite loci was performed in 15 µl reactions on a PEC 2400 thermocycler. Each reaction consisted of 14 pmol of each primer, 10X PCR buffer (10 mM Tris-hydrochloric acid [pH 8.3], 50 mM potassium chloride), 250 µM of each deoxyribonucleoside 5'triphosphates (dATP, dCTP, dGTP, dTTP) (Promega), 0.4 Unit Amplitaq DNA polymerase (Promega), and 100 ng of DNA template. The forward primer of each primer pair was labeled with a fluorescent dye phosphoramite. The PCR conditions are as follows: initial denaturation step at 94° C for 3 min; ten cycles of 94° C for 15 sec, 48 - 52° C for 15 sec, 72° C for 30 sec; followed by 20 cycles of 89° C for 15 sec, 48 - 52° C for 15 sec, 72° C for 30 sec, and terminating in a 10 min extension step at 72° C.

***Genescan Electrophoresis and Analysis***

Multiplexing of PCR products is made possible by staggering microsatellite loci having different allele size ranges and also by labeling products with the selection of tag colors. For each gel, microsatellite products generated from 4-5 different loci were electrophoresed for each individual. The gel lanes can accommodate either 36 or 64 samples; therefore several gels were run for each panel of microsatellites. In order to establish consistency across multiple plates, PCR products from 2-4 animals from each gel were run as controls across secondary and tertiary gels.

PCR products for each animal were pooled and diluted in a volume of approximately 50 µl $ddH_2O$. Due to the varying strength in fluorescent signal of each dye, different amounts of product were added to the $ddH_20$ dilution. Only 1-2 µl of product labeled with 6-FAM was added to the dilution, while 3-4 µl of the TET labeled product and 7-8 µl of the HEX products. After the PCR products were pooled, 1 µl of the

dilution was mixed with 1.5 µl of loading buffer cocktail that consists of: 2.0 µl de-ionized formamide, 1.0 µl ABI PRISM™ Genescan-350 TAMRA internal lane standard and 0.5 µl of ABI Genescan loading dye. The TAMRA lane standard is made up of a ladder of DNA fragments (350, 300, 250, 200, 160, 150, 139, 100, 75, 50), which is used in estimating PCR fragment sizes. The mixture is then denatured at 94° C for 3 min then snap-cooled on ice. The denatured products (2.0 µl) were loaded on a 6% denaturing polyacrylamide-sequencing gel housed in an automated ABI 377 Sequencer. Gel electrophoresis was run through a 1X TBE buffer solution at 2000 volts, 400 mA, and 25 W for 2.5 hours. The raw data for each sample was stored as a collection file that was created by the Gene Scan Collection software (version 1.2.2-1). The collection file was subsequently analyzed using the Genescan software package (ABI). Allele sizes were estimated using the Genotyper software package (version 1.1). The fragment lengths were called according to the Local Southern method (Elder and Southern 1987) to generate a best- fit curve from the internal lane size standards.

***Descriptive Statistical Analyses***

Genetic diversity found in the species and each subspecies was estimated as the mean number of alleles per locus (A) and heterozygosity levels both observed ($H_O$) and expected ($H_E$). The observed level of heterozygosity was compared to the expected level then analyzed by the Markov chain method (Guo and Thompson 1992) for departure from Hardy-Weinberg equilibrium using the Arlequin (v. 2.000) software package (Schneider et al. 2000).

The partitioning of genetic diversity into the two subspecies was measured by two F statistic measures. The *Fst* (Weir and Cockerham 1984) statistic can detect substructure in a population using loci that mutate under the infinite allele model. Since most

microsatellite loci are assumed to mutate under the step-wise model, an analogous statistic Rst, has been recommended to take the place of *Fst* in the measurement of substructuring (Slatkin 1995). Estimations of Rst values were carried out using the RSTCALC program (Goodman 1997).

***Clustering Analysis***

Genetic distances were calculated from allele frequency data using the MICROSAT (version 1.5) created by Minch et al. (1995). Two distance estimators, (Ps) proportion of alleles shared and (Dkf) kinship co-efficient (Bowcock et al. 1994) were chosen to estimate all pair-wise individual distances. These distance measures were chosen on the basis of their accuracy for estimating distances at population and individual levels (Takezaki and Nei 1996, Goldstein and Pollock 1997, Goldstein et al. 1999).

1. The proportion of shared alleles as distance is represented by:

$$(D_{ps}') - 1\text{-ps}$$

where ps is defined as:

$$ps = (\sum \mathbf{MIN}\ \{P[A(i)], P[B(i)]\})/n$$

for i = 1, 2, 3, ....n alleles where n is the total number of alleles for all loci.

2. Kinship co-efficient distance:

$$D_{kf} = 1\text{-kf}$$

$$kf = (\sum P[A(i)]P[B(i)]$$

where P[A(i)] is the relative frequency of allele i in taxon A for i= 1,2, 3, ...n alleles. The Kf value relates the probability that two genes taken at random from the same locus in two individuals are identical by descent.

The computed distance matrices were used in a cluster analysis employing two tree-building methods. The first method of phylogenetic reconstruction, neighbor-joining (NJ) is a method of joining the two most similar taxa (Saitou and Nei 1987). This method is best used when evolutionary rates vary among taxa. The second method unweighted pair group method with arithmetic mean (UPGMA) is normally applied when rates of evolution are assumed to be constant (Sneath and Sokal 1973, Nei 1987).

Bootstrap re-sampling was used in order to gain a confidence estimate of the tree topologies generated by all distance measures and tree building methods. The bootstrap test involves re-sampling of the data set by drawing points of data with replacement. A confidence level is given as a percentage out of 500 iterations.

***Class Assignment of Individuals***

Multi-locus genotype data for each individual was used to create a self-classification data set within the GeneClass program (Cornuet et al. 1998). Self-classification was carried by using two methods: likelihood estimates (frequency, Baysian) and distance measures (DA) average distance (Nei et al. 1983), (DAS) allele shared (Chakaborty and Jin 1983), (Dm) minimum (Nei 1972), (Ds) standard (Nei 1978), (DM) delta $mu^2$ (Goldstein et al. 1995), chord (Cavalli-Sforza and Edwards 1967). Variations of each assignment method were computed directly from the multilocus genotypes or simulated. Simulations were performed for each method incorporating 1000 simulated individuals per population with frequencies of (0.01) for each locus. After each

individual was assigned to a class, the self-classification data set was used as reference set for assigning the test individuals to an appropriate class.

***Loss of genetic diversity***

The BOTTLENECK program (Luikart and Cornuet 1998) was used to detect recent reductions in the effective population sizes (*Ne*) of both bontebok and blesbok. This method uses allele frequency estimates in order to detect loss of alleles through genetic drift or founder effect. The principle of BOTTLENECK operates under the assumption that if a population has had a constant *Ne* (under mutation-drift equilibrium) then there will be an equal probability of finding an excess or deficit in heterozygosity at a locus. In the case of reduction of *Ne*, the observed heterozygosity ($H_{OBS}$) will exceed that of the expected heterozygosity ($H_{EX}$). The heterozygosity values are used in three statistical tests (sign test, standardized differences test and a Wilcoxon sign-rank test) in order to detect heterozygosity excess (Cornuet and Luikart 1996, Luikart et al. 1997a). A reduction in the effective population size will also cause a faster loss of alleles at polymorphic loci. Therefore, the program calculates the distribution of allelic frequencies in order to detect a mode shift (indicating a bottleneck) from the expected L-shaped distribution (stable population) of the allelic range (Luikart et al. 1998b).

## Results

A panel of cattle and sheep derived primers were tested for amplification in blesbok and bontebok antelope (see Table 6). A subset of these markers failed to amplify in the antelope, which may be due to mutations in the flanking regions of the microsatellite loci. The remaining set of primers was rejected from the study due to lack

individual was assigned to a class, the self-classification data set was used as reference set for assigning the test individuals to an appropriate class.

***Loss of genetic diversity***

The BOTTLENECK program (Luikart and Cornuet 1998) was used to detect recent reductions in the effective population sizes (*Ne*) of both bontebok and blesbok. This method uses allele frequency estimates in order to detect loss of alleles through genetic drift or founder effect. The principle of BOTTLENECK operates under the assumption that if a population has had a constant *Ne* (under mutation-drift equilibrium) then there will be an equal probability of finding an excess or deficit in heterozygosity at a locus. In the case of reduction of *Ne*, the observed heterozygosity ($H_{OBS}$) will exceed that of the expected heterozygosity ($H_{EX}$). The heterozygosity values are used in three statistical tests (sign test, standardized differences test and a Wilcoxon sign-rank test) in order to detect heterozygosity excess (Cornuet and Luikart 1996, Luikart et al. 1997a). A reduction in the effective population size will also cause a faster loss of alleles at polymorphic loci. Therefore, the program calculates the distribution of allelic frequencies in order to detect a mode shift (indicating a bottleneck) from the expected L-shaped distribution (stable population) of the allelic range (Luikart et al. 1998b).

## Results

A panel of cattle and sheep derived primers were tested for amplification in blesbok and bontebok antelope (see Table 6). A subset of these markers failed to amplify in the antelope, which may be due to mutations in the flanking regions of the microsatellite loci. The remaining set of primers was rejected from the study due to lack

of polymorphism in *D. pygargus*. Therefore, out of the 25 bovine and ovine markers tested, eight loci were chosen for this population study. The loci ranged in number of alleles from 3 – 8 alleles per locus. Each locus was typed for 34 bontebok, 42 blesbok, and 9 unknown (possible hybrid) animals. The multi-locus composite genotypes are presented in Table 7. Allele frequency distributions were computed for each locus found within each subspecies (Figure 13). The eight loci together produced 41 allelic states, of which 66% were found to be specific to one subspecies (44% blesbok, 22% bontebok) and the other 34% shared between the two subspecies (Table 8).

**Table 7.a.** Microsatellite alleles found within blesbok

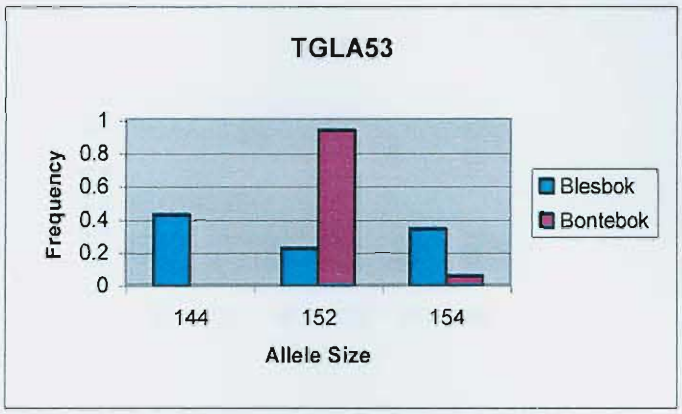
| | TGLA53 | TGLA142 | DINC | MDRB3 | OarHH64 | OarCP26 | MAF46 | MAF50 |
|---|---|---|---|---|---|---|---|---|
| BL1 | 152152 | 157161 | 106108 | 174174 | 108112 | 122122 | 103103 | 150156 |
| BL3 | 144154 | 157161 | 106106 | 000000 | 112112 | 122124 | 095095 | 150150 |
| BL4 | 144152 | 157159 | 106108 | 174174 | 108110 | 122124 | 097103 | 150150 |
| JP1 | 144154 | 157159 | 106106 | 174176 | 108108 | 122124 | 097103 | 156156 |
| JP2 | 144154 | 151153 | 106106 | 174174 | 108112 | 124124 | 097103 | 156156 |
| JP3 | 144144 | 159161 | 106106 | 174176 | 108108 | 122124 | 097103 | 156160 |
| JP4 | 154154 | 157161 | 106108 | 176176 | 108108 | 122122 | 097097 | 152160 |
| JP5 | 144154 | 161161 | 106108 | 174174 | 108112 | 122124 | 097097 | 156160 |
| JP6 | 152154 | 157159 | 108108 | 176176 | 108112 | 122124 | 097103 | 156160 |
| JP7 | 144144 | 161161 | 106106 | 000000 | 108112 | 122122 | 097103 | 156156 |
| JP8 | 144144 | 157161 | 106108 | 174174 | 108108 | 122124 | 097103 | 156160 |
| JP9 | 144154 | 157161 | 106106 | 176176 | 108112 | 122124 | 097103 | 152156 |
| JP10 | 154154 | 159161 | 106108 | 175176 | 116116 | 130130 | 107107 | 156156 |
| JP11 | 144154 | 157159 | 106108 | 174174 | 108108 | 122124 | 103103 | 156156 |
| JP13 | 144154 | 157159 | 106108 | 174174 | 108108 | 122124 | 103103 | 156156 |
| JP14 | 144144 | 159161 | 106106 | 174174 | 108112 | 122124 | 097103 | 156156 |
| JP15 | 144154 | 157161 | 106108 | 174174 | 108108 | 122122 | 097103 | 156160 |
| JP16 | 154154 | 157159 | 106108 | 176176 | 108108 | 122122 | 097103 | 156156 |
| JP17 | 154154 | 157161 | 106108 | 174176 | 108108 | 122122 | 097103 | 152156 |
| JP18 | 154154 | 157159 | 106108 | 174176 | 108112 | 122122 | 097103 | 156156 |
| JP19 | 154154 | 159161 | 108108 | 174174 | 108112 | 122124 | 097097 | 156160 |
| JP20 | 144154 | 157159 | 106106 | 176176 | 108108 | 122122 | 097097 | 152160 |
| JP21 | 144144 | 159159 | 106106 | 174174 | 108112 | 122122 | 097097 | 152156 |
| JT2 | 152154 | 159161 | 106106 | 000000 | 112112 | 122124 | 103103 | 150158 |
| JT3 | 152154 | 157161 | 106106 | 000000 | 112112 | 122124 | 095103 | 152154 |
| JT4 | 144154 | 157161 | 106106 | 174174 | 112112 | 122122 | 095095 | 156160 |
| JT5 | 144152 | 159161 | 108108 | 174174 | 112112 | 122122 | 095097 | 150150 |
| JT6 | 153154 | 157159 | 106106 | 182182 | 108112 | 124124 | 095097 | 150158 |
| DB2 | 144144 | 157157 | 106108 | 175175 | 108112 | 122124 | 095097 | 158160 |
| DB3 | 144144 | 157159 | 106106 | 175175 | 112112 | 122124 | 095097 | 158160 |
| DB4 | 144144 | 157157 | 106108 | 179179 | 108110 | 122124 | 095097 | 158160 |
| DB5 | 144144 | 157159 | 106106 | 175175 | 112112 | 122122 | 097103 | 158160 |
| SR1 | 152152 | 157157 | 106106 | 175175 | 112112 | 122122 | 095103 | 156160 |
| SR2 | 144144 | 157157 | 108108 | 000000 | 108112 | 122122 | 095097 | 158158 |
| SW1 | 144152 | 161161 | 106108 | 170175 | 112112 | 122122 | 095097 | 152158 |
| SW2 | 152154 | 161161 | 106108 | 000000 | 108112 | 122122 | 093093 | 156156 |
| GG26 | 144154 | 161161 | 106108 | 175175 | 112112 | 122124 | 097097 | 152160 |
| GG30 | 144152 | 159159 | 106108 | 175175 | 108112 | 122122 | 095097 | 152156 |
| GG31 | 144154 | 153153 | 108108 | 186186 | 112112 | 122124 | 095103 | 158160 |
| CR1 | 152152 | 159159 | 106106 | 174174 | 112112 | 126128 | 095097 | 152158 |
| CR2 | 152152 | 159159 | 106106 | 174174 | 112112 | 126128 | 097103 | 150158 |
| CR3 | 152152 | 159159 | 106106 | 174174 | 112112 | 124126 | 095103 | 150158 |

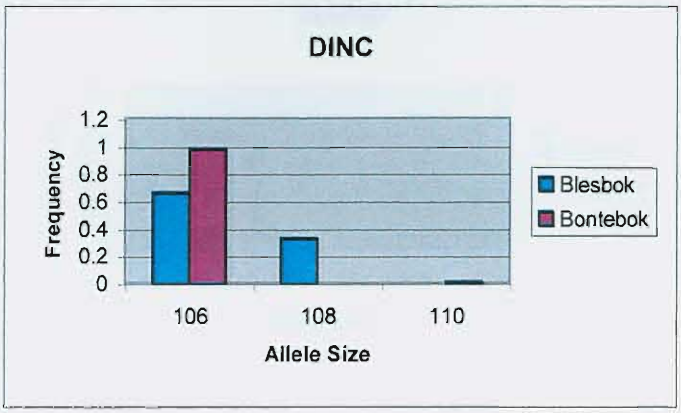
**Table. 7.b.** Microsatellite alleles found within bontebok

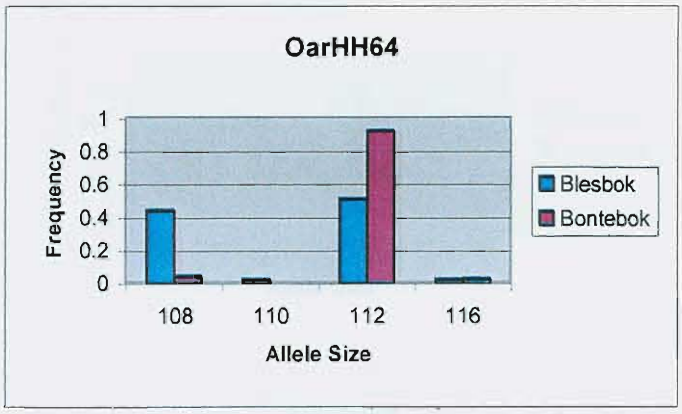
| | TGLA53 | TGLA142 | DINC | MDRB3 | OarHH64 | OarCP26 | MAF46 | MAF50 |
|---|---|---|---|---|---|---|---|---|
| BB1 | 152152 | 159159 | 106106 | 000000 | 108112 | 128128 | 095095 | 158158 |
| BB2 | 152152 | 159159 | 106106 | 186186 | 112112 | 126128 | 095095 | 158158 |
| BB3 | 152152 | 159159 | 106106 | 186186 | 108112 | 128128 | 095095 | 158158 |
| BB4 | 152152 | 159159 | 106106 | 186186 | 112112 | 126128 | 095095 | 158158 |
| BB5 | 152152 | 159159 | 106106 | 175186 | 112112 | 128128 | 095095 | 158158 |
| BB6 | 152152 | 159159 | 106106 | 175175 | 112112 | 126128 | 095095 | 158158 |
| BB7 | 152154 | 159159 | 106106 | 175186 | 112112 | 126128 | 095095 | 158158 |
| BB8 | 152154 | 159159 | 106106 | 175175 | 112112 | 128128 | 095095 | 158158 |
| BB9 | 152152 | 159159 | 106106 | 186186 | 116116 | 128128 | 095095 | 158158 |
| BB10 | 152152 | 159159 | 106106 | 000000 | 112112 | 126128 | 095103 | 158158 |
| EL3 | 152152 | 159159 | 106106 | 186186 | 112112 | 128128 | 095095 | 158158 |
| EL6 | 152152 | 159159 | 106106 | 169186 | 112112 | 126128 | 095095 | 158158 |
| EL8 | 152152 | 159159 | 106106 | 175175 | 108112 | 126128 | 095095 | 158158 |
| EL9 | 152152 | 161161 | 106106 | 000000 | 112112 | 126128 | 095095 | 158158 |
| EL10 | 152152 | 161161 | 106106 | 186186 | 112112 | 126128 | 095095 | 158158 |
| HB2 | 152152 | 159159 | 106110 | 000000 | 112112 | 126128 | 095095 | 158158 |
| JO1 | 152152 | 159159 | 106106 | 000000 | 112112 | 126128 | 095095 | 158158 |
| JO2 | 152154 | 159159 | 106106 | 186186 | 112112 | 128128 | 095095 | 158158 |
| JO3 | 152152 | 159159 | 106106 | 169169 | 112112 | 126128 | 095095 | 158158 |
| JO4 | 152152 | 159159 | 106106 | 186186 | 112112 | 128128 | 095095 | 158158 |
| JO5 | 152152 | 159159 | 106106 | 175169 | 112112 | 128128 | 095095 | 158158 |
| JO6 | 152152 | 159159 | 106106 | 175169 | 112112 | 128128 | 095095 | 158158 |
| JO7 | 152152 | 159159 | 106106 | 186186 | 112112 | 126128 | 095095 | 158158 |
| JO8 | 152152 | 159159 | 106106 | 169169 | 112112 | 126128 | 095095 | 158158 |
| WC1 | 152152 | 159159 | 106106 | 169186 | 112112 | 126128 | 095095 | 158158 |
| WC2 | 152152 | 159159 | 106106 | 186186 | 112112 | 128128 | 101101 | 158158 |
| WC3 | 152152 | 159159 | 106106 | 169186 | 112112 | 126128 | 095095 | 158158 |
| WC4 | 152152 | 159159 | 106106 | 186186 | 112112 | 128128 | 095095 | 158158 |
| WC11 | 152152 | 159158 | 106106 | 169186 | 112112 | 126128 | 095095 | 158158 |
| WC12 | 152152 | 159158 | 106106 | 186186 | 112112 | 126128 | 095095 | 158158 |
| BN1 | 152152 | 159167 | 106106 | 169169 | 112112 | 128128 | 095095 | 158158 |
| BN3 | 152152 | 159159 | 106106 | 000000 | 112112 | 126128 | 095095 | 158158 |
| BN4 | 152152 | 159167 | 106106 | 000000 | 112112 | 126128 | 095095 | 158158 |
| BN5 | 152154 | 159159 | 106106 | 000000 | 112112 | 128128 | 095095 | 158158 |

**Table 7.c.** Microsatellite alleles found within the test group.

| | TGLA53 | TGLA142 | DINC | MDRB3 | OarHH64 | OarCP26 | MAF46 | MAF50 |
|---|---|---|---|---|---|---|---|---|
| Bb1 | 152152 | 159159 | 106106 | 175175 | 112112 | 128128 | 000000 | 158158 |
| Bb3 | 152152 | 159161 | 106106 | 186186 | 112112 | 122128 | 000000 | 158158 |
| WB2 | 152152 | 159159 | 106106 | 186186 | 108112 | 128128 | 095095 | 158158 |
| WB3 | 152152 | 159159 | 106106 | 172172 | 108112 | 126126 | 095095 | 158158 |
| WB4 | 152154 | 161161 | 106106 | 175186 | 108112 | 126126 | 095095 | 158158 |
| WB5 | 154154 | 159159 | 106106 | 186186 | 108112 | 126128 | 095103 | 158158 |
| WB6 | 152152 | 159159 | 106106 | 186186 | 108108 | 126128 | 095103 | 158158 |
| WB7 | 152154 | 161161 | 106106 | 186186 | 116116 | 128130 | 101101 | 158158 |
| WB8 | 152152 | 159159 | 106106 | 186186 | 114114 | 126128 | 095095 | 158158 |



