PATTERNS OF GENETIC DIVERSITY IN VERVET MONKEYS (*CHLOROCEBUS AETHIOPS*) FROM THE SOUTH-EASTERN REGIONS OF SOUTH AFRICA

W.G. Coetzer

Dissertation submitted in fulfilment of the requirements for the degree of

Magister Scientiae

In the Faculty of Natural and Agricultural Sciences,

Department of Genetics,

University of the Free State.

Supervisor

Prof. J.P. Grobler,

UFS

Co-supervisor

Prof. T.R. Turner,

UWM

JULY 2012

Ek, Willem Gabriël Coetzer, verklaar dat die verhandeling wat hierby vir die kwalikfikasie *Magister Scientiae* aan die Universiteit van die Vrystaat deur my ingedien word, my selfstandige werk is en nie voorheen deur my vir ‘n graad aan ‘n ander universiteit / fakulteit ingedien is nie.

W.G. Coetzer

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02 Julie 2012

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the help of numerous people. I would just like to express my sincere gratitude to:

My supervisors, Prof J.P. Grobler and Prof T.R. Turner, for all their advice, encouragement and input during my project. I would not have been able to get through this without you. I cannot thank you enough for all the learning opportunities and life experiences with which you presented me. These past three years have been a great experience. Thank you for everything.

The owners, managers and staff at all the sampling locations, your support and enthusiasm during the sampling trips were much appreciated.

Christiaan Labuschagne at Inqaba Biotec for designing the primers used for my project and your assistance during the optimization stages of the lab work.

Prof Nelson B. Freimer, Director of the Center for Neurobehavioral Genetics, University of California Los Angeles (UCLA), for permission to use a limited number of samples collected during their wide-spread sampling excursions in South Africa during 2010.

The University of the Free State (UFS), for academic assistance.

The UFS Animal Ethics Committee for approval of the sampling methods.

The personnel and students of the Department of Genetics, UFS, for all your support and encouragement during my project.

The personnel of all the provincial conservation organisations.

Prof Johan Spies, head of the Department of Genetics, UFS, for his interest and support toward my project.

My parents, Johann and Karin Coetzer, thank you for all your support and interest during my nine years of studying.

Thank you all!

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LIST OF ABBREVIATIONS AND SYMBOLS

Symbols:

~	approximately
°	degree
°C	degrees Celsius
Γ	gamma
F_{ST}	genetic differentiation over subpopulations
D	genetic distance
μ	micro: 10^{-6}
Π	nucleotide diversity
%	percentage
±	plus minus
®	registered trademark
rpm	revolutions per minute
x g	times gravity
™	trademark

Abbreviations:

AFLP	Amplified Fragment Length Polymorphism
AIDS	Acquired immune deficiency syndrome
AMOVA	Analyses of Molecular Variance
BB	Blythedale Beach
BKL	Baviaanskloof
bp	base pair
CCR	central conserved region
CITES	Convention on International Trade in Endangered Species

cm	centimetre
COI	cytochrome oxidase I
CR	control region
cyt b	cytochrome b
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNS	deoksieribonukleïensuur
dNTP	deoxynucleotide triphosphate
E	East
EC	Eastern Cape
EC_Hap	Eastern Cape haplotype
ESU	evolutionary significant unit
et al.	*et alli*: and others
FS	Free State
FS_Hap	Free State haplotypes
Fw	forward (primer)
G	Gariep
HIV	Human immunodeficiency virus
HVR	hypervariable region
i.e.	*id est*: in other words, that is
IUCN	International Union for Conservation of Nature
kg	kilogram
km	kilometre
KZN_Hap	Kwa-Zulu Natal haplotypes
Lim	Limpopo
Lim_Hap	Limpopo haplotype
$MgCl_2$	magnesium chloride

