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.

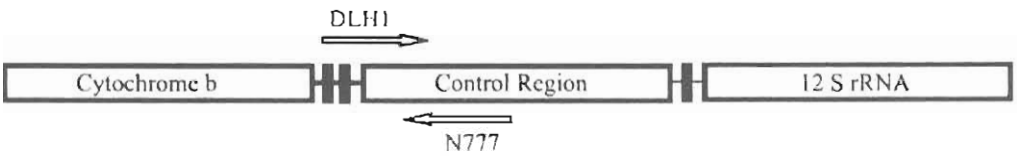
According to neutral theory, alleles in a population are either lost or gained by chance through genetic drift (Kimura 1963). Genetic variation within a species is dependent on the neutral mutation rate of the gene and the effective population size (*Ne*) of the population (Frankham 1996). This diversity 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 (δ) 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).

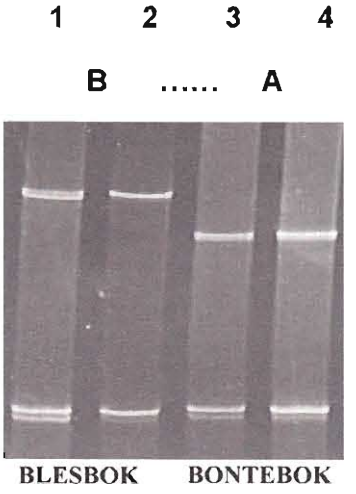


Table. 3. Control Region Sequences

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**

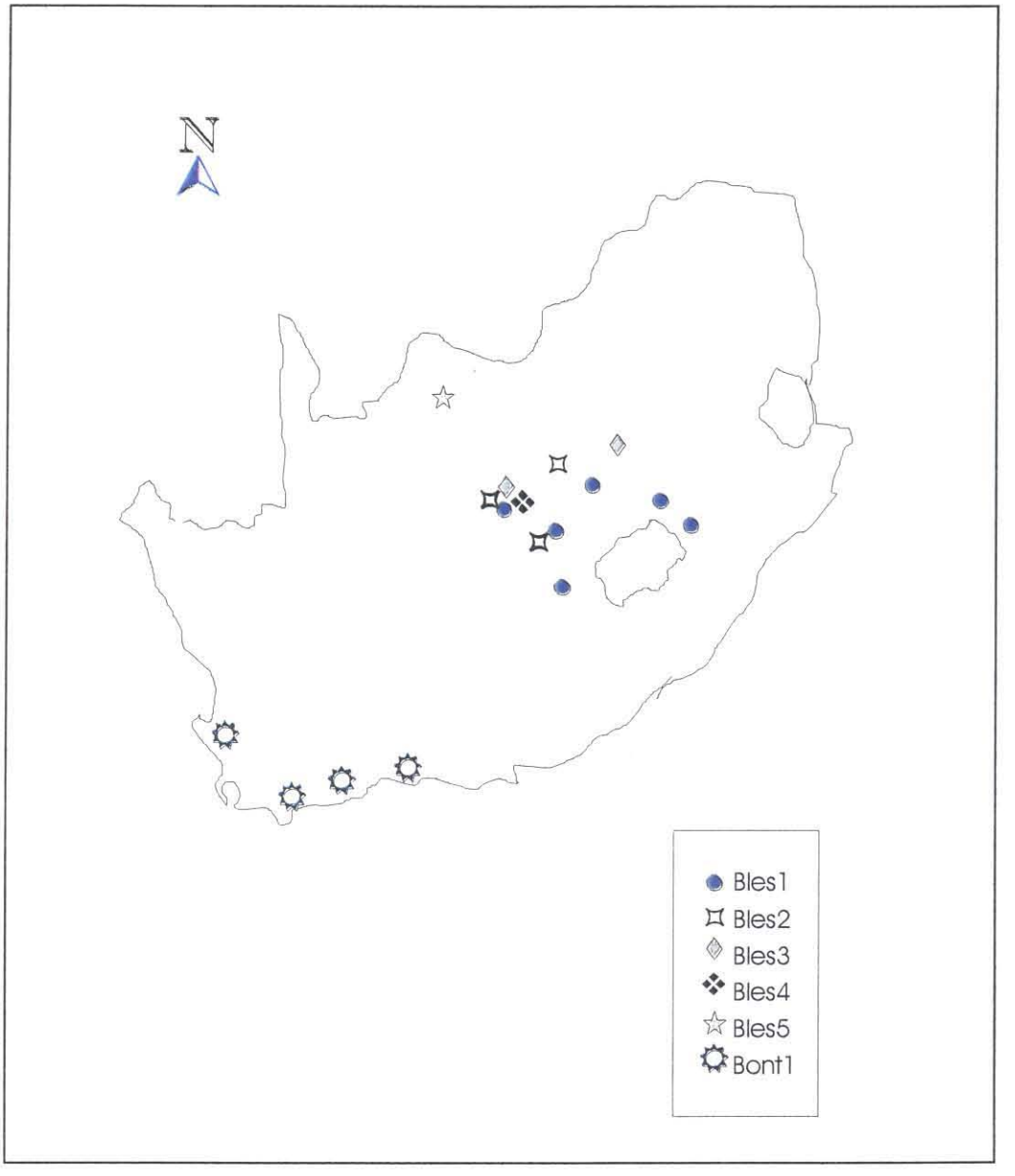


Figure 9. Distribution of control region haplotypes.