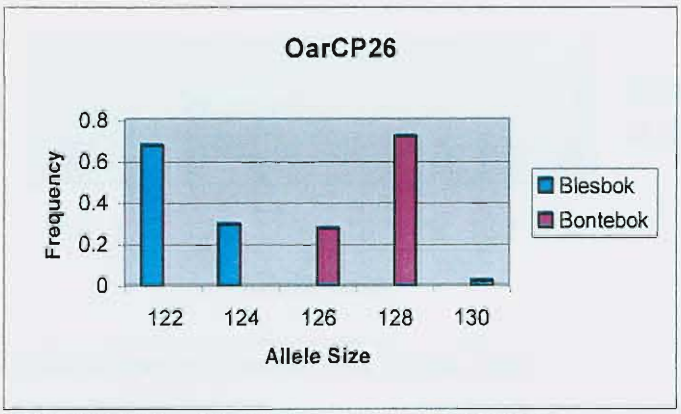
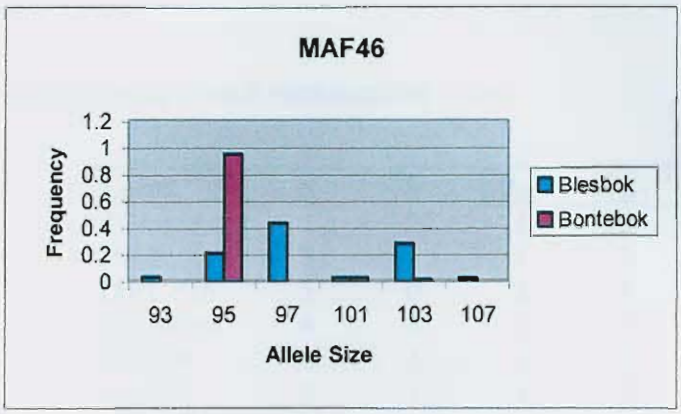
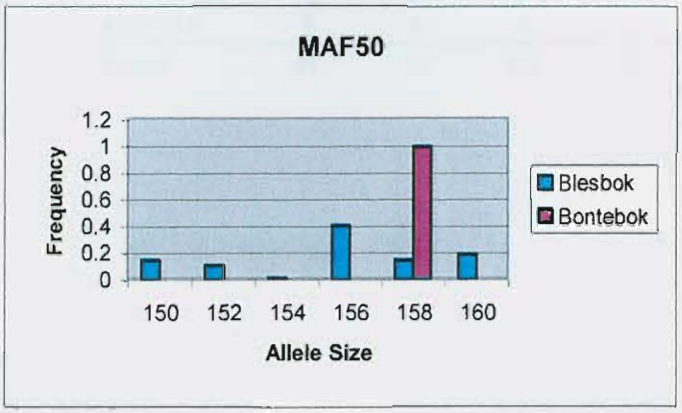
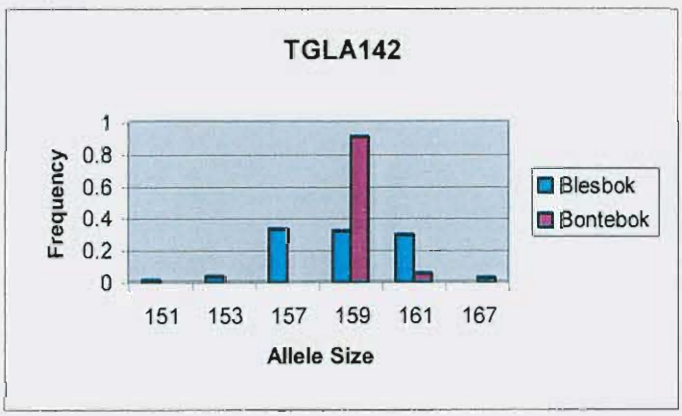
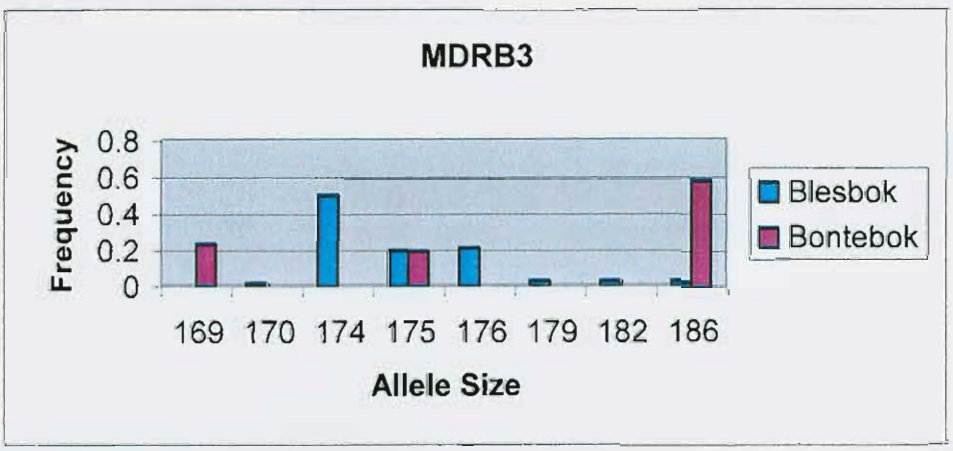




**Figure 13.a.** Allele frequency distribution




*MDRB3 is an imperfect di-nucleotide repeat locus

**Figure 13.b.** Allele frequency distribution

**Table 8.** Allelic states of each microsatellite locus.

| Locus | N | S | $P_{BL}$ | $P_{BO}$ |
|---|---|---|---|---|
| *TGLA53* | 3 | 2 | 1 | 0 |
| *DINC* | 3 | 1 | 1 | 1 |
| *OarCP26* | 5 | 0 | 2 | 3 |
| *OarHH64* | 4 | 3 | 1 | 0 |
| *MAF46* | 6 | 3 | 3 | 0 |
| *MAF50* | 6 | 1 | 4 | 1 |
| *TGLA142* | 6 | 2 | 2 | 2 |
| *MDRB3* | 8 | 2 | 4 | 2 |
| **Total** | **41** | **14** | **18** | **9** |

A comparison of genetic diversity between the two subspecies revealed a lack of polymorphism in the bontebok (Table 9a). The average observed levels of heterozygosity ($H_O$) were compared to the expected levels ($H_E$) separately for each subspecies. The observed values for the blesbok did not deviate significantly from Hardy-Weinberg equilibrium while the values for bontebok did exhibit heterozygosity deficiency across 6 out of 8 loci. Significant substructure was detected using the *Fst* and Rst calculations (Table 9b).

Genetic distances were estimated between all pairwise comparisons of individual multilocus genotypes by two measures: proportion of alleles shared (Dps) and kinship co-efficient (Dkf). Phylogenetic trees were built using both the UPGMA and NJ methods. Similar trees were reconstructed using both distance measures and phylogenetic algorithms (Figures 14-17). In all cases, the bontebok and blesbok clustered into separate groups that are supported by high bootstrap values. The bontebok cluster is characterized by shorter branch lengths and fewer bifurcations compared to that of the blesbok cluster. Microsatellite data from animals of unknown origin (Bb1, Bb3, WB2, WB3, WB4, WB5, WB6, WB7, WB8) were added to the composite multilocus genotype data from the bontebok and blesbok data set for phylogenetic analysis. All samples clustered within the bontebok group, including Bb1 and Bb3 that are derived from a hybrid herd (Figure 18).

Subspecies affiliation was executed using the variations of the GeneClass program (see Methods section). Table 10 lists each statistical method used to assign each individual to a class (subspecies). A reference data set was first computed by all statistical methods directly then under simulation. These results were then used for comparison against the test (unknown origin) sample set. All test samples were assigned to the bontebok class under all statistical methods.

**Table 9. a**. Heterozygosity values for 8 polymorphic loci.

| Blesbok | # of Alleles | Het (e) | Het (o) | Bontebok | # of Alleles | Het (e) | Het (o) |
|---|---|---|---|---|---|---|---|
| TGLA53 | 3 | 0.654 | 0.500 | TGLA53 | 2 | 0.112 | 0.118 |
| DINC | 2 | 0.450 | 0.428 | DINC | 2 | 0.029 | 0.029 |
| OarCP26 | 3 | 0.456 | 0.500 | OarCP26 | 2 | 0.409 | 0.559 |
| OarHH64 | 4 | 0.549 | 0.405 | OarHH64 | 3 | 0.141 | 0.088 |
| MAF46 | 5 | 0.685 | 0.667 | MAF46 | 3 | 0.086 | 0.029 |
| MAF50 | 6 | 0.756 | 0.667 | MAF50 | 1 | 0.000 | 0.000 |
| TGLA142 | 4 | 0.704 | 0.643 | TGLA142 | 4 | 0.221 | 0.118 |
| MDRB3 | 7 | 0.676 | 0.167 | MDRB3 | 3 | 0.588 | 0.308 |

**Table 9.b.** Microsatellite diversity indices.

| Locus | Rst | Fst |
|---|---|---|
| TGLA53 | 0.527 | 0.243 |
| DINC | 0.976 | 0.775 |
| OarCP26 | 0.066 | 0.891 |
| OarHH64 | 0.223 | 0.294 |
| MAF46 | 0.245 | 0.295 |
| MAF50 | 1.013 | 0.265 |
| TGLA142 | 6.024 | 0.584 |
| MDRB3 | 0.253 | 0.771 |