ML	maximum likelihood
μl	microlitre
μM	micromolar
Ma	million years
mg	milligram
ml	millilitre
mM	millimolar
mm	millimetres
min	minutes
mtDNA	mitochondrial deoxyribonucleic acid
mtDNS	mitochondriale deoksieribonukleïensuur
MU	management unit
NADH	nicotinamide adenine dinucleotide
NHP	non-human primate
NMMU	Nelson Mandela Metropolitan University
NR	nature reserve
P	Parys
PCR	polymerase chain reaction
PE	Port Elizabeth
PGR	Private Game Reserve
PK	Polokwane
RAPD	random amplified polymorphic DNA
Rev	reverse (primer)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S	South

SIV	simian immunodeficiency virus
S NR	Sandveld NR
SO NR	Soetdoring NR
SSC	Species Survival Commission
SSR	simple sequence repeats
St.	Saint
St. L	St. Lucia
STR	short tandem repeats
SW	Shamwari PGR
Tm	melting temperature
T NR	Tsolwana NR
TP Estate	Thorny Park Estate
tRNA	transfer ribonucleic acid
UFS	University of the Free State
U	unit
UNEP	United Nations Environment Programme
v	version
VNTR	variable number tandem repeats
WCMC	World Conservation Monitoring Centre
Y-STR	Y-chromosome short tandem repeats

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Chapter 1:

Introduction to vervet monkeys and conservation genetics

1.1 Distribution and biology of Vervet monkeys

Chlorocebus aethiops is among the most widely distributed non-human primate (NHP) species in Africa. A number of subspecies are found from Southern Africa to Ethiopia along the east coast, as well as in West Africa (Stuart *et al.,* 2007). These animals are generally grouped into three to six subspecies. *Chlorocebus aethiops aethiops* (the Grivet monkey) is generally found east of the White Nile River in Sudan, to Eritrea up to the Rift Valley in eastern Ethiopia. *Chlorocebus aethiops djamdjamensis* (the Bale mountain vervet) is located in the Bale Mountains of Ethiopia. *Chlorocebus aethiops pygerythrus* (the Vervet monkey) is found east of the Rift Valley in Ethiopia throughout eastern Africa down to South Africa. *Chlorocebus aethiops sabaeus* (the Green monkey) is found from Senegal in West Africa to the Volta River in Ghana. *Chlorocebus aethiops tantalus* (the Tantalus monkey), has a distribution ranging from the Volta River in Ghana in western Africa up to the White Nile in Sudan and stretching south east to Lake Tukana in Kenya. *Chlorocebus aethiops cynosuros* (the Malbrouck monkey) is found in southern Democratic Republic of Congo extending southward to northern Namibia and western Zambia (van der Kuyl and Dekker, 1996; Shimada *et al.*, 2002; Grubb *et al.*, 2003; Groves, 2005; Stuart *et al*., 2007). Figure 1.1 shows the geographical distribution of *Ch. aethiops* throughout Africa.

Primates of the genus *Chlorocebus* are found exclusively in Africa (Page *et al.*, 1999) and prefer savannah and riverine woodland areas, as well as coastal scrub forests, avoiding desert, high forest and open grassland habitats. Adult males can grow to a length of 100-130 cm and females 95-110 cm. The average weight of an adult male is 5.5 kg (range 4-8 kg) and the average weight of an adult female is 4 kg (range 3.5-5.5 kg) (Stuart *et al.*, 2007). They are highly social animals, and are very territorial with well-defined home range boundaries. They forage during the day and sleep at night in trees or on cliffs if sufficient tree cover is not available (Smithers, 2000; Stuart *et al.*, 2007). Vervets are omnivorous and mainly forage for a wide variety of fruits, flowers, leaves, gum and seeds, but on occasion they will prey on insects and small vertebrates, like nestlings, as well as small mammals (Smithers, 2000; Stuart *et al.*, 2007, Geser *et al.*, 2008).

Figure 1.1: The distribution range of *Chlorocebus aethiops* across Africa. (Derived from Stuart *et al.,* 2007) [**Image:** http://maps.google.com]

Vervets are highly influential on the integrity of their environments. They have both positive and negative impacts on the surrounding ecosystem. An example of one such positive impact would be their involvement in seed dispersal. It has been shown that these monkeys can influence succession in rehabilitated forest areas, due to their ability to disperse seeds from a variety of trees throughout these rehabilitation sites (Foard *et al.,* 1994).