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	*	*

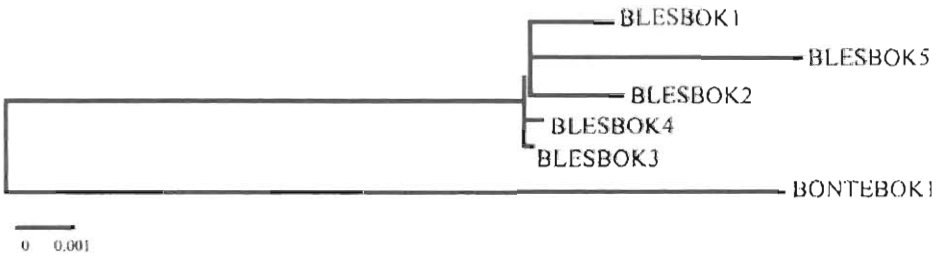
Nucleotide positions of the sequenced fragment were numbered 1 – 520. All mutations are indicated by the nucleotide number in the first row. Sequence similarity is represented by "*".

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



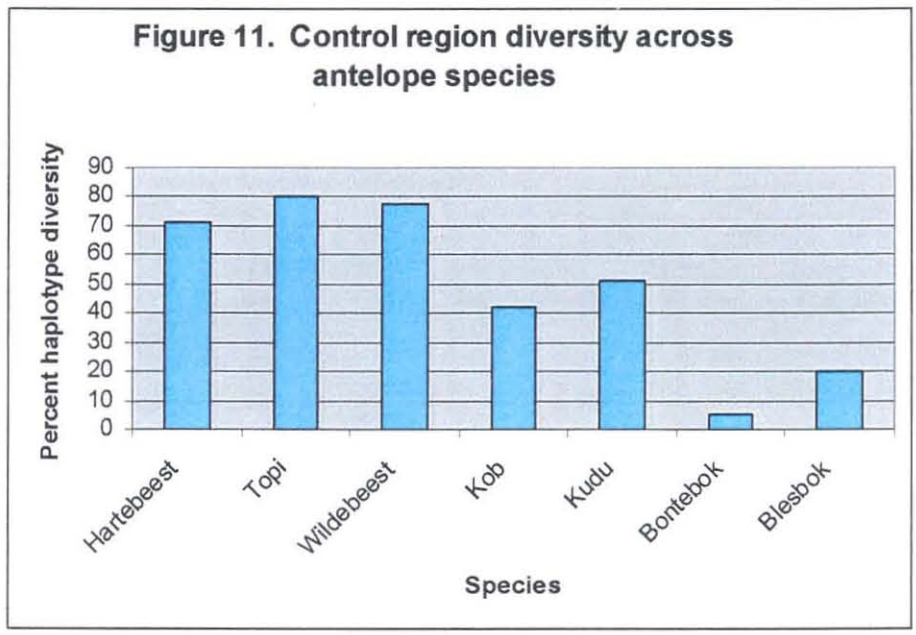


Figure 11. Control region diversity across antelope species
Percent haplotype diversity
90
80
70
60
50
40
30
20
10
0
Hartebeest
Topi
Wildebeest
Kob
Kudu
Bontebok
Blesbok
Species

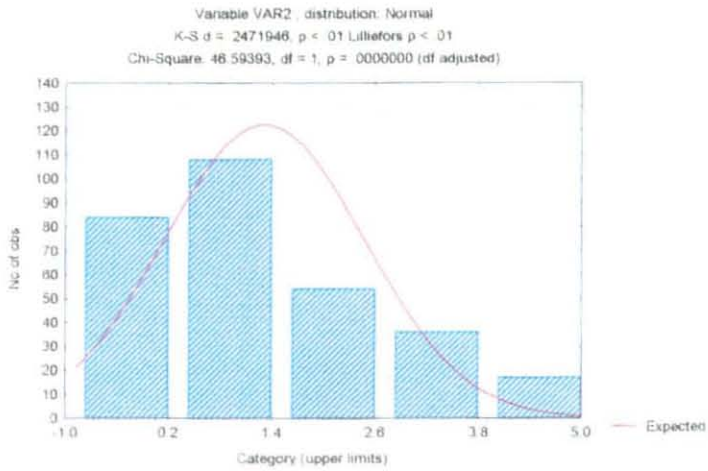


Figure 12. Mismatch distribution of control region sequences in blesbok. The histogram reveals an expanding population for this subspecies

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.