**Figure14**. UPGMA-Ps Phylogenetic tree

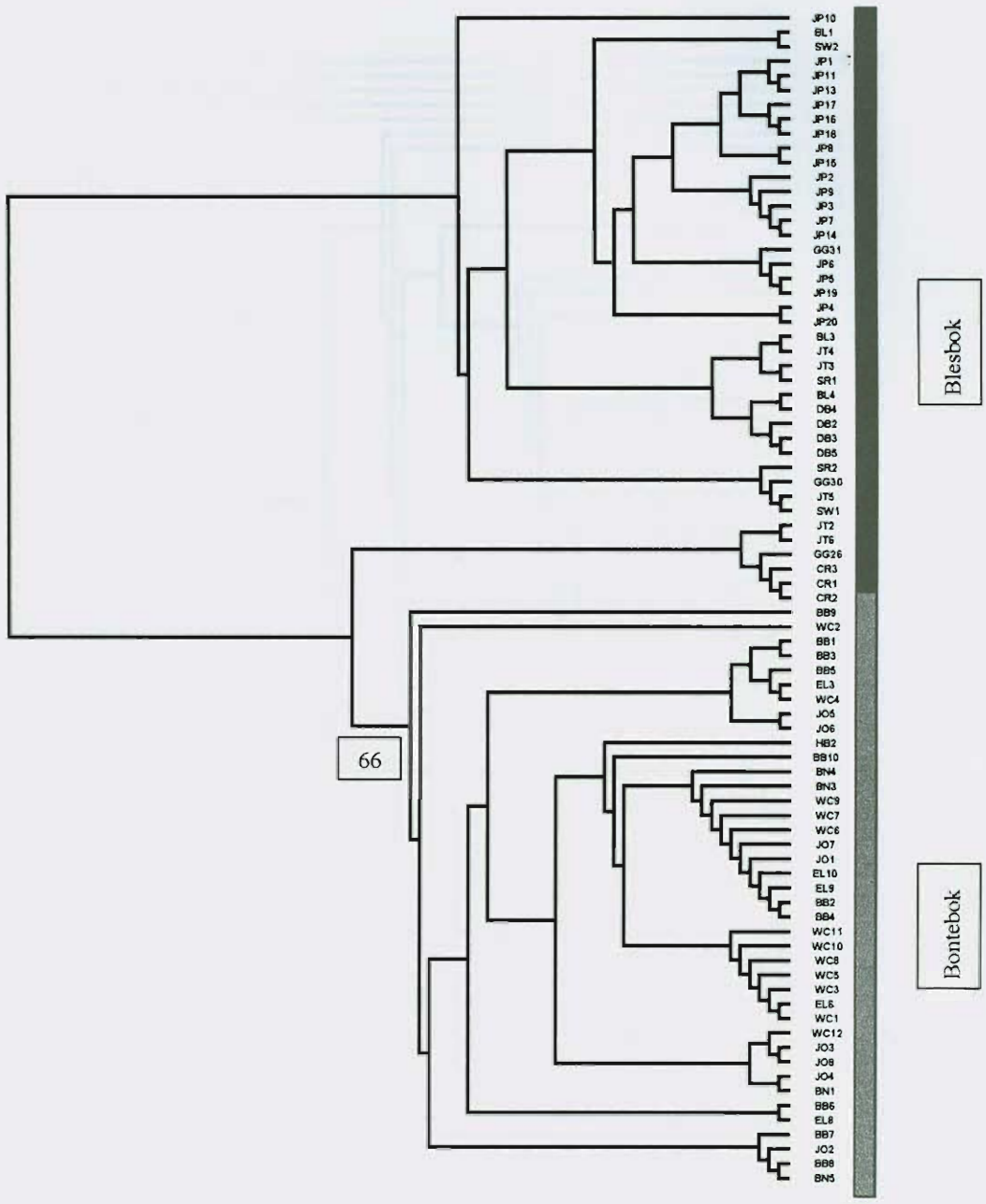


**Figure 15.** NJ-Ps Phylogenetic Tree

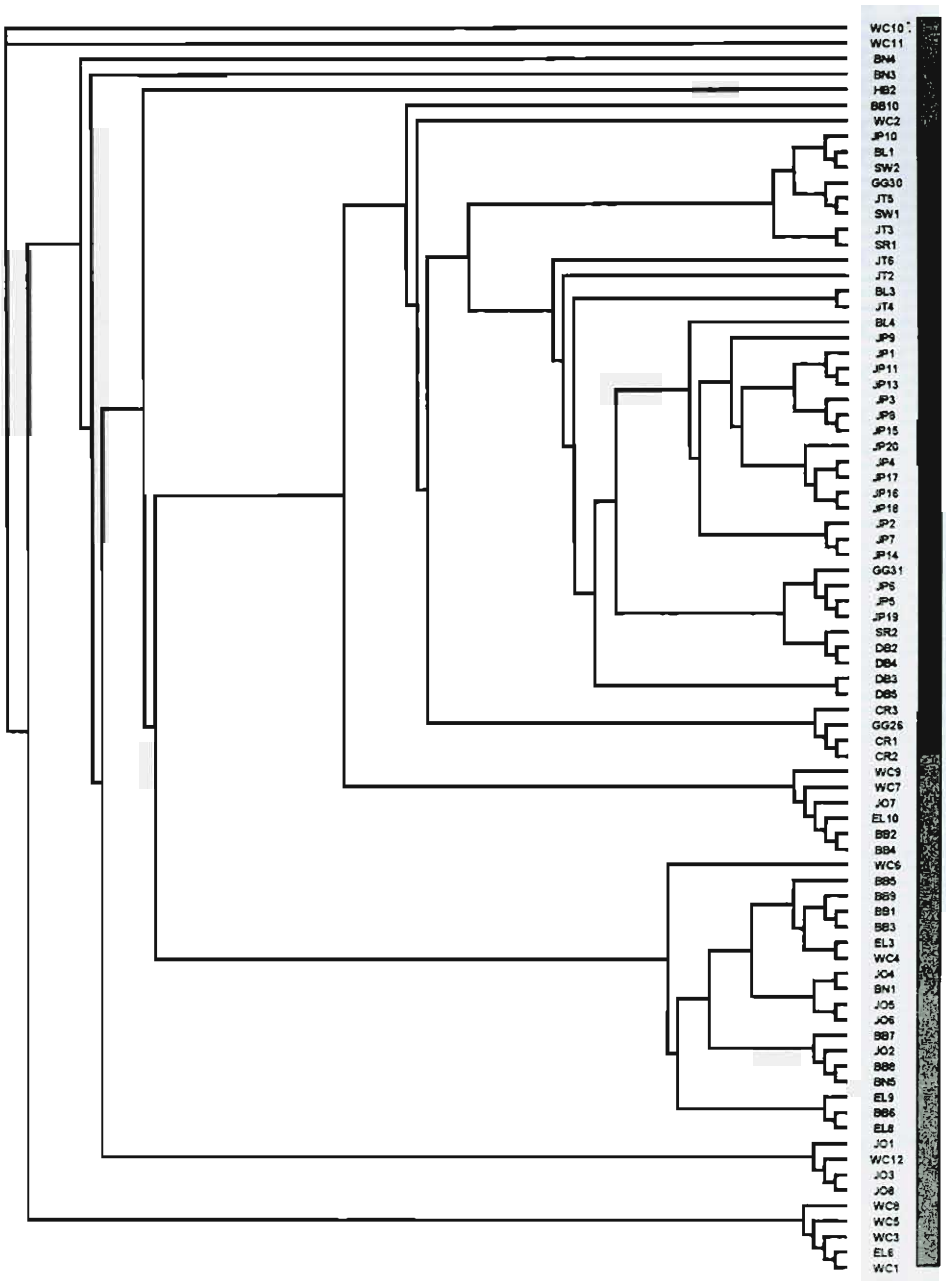


**Figure 16.** UPGMA-Kf Phylogenetic Tree

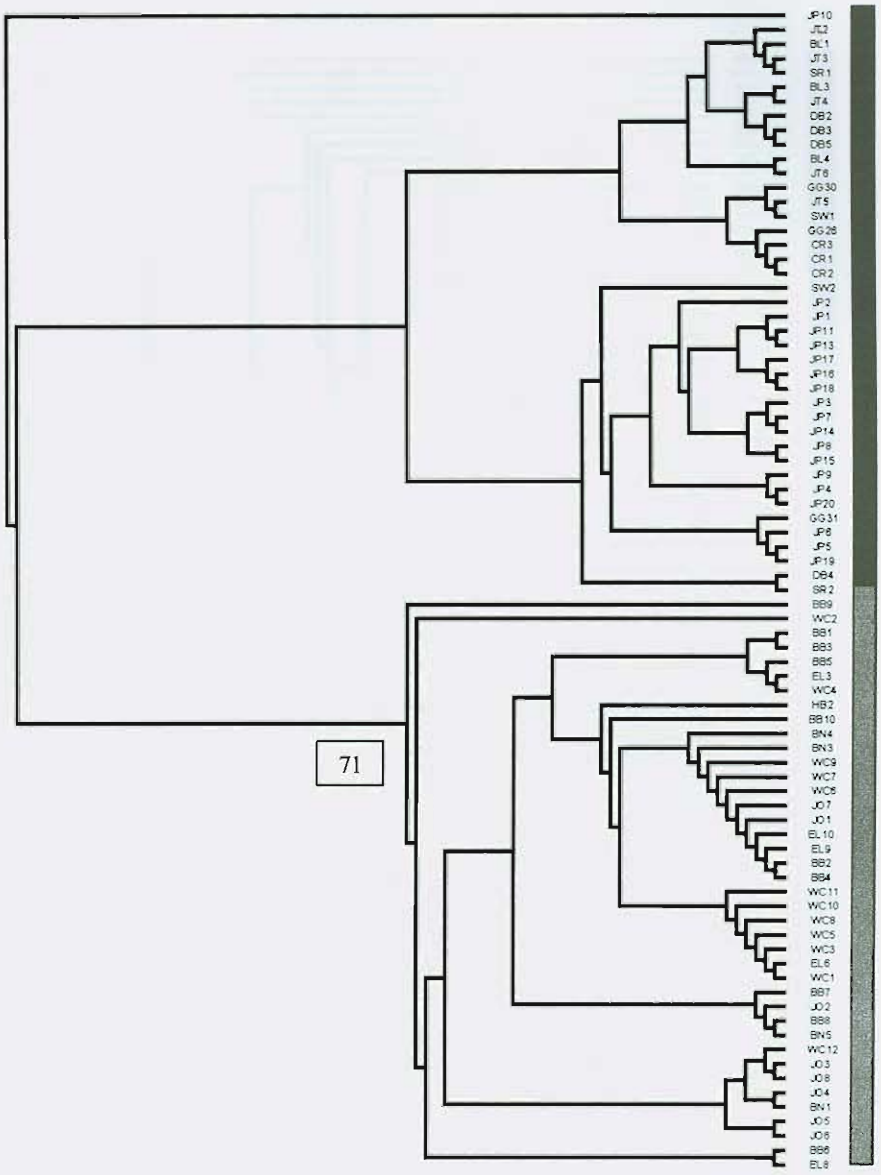


**Figure 17.** NJ-Kf Phylogenetic Tree

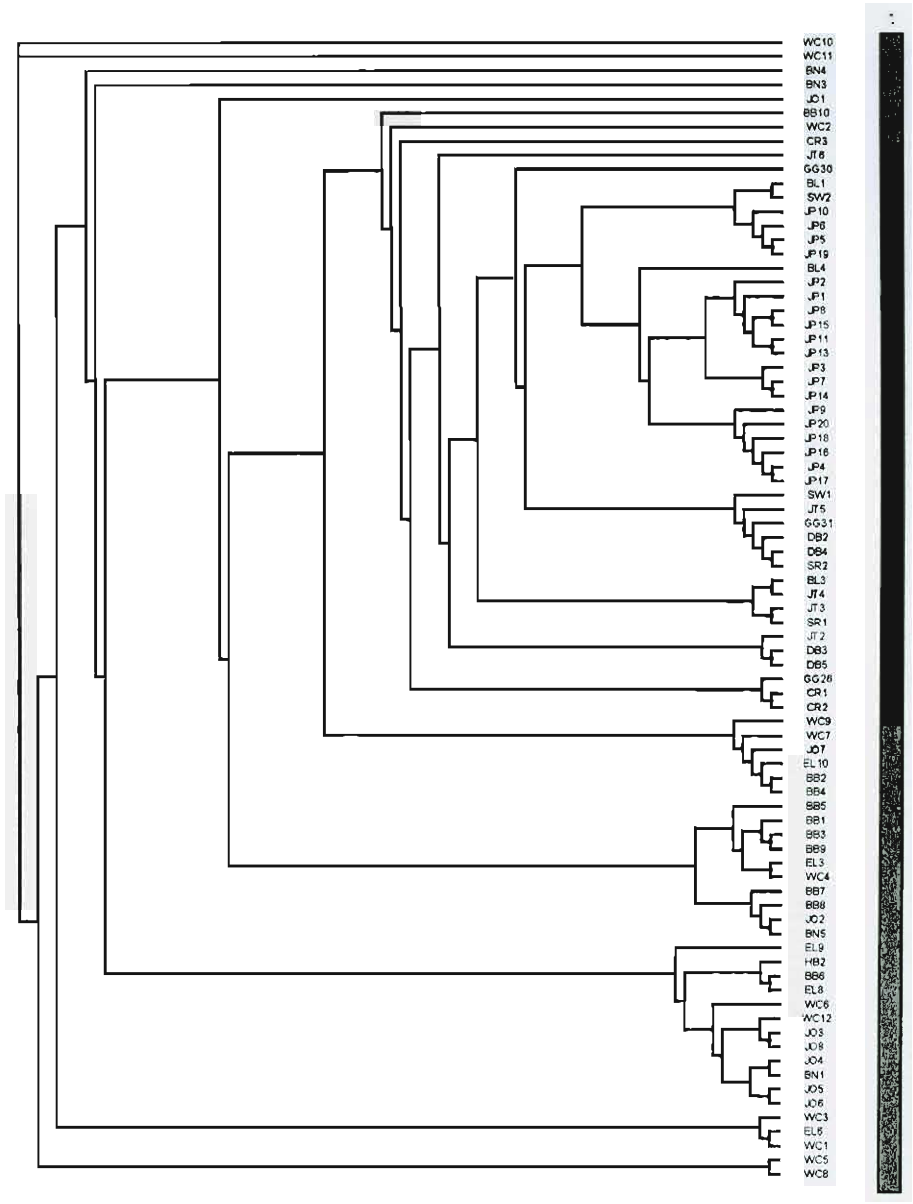


**Figure 18.** Test Animal Tree (NJ-Ps)

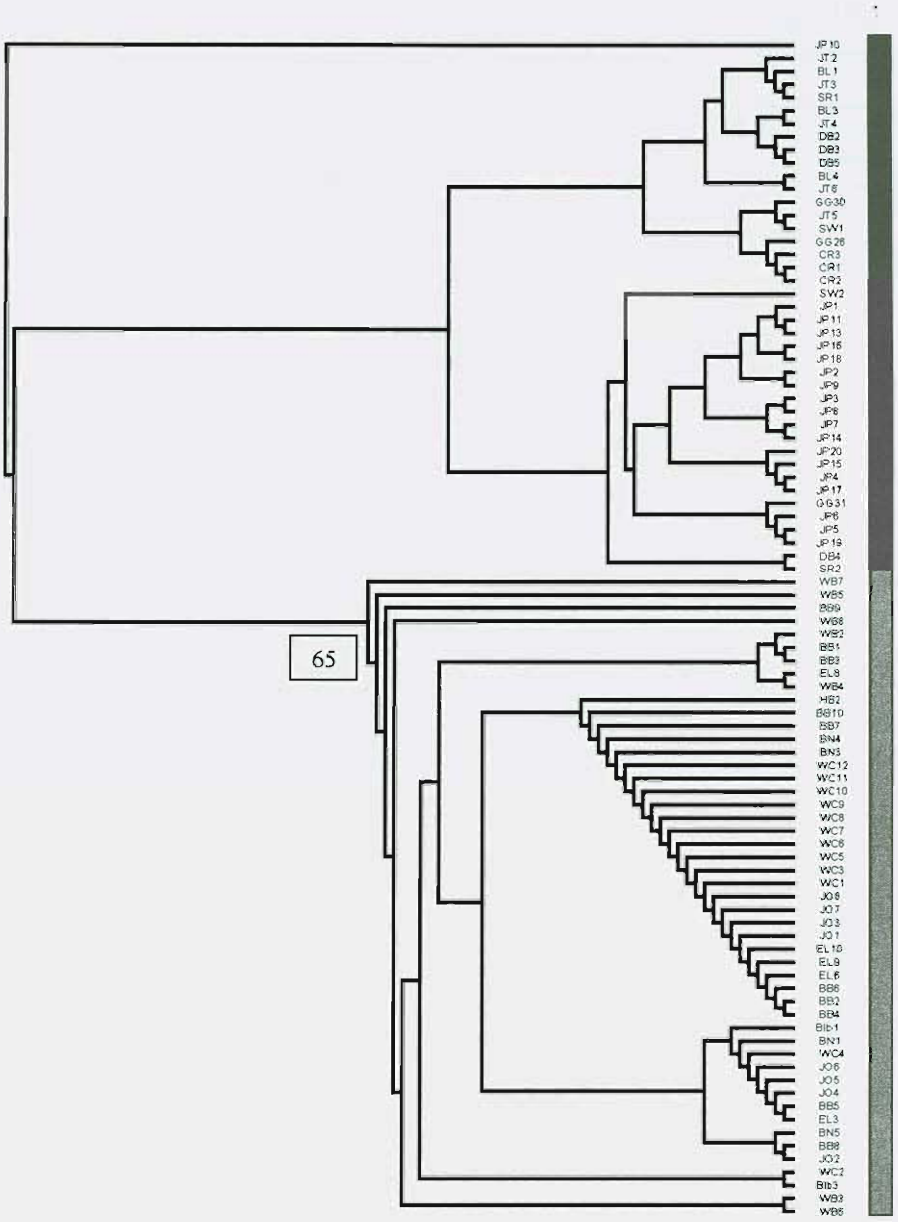


**Table 10.** GeneClass results for reference class and test group.

| Self Assignment METHOD | CLASSIFIED CORRECTLY | SAMPLES MIS-CLASSIFIED | SAMPLES NOT CLASSIFIED | | *TEST GROUP ASSIGNMENT* | |
|---|---|---|---|---|---|---|
| CHORD DISTANCE | 96% | CR1, CR2, CR3 | 0 | | All Bontebok | |
| NEI'S STANDARD | 97% | CR2, CR3 | 0 | | All Bontebok | |
| NEI'S MINIMUM | 100% | 0 | 0 | | All Bontebok | |
| NEI'S AVERAGE | 100% | 0 | 0 | | All Bontebok | |
| ALLELES SHARED | 93% | JT2, SR1, CR1, CR2, CR3 | 0 | | All Bontebok | |
| DELTA MU | 88% | JT6, GG31, EL6, JO3, JO8, WC1, WC3, WC11, BN1 | 0 | | WB3 Blesbok<br>Others bontebok | |
| FREQUENCY | 100% | 0 | 0 | | All Bontebok | |
| BAYSIAN | 100% | 0 | 0 | | All Bontebok | |
| | | | | | *TEST GROUP* | |
| **SIMULATION OF METHODS** | **CLASSIFIED CORRECTLY** | **SAMPLES MIS-CLASSIFIED** | **SAMPLES NOT CLASSIFIED** | | **NOT CLASSIFIED** | **Bontebok class** |
| CHORD DISTANCE | 100% | 0 | JP10, GG31, BB9, WC2 | | WB3-7 | Bb1, Bb3, WB2, WB8 |
| NEI'S STANDARD | 100% | 0 | JP10, JT6, DB4 GG31 | | WB3-7 | Bb1, Bb3, WB2, WB8 |
| NEI'S MINIMUM | 100% | 0 | JP10, GG31, BB9, WC2 | | WB3-7 | Bb1, Bb3, WB2, WB8 |
| NEI'S AVERAGE | 100% | 0 | JP10, JT6, DB4, GG31, BB9, EL9, EL10 WC2 | | WB3-8 | Bb1, Bb3, WB2 |
| ALLELES SHARED | 100% | 0 | JP10, GG31 | | WB4, 5, 7 | Bb1, Bb3, WB2, WB3, WB6, WB8 |
| DELTA MU | 88% samples assigned to both classes | GG31 | JP10 | | WB3 | Both classes |
| FREQUENCY | 100% | 0 | JP10, JT6, DB4, GG31, BB9, EL9, EL10 WC2 | | WB3-8 | Bb1, Bb3, WB2 |
| BAYSIAN | 100% | 0 | JP10, JT6, DB4, GG31, BB9, EL9, EL10 WC2 | | WB 3-8 | Bb1, Bb3, WB2 |

*Note – Two assignment methods (self assignment and simulation) were performed to categorize samples into subspecies groups. Both types of assignment used either a distance-based or a likelihood-based method (frequency or Baysian). Seven distance estimators were used to assign an individual to the population that is genetically closest to it. The likelihood methods assign the individual to the group that has the highest likelihood compared to all other populations. The first 4 columns indicate the assignment of all samples from the bontebok and blesbok populations. The last 2 columns represent the classification of the test animals using all methods.

Microsatellite allele frequencies were imported into the Bottleneck program to quantitatively estimate the amount of genetic diversity lost through the demographic reductions in both subspecies. The blesbok data set did not show a highly significant excess of heterozygosity under the SMM model when tested by both the sign and Wilcoxon methods (Table 11.a). Within the bontebok data set, two loci (OarCP26, MAF50) demonstrated heterozygosity excess under the SMM model ($P = 0.028$, $P = 0.01$, respectively. The results of the sign and Wilcoxon tests do not show significant excess of heterozygosity under any model (Table 11.b.). The mode-shift test produced an L-shaped allele distribution that was expected under equilibrium for blesbok; however a slight mode shift was observed in the distribution of alleles found in bontebok (Figure 19).

| | | Observed | | Under the I.A.M. | | | | Under the S.M.M. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Locus** | **n** | **ko** | **He** | **Heq** | **S.D.** | **DH/sd** | **Prob** | **Heq** | **S.D.** | **DH/sd** | **Prob** |
| TGLA 53 | 84 | 4 | 0.659 | 0.450 | 0.169 | 1.238 | 0.0890 | 0.589 | 0.111 | 0.628 | 0.2990 |
| TGLA 142 | 84 | 5 | 0.704 | 0.523 | 0.156 | 1.163 | 0.0920 | 0.675 | 0.082 | 0.356 | 0.4390 |
| DINC | 84 | 2 | 0.450 | 0.196 | 0.169 | 1.502 | 0.1370 | 0.227 | 0.168 | 1.330 | 0.1700 |
| MDRB3 | 72 | 7 | 0.676 | 0.652 | 0.122 | 0.193 | 0.4870 | 0.774 | 0.054 | -1.831 | 0.0550 |
| OarHH64 | 84 | 4 | 0.549 | 0.433 | 0.175 | 0.665 | 0.3110 | 0.588 | 0.112 | -0.348 | 0.2900 |
| OarCP26 | 84 | 3 | 0.456 | 0.335 | 0.181 | 0.664 | 0.3480 | 0.466 | 0.136 | -0.074 | 0.3760 |
| MAF46 | 84 | 5 | 0.685 | 0.515 | 0.159 | .064 | 0.1370 | 0.579 | 0.083 | 0.072 | 0.4380 |
| MAF50 | 84 | 6 | 0.756 | 0.587 | 0.145 | 1.166 | 0.0800 | 0.729 | 0.068 | 0.407 | 0.4240 |

**SIGN TEST**

Assumptions: all loci fit I.A.M., mutation-drift equilibrium.
Expected number of loci with heterozygosity excess: **4.41**
0 loci with heterozygosity deficiency and 8 loci with heterozygosity excess.
Probability: 0.00811

Assumptions: all loci fit S.M.M., mutation-drift equilibrium.
Expected number of loci with heterozygosity excess: **4.66**
3 loci with heterozygosity deficiency and 5 loci with heterozygosity excess.
Probability: 0.55265

**WILCOXON TEST**

Assumptions: all loci fit I.A.M., mutation-drift equilibrium.
Probability (one tail for H deficiency): 1.00000
Probability (one tail for H excess): 0.00195
Probability (two tails for H excess and deficiency): 0.00391

Assumptions: all loci fit S.M.M., mutation-drift equilibrium.
Probability (one tail for H deficiency): 0.76953
Probability (one tail for H excess): 0.27344
Probability (two tails for H excess or deficiency): 0.54688

**Table 11.a.** Bottleneck test simulations for blesbok. The number of alleles for each locus is represented by (n). Expected heterozygosity values were generated from the observed number of alleles (k) for IAM and SMM. Standard deviations (SD) were computed. The standard difference is given as ((Hobs/Hexp)/SD). All tests indicated that the *Ne* of blesbok have not experienced a demographic reduction.

| | | Observed | | Under the I.A.M. | | | | Under the S.M.M. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Locus** | **n** | **ko** | **He** | **Heq** | **S.D.** | **DH/sd** | **Prob** | **Heq** | **S.D.** | **DH/sd** | **Prob** |
| TGLA 53 | 68 | 2 | 0.112 | 0.205 | 0.166 | -0.559 | 0.4500 | 0.240 | 0.170 | -0.751 | 0.3700 |
| TGLA 142 | 68 | 5 | 0.221 | 0.541 | 0.151 | -2.109 | 0.0410 | 0.680 | 0.081 | -5.645 | 0.0000 |
| DINC | 68 | 2 | 0.029 | 0.201 | 0.168 | -1.022 | 0.2230 | 0.245 | 0.167 | -1.296 | 0.1360 |
| MDRB3 | 52 | 3 | 0.588 | 0.374 | 0.173 | 1.236 | 0.1020 | 0.481 | 0.132 | 0.815 | 0.2180 |
| OarHH64 | 68 | 3 | 0.141 | 0.354 | 0.176 | -1.213 | 0.1890 | 0.470 | 0.137 | -2.409 | **0.0280** |
| OarCP26 | 68 | 2 | 0.409 | 0.203 | 0.169 | 1.217 | 0.2010 | 0.253 | 0.170 | 0.915 | 0.2820 |
| MAF46 | 68 | 3 | 0.086 | 0.347 | 0.174 | -1.492 | 0.1040 | 0.471 | 0.134 | -2.873 | **0.0100** |
| MAF50 | 68 | 1 | 0.000 | MONOMORPHIC LOCUS. | | | | | | | |

**SIGN TEST**

Assumptions: all loci fit I.A.M., mutation-drift equilibrium.
Expected number of loci with heterozygosity excess: **3.42**
5 loci with heterozygosity deficiency and **2** loci with heterozygosity excess.
Probability: 0.24249

Assumptions: all loci fit S.M.M., mutation-drift equilibrium.
Expected number of loci with heterozygosity excess: **3.82**
5 loci with heterozygosity deficiency and **2** loci with heterozygosity excess.
Probability: 0.15553

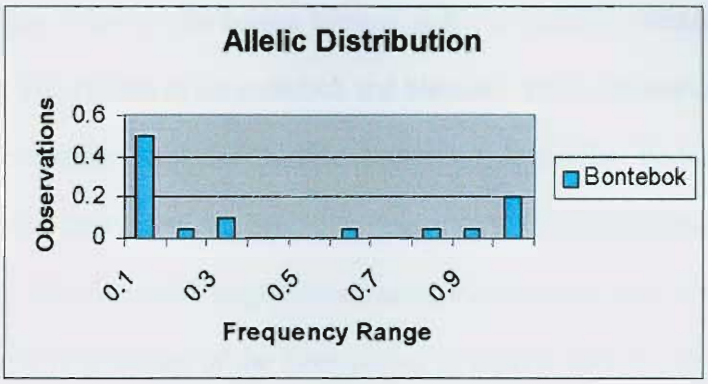
**WILCOXON TEST**

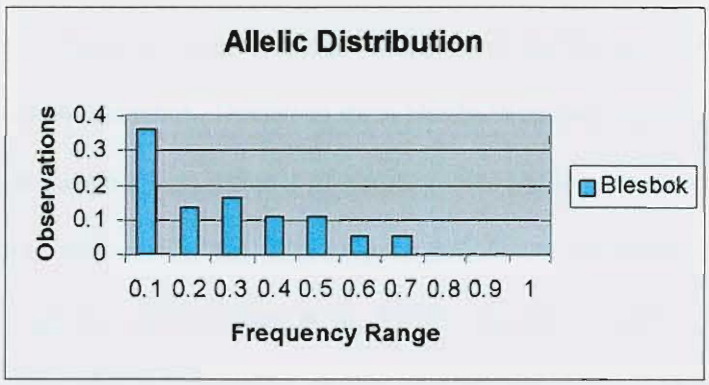
Assumptions: all loci fit I.A.M., mutation-drift equilibrium.
Probability (one tail for H deficiency): 0.23438
Probability (one tail for H excess): 0.81250
Probability (two tails for H excess and deficiency): 0.46875

Assumptions: all loci fit S.M.M., mutation-drift equilibrium.
Probability (one tail for H deficiency): **0.03906**
Probability (one tail for H excess): 0.97266
Probability (two tails for H excess or deficiency): 0.07813

**Table 11.b.** Bottleneck test simulations for bontebok. All tests indicated that the *Ne* of bontebok have not experienced a demographic reduction.

**Figure 19.** Distribution of microsatellite alleles for all loci



**Discussion**

A panel of bovine and ovine primers was tested for cross-species amplification in *D. pygargus*. Many of the bovine primers failed to amplify, produce strong products or exhibit polymorphism in the bontebok and blesbok. The ovine markers proved to amplify more successfully than the bovine primers. A similar finding was shown in a microsatellite study of the closely related hartebeest (*Alcelaphus buselaphus*) (Flagstad et al. 1999). The successful amplification using the ovine primers is most likely explained by the close relationship of the alcelaphines to caprine species rather than to the bovids (Matthee and Robinson 1999a).

The microsatellite diversity ranged in the amount of variation found within subspecies. The lack of genetic diversity appears to be the result of the bottleneck events experienced by bontebok. Deviation from Hardy-Weinberg equilibrium was not found in bontebok although a high number of homozygotes per locus was observed. A number of events could account for the heterozygote deficiency, including selection, non-random mating or genetic drift (Callen et al. 1993). Given the demographic history of the bontebok and lack of variation at the control region, it is most plausible that the bottlenecks have caused a great loss of allelic diversity within microsatellite loci.

In order to assess the loss of nuclear genetic variation in the bontebok, the outbred blesbok subspecies should provide a reliable indicator of diversity. The estimated diversity may be slightly reduced, partially biased, since the microsatellite primers were designed from homologous loci in cattle and sheep (Goldstein and Pollock 1997). However, this ascertainment bias should not influence the relationship between the amount of variation that is maintained within each subspecies. Blesbok demonstrated a moderate level of allelic diversity and heterozygosity. Table 12 illustrates the range of heterozygosity values for ungulate species having different demographic histories.

**Table 12.** Microsatellite heterozygosity values and demographic histories of various ruminant species.

| Taxa | # Loci Typed | Average H | Demographic history | Reference |
|---|---|---|---|---|
| Moose (Canada) | 5 | 0.219 | Founder event with 6 individuals | Broders et al. 1999 |
| African buffalo | 6 | 0.648 | Slight bottleneck event | Simonsen et al. 1998 |
| Hartebeest | 16 | 0.700 | Outbred | Flagstad et al. 1999 |
| Arabian oryx | 6 | 0.544 | Captive herd, founder effect | Marshall et al. 1999 |
| Muskox | 12 | 0.059 | Possible historic or prehistoric bottleneck | Holm et al. 1999 |
| Blesbok | 8 | 0.623 | Outbred | This study |
| Bontebok | 8 | 0.201 | Bottleneck events | This study |

Clustering methods and assignment tests were preformed for the assignment of individuals into subspecies classes. The phylogenetic trees depicted clustering of individuals according to their subspecies designation. Unknown samples were placed within the bontebok subspecies class. It appears that the genotypes of the test samples consisted of many of the shared alleles rather than the subspecies specific (private) alleles. Samples (Bb1, Bb3) were derived from a "bontebok" population that is suspected to contain hybrid animals based on the presence of blesbok control region haplotypes. The clustering test places them within the bontebok class since no blesbok private alleles were evident.

Multilocus genotypes were used for assignment using distance estimates and likelihood scores. The reference data set showed high success in correct classification using all variations of each method (96%-100%) with delta $mu^2$ being the exception displaying the lowest percentage (88%). Delta $mu^2$ is successfully used to estimate distances between taxa at the species level (Goldstein et al. 1995a,b). The samples that were mis-classified (CR1, CR2, CR3) are derived from a blesbok population located within the Eastern Cape. This population could possess old bontebok alleles from the time before the species split into two subspecies. A more likely scenario is that bontebok were translocated into that herd of blesbok. The data set and each method was simulated and shown to classify individuals correctly (100%), however, the methods failed to place several samples into a category (see Table 10). The genotypes of many of the un-classified animals consist of rare, low frequency alleles. The rare alleles may create larger distances and smaller likelihood values for these animals and make placement difficult compared to all other samples. The test group data set was compared to the reference set and all samples were classified to the bontebok subspecies by direct

assignment. The simulated test showed similar results but failed to classify animals "test" animals (WB 3 - WB7) from one population.

The classification tests and phylogenetic clustering methods demonstrate that individuals of known origin can be correctly placed into their subspecies category. However, the degree to which the assignment tests were able to successfully assign an unknown was not confirmed due to the limitations of the study. More animals documented as hybrids are required to serve as positive controls. In this study, animals were only suspected as being hybrid by morphological characteristics. Secondly, a higher number of polymorphic loci (10 – 20 loci) would provide greater allocation success of known animals (Blott et al. 1999).

It has been predicted that bottlenecked populations will demonstrate excess in heterozygosity that will be higher than expected at equilibrium (Luikart et al. 1998a,b). The blesbok data did not display significant heterozygosity excess when tested under the assumptions of the SSM model. Furthermore, the distribution of allele frequencies did not show the signature shift that is indicative of a bottlenecked population. These results suggest that blesbok have had a stable demographic history.

Two loci demonstrated significant heterozygosity excess within the bontebok data set. The results of the sign and Wilcox tests do not indicate a bottleneck event in bontebok, although historical records have recorded two severe reductions in (*Ne*). Three possible explanations may elucidate the results of the bottleneck tests. Firstly, the historical records may have been incorrect and the actual founding population of bontebok may have been larger than the 20-30 individuals estimated. Secondly, the power of bottleneck detection relies more on the assumption of heterozygosity excess than on allelic diversity estimates. It has been suggested that reduction in (*Ne*) has a greater impact on the allelic diversity of a population than on heterozygosity (Nei et al.

1976). Therefore, the distribution of alleles appears to be a more powerful in detecting recent and severe bottleneck events. Lastly, the bottleneck tests may be dependent on the size of the bottleneck and the number of generations that have passed since the time of the event. Luikart and colleagues (1998a) have simulated bottleneck events based on varying number of founders and have reported that small bottleneck sizes (<20) are more likely to be detected.

A genetic bottleneck event will quickly result in the loss of low frequent alleles, which will in turn, cause the increase of the intermediate and high frequency alleles (Nei et al. 1975, Luikart et al. 1998a). Figure 19 clearly illustrates the loss of rare and intermediate alleles in the distribution of bontebok microsatellite alleles compared to that of blesbok. The graphical representation also reveals a distortion of allele frequencies, however, the results of the Bottleneck program indicate that the distribution of bontebok alleles is L-shaped. Clearly, there can be other alternatives to distribution shape other than the L-shape and mode-shift distributions. The generated data set from Luikart et al. 1998a also revealed that a mode shift is only detected after 40 generations with 20 founders. I predict that there is a mode shift in the distribution and will be apparent once the number of generations exceeds 40.

Microsatellite diversity was examined in order to assess the nuclear gene variation within each subspecies. The results of this study confirm the mtDNA findings that revealed extremely low levels of genetic variation in bontebok and moderate levels within blesbok and a high degree of substructure between the subspecies. Both studies revealed patterns of genetic evolution at non-coding loci. In the next chapter, I present the results from a coding nuclear gene that is presumed to evolve under selective forces in vertebrate species.