Primates in general also act as a reservoir for a variety of pathogens, some of which can be fatal to non-host mammalian species. Most notably, vervet monkeys are the largest reservoir of simian immunodeficiency virus (SIV) (Allan *et al.,* 1991). It has been found that the host species do not develop disease symptoms, but non-natural hosts develop disease symptoms which can lead to death of the non-natural host (Broussard *et al.*, 2001).

1.2 Phylogeography of primates and the taxonomy of Vervet monkeys

Phylogeography

According to Masters (2006), the biggest problem for the primate evolutionary biologist is to explain how primates migrated to their current distribution ranges, and from where they originated. There is continuous disagreement surrounding the evolution of primates. The main arguments are focused on the centre of origin of primates, which varies between Africa, Asia and the Americas (Heads, 2010). There is also disagreement surrounding the time frame of primate evolution. Fossil-based dates place the origin of primates in the Paleocene (~56 Ma), with Cretaceous dates (~90 Ma) obtained from fossil-calibrated molecular clocks (Bloch *et al.*, 2007; Janečka *et al.,* 2007). There is also some controversy surrounding the origins of the main primate clades. There are two main clades, consisting of the Strepsirrhines (Lemurs; Lorises and Galagos), the Haplorhines (Tarsiers; Anthropoids: catarrhines (Old World monkeys) and the Platyrrhines (New World monkeys)). Strepsirrhines are found in Africa, including Madagascar and Asia, and Haplorhines are widespread in South America, Asia and Africa, excluding Madagascar. It was proposed by Matthew (1915) that primate evolution can be explained by a central place of origin theory, and that a more or less modern land and sea arrangement provided the stage for development and dispersal. The primates of South America and Madagascar produce a rather interesting problem, namely how did they get to their respective areas of distribution, from where did they originate and why are they restricted to these areas? The only explanations, from a centre of origin viewpoint, are dispersal through mechanisms such as land bridges, dispersal over open ocean on vegetation rafts and intermediate island hopping. These are all excepted mechanisms for distribution, but it cannot be explained why these dispersals only

happened once during the history of primate evolution (Heads, 2010). Another model recently developed to explain primate evolution is discussed by Heads (2010). This model is based on the vicariance (the separation of a group of organisms by a geographic barrier) of an already-widespread common ancestor, through the occurrence of continental drift. This model explains the occurrence of Lemurs in Madagascar, but nowhere else in Africa, Asia or America. The occurrence of the New World monkeys is also explained by this model (Heads, 2010).

Taxonomy of Vervet monkeys

Vervet monkeys form part the Cercopithecoidea superfamily known as the Old World Monkeys (OWM), and fall under the subfamily Cercopithecinae, or cheek-pouch monkeys (Grubb *et al.,* 2003). It was estimated that the split between OWM and hominoids (apes and humans) ranges from between 26.9 to 36.4 million years ago. The cercopithecoids are also the closest family to the hominoid family (Steiper and Young, 2006).

Vervet monkeys have a very wide distribution, which stretches over a number of different ecological areas. The animals in these different areas tend to show phenotypic differentiation, ranging from various fur colourations to length variation of the whiskers. These differences have lead to many taxonomical debates. These OWM have been separated into to as many as 25 different subspecies (Table 1.1), and have the highest number of nominal subspecies among the OWM (Grubb *et al.,* 2003).