# CHAPTER 4

# MHC-DRB EVOLUTION

## Chapter 4: MHC-DRB Characterization and Evolution

### *Introduction*

The major histocompatibility complex (MHC) is a multigene family of tightly linked homologous genes. The genes of the MHC encode for glycoproteins that function as receptors presenting self and foreign peptides to circulating T cells (Klein 1986). The complex is divided into three classes (I, II, III) of tightly linked genes (Figure 20). Class I genes encode receptor molecules which are comprised of an α chain and non-covalently associated β microglobulin component. These receptors are found on all nucleated cells and function to present self-peptides to the immune system for self-recognition. If a somatic cell is infected by a virus or becomes neoplastic, the foreign derived peptide is loaded into the class I receptor so that circulating cytotoxic T cells (CD8+) can recognize the "diseased" cell and eliminate it. Class II genes encode for a heterodimers (composed of α and β chains) that are exhibited on the cell surface of antigen presenting cells such as macrophages, B-cells, dendritic cells (Figure 21). Once an antigen-presenting cell, for instance a macrophage, is infected by a pathogen (bacteria, fungi, protist), the cell internally processes the parasite and presents a small antigen in the peptide-binding region (PBR) on the MHC receptor. This binding groove is composed of approximately 50 amino acid residues and presents peptides that are usually 11-19 residues in length (Blum and Creswell 1998). The presentation of the foreign peptide is then recognized by a T-cell receptor (TCR) that is specific to the antigen and MHC molecule. The interaction of receptors stimulates the proliferation of T cell expansion, release of cytokines and secretion of antibodies. The immune cascade described enables for the clearing of infection in the animal. Class II genes also have important roles for the processing of antigen (peptide) and immune response. For example, peptide degradation

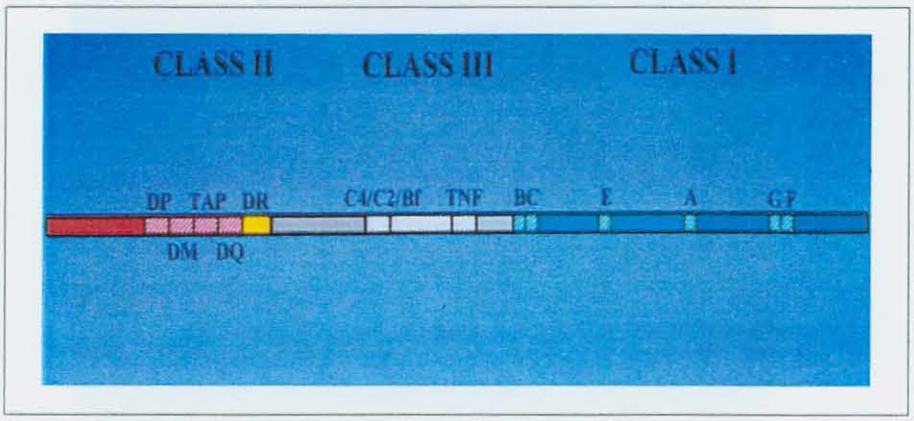



**Figure 20.** Major Histocompatibility Complex. The illustration depicts the gene organization of mammalian MHC genes. The DR locus is located within Class II.

**Figure 21.** Three-dimentional structure of the DRB allele Dama*16. The amino acid chain consists of an α-helix and β-sheet. A pocket of amino acids are involved in antigen binding.

and processing is enabled through the binding of a low molecular mass polypeptide (LMP). After the peptide is processed, it is transported into the endoplasmic reticulum (ER) all of which is facilitated by the proteins encoded by the TAP genes of class III (Neefjes et al. 1993).

The genes that encode the MHC protein receptors are the most polymorphic loci of all the nuclear encoding genes in vertebrate species (Hughes and Hughes 1995). The extreme polymorphism takes the form in a high number of alleles found at an individual locus, a high number of amino acid substitutions and large sequence divergence between alleles. Recovery from disease may be related to the MHC profile of the animal. This dependence or "restriction" of MHC is often characterized as co-evolving with parasites. Although the receptors are restricted in a sense, the binding grooves are able to display a range of peptides and are not limited to only one peptide. The binding is most likely dependent upon the physiochemical nature of the amino acids of the foreign peptide.

A selection of HLA alleles in humans has been associated with susceptibility to autoimmune diseases such as lupus (Gladman et al. 1999), Grave's disease (Chen et al. 1999) and subtypes of multiple sclerosis (Yamasaki et al. 1999). Other studies have found strong evidence for specific HLA alleles offering protection against infectious diseases including malaria in humans (Hill et al. 1991, Gilbert et al. 1998), Marek's disease in chickens (Briles et al. 1977) and bovine leukemia in cattle (Xu et al. 1993). In contrast, other MHC haplotypes tend to accelerate disease progression of hepatitis B (Thurz et al.1997) and AIDS (Carrington et al. 1999). Specific MHC allele polymorphisms are also being used to design polyepitope vaccines against foot and mouth disease virus in cattle (Glass and Miller 1994) and melanoma in humans (Mateo et al. 1999).

Balancing selection has been regarded as the strongest force acting on MHC loci to maintenance of high allelic diversity. The two types of balancing selection are

overdominant selection (Hughes and Nei 1989) and frequency dependent selection (Takahata and Nei 1990). Positive (overdominant) selection is evident when the number of nonsynonymous ($d_N$) substitutions exceeds the number of synonymous ($d_S$) substitutions (Maruyama et al. 1981), which can be represented as ($d_N > d_S$). The peptide binding region (PBR) of MHC molecules display a large number of amino acid substitutions (Hughes and Nei 1989, Hughes et al. 1990). The diversity of amino acids at these binding positions enables a wide variety of pathogen-derived peptides to be displayed to the immune system (Doherty and Zinkernagel 1975). Selection pressure on peptide binding residues should be greatest during speciation, when organisms move into new environments and their MHC molecules encounter new pathogens (Dixon et al 1996).

There are two approaches to the theory of polymorphism by way of frequency dependent or pathogen-driven selection. The heterosis model explains that heterozygous individuals have a selective advantage over those that are homozygous because a wider array of pathogens can be presented to the immune system. This type of selection has been shown in the study of hepatitis B prevalence in West Africans (Gambia) where individuals heterozygous for specific alleles were resistant (Thurz et al. 1997). In theory, this selection pressure seems likely given the constant co-evolution between host and parasite over time. The other approach to parasite driven selection assumes that an individual can acquire a selective advantage if he/she possesses an allele that has arisen recently in the population. In this case, the new antigen protects the host because the pathogen has not had time to evolve against it (Bodmer 1972). Although, parasite driven selection has been offered as an important force that has shaped HLA frequencies in human populations (Hill et. al. 1991), there is a paucity of field data that supports this theory (Hughes and Nei 1992). Moreover, it is difficult to separate the forces of over-

dominant (heterozygote advantage) and parasite driven selection that may work together in maintaining polymorphisms in populations.

The hypothesis that polymorphism of alleles is maintained by some form of balancing selection is able to explain both the diversity and ancient persistence of MHC alleles (Hedrick and Thompson 1983, Hughes and Nei 1989). The discovery of ancient polymorphisms within MHC allelic lineages provides more evidence for their evolution under balancing selection rather than neutrality. Polymorphism is maintained in allelic lineages that evolve in a trans-specific manner, whereby MHC alleles are passed from one species to another descendant species (Klein 1980). Evidence of ancient polymorphisms from primates (Mayer et al. 1988), rodents (Figueroa et al. 1988), pinnipeds (Hoelzel et al. 1999) and felids (Yuki and O'Brien 1999) supports the trans-specific mode of evolution of MHC loci.

Much of research that has resulted in the elucidation of MHC evolution and organization has been carried out of humans and domestic animals (Bodmer et al. 1998, Lewin et al. 1999). The extremely polymorphic nature of the MHC loci makes them appropriate makers for estimating genetic diversity in populations (Potts et al. 1992). Recently, these genes have been investigated to assess genetic diversity in populations of exotic species. High allelic diversity has been documented in MHC genes in most outbred terrestrial species (see review in Edwards and Hedrick 1998), with some exceptions (e.g. Murray et al. 1995). Demographic crashes can result in either severe loss of MHC alleles as demonstrated in populations of moose (Mikko and Andersson 1996), cheetah (Yuhki and O'Brien 1990) and northern elephant seal (Hoelzel et al. 1999), or a slight reduction in polymorphism as seen in populations of African buffalo (Wenink et al. 1998). Given the paramount role of MHC molecules to species survival, it has been

recommended that diversity at these loci should be maintained in natural populations (Hughes1991, Potts et al. 1994).

In the previous chapters, non-coding loci were examined to assess genetic diversity in each subspecies. The aim of this study was to characterize the DRB locus in order to reveal the pattern of polymorphism found in the exotic African bovid species, *Damaliscus pygargus*. Variation at the sequence level was investigated to define the regions of functional significance involved in antigen presentation. DRB sequences derived from distantly related ungulate species were aligned with Dama alleles so that the mode of evolution and relationships of alleles could be revealed in a phylogenetic analysis.

## MATERIALS AND METHODS

### *Samples and Collection Sites*

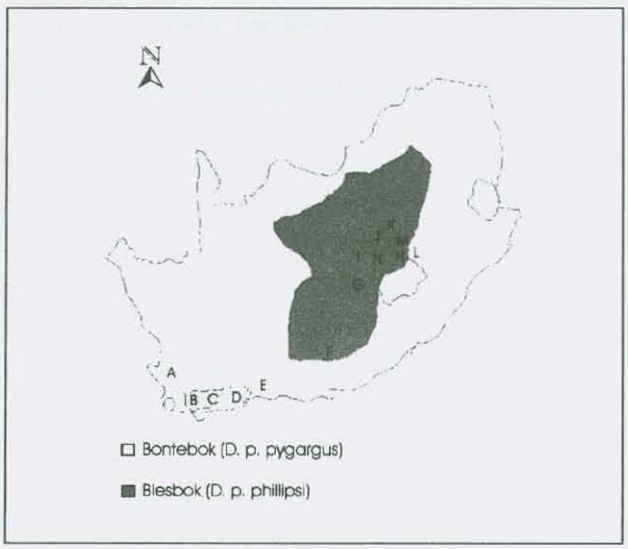
See Figure 22.

### *PCR-SSCP of the DRB locus*

Cattle-specific primers HL030 and HL031 were used to amplify a 284 bp fragment of exon 2 of the DRB locus from genomic DNA as described by van Eijk et al. (1992). PCR product by thermocycling for one cycle at 94° C for 4 minutes, 30 cycles at 94° C for 1 minute, 30 seconds at 65° C, and 72° C, followed by an extension for 5 minutes at 72 ° C.

PCR products (8 µl) were mixed with a 32 µl volume of a low ionic strength buffer (LIS) and subjected to heat denaturation at 97 C ° for 2 minutes. The single strands were formed within the LIS sugar matrix (10% saccharose, 0.01% bromophenol blue, 0.01% xylene cyanol FF) and remained stable at room temperature (Maruya et al. 1996).

**Figure 22.** Sample Distribution




Sampling locations and number of animals genotyped are as follows: A = West Coast National Park (10), B = Overberg Farm (8), C = Bontebok National Park (14), D = Heidleberg Farm (2), E = Elandsberg Farm (4), F = Cradock Farm (2), G = De Brug Farm (2), H = Golden Gate National Park (3), I = Bloemhof Farm (3), J = Parys Farm (25), K = Suikersbosrand Reserve (2), L = Sterkfontein Dam Reserve (2), M = TDR Farm (5) and N = Fairview Farm (2).

Single-stranded products were then subjected to electrophoresis through a 10% non-denaturing polyacrylamide gel (1.4% cross-linking) in 1X TBE (Tris Borate EDTA) buffer at room temperature for 18 hours (Glavac and Dean 1993). After electrophoresis, the gel was incubated for 30 minutes with the fluorescent GelStar Nucleic Acid Gel Stain (BioWhittaker). Alleles were visualized by UV illumination and image capture with a Kodak digital camera. Allele patterns were scored for each animal and genotypes assigned. Unique patterns of allelic variation were investigated by sequence analysis. In most cases, gel fragments were excised and purified for sequencing (Sambrook et al. 1989).

***Cloning and Sequencing***

A subset of alleles as revealed by SSCP mobilities were chosen for cloning and sequencing. Homozygous individuals were directly sequenced from purified DRB PCR products (see Chapter 2-Materials and Methods). Heterozygous genotypes were cloned into a pGEM vector (Promega) using a TA cloning kit. Ten recombinant clones were purified by mini-prep filter columns (Qiagen) and sequenced in the forward and reverse directions using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI). The labelled PCR fragments were analysed using an ABI 377 automated sequencer.

***Statistical and Phylogenetic Analyses***

All Dama-DRB nucleotide sequences and one cow reference sequence (Bota-1) were aligned and translated into the corresponding amino acid sequences using MEGA software package (Kumar et al. 1993). Amino acid positions involved in peptide binding were identified by comparison with the peptide binding groove structure of the human class II molecule (Brown et al. 1993). Relative frequencies of nonsynonymous (dN) and

synonymous (dS) substitutions were calculated for the peptide binding region (PBR) and non-PBR according to Nei and Gojobori (1986) using the Jukes-Cantor correction. (1969). All dN and dS frequencies plus standard errors were estimated using the MEGA computer package. Significance levels were determined by the student's t-test where t = d/ s(d). Heterozygosity levels were determined assuming random mating within subpopulations and deviation from Hardy-Weinberg equilibrium was tested using Markov chain permutation test of 100,000 steps in Genepop. Genetic distances were estimated for the nucleic acid sequences by applying the Kimura two-parameter method. Neighbor-joining trees were constructed from distance matrices of ungulate alleles using the MEGA program and PAUP* (Swofford et al. 1998).

***Genebank Sequences***

Cattle sequences representing 39 of the major BLA allelic lineages were acquired through the BoLA Nomenclature Website: http://www2.ri.bbsrc.ac.uk.bola/) and used as in a phylogenetic analysis of cattle DRB exon 2 sequences: AF144544, AF144545, AJ001999, AJ002001, U77067, U78548, X87643 – X87645, X87647 – X87650, X87652, X87655, X87656, X87658, X87659, X87662, X87665 – X87668, X87670, Z30650, Z30649, Z36538, Z36542, Z82023 – Z82025, Z82027, Z82029, Z82032 - Z82035. DRB sequences derived from horse, ELA-DRB*9 (L7674) and moose, ALA1DRB1*8 (X83284) were used as outgroup taxa for the phylogenetic analyses.

## RESULTS

The Dama-DRB homolog to the cow BoLA-DRB3 locus was characterized by SSCP analysis followed by cloning and direct sequencing of alleles. I am confident that only one DRB locus was amplified as no more than two sequences were revealed in each animal. Cloned PCR products revealed only two alleles through sequence analysis of a minimum of eight recombinant clones for any heterozygote. Alignment of all Dama alleles demonstrates close homology to the cattle (BoLA) DRB3 expressed locus. This close homology and the absence of stop codons within the nucleotide sequences provide evidence that the expressed DRB locus was isolated in *D. pygargus*.

Sequence variation of exon 2 was examined by SSCP analysis revealing 27 different alleles in the typed individuals (Figure 23, Table 13). Genotype frequencies are given in Table 14. Extensive polymorphism was found for the blesbok subspecies (22 alleles) compared to the level of polymorphism found for the bontebok subspecies (6 alleles). Two alleles are shared between subspecies (Dama*7 and *8). The sample Bb1 from the Fairview hybrid population displayed alleles from both subspecies. Heterozygosity values are shown in Table 15.

Polymorphism at class II loci occurs predominately in exon 2, which encodes a majority of the peptide region (PBR). The exon translates into a sequence length of 83 amino acids having 16 possible binding sites for foreign peptide presentation (Brown et al. 1993). In an alignment of all inferred exon amino acid sequences, 39 sites (47.0%) were variable while the other sites were conserved. The majority of amino acid site changes were found within the PBR or in positions that are adjacent to these binding sites, such as sites 23, 24 and 52 (Figure 24). Table 16 illustrates the number of amino acid differences between each allele. The relative frequency of nonsynonymous substitutions (dN = 0.299; SE = 0.066) was significantly higher than the frequency of synonymous

**Figure 23.a.**

SSCP analysis of DRB alleles in blesbok

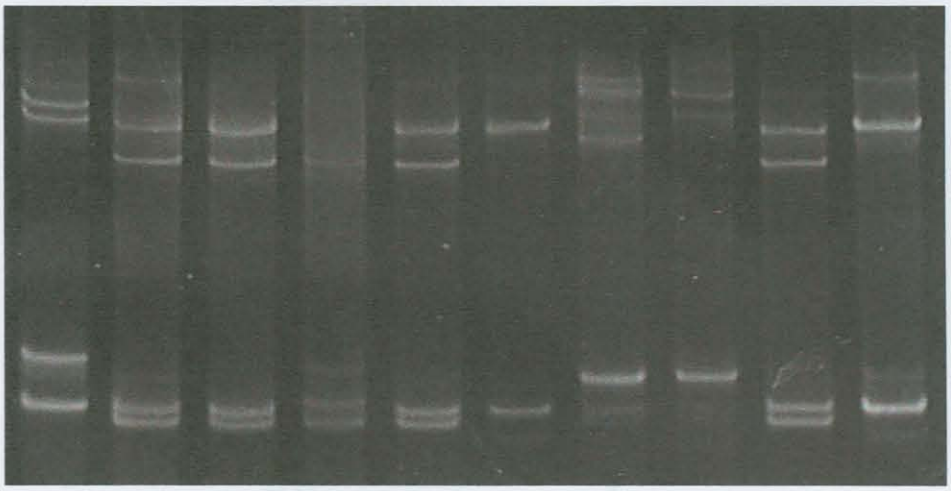


**Figure 23.b.**

SSCP analysis of DRB alleles in bontebok

| Animal | Allele 1 | Allele 2 |
|---|---|---|
| JP1 | 1 | 19 |
| JP2 | 1 | 19 |
| JP3 | 19 | 19 |
| JP4 | 3 | 4 |
| JP5 | 18 | 20 |
| JP6 | 13 | 21 |
| JP7 | 20 | 20 |
| JP8 | 4 | 8 |
| JP9 | 2 | 3 |
| JP10 | 1 | 6 |
| JP11 | 1 | 4 |
| JP13 | 1 | 4 |
| JP14 | 1 | 4 |
| JP15 | 1 | 4 |
| JP16 | 4 | 5 |
| JP17 | 1 | 2 |
| JP18 | 1 | 2 |
| JP19 | 1 | 4 |
| JP20 | 2 | 4 |
| JP21 | 2 | 4 |
| JP22 | 1 | 4 |
| JP23 | 2 | 4 |
| JP24 | 1 | 1 |
| JP26 | 1 | 2 |
| SR1 | 1 | 4 |
| SR2 | 1 | 1 |
| JT2 | 26 | 27 |
| JT3 | 15 | 15 |
| JT4 | 17 | 26 |
| JT5 | 15 | 17 |
| JT6 | 15 | 15 |
| BL2 | 6 | 12 |
| BL3 | 15 | 15 |
| BL4 | 1 | 4 |
| DB2 | 2 | 3 |
| DB3 | 17 | 3 |
| ST3 | 4 | 9 |
| ST4 | 10 | 11 |
| Bb2 | 24 | 25 |
| CR1 | 2 | 3 |
| CR3 | 2 | 3 |
| GG26 | 7 | 8 |
| GG27 | 8 | 8 |
| GG31 | 8 | 8 |

**Table 13.a.** DRB genotypes of blesbok samples.

| Animal | Allele 1 | Allele 2 |
|---|---|---|
| BB1 | 8 | 8 |
| BB2 | 8 | 8 |
| BB3 | 8 | 8 |
| BB4 | 8 | 8 |
| BB5 | 7 | 23 |
| BB6 | 14 | 16 |
| BB7 | 14 | 22 |
| BB9 | 14 | 22 |
| BB10 | 14 | 22 |
| EL3 | 8 | 8 |
| EL5 | 7 | 8 |
| EL8 | 7 | 14 |
| EL10 | 8 | 8 |
| JO1 | 7 | 8 |
| JO2 | 8 | 8 |
| JO4 | 8 | 8 |
| JO5 | 7 | 8 |
| JO6 | 7 | 14 |
| JO7 | 8 | 8 |
| JO8 | 8 | 8 |
| HB1 | 8 | 8 |
| HB2 | 8 | 8 |
| DH2 | 14 | 16 |
| DH8 | 8 | 8 |
| DH6 | 14 | 16 |
| DH11 | 14 | 14 |
| WC1 | 14 | 23 |
| WC2 | 14 | 16 |
| WC3 | 14 | 23 |
| WC4 | 14 | 23 |
| WC5 | 14 | 16 |
| WC6 | 14 | 14 |
| WC8 | 14 | 14 |
| WC10 | 14 | 14 |
| WC11 | 14 | 14 |
| WC12 | 8 | 8 |
| BN1 | 14 | 23 |
| BN2 | 14 | 16 |
| BN3 | 8 | 7 |
| BN4 | 8 | 7 |
| BN5 | 14 | 14 |
| BN6 | 14 | 14 |
| WB1 | 8 | 8 |
| WB8 | 14 | 23 |
| Bb1 | 14 | 23 |

**Table 13.b.** DRB genotypes for bontebok samples.

**Table14.** DRB allele frequencies found within each subspecies.

| DRB Allele | Blesbok | Bontebok |
|---|---|---|
| Dama*01 | 0.204 | 0.000 |
| Dama*02 | 0.113 | 0.000 |
| Dama*03 | 0.068 | 0.000 |
| Dama*04 | 0.170 | 0.000 |
| Dama*05 | 0.011 | 0.000 |
| Dama*06 | 0.023 | 0.000 |
| Dama*07 | 0.011 | 0.090 |
| Dama*08 | 0.068 | 0.390 |
| Dama*09 | 0.011 | 0.000 |
| Dama*10 | 0.011 | 0.000 |
| Dama*11 | 0.011 | 0.000 |
| Dama*12 | 0.011 | 0.000 |
| Dama*13 | 0.011 | 0.000 |
| Dama*14 | 0.000 | 0.344 |
| Dama*15 | 0.079 | 0.000 |
| Dama*16 | 0.000 | 0.067 |
| Dama*17 | 0.034 | 0.000 |
| Dama*18 | 0.011 | 0.000 |
| Dama*19 | 0.045 | 0.000 |
| Dama*20 | 0.034 | 0.000 |
| Dama*21 | 0.011 | 0.000 |
| Dama*22 | 0.000 | 0.033 |
| Dama*23 | 0.000 | 0.078 |
| Dama*24 | 0.011 | 0.000 |
| Dama*25 | 0.011 | 0.000 |
| Dama*26 | 0.023 | 0.000 |
| Dama*27 | 0.011 | 0.000 |

**Table 15.** Allelic diversity values estimated for blesbok and bontebok.

| Subspecies | N | # of Alleles | D | $H_{exp}$ | $H_{obs}$ |
|---|---|---|---|---|---|
| blesbok | 44 | 22 | 10.7(2.5) | 0.89 | 0.98 |
| bontebok | 45 | 6 | 5.0(1.6) | 0.71 | 0.41 |

N indicates the total number of samples genotyped for each subspecies. The average genetic distance between alleles (Jukes-Cantor) are represented as percentage D values by MEGA software (Kumar et al. 1993). Standard errors of calculated distances are indicated in parentheses. Heterozygosity levels are given for observed and expect (Nei 1987).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.0 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 10.0 | 0.0 | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 9.0 | 1.0 | 0.0 | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 14.0 | 16.0 | 15.0 | 0.0 | | | | | | | | | | | | | | | | | | | | | | |
| 5 | 15.0 | 17.0 | 16.0 | 1.0 | 0.0 | | | | | | | | | | | | | | | | | | | | | |
| 6 | 14.0 | 16.0 | 15.0 | 0.0 | 1.0 | 0.0 | | | | | | | | | | | | | | | | | | | | |
| 7 | 8.0 | 7.0 | 6.0 | 16.0 | 17.0 | 16.0 | 0.0 | | | | | | | | | | | | | | | | | | | |
| 8 | 7.0 | 16.0 | 15.0 | 14.0 | 15.0 | 14.0 | 12.0 | 0.0 | | | | | | | | | | | | | | | | | | |
| 9 | 1.0 | 11.0 | 10.0 | 15.0 | 16.0 | 15.0 | 9.0 | 8.0 | 0.0 | | | | | | | | | | | | | | | | | |
| 10 | 15.0 | 17.0 | 16.0 | 1.0 | 2.0 | 1.0 | 17.0 | 15.0 | 16.0 | 0.0 | | | | | | | | | | | | | | | | |
| 11 | 14.0 | 15.0 | 14.0 | 3.0 | 4.0 | 3.0 | 15.0 | 13.0 | 15.0 | 4.0 | 0.0 | | | | | | | | | | | | | | | |
| 12 | 14.0 | 16.0 | 15.0 | 0.0 | 1.0 | 0.0 | 16.0 | 14.0 | 15.0 | 1.0 | 3.0 | 0.0 | | | | | | | | | | | | | | |
| 13 | 14.0 | 16.0 | 15.0 | 1.0 | 2.0 | 1.0 | 16.0 | 14.0 | 15.0 | 2.0 | 3.0 | 1.0 | 0.0 | | | | | | | | | | | | | |
| 14 | 8.0 | 16.0 | 15.0 | 14.0 | 15.0 | 14.0 | 13.0 | 1.0 | 9.0 | 15.0 | 13.0 | 14.0 | 14.0 | 0.0 | | | | | | | | | | | | |
| 15 | 8.0 | 15.0 | 14.0 | 13.0 | 14.0 | 13.0 | 12.0 | 4.0 | 9.0 | 14.0 | 13.0 | 13.0 | 13.0 | 3.0 | 0.0 | | | | | | | | | | | |
| 16 | 8.0 | 17.0 | 16.0 | 15.0 | 16.0 | 15.0 | 13.0 | 1.0 | 9.0 | 16.0 | 14.0 | 15.0 | 15.0 | 2.0 | 5.0 | 0.0 | | | | | | | | | | |
| 17 | 5.0 | 11.0 | 10.0 | 15.0 | 16.0 | 15.0 | 8.0 | 8.0 | 6.0 | 16.0 | 13.0 | 15.0 | 15.0 | 7.0 | 8.0 | 9.0 | 0.0 | | | | | | | | | |
| 18 | 5.0 | 14.0 | 13.0 | 11.0 | 12.0 | 11.0 | 12.0 | 8.0 | 6.0 | 12.0 | 11.0 | 11.0 | 11.0 | 9.0 | 9.0 | 9.0 | 8.0 | 0.0 | | | | | | | | |
| 19 | 9.0 | 2.0 | 1.0 | 15.0 | 16.0 | 15.0 | 6.0 | 15.0 | 10.0 | 16.0 | 14.0 | 15.0 | 15.0 | 15.0 | 14.0 | 16.0 | 10.0 | 13.0 | 0.0 | | | | | | | |
| 20 | 14.0 | 16.0 | 15.0 | 0.0 | 1.0 | 0.0 | 16.0 | 14.0 | 15.0 | 1.0 | 3.0 | 0.0 | 1.0 | 14.0 | 13.0 | 15.0 | 15.0 | 11.0 | 15.0 | 0.0 | | | | | | |
| 21 | 14.0 | 17.0 | 16.0 | 2.0 | 3.0 | 2.0 | 16.0 | 14.0 | 14.0 | 3.0 | 5.0 | 2.0 | 3.0 | 14.0 | 13.0 | 15.0 | 15.0 | 11.0 | 16.0 | 2.0 | 0.0 | | | | | |
| 22 | 8.0 | 16.0 | 15.0 | 14.0 | 15.0 | 14.0 | 13.0 | 2.0 | 9.0 | 15.0 | 14.0 | 14.0 | 14.0 | 1.0 | 3.0 | 3.0 | 8.0 | 9.0 | 15.0 | 14.0 | 14.0 | 0.0 | | | | |
| 23 | 7.0 | 16.0 | 15.0 | 14.0 | 15.0 | 14.0 | 12.0 | 1.0 | 8.0 | 15.0 | 14.0 | 14.0 | 14.0 | 2.0 | 4.0 | 2.0 | 9.0 | 8.0 | 15.0 | 14.0 | 14.0 | 1.0 | 0.0 | | | |
| 24 | 7.0 | 15.0 | 14.0 | 13.0 | 14.0 | 13.0 | 12.0 | 2.0 | 8.0 | 14.0 | 12.0 | 13.0 | 13.0 | 1.0 | 2.0 | 3.0 | 6.0 | 8.0 | 14.0 | 13.0 | 13.0 | 2.0 | 3.0 | 0.0 | | |
| 25 | 4.0 | 13.0 | 12.0 | 14.0 | 15.0 | 14.0 | 11.0 | 3.0 | 5.0 | 15.0 | 12.0 | 14.0 | 14.0 | 4.0 | 7.0 | 4.0 | 5.0 | 5.0 | 12.0 | 14.0 | 14.0 | 5.0 | 4.0 | 5.0 | 0.0 | |
| 26 | 8.0 | 15.0 | 14.0 | 13.0 | 14.0 | 13.0 | 12.0 | 3.0 | 9.0 | 14.0 | 12.0 | 13.0 | 13.0 | 2.0 | 1.0 | 4.0 | 7.0 | 9.0 | 14.0 | 13.0 | 13.0 | 3.0 | 4.0 | 1.0 | 6.0 | 0.0 |
| 27 | 6.0 | 12.0 | 11.0 | 14.0 | 15.0 | 14.0 | 9.0 | 7.0 | 7.0 | 15.0 | 12.0 | 14.0 | 14.0 | 6.0 | 7.0 | 8.0 | 1.0 | 7.0 | 11.0 | 14.0 | 14.0 | 7.0 | 8.0 | 5.0 | 4.0 | 6.0 |

**Table 16.** Distance matrix computed using the number of amino acids differences found between pairwise comparisons of each OTU. All Dama alleles are num 1 – 27 and highlighted in colors: (yellow = private blesbok alleles, light blue = private bontebok alleles, and green = shared alleles.

substitutions (dS=0.047; SE = 0.040) in the PBR for all alleles (Table 17). In the non-PBR region, substitutions were found to occur much less frequently (dN = 0.049; SE = 0.011 : dS = 0.019; SE = 0.012).

The number of replacement sites in the α-helix (positions 174-250) of exon 2 was compared to that of the β-sheet (positions 1-168) in order to define the region where selection pressure is greatest. In both subspecies, the number of replacement sites in the PBR was nearly equal in both the α-helix and the β-sheet. Moreover, the number of synonymous changes within exon 2 of Dama alleles falls far below the rate substitution of cattle and most other ruminants (Mikko et al. 1999). This result suggests that diversity of Dama alleles has evolved rapidly compared to cattle and other species (Table 18).

Neighbor-joining (minimum evolution), maximum likelihood and maximum parsimony trees based on 249 bp of exon 2 were constructed using Dama alleles and outgroup sequences derived from horse, cow and moose. Table 19 lists the sequence parameters derived from a maximum likelihood analysis that were incorporated for subsequent phylogenetic inference. Genetic distances were estimated by using the HKY model (Hasegawa et al. 1985) and the Kimura 2-parameter model (Kimura et al. 1980) for the minimum evolution (ME) analysis. Both distance measures produced strongly supported trees of equal topology revealing three evolutionary lineages of Dama alleles (Figure 25). The phylogeny constructed from the maximum likelihood estimation recapitulates this evolution. Most of the alleles found in the bontebok subspecies cluster together within group A (Dama*8, 14, 16, 22, 23), while Dama*7 clusters in C. The maximum parsimony analysis estimated 1005 equally parsimonious trees due to the lack of informative sites (39 out of 249 total sites). Although the number of informative sites was low, the MP analysis estimated a consensus tree topology (using 50% majority rule) similar to the ME and ML trees (tree not shown).

**Table 17.** Jukes-Cantor corrected proportion of dS and dN substitutions.

| Exon 2 | PBR dN | PBR dS | P-value | Non-PBR dN | Non-PBR dS | P-value |
|---|---|---|---|---|---|---|
| Full length | 29.8(6.6) | 4.7(4.0) | ** | 4.9(1.0) | 1.9(1.1) | ns |
| β sheet | 32.1(11.1) | 1.9(2.7) | ** | 5.4(1.4) | 2.3(1.9) | ns |
| α helix | 32.8(11.6) | 8.0(8.3) | * | 3.0(1.6) | 0.5(0.5) | ns |

*Note - The positions involved in the peptide binding region (PBR) were inferred using human DRB1 sequence data (Brown et al. 1993). Exon 2 was divided according to the coding regions for the β sheet (postions 1-168) and the α helix (postions 174-249). Significance values were estimated using the student t-test and are indicated by * <0.1, ** <0.05, *** <0.001 and ns (non-significant).

**Table 18**. Species comparison of exon 2 diveristy within homologous DRB loci.

| Species | # of alleles | PBR dN | PBR dS | Non-PBR dN | Non-PBR dS | P value |
|---|---|---|---|---|---|---|
| Cattle | 68 | 41.1(5.1) | 13.0(6.6) | 4.4(0.7) | 2.2(1.2) | <0.001 |
| Sheep | 20 | 29.8(5.6) | 5.2(5.5) | 3.1(0.6) | 1.5(0.7) | <0.01 |
| Moose | 10 | 11.3(3.2) | 0 | 0 | 1.1(1.1) | <0.001 |
| Roe deer | 3 | 23.5(7.4) | 2.5(4.6) | 1.8(0.9) | 2.8(2.0) | <0.05 |
| Reindeer | 11 | 28.0(5.9) | 9.8(7.4) | 3.9(1.0) | 3.1(1.7) | <0.05 |
| Red deer | 49* | 42.7(5.5) | 10.5(4.8) | 6.4(0.9) | 6.0(1.8) | <0.001 |
| Blesbok/Bontebok | 27 | 29.9(6.6) | 4.7(4.0) | 4.9(1.0) | 1.9(1.1) | <0.01 |

*Note - Percentage relative frequencies of non-synonymous (replacement site) and synonomous (silent) substitutions within the peptide binding region (PBR) and non binding regions (non-PBR). The * indicates that alleles were derived from more than one locus. Adapted from Mikko et al. 1999.

**Table 19.** Estimation of nucleotide sequence parameters of Dama alleles for phylogenetic analysis.

| Parameters | Estimates |
|---|---|
| Base frequencies | |
| A | 0.225760 |
| C | 0.234422 |
| G | 0.348934 |
| T | 0.190884 |
| Ts:Tv ratio | 0.778616 |
| Proportion of invariant sites | 0.290924 |
| α shape parameter | 0.811716 |
| # informative sites | 39.0 |

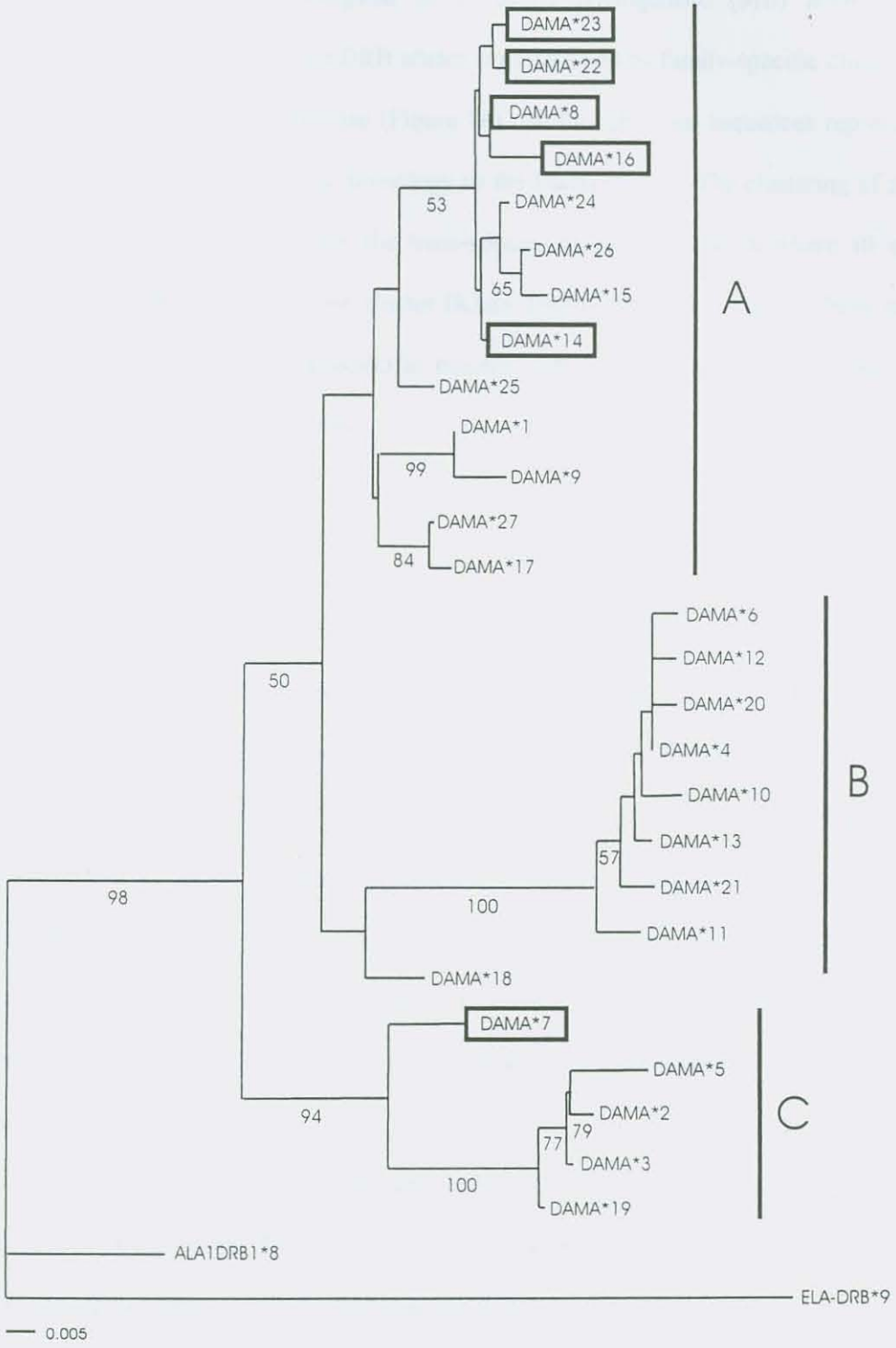



**Figure 25.** Minimum evolution tree of Dama alleles rooted by horse (ELA-DRB*9) and moose (ALA1DRB1*8) sequences. The tree topology displays three evolutionary lineages of Dama alleles. Bootstrap vaules (1000 iterations) are indicated at the nodes. Bontebok alleles are indicated by boxes.

The relationship between Dama alleles and the domestic cow (*Bos indicus* and *Bos tarus* = BoLA) alleles was investigated in a second phylogenetic (ME) analysis. The evolutionary relationship of the DRB alleles is represented by family-specific cluster as all sequences cluster into Bovidae (Figure 26). In this analysis, sequences representing 39 major BoLA lineages strong homology to the Dama alleles. The clustering of alleles from the bovid species displays the trans-species mode of evolution where all alleles derived from Bovidae form one cluster (Klein 1986). However, Dama alleles cluster strongly together in a species-specific manner, which is indicative of the large time divergence between these species.

## DISCUSSION

The DRB3 homologue was characterized in *Damaliscus pygargus* in order to study the evolution of this MHC class II locus in an exotic species of bovid, and to reveal the impact of population collapse on DRB3 polymorphism in the bontebok subspecies. In this study I have presented 27 novel ungulate DRB alleles from a threatened species of antelope that are distantly related to the cow. The relative levels of allelic diversity in the two subspecies were consistent with their different demographic histories as well as their geographic subdivision. Furthermore, this investigation provides evidence for the mechanisms of trans-species evolution and balancing selection acting on the DRB locus in a new species.

The DRB profiles of each subspecies reflect past demographic histories and geographic partitioning. The blesbok subspecies had large and sustainable populations throughout the South African grasslands since the first recorded European settlement in the 17$^{th}$century. However, the Great Trek brought the settlers and their guns northward, and with this human migration, blesbok numbers sharply declined due to over hunting.

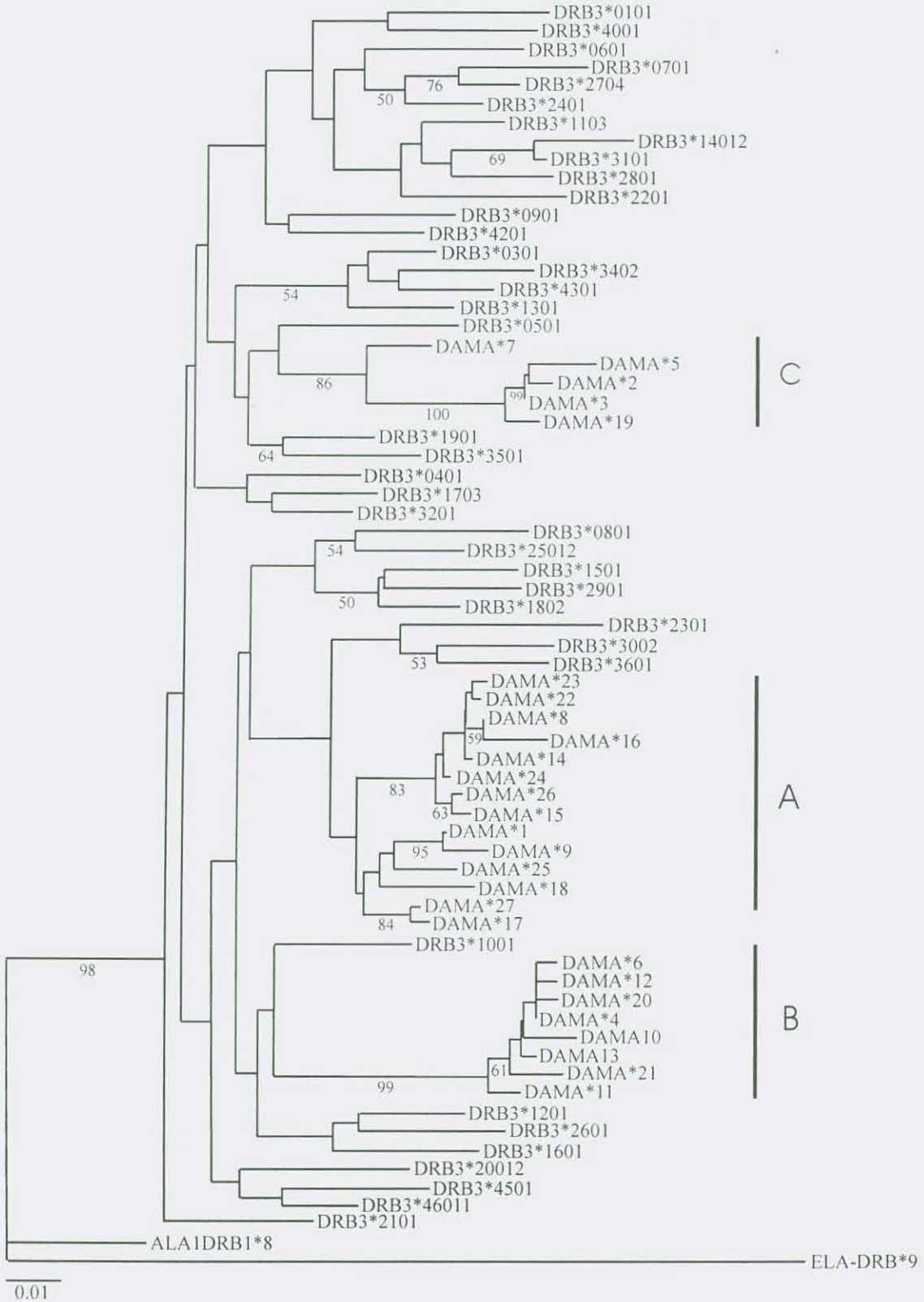



**Figure 26.** Minimum evolution tree of Dama and Bola-DRB alleles. The tree topology demonstrates the trans-species mode of evolution at this locus as well as species specific clustering of Dama alleles. Bootstrap values <50 are indicated at tree nodes.

The DRB3 data suggests that much of the allelic variation, unlike the mitochondrial diversity, has been retained. The considerable degree of polymorphism observed from our analysis suggests that the DRB diversity must have been generated through large numbers of breeding herds over a long period of time. Historical records have documented a drastic reduction of blesbok numbers throughout their range during the late 1800's; however, an exact census was never known. The founding population was most likely in the hundreds or even thousands in order for the population to increase to 64,000 individuals in the 1960's. The populations that survived into the 20$^{th}$ century were fenced off to prevent further hunting pressure but in doing so, also prohibited the exchange of new alleles between populations. Although there are no existing natural migrations of blesbok, movement of animals has been facilitated by translocation events. This type of management has led to the introduction of alleles from allopatric populations thus increasing diversity.

However, the relict population of bontebok in the southwestern region of the country appears to have maintained only six alleles from the recent founding population. Historic bontebok populations were restricted to a small region bound by a mountain range and ocean with no opportunity for migration. More recently, genetic variation was depleted by population collapse leaving only a residual amount of diversity from the 27 founding individuals that were saved in 1839. The effective population size (*Ne*) was not sufficient enough to re-establish polymorphism (Nei, 1975).