Within the *Cercopithecus aethiops* group, three species were defined by Dandelot (1959), based on morphological characteristics such as cheek whiskers and the colour of male genitalia. They were: *Cercopithecus sabaeus, C. aethiops* (containing grivets (*C. a. aethiops*) and tantalus (*C. a. tantalus*) subsections), and *C. pygerythrus* (containing vervet (*C. p. pygerythrus*) and malbrouck (*C. p. cynosures*) subsections) (Dandelot, 1959 cited in Grubb *et al.,* 2003). Some authors also tend to group these monkeys into one highly polytypic species. Grubb *et al.* (2003) placed this group of monkeys under the species group *Cercopithecus aethiops* with six subspecies following Napier's (1981) classification. This reorganization was done on the

grounds of the general uncertainty surrounding the existing boundaries between the species / subspecies, as well as the work done by Struhsaker (1970). This author found no vocal differences between tantalus and vervet monkeys, and he also did not agree with Dandelot's (1959) discrimination made on grounds of the colour of male genitalia. The subspecies grouping put forward by Grubb *et al.* (2003) is: *C. a. aethiops, C. a. djamdjamensis, C. a. tantalus, C. a. sabaeus, C. a. cynosuros* and *C. a. pygerythrus.*

The *Cercopithecus aethiops* group was placed in the genus *Chlorocebus* by Groves (1989, 2001, 2005) on the basis of synapomorphic cranial characteristics. Instead of forming six subspecies this author formed six separate species: *Chlorocebus aethiops, Ch. djamdjamensis, Ch. tantalus, Ch. sabaeus, Ch. cynosuros* and *Ch. pygerythrus* (Groves, 1989, 2001, 2005). The placement of vervets in the genus *Chlorocebus* was also supported through genetic evidence by Tosi *et al.* (2003) and by postcranium and long bone measurements done by Sargis *et al.* (2008).

The *Cercopithecus pygerythrus* group was divided into 15 subspecies by Dandelot (1959, 1968, 1974). Vervet monkeys (*C. pygerythrus*) were separated from Grivet monkeys *(C. aethiops*) as a species by Dandelot (1959), Kingdon (1997) and Groves (2001). As stated above, vervet monkeys were also previously grouped with the Malbrouck monkeys (*C. p. cynosuros*) (Dandelot, 1974). Groves (2001) and Kingdon (2008) separated the two groups and recognized them as separate species, *Chlorocebus pygerythrus* and *Ch. cynosuros*. Vervet monkeys were regarded as a subspecies of the *Cercopithecus aethiops* group by Grubb *et al.* (2003): *C. a. pygerythrus*. Throughout the rest of this paper, vervet monkeys will be referred to as *Chlorocebus aethiops pygerythrus*. By doing this, the ground living clade, containing the vervet, patas, and l'Hoest's monkeys, is recognized (Sargis *et al., 2008*) and the polytypic species status recognized by Struhsaker (1970), Napier (1981) and Grubb *et al.* (2003) is also considered.

Table 1.1: The classification of the *Cercopithecus (Chlorocebus) aethiops* group.
[*Note:* Nominal species are those recognized in the classification of Dandelot (1974), as modified by Groves (2001). Subspecies are those recently recognized as valid by various authors. Alternative opinions on systematic treatment are taken from Dandelot (1974), Hill (1966), Napier (1981), and Groves (2001)] (Derived from Grubb *et al.*, 2003)