The allelic diversity of each subspecies demonstrates a significant amount of genetic substructure within the species. Two alleles (Dama*7 and *8) were shared between the subspecies while all others (n = 25) were private to each subspecies. MHC diversity measured in other species including chinook salmon runs (Kim et al. 1999) and beluga whale populations (Murry et al. 1999) was reported as significantly differentiated.

The genetic differences at this locus can be used to differentiate between bontebok and blesbok subspecies as well as identify hybrid animals. In one case, blesbok and bontebok alleles were found in a hybrid population indicating that at some point there had been genetic admixture in that herd.

There are several possible explanations for the robust structuring of the DRB locus, an unexpected result given the trans-specific pattern of evolution observed for MHC loci (see Klein 1987;below). First, private alleles in one subspecies may be present in the other but failed to be sampled in this analysis. However, the sample size in this study was adequate (178 chromosomes examined) and the populations sampled were representative of the species distribution across South Africa. Of course, extensive sampling could ultimately uncover more shared alleles as well as additional novel alleles. Second, the partitioning of DRB alleles may represent the phylogeography of the species as bontebok and blesbok populations have been separated by the expanse of the Karoo Desert. Some lineage sorting could have occurred in these geographically isolated populations as a result of genetic drift, especially given the population contractions experienced by the bontebok (cf. Yuhki and O'Brien 1990). This scenario is probably the most likely, given the relatively small number of alleles found in bontebok. Some novel alleles may represent new mutations, possibly maintained by heterotic selection. Lastly, the private alleles may have had adaptive value to the subspecies and signify historic selective event. Because the bontebok subspecies experienced a second bottleneck event that was caused partly by parasitic infection, certain alleles may have been selected in response to presentation of the foreign antigen (Hamilton 1980).

A comparison of amino acid sequences showed extensive polymorphism within the PBR. The relative frequencies of nonsynonymous substitutions exceed that of synonymous substitutions in the PBR, suggesting that positive selection has maintained

the allelic polymorphism in this species (Hughes and Nei 1989). The number of replacement sites in the α-helix (positions 174-250) of exon 2 was compared to that of the β-sheet (positions 1-168) in order to define the region where selection pressure is greatest (Schwaiger et al. 1993). The similar values and tree topologies (trees not shown) indicate that selection pressure is nearly equal in both sequence regions. Moreover, the bovid phylogeny illustrates the conservation of ancestral polymorphisms that could not exist under a neutral model of evolution (Takahata and Nei 1990). The close homology of Dama alleles to other bovid sequences reflects the transpecies mode of evolution of this MHC locus that has been documented in primates (Mayer et al. 1988), ungulates (Andersson 1994), felines (Yuhki and O'Brien 1997), pinnepeds (Hoelzel et al. 1999) and rodents (Figuera et al. 1988).

These results further indicate that the selective retention of diversity may be through heterosis whereby heterozygous individuals have a selective advantage. It is assumed that allelic diversity at Class II MHC genes evolves to promote pathogen resistance (Doherty and Zinkernagel 1975, O'Brien and Evermann 1988). The striking difference between the levels of the neutral nuclear loci and the coding DRB locus indicates that MHC heterozygosity is generated and maintained by a selective force (Hedrick and Thompson 1983). This pattern of diversity, displaying elevated numbers of heterozygotes for polymorphic MHC class II genes, has been well documented in many outbred species and in humans (Hedrick 1994, Markow et al. 1993).

Disassortative (non-random) mating has been proposed to play a role in selection of alleles whereby animals with dissimilar genotypes mate in order to avoid inbreeding within the population (Potts et al. 1994). Mate selection according to MHC genotypes has been documented in several vertebrate species (see Jordon and Bruford 1998 for review); however, MHC-based mating patterns have been excluded as a mechanism for

generating allelic diversity in Soay sheep (Paterson and Pemberton 1997). There is no evidence to describe disassortive mating in bontebok, and other authors question the capacity for this selective force to account for the large number of amino acid site changes found within the PBR (Yeager and Hughes 1999). It is most likely that a combination of evolutionary mechanisms has contributed to the enrichment of diversity found within both subspecies.

The phylogeny of full-length exon 2 Dama alleles revealed three distinct lineages of the DRB locus in this species. Alleles of each lineage are characterized by short branch lengths, which indicate small genetic distances and a recent origin and evolution. The genetic distances among the clusters were greater. Exon 2 was divided into α-helix and the β-sheet coding regions. An equal number of replacement substitutions were found within the α-helix and the β-sheet coding regions of exon 2. This finding contrasts that of the comparisons made in other ruminant species where far more replacement sites are found within the α-helix regions of cattle, sheep and goats (Schwaiger et al. 1994). It is expected that if the selective force drives the evolution of each region equally, then similar tree topologies will be reconstructed for each region (Schwaiger et al. 1993, Swarbrick et al. 1996).

In a further phylogenetic analysis, Dama alleles were aligned with other ruminant DRB alleles and it was showed that all bovid alleles are monophyletic. The inter-leaving of bovid sequences suggests that the Bovidae lineages were generated prior to the divergence of bovids from a common ancestor 20 million years ago (Vrba 1979). Dama alleles, although interleaved throughout the phylogeny, displayed more species-specific clustering, reminiscent of a phylogeny reconstructed with other ruminant species by Mikko and colleagues (1999). This species-specific clustering suggests that gene diversity of the Dama lineages evolved after the split between the other bovid taxa. This suggests a

possible difference in the selective environment for this species, perhaps especially during the relatively recent radiation of alleles.

High allelic diversity at MHC loci is thought to establish a stronger host defence, thereby increasing individual fitness (Hughes and Nei 1988, Takahata and Nei 1990). So far there are few empirical data sets to substantiate this assumption, and many studies have shown that species having low MHC diversity are still viable (Hoelzel et al. 1999, Mikko et al. 1999). Although bontebok populations are indeed viable and show no apparent morphological signs of inbreeding depression, they are still at risk to diseases that may be transmitted by cattle existing in close proximity to the reserves. Given this possible threat of cattle disease transmission and the lack of MHC variation, bontebok may have a smaller chance of mounting immune responses against pathogens in the long term. One very important difference between the bontebok and other species with low MHC diversity is that population size of this antelope has only expanded to 2,500 – 3,000 individuals since the first bottleneck (1800s). Other species that have undergone severe population crashes such as the Northern elephant seal, American bison and African buffalo have recovered to roughly 100,000 to 1,000,000 animals (Hoelzel et al. 1998; Mikko et al. 1997, Wenink et al. 1998). Although, bontebok are protected against hunting pressure, the status of the subspecies is still critical. Stochastic events (disease, drought) as well as human mediated events (hybridization, poaching) are possible threats to the survival of the remaining populations.

# CHAPTER 5

# CONCLUSIONS

## Chapter 5: Conservation Relevance and Management Recommendations

### *Synthesis of Genetic Findings*

*D. pygargus* has been restricted to the grasslands and coastal plains that lie within the boundaries of South Africa since its origin during the Pleistocene. The evolution of this antelope has been influenced by climatic fluctuations during this epoch along with its tribe members (Arctander et al. 1999). In turn, these periods of change have caused a division of the species into two allopatric groups approximately 1 - 2 million years ago. Moreover, the harsh conditions of the past geologic ice age plus the more recent impact of over-hunting appear to have had drastic effect on the demography of the species reducing the number of mitochondrial lineages to very few. The low level of genetic diversity found at the fast evolving loci (control region and microsatellites) suggests that this alcelaphine species was not as successful as its conspecifics. In contrast, extensive diversity was revealed within the MHC-DRB locus. This high level of DRB polymorphism is far greater than the diversity reported in any other exotic ungulate.

### *Conservation and Evolutionary Units for Taxonomy*

The combination of genetic data can be applied to a conservation structure that is able to satisfy the viewpoints of all competing theories of conservation policy. Firstly, the data revealed strong genetic partitioning between bontebok and blesbok, which supports the recognition of as distinct taxa deserving, separate management. This line of evidence fulfils the systematic conservation perspective in that each taxon is an evolutionary distinct lineage. The conservation efforts would include the continued

management of each subspecies in their respective habitats and, most importantly, the restriction of hybridization. Secondly, the subspecies inhabit different ecosystems that support a diverse array of fauna and flora. The bontebok inhabit renoster shrubland that is part of the fynbos ecosystem of the western Cape. This ecosystem supports a unique kingdom of flora endemic to South Africa. The open grasslands that support blesbok populations throughout South Africa are disappearing at an alarming rate to support mono-culture agriculture crops, commercial forests and cattle farms. The negative consequences of grassland removal will result in the erosion of soil, desertification and extinction of endemic species within this ecosystem. The management of each subspecies in its ecosystem will in effect sustain the biological processes that support life. Lastly, the molecular results satisfy the criteria of evolutionary conservation to preserve genetic diversity of both subspecies. The genetic paucity of the species is evident from the complete monomorphism of mtDNA in bontebok and low amount of diversity in blesbok. Evolutionary theory predicts that genetic diversity permits a species to respond to stochastic changes. From the control region data, I predict that *D. pygargus* experienced demographic crashes possibly during both the Pleistocene and recent times.

***Inbreeding Depression***

Since the early 1980's, biologists have supported the genetic theory of inbreeding between closely related individuals leads to increased risk of extinction of the population. Inbreeding has been suggested to cause fixation of deleterious alleles and well as the loss of adaptive genetic variation (Lande 1988). Strong evidence from empirical studies of captive and laboratory populations (Ralls et al. 1979, Frankham et al. 1999) has correlated the cause of extinction in populations due to inbreeding depression. Moreover, the significance of inbreeding depression in wild populations has been demonstrated in song

sparrows (Keller et al. 1994) and butterflies (Saccheri et al. 1998). Uncovering a direct link between reductions in evolutionary potential within inbred mammal species, however, has been far more difficult to determine (Mikko et al 1999.)

Presently, conservation geneticists are still confronted with the contentious issue of relating depletion of genetic variation and species survival (Crnokrak and Roff 1999). The genetic data from this study clearly indicate a loss genetic diversity within bontebok at both nuclear and mitochondrial loci; however, there are no apparent signs of inbreeding depression in the present populations. Similar findings have been demonstrated in other mammalian species where inbred populations were able to recover, reproduce and flourish, despite low genetic variation (Hoelzel et al. 1993, Broders et al. 1999, Le Page et al. 2000).

***Fitness traits***

In order to relate the genetic variation of inbreeding depression to the phenotypic traits in a population, polymorphic neutral loci are chosen for analysis. It has been argued that since these genetic "markers" are non-coding, they are not a good indication of fitness (Caro et al. 1994). However, this argument does not consider the fact that inbreeding will reduce heterozygosity levels at the marker loci and other gene regions including those that control fitness traits.

Long-term behavioral and demographic studies of a species are needed in order to make assumptions about the relationship of genetic data to fitness traits of individuals. This present study focussed on only the genetic aspects of bontebok; however, I will attempt to describe the current population fitness measurements with respect to the genetic data.

The low molecular diversity uncovered within bontebok was expected given its demographic history. The subspecies' population size has remained relatively low compared to other ruminant species that have undergone severe bottleneck or founder events. For example, a population of moose in Newfoundland, Canada was founded by merely 6 individuals in the early 1900's and has since exploded to over 500,000 animals (Broders et al. 1999). Many factors including environmental conditions, lack of predators, stable sex ratios and reproductive success may have promoted the expansion of this moose population. According to the recent demographic history of bontebok, one or more of these factors may have prevented the growth of its population size.

It has been suggested that high inbreeding in animals may have a strong negative affect on sperm morphology and ejaculate quality (Roldan et al. 1999) and ultimately influence the success rate of fertilization. A cursory study of bontebok sperm morphology was reported by Skinner et al. (1980) in response to the low lambing rate within BNP. Unfortunately, this study was not a thorough investigation of sperm traits that would have led to a conclusion regarding fertilization success of bontebok rams (see Gomendio et al. 2000). Further sound reproductive studies may be able to link the genetic data with individual fitness so that the low lambing rate may be explained.

This investigation has provided a comprehensive genetic survey of bontebok that can be used to monitor populations for future management. Although, I have inferred that inbreeding has occurred within the subspecies, one cannot predict the effect which low genetic variation will have on the future evolutionary potential. The descendents of the bottleneck populations were able to survive and persist over time suggesting that the residual genetic variation was suitable to support viable populations. However, in the present, species are challenged with unnatural human mediated pressures such as habitat fragmentation, pollution, hunting and translocations. These new destructive forces create

a situation where the adaptive potential of the species is stretched to the limit, where genetic variation is crucial for long-term survival.

***Hybridization Detection***

Illegal translocation events have led to the hybridization between bontebok and blesbok subspecies. Although movements of animals are now tightly regulated between provinces, the initial translocation events have produced a large number of hybrids throughout South Africa. This has caused numerous problems in the conservation and management of both subspecies for conservation agencies. One of the objectives of bontebok conservation is to preserve the genetic integrity of the subspecies. In order to do so, the genetic purity of both bontebok and blesbok herds needs to be confirmed before animals can be moved between populations. Purity of bontebok herds located within national reserves and most private farms is verified by documentation. A discriminant analysis test of phenotypic traits has also been used to differentiate between hybrids and pure subspecies. In cases where there is doubt of classification, animals were tested by genetic markers.

Bontebok from two farms, Wag 'n Beitjie (WB=10) and Fairview (Bb=5) were analyzed for genetic purity using three classes of molecular markers. The control region was used to detect maternal introgression within each herd. The disadvantage of this method, of course, is that this mitochondrial gene is inherited maternally and would not pick up paternal introgression. The DRB gene provided private alleles for each subspecies and is a potential gene marker for detecting hybridization events from both sexes. Hybrids at the F1 level could readily be identified using only one marker, however, hybridization within further generations (F2, F3, ...) would require a panel of

genetic markers to uncover admixture. In this regard, I used a set of polymorphic microsatellite loci with relatively high heterozygosity and Fst values to detect possible hybrids. A large proportion of private alleles were found within each subspecies. These allele frequencies were used in two methods of classification. Individuals were assigned to subspecies designations based on each multilocus genotypes. The methods of classification included a clustering analysis and a likelihood approach. In both cases, suspected hybrids were places within the bontebok category.

The results from all WB bontebok samples indicate genetic purity. The microsatellite analysis picked up a few rare alleles that were not found within either subspecies. However, the microsatellite test was not able to accurately detect hybridization within the Bb samples or correctly classify test animals in simulation tests. Two blesbok haplotypes were found with the population, clearly indicating maternal introgression. The DRB genotype of Bb3 revealed alleles not found within bontebok, which may be blesbok specific.

The small sample size of the test group is problematic in determining the strength of the genetic tests for hybrid detection. Many of the bontebok specific alleles occurred at low frequencies while alleles shared between subspecies were found at high frequencies. The large reduction of bontebok *Ne* may have caused the loss of potentially private alleles. Additional microsatellite loci could enhance the power of hybrid detection. Furthermore, microsatellites linked to coding genes may show a greater degree of fixation than the neutral evolving microsatellite markers.

### *Management Recommendations*

I have identified two evolutionary lineages of *D. pygargus* and recommend that each be managed separately. The genetic evidence reported here supports the

classification of subspecies as units for conservation. This suggestion is based in part from the genetic evidence that confirms that they are historically isolated and evolve independently from one another. Furthermore, the DRB data demonstrated possible adaptive significance between the different subspecies specific alleles. In this regard, conservation of each subspecies will maintain the ecological and evolutionary processes of both bontebok and blesbok.

The genetic diversity remaining in bontebok should be conserved for future evolutionary change. In this regard, efforts should focus on enhancing population numbers as well as genetic exchange between existing populations. The controversy still remains as to how large a population size must be in order to preserve evolutionary potential. Results of some quantitative genetic studies have shown that small populations (100 individuals) are extremely susceptible to fixation of deleterious alleles and loss of adaptive potential. In addition, these studies suggest that a minimum of 1,000 breeding individuals is needed to avoid rare deleterious mutations from fixating in the population. The total population size should be one order of magnitude greater than the effective population size (*Ne*). In general, an (*Ne*) of approximately 500-1,000 individuals would allow for retention of evolutionary potential (Franklin and Frankham 1998). The present population size of bontebok is 2,500-3,000, which is well within the projected limit.

***Biodiversity in South Africa***

The successful recovery of bontebok is shadowed by the loss of another endemic South African antelope species called the blue antelope (*Hippotragus leucophaeus*), which went extinct in 1800. It has been suggested that changes in climate during the late Pleistocene reduced viable grasslands in the cape region for the blue antelope and

bontebok (Klein 1983). More recently, habitat destruction before European settlement may also have attributed to their decline (Robinson et al 1996). From the historical data, it appears that both antelope lived in similar conditions and experienced severe reductions in population size. Fortunately, the bontebok was saved from extinction and continues to contribute to the biodiversity of the cape region.

## SUMMARY

A comprehensive molecular study was conducted in order to reveal levels of genetic polymorphism in bontebok and blesbok antelope and to define the degree of genetic substructure between the two subspecies. Gene markers were also used to assign individuals to specific categories (subspecies, hybrid). The results of this study can be used in further management of each subspecies.

The levels of genetic diversity revealed in the control region reflected each subspecies demographic history. Only one control region lineage was revealed by SSCP and sequence analysis in bontebok while five others were found in blesbok. The subspecific control region haplotypes demonstrated the unique evolutionary lineages of the species. Sequence divergence estimates were used to approximate a time divergence (1 – 2 MYA) since the two subspecies shared a common ancestor.

Haplotypic diversity of blesbok was compared to other antelope species and found to substantially lower. This finding may be explained by evidence that blesbok failed to expand a range out of South Africa throughout their evolution during the Pleistocene. This endemic antelope may have not been as successful as its conspecifics (topi, wildebeest, hartebeest) in withstanding severe climate changes and as a result declined. However, the control region data does reflect population expansion in the present blesbok populations.

Microsatellite data confirms the genetic partitioning and differing levels of allelic diversity found at the control region. Both sets of gene markers are neutral and expected to be strongly influenced by the effects of genetic drift. Analysis of eight polymorphic

microsatellite loci also revealed many private (subspecies specific) alleles that can be used to assign individuals into subspecies classes through cluster and likelihood analyses. Hybrid detection in a test group of animals was impaired by the high frequency of shared alleles between subspecies. Distributions of allelic frequencies were used to reveal the demographic histories of each subspecies.

The class II major histocompatibility complex (MHC) DRB locus was characterized by SSCP and sequence analysis in both subspecies. Allele sequences were generated in order to assess polymorphism at a coding nuclear gene and uncover mechanisms that govern maintenance of polymorphism. The results of this study have revealed that the allelic diversity of the African antelope species far exceeds the reported polymorphisms of any other wild ungulate species. High levels of polymorphism were found within the blesbok (23 alleles) while a lack of diversity was recorded in bontebok (6 alleles). These levels of diversity reflect past demographic events of both subspecies. A majority of the polymorphism was found at the antigen binding sites where nonsynonymous changes were significantly greater than synonymous changes. This and the apparent trans-species relationship among alleles in a bovid phylogeny suggest the evolution of diversity by heterosis or frequency dependent selection.

Bontebok antelope have economic and ecological value to South Africa and should be regarded as a conservation priority. The polymorphism remaining in bontebok populations should be conserved through careful breeding and management plans. I suggest that translocation of bontebok between small populations should proceed to establish gene flow and prevent further breeding between closely related individuals.

# CHAPTER 6

# LITERATURE CITED

## Chapter 6: Literature Cited

Allardice, R. and I. G. Gaigher. 1979. Report on the results of a questionnaire survey of distribution, abundance and genetic purity of the bontebok. Cape Dept. of Nature and Environmental Conservation.

Allendorf, F.W. 1983. Gene flow, genetic differentiation amoung populations. In: Genetics and Conservation: A Reference for Managing Wild Animal and Plant Populations C.M. Schonewald-Cox, S.M. Chambers, F. McBryde and L. Thomas (Eds.), Benjamin/Cummings, Menlo Park, CA. pp. 241-261.

Ammer, H., F. Schwaiger, C. Kammerbauer, M. Gomolka, A. Arriens, S. Lazary and J. T. Epplen. 1992. Exonic polymorphism vs intronic simple repeat hypervariability in MHC-DRB genes. Immunogenetics 35:332-340.

Amos, B. and A.R. Hoelzel. 1991. Long term preservation of whale skin for DNA analysis. Rep. Int. Whaling Comm. (Special Issue)13:99-104.

Anderson, S., M.H.L. de Bruin, A.R. Coulson, I.C. Eperon, E. Sanger and I.G. Young. 1982. Complete sequence of bovine mitochondrial DNA: conserved features of the mammalian mitochondrial genome. J. Mol. Biol. 156:683-717.

Andersson, L., S. Sigurdardottir, C. Borsch and K. Gustafsson. 1991. Evolution of MHC polymorphism: extensive sharing of polymorphic sequence motifs between human and bovine DRB alleles. Immunogenetics. 33:188-193.

Ansell 1972, W. F. H. Family Artiodactyla. In: The Mammals of Africa: An Identification Manual. J. Meester and H. W. Setzer (Eds.), Smithsonian Institue Press, Washington D.C., pp. 15-83.

Arctander, P., C. Johanson and M. Coutellec-Vreto. 1999. Phylogeography of three closely related African bovids (Tribe Alcelaphini). Mol. Biol. Evol. 16(12):1724-1739.

Avise J. 1989. A role for molecular genetics in recognition and conservation of endangered species. TREE. 4:279-281.

Avise J. 1994 Conservation genetics. In: J. Avise (Ed.), Molecular Markers, Natural History and Evolution. Chapman and Hall, NY, pp. 361-399.

Ballard, J.W. and M. Kreitman. 1995. Is mitochondrial DNA a strictly neutral marker? TREE. 10(12):485-488.

Barnard, P. J. and K. van der Walt. 1961. Translocation of the bontebok (*Damaliscus pygargus*) from Bredasdorp to Swellendam. Koedoe 4:105-109.

Bigalke, R. 1955. The bontebok *Damaliscus pygargus* (Pall.) with special reference to its history and preservation. Fauna and Flora 6:95-116.

Blott, S. C., J. L. Williams and C. S. Haley. 1999. Discriminating among cattle breeds using genetic markers. Heredity 82:613-619.

Blum, J.S. and P. Creswell. 1998. Role for intracellular proteases in the processing and transport of class II HLA antigens. Proc. Natl. Acad. Sci. USA. 85:3975-3979.

Bodmer, W. 1972. Evolutionary significance of the HLA system. Nature 237:139-145.

Borries, C., K. Launhardt, C. Epplen, J. T. Epplen and P. Winkler. 1999. DNA analyses support the hypothesis that infanticide is adaptive in langur monkeys. Proc. R. Soc. Lond. B. 266:901-904.

Bowcock, A., Ruiz-Linares, J. Tomforhde, E. Minch, J. R. Kidd and L. L. Cavalli-Sforza. 1994. High resolution of human evolutionary trees with polymorphic microsatellites. Nature. 368:455-457.

Bowman and Falconer 1960. Inbreeding depression and heterosis of litter size in mice. Genet. Res. 1:262-274.

Boyce, W., P. Hedrick, N. Muggli-Crockett, S. Kalinowski, M. Penedo and R. Ramey II. 1997. Genetic variation of the major histocompatibility complex and microsatellite loci: a comparison in bighorn sheep. Genetics. 145:421-433.

Boyce, W., R. Ramey II, T. Rodwell, E. Rubin and S. Singer. 1999. Population subdivision among desert bighorn sheep (*Ovis canadensis*) ewes revealed by mitochondrial DNA analysis. Mol. Ecol. 8:99-106.

Briles, W., H. Stone and R. Cole. 1977. Marek's disease: effects of B histocompatibility alloalleles in resistant and susceptible chicken lines. Science 195:193-195.

Broders, H. G., S. P. Mahoney, W.A. Montevecchi and W. S. Davidson. 1999. Population genetic structure and the effect of founder events on the genetic variability of moose, Alces alces, in Canada. Molecular Ecology 8:1309-1315.

Brown J.H., T.S. Jardetzky, J.C. Gorda, L.J. Stern , R.G. Urban, J.L. Strominger and D.C. Wiley. 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364: 33-39.

Brown, W.M., M. George Jr, A.C. Wilson. 1979. Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. USA 74:3942-3946.

Brown, W.M., E.M. Prager, A. Wang and A.C. Wilson. 1982. Mitochodrial DNA sequences of primates: tempo and mode of evolution. J. Mol. Evol. 18:225-239.

Bryden, H.A. 1893. Gun and camera in Southern Africa. London, Eduard Stanford, pp. 544.

Buford, M. W. and R. K. Wayne. 1993. Microsatellites and their application to population genetic studies. Cur. Op. Gen. and Dev. 3:939-943.

Callen, D. F., A. D. Thompson, Y. Shen, H. A. Phillips, R. I. Richards, J. C. Mullens and G. R. Sutherland. 1993. Incidence and origin of null alleles in the (AC)n microsatellite markers. Am. J. Hum. Genet. 52:922-927.

Cann, R.L., W.M. Brown and A.C. Wilson. 1984. Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. Genetics 325:31-36.

Caro, T. M. and M. K. Laurenson 1994. Ecological and genetic factors in conservation: a cationary tale. Science 263:485-486.

Cassens, I., R. Tiedemann, F. Suchentrunk and G.B. Hartl. 2000. Mitochondrial DNA variation in the European otter (*Lutra lutra*) and the use of spatial autocorrelation analysis in conservation. J. Heredity. 91(1):31-35.

Cavalli-Sforza, L. L. and A. W. F. Edwards. 1967. Phylogenetic analysis: models and estimation procedures. Am .J. Hum. Genet. 19:233-257.

Cerling, T. E. 1992. Development of grasslands and savannas in East Africa during the Neogene. Palaeologeogr. Palaeoclimatol. 97:241-247.

Charlesworth , D. and B. Charlesworth 1987. Inbreeding depression and its evolutionary consequences. A. Rev. Ecol. Syst. 18:237-268.

Chen QY, W. Huang, J.X. She, F. Baxter, R. Volpe and N.K. Maclaren. 1999. HLA-DRB1*08, DRB1*03/DRB3*0101, and DRB3*0202 aresusceptibility genes for Graves' disease in North American Caucasians, whereas DRB1*07 is protective. Clin. Endocrinol. Metab. 84(9):3182-6

Crnokrak , P. and D. A. Roff 1999. Inbreeding in the wild. Heredity 83:260-270.

Coltman, D. W., W. D. Bowen and J. M. Wright. 1998. Birth weight and neonatal survival of harbor seal pups are positively correlated with genetic variation measured by microsatellites. Proc. R. Soc. Lond. B. 265:803-809.

Coltman, D., J. Pilkington, J. Smith and J. Pemberton. 1999. Parasite-mediated selection against inbred Soay sheep in a free-living island population. Evolution. 53(4):1259-1267.

Cornuet, J., S. Piry, G. Luikart, A. Estoup and M. Solignac. 1999. New methods in employing multilocus genotypes to select or exclude populations as origins of individuals. Genetics 158:1989-2000.

Coulson, T., J. M. Pemberton, S. D. Albon, M. Beaumont, T. C. Marshall, J. Slate, F. E. Guinness and T. H. Clutton-Brock. 1998. Microsatellites reveal heterosis in red deer. Proc. R. Soc. Lond. B. 265:489-495.

Coulson, T., S. Albon, J. Slate and J. Pemberton. 1999. Microsatellite loci reveal sex-dependent responses to inbreeding and outbreeding in red deer calves. Evolution 53(6):1951-1960.

Crandall, K. A., O. R. P. Dininda-Emonds, G. M. Mace and R. K. Wayne. 2000. Considering evolutionary processes in conservation biology. TREE 15(7):290-295.

Crow, J. F. 1948. Alternative hypotheses to hybrid vigor. Genetics 33:477-487.

Dallas, J. F. 1992. Estimation of microsatellite mutation rates in recombinant inbred strains of mouse. Mammalian Genome 3:452-456.

David, J. H. M. 1973. The behavior of the bontebok (*Damaliscus dorcas dorcas*), (Pallas 1766), with special reference to territorial behavior. Z. Tierpsychol. 33:38-107.

David J.H. M. 1997. Family Bovidae,blesbok In: *The complete book of Southern African mammals* (ed. Mills G., Hes L), pp. 257. Struik Publishing Group (Pty) Ltd Cape Town South Africa.

Di Rienzo, A. A.C. Peterson, J.C. Garza, A.M. Valdes, M. Slatkin and N.B.Freimer. 1994. Mutational processes of simple-sequence repeat loci in human populations. Proc. Natl. Acad. Sci. USA. 91:3166-3170.

Doherty P.C. and R.M. Zinkernagel. 1975. Enhanced immunological surveillance in mice heterozyogus at the H-2 gene complex. Nature 256:50-52.

Douzery, E. and E. Randi. 1997. The mitochondrial control region of Cervidae: evolutionary patterns and phylogenetic content. Mol. Biol. Evol. 14(11):1154-1166.

Drobnis, E. Z. and J. W. Overstreet. 1992. Natural history of mammalian spermatozoa in the female reproductive tract. Oxford. Rev. Reprod. Biol. 14:1-45.

Edwards, S. and P. Hedrick. 1998. Evolution and ecology of MHC molecules: from genomics to sexual selection. TREE. 13(8):305-311.

Elder, J.K. and E.M. Southern. Computer assisted analysis of one-dimensional restriction fragment gels. In: Nucleic Acid and Protein Sequence Analysis- A Practical Approach. (Eds.) Bishop, M.J.and C.J. Rawlings. Oxford:IRL Press. pp.:165-172.

Ellegren, H., C.J. Davies and L. Andersson. 1993. Strong association between polymorphisms in an intronic microsatellite and in the coding sequence of the BoLA-DRB3 gene: implications for microsatellite stability and PCR-based DRB3 typing. Animal Genetics 24:269-275.

Ellis, S. and K. Ballingall. 1999. Cattle MHC: evolution in action? Immunological Rev. 167:159-168.

Epifanio, J. M. and D. P. Phillip. 1997. Sources of misclassifying genealogical origins in mixed hybrid populations. J. Heredity 88(1):62-65.

Estes R. 1991. The behavior guide to African mammals. University of California Press, Berkley CA., pp. 270-272.

Estoup, A., L., Garnery, M. Solignac and J. Cornuet. 1995. Microsatellite variation in honey bee (Apis mellifera L.) populations: heirarchical genetic structure and test of the infinite alleles and stepwise mutation models.

Estoup, A., J. Cornuet, F. Rousset and R. Guyomard. 1999. Juxtaposed microsatellite systems as diagnostic markers for admixture: theoretical aspects. Mol. Biol. Evol. 16(7):898-908.

Fabricius C., D. Lowery and P. van den Berg. 1988. Fecund black x blue wildebeest hybrids. S. Afr. J. Wildl. Res. 18:35-37.

Fabricius, C. D., H. J. van Hensbergen and W. Zucchini. 1989. A discriminant function for identifying hybrid bontebok x blesbok populations. S. Afr. J. Wildl. Res. 19(2):61-66.

Felsenstein, J. 1993. PHYLIP (Phylogenetic Inference Package) version 3.5. Department of Genetics, University of Washington, Seattle.

Fernando, P., M. Pfrender, S. Encalada and R. Lande. 2000. Mitochondrial DNA variation, phylogeography and population structure of the Asian elephant. Heredity. 84:362-372.

Figuera, F., G. Eberhart and J. Klein. 1988. MHC polymorphism pre-dating speciation. Nature. 355:265-267.

Fisher, R.A. 1930. The Genetical Theory of Natural Selection. Oxford University Press, Oxford.

FitzSimmons, N., C. Moritz, S. S. Moore. 1995. Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution. Mol. Biol. Evol. 12(3):432-440.

Flagstad, O., I. Olsaker and K. Roed. 1999. The use of heterologous primers for analysing microsatellite variation in hartebeest Alcelaphus buselaphus. Hereditas 130:337-340.

Frankham, R. 1996. Relationship of genetic variation to population size in wildlife. Conservation Biology :1500-1508.

Frankham, R., K. Lees, M. E. Montgomery, P. R. England, E. H. Lowe and D. A. Briscoe. 1999. Do population size bottlenecks reduce evolutionary potential. Animal Conservation 2:255-260.

Gagneux, P., C. Willis, U. Gerloff, D. Tautz, P. Morin, C. Boesch, B. Fruth, G. Hohmann, O. Ryder and D. Woodroof. 1999. Mitochondrial sequences show diverse evolutionary histories of African hominoids. Proc. Natl. Acad. Sci. USA. 96:5077-5082.

Garza, J., M. Slatkin and N. Freimer. 1995. Microsatellite allele frequencies in human and chimpanzees, with implications for constraints on allele size. Mol. Biol. Evol. 12(4):594-603.

Gatsey, J., G. Amato, E. Vrba, G. Schaller and R. DeSalle. 1997. A cladistic analysis of mitochondrial ribosomal DNA from Bovidae. Mol. Phyl. Evol. 7:303-319.

Gerloff, U., B. Hartung, B. Fruth, G. Hahmann and D. Tautz. 1999. Intracommunity relationships, dispersal pattern and paternity success in a wild living community of Bonobos (*Pan paniscus*) determined from DNA analysis of faecal samples. Proc. R. Soc. Lond. B. 266:1189-1195.

Gentry A. 1992. The subfamilies and tribes of the family Bovidae. Mammal Rev. 22:1-32.

Gilbert, S.C., M. Plebanski, S. Gupta, J. Morris, M. Cox, M. Aidoo, D. Kwiatkowski, B.M. Greenwood, H.C. Whittle and A.V.S. Hill. 1998. Association of malaria parasite population structure, HLA, and immunological antagonism. Science 279:1173-1177.

Gladman D.D, M.B. Urowitz and G.A. Darlington . 1999. Disease expression and class II HLA antigens in systemic lupus erythematosus. Lupus 8(6):466-70.

Glass E.J. and P. Miller. 1994. Induction of effective cross-reactive immunity by FMDV peptides is critically dependent upon specific MHC-peptide-T-cell interactions. Immunology 82:1-8.

Glavac D. and M. Dean. 1993. Optimization of the single-strand conformation polymorphism (SSCP) technique for detection of point mutations *Human Mutation* 4:404-414.

Goldstein, D., A. Linares, L. Cavalli-Sforza and M. Feldman. 1995a. An evaluation of genetic distances for use with microsatellite loci. Genetics. 139:463-471.

Goldstein, D., A. Linares, L. Cavalli-Sforza and M. Feldman. 1995b. Genetic absolute dating based on microsatellites and the origin of modern humans. Proc. Natl. Acad. Sci. USA. 92:6723-6727.

Goldstein, D. B. and D. D. Pollock. 1998. Launching microsatellites: a review of mutation processes and methods of phylogenetic inference. J. Heredity 88:335-342.

Golstein, D., G. W. Roemer, D. A. Smith, D. E. Reich, A. Bergman and R. Wayne. 1999. The use of microsatellite variation to infer population structure and demographic history in a natural model system. Genetics 151:797-801.

Gomendio, M., J. Cassinello and E.R.S. Roldan. 2000. A comparative study of ejaculate traits in three endangered ungulates with different levels of inbreeding: fluctuating asymmetry as an indicator of reproductive and genetic stress. Proc R. Soc. Lond. B. 267:875-882.

Goodman, S. J. 1997. Rst Calc: a collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and determining their significance. Molecular Ecology 6:881-885.

Goodman, S., N. Barton, G. Swanson, K. Abernathy and J. Pemberton. 1999. Introgression through rare hybridization: a genetic study of a hybrid zone between red and sika deer (Genus *Cervus*) in Argyll, Scotland. Genetics. 152:355-371.

Goostrey, A., D. N. Carss, L. R. Noble and S. B. Piertney. 1998. Population introgression and differentiation in the great cormorant *Phalacrocorax carbo* in Europe. Molecular Evolution 7:329-338.

Gottelli, D., C. Sillero-Zubiri, G. D. Applebaum, M. S. Roy, D. J. Girman, J. Garcia-Moreno, E. A. Ostrander and R. K. Wayne. 1994. Molecular genetics of the most endangered canid: the Ethiopian wolf, *Canis simensis*. Molecular Ecology 3:301-312.

Gou, S. and E. Thompson. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 48:361-372.

Green, W. C. H. and A. Rothstein 1997. Translocation, hybridization and the endangered black-faced impala. Conservation Biology 12(2):475-480.

Grubb, P. 1993. Family Bovidae. In: D. E. Wilson and D. M. Reeder (Eds.), Mammal Species of the Word. A Taxonomic and Geographic Reference. Smithsonian Institute Press, Washington, pp. 393-414.

Hamada, H. M. G. Pertino, and T. Kakunga. 1982. A novel repeated element with Z-DNA forming potential is widely found in evolutionarily diverse eukaryotic genomes. Proc. Natl. Acad. Sci. U.S.A. 79:6465-6469.

Hamilton W.D. (1980) Sex versus non-sex parasite. *Oikos* 35,282-290.

Harper, F. 1940. The nomenclature and type localities of certain Old World mammals. J. Mammology. 21:322-323.

Hasegawa, M., H. Kashino and T. Yano T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 21:160-174.

Hassanin, A. and E. Douzery. 1999. The tribal radiation of the family Bovidae (Artiodactyla) and the evolution of the mitochondrial cytochrome b gene. Mol. Phyl. Evol. 13(2):227-243.

Hatfield, T. and D. Schluter. 1999. Ecological speciation in sticklebacks: environment-dependent hybrid fitness. Evolution 53:866-873.

Hedrick, P. W. 1994. Evolutionary genetics of the major histocompatibility complex. American Naturalist. 143(6):945-964.

Hedrick, P. W. 1999. Highly variable loci and their interpretation in evolution and conservation. Evolution 53(2):313-318.

Hedrick P.W. and G. Thomson. 1983. Evidence for balancing selection at HLA. Genetics 104: 449-456.

Hill, A.V.S, C.E.M. Allsopp, D. Kwiatkowski, N.M Anstey, P. Twumasi, P.A. Rowe, S. Bennett, D. Brester, A.J. MacMichael and B.M. Greenwood. 1991. Common West African HLA antigens are associated with protection from severe malaria. Science 352:595-600.

Hoelzel, A.R., J.M. Hancock and G.A. Dover. 1991. Evolution of the cetacean mitochondrial d-loop region. Mol. Biol. Evol. 8:475-493.

Hoelzel, A. R. 1993. Evolution by DNA turnover in the control region of vertebrate mitochondrial DNA. Cur. Op. Gen. and Dev. 3:891-895.

Hoelzel, A. R., J. Halley, S. J. O'Brien, C. Campagna, T. Arnbom, B. Le Boef, K. Ralls and G. Dover. 1993. Elephant seal genetic variation and the use of simulation models to investigate historical population bottlenecks. Jour. of Heredity. 84:434-449.

Hoelzel, A. R., M. Dahlheim and S. J. Stern. 1998. Low genetic variation among killer whales (*Orcinus orca*) in the eastern north Pacific and genetic differentiation between foraging specialists. Journ. of Heredity. 89:121-128.

Hoelzel, A.R., J.C. Stephens, S.J.O'Brien. 1999. Molecular genetic diversity and evolution at the MHC DQB locus in four species of pinnipeds. Mol. Biol. Evol. 16(5): 611-618.

Holm, L. E., M. C. Forchhammer and J. J. Boomsma. 1999. Low genetic variation in muskoxen (*Ovibos moschatus*) from western Greenland using microsatellites. Molecular Ecology 8:675-679.

Hughes, A. L. and M. Nei. 1989. Nucleotide substitution at major histocompatibility complex class II loci: evidence for overdominant selection. Proc. Natl. Acad. Sci. USA. 86:958-962.

Hughes, A.L. and M. Nei. 1992. Maintenance of MHC polymorphism. Nature (London) 355:167-170.

Hughes, A.L., T. Ota and M. Nei. 1990. Positive Darwinian selection promotes charge profile diversity in the antigen-binding cleft of class I major-histocompatibility-complex molecules. Mol. Biol. Evol. 7:515-524.

Hughes, A. and M. Hughes. 1995. Natural selection on the peptide-binding regions of major histocompatibility complex molecules. Immunogenetics. 42:233-243.

Johnson, W.E. and S.J. O'Brien. 1997. Phylogenetic reconstruction of the Felidae using 16S rRNA and NADH-5 mitochondrial genes. J. Mol. Evol. 44:S98-116.

Jordon W.C. and M.W. Bruford. 1998. New perspectives on mate choice and the MHC. Heredity 81: 239-245.

Jukes T.H., and C.R. Cantor. 1969. Evolution of protein molecules In: Mammalian Protein Metabolism (ed. HN Munro),. Academic Press, New York. pp 21-132.

Keller, L.F., P. Arcese, J.N. Smith, W.M Hochachka and S.C. Stearns. 1994. Selection against inbred song sparrows during a natural populationsbottleneck. Nature, Lond. 372:356-357.

Kettlitz, W.K. 1967. The blesbok with special reference to the herd in the Percy Fyfe National Park. Fauna and Flora 18: 36-46.

Kim, T.J., K.M. Parker and P.W. Hedrick. 1999. Major histocompatibility complex differentiation in Sacramento River chinook salmon. Genetics 151:1115-1122.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111-120.

Kimura, M and J. F. Crow. 1964. The number of alleles that can be maintained in a finite population. Genetics 49:725-738.

Kimura, M and T. Ohta. 1978. Stepwise mutation model and distribution of allelic frequencies in a finite population. Proc. Nat. Acad. Sci. U.S.A. 75:2868-2872.

Klein, J. 1980. Generation of diversity at MHC loci: Implications for T-cell receptor repertoires. In: M. Fougereau and J. Dausset (Eds.), Immunology 80. Academic Press, London, pp. 239-253.

Klein J. 1986. Natural History of the Major Histocompatibility Complex. John Wiley and Sons, New York.

Klein, J. and F. Figuero. 1986. Evolution of the major histocompatibility complex. CRC Crit. Rev. Immunology. 6:295-386.

Kohn, M. H., E. C. York, D. A. Kamradt, G. Haught, R. M. Sauvajo and R. K. Wayne. 1999. Estimation population size by genotyping faeces. Proc. R. Soc. Lond. B. 266:657-663.

Krings, M., H. Geisert, R. Schmitz, H. Krainitzki and S. Paabo. 1999. DNA sequence of the mitochondrial hypervariable region II from the Neanderthal type sequence. Proc. Natl. Acad. Sci. USA. 96:5581-5585.

Kumamoto, A. T., S. J. Charter. M. L. Houck and M. Frahm. 1996. Chromosomes of Damaliscus (Artiodactyla, Bovidae): simple and complex centric fusion arrangements. Chromosome Res. 8:614-621.

Laikre, L. 1999. Conservation genetics of Nordic carnivores: lessons from zoos. Hereditas 130:203-216.

Lande, R. 1988. Genetics and demography in biological conservation. Science. 241:1455-1460.

Lande R. 1998. Risk of population extinction from fixation of deleterious and reverse mutations. Genetica 102/103:21-27.

Lewin, H., G. Russell and E. Glass. 1999. Comparative organization and function of the major histocompatibility complex of domesticated cattle. Immunological Rev. 167:145-158.

Litt, M. and J. A. Luty. 1989. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am. J. Hum. Genet. 44:397-401.

Lopez, J., M. Culver, J. C. Stephens, W. Johnson and S. J. O'Brien. 1996. Rates of nuclear and cytomplasmic mitochondrial DNA sequence divergence in mammals. Mol. Biol. Evol.

Luikart, G., W. B. Sherwin, B. M. Steele and F. W. Allendorf. 1998a. Usefulness of molecular markers for detecting population bottlenecks via monitoring genetic change. Molecular Ecology 7:963-974.

Luikart, G., F. W. Allendorf, J. M. Cornuet and W. B. Sherwin. 1998b. Distortion of allele frequency distributions provides a test for recent population bottlenecks. J. Heredity 89:238-247.

Lynch, C. D. 1971. A behavioral study of, *Damaliscus dorcas phillipsi*, with special referernce to territoriality. M.Sc. thesis. University of Pretoria.

MacHugh, D. E., M. D. Shriver, R. T. Loftus, P. Cunningham and D. G. Bradley. 1997. Microsatellite DNA variation and the evolution, domestication and phylogeography of taurine and zebu cattle (Bos taurus and Bos indicus). Genetics 146:1071-1086.

Markow T,. P.W. Hedrick, K. Zuerlein, J. Danilovs, J. Martin, T. Vyvial and C. Armstrong. 1993. HLA polymorphism in the Havasupai, evidence for balancing selection. Am. J. Hum. Genet. 53:943-952.

Marshall, T. C., P. Sunnucks, J. A. Spalton, A. Greth and J. M. Pemberton. 1999. Use of the genetic data for conservation management: the case of the Arabian oryx. Animal Conservation 2:269-278.

Maruya, E., H. Saji and S. Yokoyama. 1996. PCR-LIS (Low ionic strength single-stranded conformation polymorphism)—a simple method for high resolution allele typing of HLA-DRB1, DQB1 and DPB1. Genome Res. 6:51-57.

Mateo L, J. Gardner, Q. Chen, C. Schmidt, M. Down, S.L. Elliott, S.J. Pye, H. Firat, F.A. Lemonnier, J. Cebo, and A. Suhrbier. 1999. An HLA-A2 Polyepitope Vaccine for Melanoma Immunotherapy. Immunology 163(7):4058-4063.