Nominal taxa:	**Alternative systematic treatment:**
***Cercopithecus aethiops* section:**	
C. a. aethiops	
C. a. hilgerti	Transferred to *C. pygerythrus* as *C. p. hilgerti* (Groves)
C. a. ellenbecki	Synonym of *C. a. hilgerti* (Dandelot, Napier) or of *C. p. hilgerti* (Groves)
C. a. matschiei	Synonym of *C. a. ellenbecki* (Hill), or of *C. a. aethiops* (Groves)
***Cercopithecus djamdjamensis* section (assigned to *Cercopithecus aethiops* section by Dandelot):**	
C. djamdjamensis	Synonym of *C. a. ellenbecki* (Hill); raised to species status (Groves)
***Cercopithecus tantalus* section:**	
C. t. tantalus	
C. t. budgetti	
C. t. marrensis	
***Cercopithecus sabaeus* section:**	
C. sabaeus	
***Cercopithecus pygerythrus* section (part of *Cercopithecus cynosuros* section of Dandelot):**	
C. p. pygerythrus	
C. p. cloetei	Omitted (Dandelot); synonym of *C. p. pygerythrus*(Napier, Groves)
C. p. helvescens	Omitted (Hill); synonym of *C. cynosuros* (Groves)
C. p. ngamiensis	Synonym of *C. p. pygerythrus* (Groves)
C. p. marjoriae	Synonym of *C. p. pygerythrus* (Groves)
C. p. whytei	Synonym of *C. p. rufoviridis* (Napier, Groves)
C. p. rufoviridis	
C. p. johnstoni	Synonym of *C. p. hilgerti* (Groves)
C. p. rubellus	Synonym of *C. p. johnstoni* (Dandelot), or of *C. p. hilgerti* (Groves)
C. p. centralis	Synonym of *C. p. rufoviridis* (Groves)
C. p. callidus	Synonym of *C. p. hilgerti* (Groves)
C. p. nesiotes	
C. p. excubitor	
C. p. arenarius	Synonym of *C. p. hilgerti* (Groves)
C. p. zavattarii	Synonym of *C. p. arenarius* (Dandelot), or of *C. a. aethiops* (Groves)
***Cercopithecus cynosuros* section (part of *Cercopithecus cynosuros* section of Dandelot):**	
C. cynosuros	Raised to species status (Groves)

1.3 Conservation genetics and conservation units

Conservation genetics

As can be derived from the name, conservation genetics is a combination of genetics concepts and tools which then are used to solve problems in conservation biology. The molecular techniques discussed in section 1.6 are the most commonly used techniques in conservation genetics. A summary of the uses of genetics in conservation biology is presented in Table 1.2.

Identifying genetic variation within and among populations is very important for the prevention of inbreeding in endangered species. It will also make it possible to identify the presence and effect of genetic drift, and identify whether the population in question has undergone selection or a recent genetic bottleneck. Pedigree analysis is another aspect of conservation genetics which can be used to create adequate breeding programs for captive animal populations and to prevent inbreeding (Hedrick and Miller, 1992; Oyler-McCance and Leberg, 2005).

For hundreds of years taxonomic classifications have been done on the basis of morphological and behavioural characteristics, but it has been found that classifications relying solely on morphological characterization can be erroneous (Avise, 1989). Using genetic data in combination with morphological and behavioural characteristics can resolve inconsistencies and provide refined taxonomic definitions (Oyler-McCance and Leberg, 2005). The two most commonly used species concepts can also be addressed through genetic analysis; namely the biological species concept (Dobzhansky, 1937) and the phylogenetic species concept (Cracraft, 1983). Identifying the level of gene flow occurring among populations can be used, in combination with morphological and behavioural data, to identify the delineation of a species. Genetic analysis also can be used to construct phylogenetic relationships among populations to identify the presence or absence of a monophyletic group (Oyler-McCance and Leberg, 2005).

Table 1.2: The different uses of genetics in conservation biology. (Derived from DeSalle and Amato, 2004)

Role in conservation biology	Sub-disciplines
Minimizing inbreeding and loss of genetic variation	Population genetics
Identifying populations of concern	Population genetics
Resolving population structure	Population genetics
Resolving taxonomic uncertainty	Systematics
Defining management units within species	Systematics
Detecting hybridization (genetic pollution)	Population genetics / systematics
Detecting and defining invasive species	Population genetics / systematics
Defining sites and genotypes for re-introduction	Population genetics / systematics
Use in conservation forensics	Systematics
Estimation population size and sex ratio	Population genetics
Establishing parentage; pedigree analysis	Population genetics
Understanding population connectivity	Population genetics / systematics
Use in the management of captive populations	Population genetics
Understanding relationships of focal groups of taxa	Systematics
Implementing genotoxicity studies	Population genetics
Increasing the reproductive capacity of organisms	Population genetics

Conservation units

The evolutionary significant unit (ESU) concept (and related concepts) has become very prominent in the conservation of natural and captive animal populations. The term was first used by Ryder (1986). The goal of defining ESUs is to ensure that historical and geographical genetic variants are recognized and protected and that the evolutionary processes within ESUs are maintained. Some authors advise against the translocation of individuals between ESUs (Avise, 1994; Ryman, 1991), thus it is important to identify ESUs within a species in order to avoid mixing of populations which are on different evolutionary pathways.

The ESU concept has seen many changes over the years. It was first described as population units which 'represent significant adaptive variation' derived from harmonious data sets obtained from different techniques (Ryder, 1986). At a later stage, ESUs were described as populations which are reproductively separate from others, with unique or different adaptations (Waples, 1991). Moritz (1994) focused on evolutionary history; thus, populations isolated for a long period of time have the potential to develop into distinct population groups or to form populations which are uniquely adapted to their current environmental conditions. It was therefore argued that ESUs should be recognized as reciprocally monophyletic groups. This approach would ensure the maintenance of the evolutionary heritage within species, through the separate management of the populations (Moritz, 1994). Crandall *et al.* (2000) discussed an exchangeability model focusing on historical and recent genetic and ecological exchangeability between populations. The reason for the inclusion of the ecological factor is that by solely basing ESUs on genetic isolation might have a limiting influence on the available options for gene flow and adaptation through natural selection (Crandall *et al.*, 2000).

Waples and Gaggiotti (2006) reviewed two commonly used population definitions, namely the ecological paradigm and the evolutionary paradigm. Their definitions of these two concepts were:

> "Ecological paradigm: A group of individuals of the same species that co-occur in space and time and have an opportunity to interact with each other."

> "Evolutionary paradigm: A group of individuals of the same species living in close enough proximity that any member of the group can potentially mate with any other member."

It was concluded that neither of these concepts are truly operational, and these authors suggested several quantitative criteria to aid in the identification of separate populations. No agreeable quantitative definition could be found regarding "population". But, the concept of a population does have meaning under each of the discussed paradigms, and probably at different levels within each paradigm (Waples and Gaggiotti, 2006).

Another conservation unit of interest is the management unit (MU). A MU is defined as a population of conspecific individuals with relatively low levels of connectivity among populations, in which case each population should be monitored and managed on its own (Taylor and Dizon, 1999). The most commonly used criterion used to define MUs was derived from the definition of a MU used by Moritz (1994), "populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles..." (Moritz, 1994). It was taken from this definition that to give a population MU status, the presence of panmixia should be statistically rejected when using population genetic data (Palsbøll *et al.*, 2006). It was suggested by Palsbøll *et al.* (2006) that instead of focusing on the rejection of panmixia, researchers should rather focus on the levels of genetic divergence at which populations become demographically independent. Various problems could be encountered when focusing on the rejection of panmixia for the identification of MUs, which could lead to the erroneous assignment of populations to the same MU or populations which should be managed as one MU can be divided into multiple MUs. These issues can be avoided when focusing on the level of divergence of a population's alleles, which is in turn linked to population dispersal rates, than on the rejection of panmixia (Palsbøll *et al.*, 2006).

1.4 Vervet monkeys as model organisms for processes in humans

Non-human primates (NHP) are generally highly social animals, with many similarities in behaviour to humans. The cercopithecoids, which include the vervet monkeys, are viewed as the closest family to the hominoid family (apes and humans). This relatively close relation to humans, as well as their ease of handling, makes vervet monkeys important model animals for various research areas (Jasinska *et al.,* 2007).

The most common areas of research in which NHPs are used include immunology (including HIV/AIDS response), neuroscience, biochemistry/chemistry (Carlsson *et al.,* 2004; Hau, 2006) and behaviour (Jasinska *et al.,* 2007). Carlsson *et al.* (2004) performed a study on the use of NHPs in research by reviewing research papers published during 2001. It was found that *Chlorocebus aethiops* is the most commonly used NHP species (19% of all NHPs) used in biomedical research.