Matthee, C. and T. J. Robinson. 1999a. Cytochrome b phylogeny of the family Bovidae: resolution within the Alcelaphini, Antilopini, Neotragini and Tragelaphini. Mol. Phyl. Evol. 12(1):31-46.

Matthee, C. and T.J. Robinson. 1999b. Mitochondrial DNA population structure of roan and sable antelope: implications for the translocation and conservation of the species. Mol. Ecol. 8:227-238.

Mayer, W.E., D. Jonker, D. Klein, P. Ivanyi, G. van Seventer and J. Klein. 1988. Nucleotide sequence of chimpanzee MHC class I alleles: evidence for transpecies mode of evolution. EMBO 7:2765-2774.

Mayr, E. 1963. Geographic variation. In: Populations, Species and Evolution. Harvard Press, pp. 186-204.

Mayr, E. 1999. Understanding evolution. TREE 14(9):372-373.

McNeely, J.A., K.R. Miller, W.V. Reed, R.A. Mittermeier and T.M. Werner 1990. Conserving the World's Bological Diversity. IUCN. Gland, Switzerland.

Menotti-Raymond, M.A. and S. J. O'Brien. 1995. Evolutionary conservation of ten microsatellite loci in four species of Felidae. J. Heredity 86:319-322.

Miller, L. M. 2000. Classifying genealogical origins in hybrid populations using dominant markers. Jour. of Heredity. 91(1):46-49.

Mikko, S. and L. Andersson. 1995. Low major histocompatibility complex class II diversity in European and North American moose. Proc. Natl. Acad. Sci. USA. 92:4259-4263.

Mikko, S. M. Spencer, B. Morris, S. Stabile, T. Basu, C. Stormont and L. Andersson. 1997. A comparative analysis of the MHC DRB3 polymorphism in the American bison (*Bison bison*). J. Heredity. 88:499-503.

Mikko, S., K. Roed, S. Shmutz and L. Andersson. 1999. Monomorphism and polymorphism at MHC DRB loci in domestic and wild ruminants. Immunological Rev. 167:169-178.

Miller, L. M. 2000. Classifying genealogical origins in hybrid populations using dominant markers. J. Heredity 91(1):46-49.

Minch, E., A. Ruiz-Linares, D. Goldstein, M. Feldman and L. L. Cavalli-Sforza. 1995-1997. MICROSAT (version 1.5d): a computer software program for calculating various statistics on microsatellite allele data. Stanford, CA: Stanford University.

Miththapala, S., J. Seidensticker and S. J. O'Brien. 1995. Molecular genetic diversity within and between subspecies of leopards (*Panthera pardus*)

Mitton, J. B. 1993. Theory and data pertinent to the relationship between heterozygosity and fitness. In: The Natural History of Inbreeding and Outbreeding (Eds.) N.W. Thornhill., pp. 17-41. University of Chicago Press.

Moore, S. S., L. L. Sargeant, T. J. Kings, J. S. Mattick, M. Georges, and D. J. S. Hetzel. 1991. The conservation of dinucleotide microsatellites among mammalian genomes allows the use of heterologous PCR primer pairs in closely related species. Genomics 10:654-660.

Moreau, R. E. 1962. Vissitudes of the African biomes in the late Pleistocene

Morin, P. A., J. J. Moore, R. Chakraborty, L. Jin, J. Goodall and D. Woodruff. 1994. Kin selection, social structure, gene flow, and the evolution of chimpanzees. Science. 265:1193-2101.

Morton, N. E., J. F. Crow and J. H. Miller. 1955. An estimate of the mutational damage in man from data on consanguineous marriages. Proc. Natl. Acad. Sci. USA 42:855-863.

Moritz, C. 1994a. Defining 'evolutionary significant units' for conservation. TREE 9(10):373-375.

Moritz, C. 1994b. Applications of mitochondrial DNA analysis in conservation: a critical review. Mol. Ecol. 3:403-413.

Moritz, C. 1999. Conservation units and translocations: strategies for conserving evolutionary units. Hereditas. 130:217-228.

Murry, B., R. Michaud and B. White. 1999. Allelic and haplotypic variation of major histocompatibility complex class II DRB1 and DQB loci in the St. Lawrence beluga (Delphinapterus leucas). Mol. Ecol. 8:1127-1139.

Nason, J. D. and N. C. Ellstrand 1993. Estimating the frequencies of genetically distinct classes of individuals in hybridized populations. J. Heredity 84:1-12.

Nauta, M. J. and F. J. Weissing. 1996. Constraints on allele size at microsatellite loci: implications for genetic differentiation. Genetics 143:1021-1032.

Neefjes, J.J., F.Momburg and G.J. Hammerling. 1993. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. Science 261:769-771.

Nei, M. 1972. Genetic distance between populations. American Naturalist 106:283-292.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genomics 41:225-233.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Nei, M., T. Maruyama and R. Chakraborty. 1975. The bottleneck effect and genetic variability in populations. Evolution. 52:1-10.

Nei M. and T. Gojobori T. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. 3: 418-426.

Nei, M. and N. Takezaki. 1996. The root of the phylogenetic tree of human populations. Mol. Biol. Evol. 13(1):170-177.

O'Brien, S.J., M. E. Roelke, A. Newman, C. A. Winkler, D. Meltzer, L. Colly, J. F. Evermann, M. Bush and D. E. Wildt. 1985. Genetic basis for species vulnerability in the cheetah. Science 227:1428-1434.

O'Brien, S. J. and J. F. Evermann. 1988. Interactive influences of infectious disease and genetic diversity in natural populations. TREE 3:254-259.

O'Brien, S. J. and E. Mayr. 1991. Species hybridization and protection of endangered animals. Science 253:251-252.

O'Brien, S. J. 1994a. A role for molecular genetics in biological conservation. Proc. Natl. Acad. Sci. USA. 91:5748-5755.

O'Brien, S. J. 1994b. Perspective on conservation genetics. In: (Eds) Schierwater, Streit, Wagner and De Salle. Molecular ecology and evolution: approaches and applications. Birkhauser Verlang Basel, Switzerland. Pp. 275-280.

O'Brien, S. and N. Yuhki. 1999. Comparative genome organization of the major histocompatibility complex: lessons from Felidae. Immunological Rev. 167:133-144.

Ohta, T. and M. Kimura. 1973. The model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a genetic population. Genetical Res. 22:201-204.

Osterhoff, D. R., Ward-Cox I. S. and V. Emslie. 1972. A study of genetic markers in the South African blesbok (*Damaliscus dorcas phillipsi*). Koedoe. 15:49-53.

Paabo, S. 2000. Of bears, conservation genetics and the value of time travel. Proc. Natl. Acad. Sci. USA. 97(4):1320-1321.

Pallas, P. S. 1766. Miscellanea Zoologica. Hagae Comitum. P van Cleet.

Pallas, P. S. 1767. Spielegia Zoologica. Berloni. G. A. Lange.

Palmer, A. R. and C. Strobeck. 1986. Fluctuating asymmetry: measurement, analysis, patterns. Annual Review of Ecology and Systematics 17:391-421.

Palumbi, S. 1989. Rates of molecular evolution and the fraction of nucleotide positions free to vary. J. Mol. Evol. 29:180-187.

Paterson, S. 1998. Evidence for balancing selection at the major histocompatibility complex in a free-living ruminant. J. Heredity. 89:289-294.

Paterson S. and J.M. Pemberton. 1997. No evidence for major histocompatibility-dependent mating patterns in a free-living population. Proc. Royal Soc. Lond, B; 264:1813-1819.

Penrith, M.L., R.C. Tustin, D.J. Thorton and P.D. Burdett. 1996. Swayback in blesbok (*Damaliscus dorcas phillipsi*) and a black wildebeest (*Connochaetes gnou*). J. S. Afr. Vet. Assoc. 67(2):93-96.

Pennock, D. and W. Dimmick. 1997. Critique of the evolutionary significant unit as a definition for distinct population segments under the U.S. endangered species act. Con. Biol. 11(3):611-619.

Pepin, L, Y. Amigues, A. Lepingle, J. Berthier, A. Bensaid and D. Vaiman. 1997. Sequence conservation of microsatellites between Bos taurus (cattle), Capra hircus (goat) and related species. Examples of use in parentage testing and phylogeny analysis. Heredity 74:53-61.

Petren, K., B. R. Grant and P. R. Grant. 1999. A phylogeny of Darwin's finches based on microsatellite DNA length variation. Proc. R. Soc. Lond. B. 266:321-329.

Potts W.K., C.J. Manning and E.K. Wakeland. 1991. Mating patterns in seminatural populations of mice influenced by MHC genotype. Nature 352, 619-621.

Potts WK, C.J. Manning and E.K, Wakeland. 1994. The role of infectious disease inbreeding and mating preferences in maintaining MHC genetic diversity, an experimental test. Phil. Trans. Royal Soc. B. 346:369-378.

Prithiviraj, F., M.E. Pfrender, S.E. Encalada and R. Lande. 2000. Mitochondrial DNA variation, phylogeography and population structure of the Asian elephant. Heredity 84:362-372.

Ralls, K, K. Brugger and J. Ballou. 1979. Inbreeding and juvenile mortality in small populations of ungulates. Science 206:1102-1103.

Ralls, K, J. Ballou and A. Templeton. 1988. Estimates of lethal equivalents and the cost of inbreeding in mammals. Conserv. Biol. 2(2):185-193.

Rassmann, K., D. Tautz, F. Trillmich and C. Gliddon. 1997. The microevolution of the Galapagos marine iguana *Amblyrhynchus cristatus* assessed by nuclear and mitochondrial genetic analyses. Mol. Ecol. 6:437-452.

Rebholz, W.E.R. and E.H. Harley. 1997. Cytochrome b sequences from the endangered Saudi gazelle (*Gazella saudiya*) suggest hybridization with chinkara (*G. bennetti*). Cons. Biol. 11(1):251-255.

Reich, D.E., R.K. Wayne and D.B. Goldstein. 1999. Genetic evidence for a recent origin by hybridization of red wolves. Mol. Ecol. 8:138-144.

Robinson, T. J., D. J. Morris and N. Fairall. 1991. Interspecific hybridization in the Bovidae: Sterility of *Alcelaphus buselaphus* X *Damaliscus dorcas* F1 progeny. Biol. Cons. 58:345-356.

Robinson, T. J. and E. H. Harley. 1995. Absence of geographic chromosomal variation in the roan and sable antelope and the cytogenetics of a naturally occurring hybrid. Cytogenet. Cell. Genet. 71(4):363-369.

Robinson, T. J., A. Bastos, K. Halanych and B. Herzig. 1996. Mitochondrial DNA sequence relationships of the extinct blue antelope *Hippotragus leucophaeus*. Naturwissenschaften. 83:178-181.

Roelke, M., J. Martenson and S. J. O'Brien. 1993. The consequences of demographic reduction and genetic depletion in the endangered Florida panther. Curr. Biol. 3(6):340-350.

Rogers, A.R. and H. Harpending. 1992. Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. 9:552-569.

Rogers, A.R. 1995. Genetic evidence for a Pleistocene population explosion. Evolution 49:608-615.

Roldan, E. R. S. and J. Cassinello, T. Abaigar and M. Gomendio. 1999. Inbreeding, fluctuating asymmetry and ejaculate quality in an endangered ungulate. Proc. R. Soc. Lond. B. 265:243-248.

Rookmaaker, L. C. 1991. The scientific name of the bontebok. Z. Saugetierkunde 56:190-191.

Roques, S., P. Duchesne and L. Bernatchez. 1999. Potential of microsatellites for individual assignment: the North Atlantic redfish (genus Sebastes) species complex as a case study. Molecular Ecology 8:1703-1717.

Rowe, G., T. J. C. Beebee and T. Burke. 1999. Microsatellite heterozygosity, fitness and demography in natterjack toads Bufo calamita. Animal Conservation 2:85-92.

Roy, M. S., E. Geffen, D. Smith and R. K. Wayne. 1996. Molecular genetics of pre-1940 red wolves. Conservation Biology 10(5):1413-1424.

Ryder, O. A. 1986. Species conservation and systematics: the dilemma of subspecies. TREE 1:9-10.

Saccheri, I., M. Kuussaari, M. Kankare, P. Villman, W. Fortlius and I. Hanski. 1998. Inbreeding and extinction in butterfly metapopulations. Nature, Lond. 392:491-494.

Saccone, C., M. Attimonelli, E. Sbisa. 1987. Structural elements highly preserved during the evolution of the D-loop containing region in vertebrate mitochondrial DNA. J. Mol. Evol. 26:205-211.

Saitou, D and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sarmiento, U. and R. Storb. 1990. Nucleotide sequence of a dog DRB cDNA clone. Immunogenetics. 31:396-399.

Scatler, P. and O. Thomas. 1894-1900. The book of antilopes. Vol. 1. R. H. Posser (Ed.) London.

Schneider, S., J. Kueffer, D. Roessli and L. Excoffier. 1999. Arlequin ver.2.0: A software for population genetic data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Schwaiger, F., J. Buitkamp, E. Myers and J.T. Epplen. 1993a. Typing of artiodactyl MHC-DRB genets with the help of intronic simple repeated sequences. Mol. Ecol. 2:55-59.

Schwaiger, F., E. Weyers, C. Epplen, J. Brun, G. Ruff, A. Crawford and J.T. Epplen. 1993. The paradox of MHC-DRB exon-intron evolution—alpha-helix and beta-sheet encoding regions diverge while hypervariable intronic simple repeats co-evolve with beta-sheet codons. J. Mol. Evol. 37: 260-272.

Schwaiger F., E. Weyers, J. Buitkamp, A.J. Ede, A. Crawford, and J.T. Epplen. 1994. Interdependent MHC-DRB exon-plus-intron evolution in artiodactyls. Mol. Biol. Evol. 11: 239-249.

Seddon, J. M. and P. R. Baverstock. 1999. Variation on islands: major histocompatibility complex (Mhc) polymorphism in populations of the Australian bush rat. Molecular Ecology 8:2071-2979.

Shriver, M. D., L. Jin, R. E. Ferrel and R. Deka. 1997. Microsatellite data support an early population expansion in Africa. Genome Res. 7:586-591.

Simonsen, B., H. Siegismund and P. Arctander. 1998. Population structure of African buffalo inferred from mtDNA sequences and microsatellite loci: high variation but low differentiation. Molecular Ecology 7:225-237.

Skead, C. J. 1980. Historical mammal incidence in the Cape Provence. Vol.1. Cape Town. Dept. of Nature and Environmental Conservation

Skinner, J. D., H. M. Dott, V. de Vos and R. P. Millar. 1980. On the sexual cycle of mature bachelor bontebok rams at the Bontebok National Park, Swellendam. S. Afr. J. Zool. 15(2):117-119.

Skinner J. D. and R. H. N. Smithers. 1990. Alcelaphini. In: The mammals of southern *African subregions*. University of Pretoria Press, Pretoria, South Africa, pp. 612-634.

Slatkin, M. 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics 139:457-463.

Sneath, P.H.A. and R.R. Sokal. 1973. Numerical taxonomy. San Francisco. W. H. Freeman.

Sunnocks, P. Efficient genetic markers for population biology. TREE 15(5):199-203.

Swarbrick, P., F. W. Schwaiger, J. Epplen, G. Buchan, J. Frank, T. Griffen and A. Crawford. 1996. Cloning and sequencing of expressed DRB genes of the red deer (*Cervus elephus*) MHC. Immunogenetics. 42:1-9.

Swofford D.L. 1998. Phylogenetic Analysis Using Parsimony (PAUP) version 4.0 University of Illinois, Champaign Illinois.

Taberlet, P, J-J. Camarra, S. Griffen et al. 1997. Non-invasive genetic tracking of the endangered Pyrenean brown bear population. Molecular Ecology 6:869-876.

Takahata, N. and M. Nei. 1990. Allelic genealogy under overdominant selection and frequency-dependent selection and polymorphism of major histocompatibility complex loci. Genetics. 124:967-978.

Takezaki, N. and M. Nei. 1996. Genetic distances and reconstruction of phylogenetic trees from microsatellite data. Genetics 144:389-399.

Tautz, D. 1989. Hypervariability of simple sequences as a general source of polymorphic DNA markers. Nucleic Acids Res. 17:6463-6471.

Taylor, B.L. and A.E. Dizon. 1999. First policy then science: why a management unit based solely on genetic criteria cannot work. Mol. Ecol. 8:S11-S16.

Templeton, A. 1987. Inferences on natural population structure from genetic studies on captive mammalian populations. In: Chepko-Sade, B.D. and Halpin, Z. T. (eds) Mammalian Dispersal Patterns: the Effects of Social Structure on Population Genetics, pp. 257-272. University of Chicago Press, Chicago.

Thornhill, N. W. 1993. The Natural History of Inbreeding and Outbreeding: Theoretical and Empirical Perspectives. University of Chicago Press, Chicago.

Thurz, M.R., H.C. Thomas, B.M. Greenwood and A.V.S. Hill. 1997. Heterozygote advantage for HLA class-II type in hepatitis B virus infection. Nat. Genet. 17:11-12.

Van Eijk MJT, J.A. Stewart-Haynes and Lewin. 1992. Extensive polymorphism of the BoLA-DRB3 gene distinguished by PCR-RFLP. Animal Genetics 23: 483-496.

Van der Merwe, N. J. 1968. Die bontebok. Koedoe 11:161-168.

Van Haeringen, W.A., P.S. Gwakisa, S. Mikko, E. Eythorsdottir, L.E. Holm, I. Olsaker, P. Outteridge and L. Andersson. 1999. Heterozygosity excess at the cattle DRB locus revealed by large scale genotyping of two closely linked microsatellites. Animal Genetics 30:169-176.

Van Valen, L. 1962. Study of fluctuating asymmetry. Evolution 16:125-142.

Vila, C., J. E. Maldonado and R. K. Wayne. 1999. Phylogenetic relationships, evolution and genetic diversity of the domestic dog. J. Heredity. 90(1):71-77.

Vrba E. S. 1979. Phylogenetic analysis and classification of fossil and recent Alcelaphini Mammalia: Bovidae. Biol. J. Linn. Soc. 11:207-228.

Vrba E. S. 1995. The fossil records of African antelopes (Mammalia, Bovidae) in relation to human evolution and paleoclimate. In: E.S. Vrba, G. H. Denton, T. C. Partridge and L. H. Burckle (Eds.), Paleoclimateand evolution, with emphasis on human origins. Yale University Press, New Haven, CT. pp. 385-424.

Wagner, J,. R. Burnett and R. Storb. 1999. Organization of the canine major histocompatibility complex: current perspectives. J. Heredity 90:35-38.

Waples, R.S. 1991. Pacific salmon, Oncorhynchus spp., and the definition of "species" under the endangered species act. Mar. Fish. Rev. 53:11-22.

Wayne, R.K. and S.M. Jenks. 1991. Mitochondrial DNA analysis implying extensive hybridization of then endangered red wolf *Canis rufus*. Nature 351:565-568.

Wayne, R.K., N. Lehman, M.W. Allard and R.L. Honeycutt. 1992. Mitochondrial DNA variability of the gray wolf: genetic consequences of population decline and habitat fragmentation. Cons. Biol. 6(4):559-569.

Weber, J.L. and P.E. May. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am. J. Hum. Genet. 44:388-396.

Weber, J. L. and C. Wong. 1993. Mutation of human short tandem repeats. Human Molecular Genetics 2:1123-1128.

Wier, B. S. and C. C. Cockeram. 1984. Estimating $F$-statistics for the analysis of population structure. Evolution 38:1358-1370.

Wenink, P., A. Groen, M. Roelke-Parker and H. H. T. Prins. 1998. African buffalo maintain high genetic diversity in the major histocompatibility complex in spite of historically known population bottlenecks. Mol. Evol. 7:1315-1322.

Wentzel, J., J. C. Stephens, W. Johnson, M. Menotti-Raymond, J. Pecon Slattery, N. Yuhki, M. Carrington, H. Quigley, D. Miquelle, R. Tilson, J. Manansang, G. Brady, L. Zhi, P. Wenshi, H. Shi-Quiang, L. Johnston, M. Sunquist, U. Karanth,and S.J. O'Brien. 1998. Molecular genetic ascertainment of subspecies affiliation in tigers. In: Riding the

tiger: Tiger conservation in human-dominated landscapes, (Eds) Seidensticker, Christie, and Jackson. Cambridge University Press. pp.40-49.

Wildt, D. E., M. Bush, J. G. Howard, S. J. O'Brien, D. Meltzer, A. van Dyke, H. Ebedes and D. J. Brand. 1983. Unique seminal quality of the South African cheetah and a comparative evaluation of the domestic cat. Biol. Reprod. 29:1019-1025.

Wildt, D. E., M. Bush, K. L. Goodrowe, C. Packer, A. E .Pusey, J. L. Brown, P. Joslin and S. J. O'Brien. 1987. Reproductive and genetic consequences of founding isolated lion populations. Nature 329(6137):328-331.

Wilson, A.C., R. L. Carr, S. M. Carr, et al. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. Biol. J. Linn. Soc. 26:375-400.

Wise, C., M. Smith, D. Rubinstein and S. Easteal. 1997. Comparative nuclear and mitochondrial genome diversity in humans and chimpanzees. Mol. Biol. Evol. 14(7):707-716.

Wright, S. 1922. Coefficients of inbreeding and relationship. Am. Naturalist 56:330-338.

Wright , S. 1931. Evolution in Mendelian populations. Genetics 16:97-159.

Wright, S. 1977. Evolution and the genetics of populations. (Vol 3). Experimental results and evolutionary deductions. Chicago, University of Chicago Press.

Xu, A., M.J.T. van Eijk, C. Park and H. Lewin. 1993. Polymorphism in BoLA-DRB3 exon 2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia virus. J. Immunology 151(12):6977-6984.

Yamasaki K, I. Horiuchi, M. Minohara, Y. Kawano, Y. Ohyagi, T. Yamada, F. Mihara, H. Ito, Y. Nishimura and J. Kira. 1999. HLA-DPB1*0501-associated opticospinal multiple sclerosis: Clinical,neuroimaging and immunogenetic studies. Brain 122 (Pt9):1689-1696.

Yeager M. and A.L. Hughes. 1999. Evolution of the mammalian MHC, natural selection recombination and convergent evolution. Immunol. Rev. 167:45-58.

Yuhki, N. and S.J. O'Brien. 1990. DNA variation of the mammalian major histocompatibility complex reflects genomic diversity and population history. Proc. Natl. Acad. Sci. USA. 87: 836-840.

Yuhki N. and S.J. O'Brien. 1997. Nature and origin of polymorphism in feline MHC class II DRA and DRB genes. J. Immunology 158: 2822-2833.

Zuckerkandl, E. and L. Pauling. 1962. Molecular disease, evolution, and genetic heterogeneity. In: M. Kasha and B. Pullman (Eds.). Horizons in Biochemistry. New York, Academic Press. pp. 189-225.