The human immunodeficiency viruses, HIV-1 and HIV-2, are known as zoonotic viruses, as humans are not the natural host organism of these viruses. It is now known that these viruses were transmitted to the human population through cross-species transmission from NHPs. Vervets are especially important in HIV/AIDS research. These NHPs are carriers of SIV, which is closely related to HIV (Hahn *et al.*, 2000). Interestingly, the natural hosts of these viruses do not show any AIDS-like symptoms (Heeney *et al.,* 1993; Norley *et al.*, 1999), which forms the main basis for the HIV/AIDS research conducted on NHPs (Broussard *et al.*, 2001).

1.5 Vervet monkeys and sanctuaries

Vervet monkeys are extremely adaptable and are frequently found in suburban areas which overlap with their home ranges, where they come in frequent contact with humans (Figure 1.2). This behaviour frequently leads to human / non-human primate conflict. Such conflict also arises with farmers, as vervet monkeys have been blamed for considerable damage to orchards and crops, as well as mortalities in poultry. Animals often end up in primate sanctuaries due to injury or being orphaned as a result of such conflict. The illegal pet trade is also a source of orphaned animals. When confiscated, these animals are frequently sent to conveniently close sanctuaries. Currently there are several hundred vervet monkeys in sanctuaries across South Africa.

The aim of some of these centers is to re-introduce rehabilitated animals back into the wild. Limited space and funding at sanctuaries means that there is little room to add a genetic component to rehabilitation efforts, and centers usually form troops by mixing animals from different areas (Grobler *et al.,* 2006).

Several measures should be taken into consideration before re-introduction programs are initiated. Such guidelines were provided by the IUCN's Species Survival Commission (SSC). These recommendations focus on habitat, behaviour, socioeconomic, financial and legal issues and release stock status; and also include genetic assessment of the animals (Baker, 2002).

Figure 1.2: An example of vervet – human contact. These animals are highly adaptable and will quickly learn to obtain food from any human environment. [**Photo** by W.G. Coetzer]

The status of the habitat at the release site is very important. It is recommended that the release site should be within the species' historic distribution range. The availability of food, shelter and water also should be considered (Baker, 2002). It is also advised that such areas be situated within protected areas, such as reserves or national parks with no conspecific populations or at least a very small resident conspecific population (Kleinman, 1989). All aspects of the species' behavioural patterns, social structures and habitat preferences should be studied, focusing on wild populations. The integrity of the release stock or rehabilitated animals should be assessed. Captive populations should be under sound management, considering both demographic and genetic background. The behaviour of the captive animals should also be focused on, since these animals might have acquired behavioural characteristics which could influence their survival in the wild. The group composition of the captive population should resemble that of the species' wild groupings.

Conservation authorities in some regions of South Africa require (or formerly required) that genetic assessment should be made of the animals to be released, as well as of the wild populations in the area of the release site, to ensure that no mixing of distinct lineages would occur. On the other hand, high levels of genetic variation are also very important for the survival of the newly introduced population, as inbreeding can be highly detrimental to the animals' likelihood of survival. The likelihood of inbreeding can be managed through the knowledge of the individual animals' pedigrees (Stanley-Price, 1989; Sarrazin and Barbault, 1996; Baker, 2002).

Strict quarantine and veterinary procedures are also of high importance for the detection and control of diseases. Captive animals potentially can contract diseases that they would not normally be exposed to through contact with their human caretakers. These diseases then can be transmitted to their wild counterparts (Karesh, 1995; Baker, 2002). Tuberculosis is one such pathogen. This disease has been detected among numerous species of captive non-human primates. Such animals include orang-utans (*Pongo pygmaeus* and *Pongo abelii*) (Russon, 2009), chimpanzees (*Pan troglodytes*) (Griffith, 1928, Michel *et al.,* 2003) and Chacma baboon (*Papio ursinus*) (Fourie and Odendaal, 1983). A post-release monitoring system should be implemented to ensure the survival and successful adaptation of the re-introduced population (Baker, 2002). Specific guidelines for primate rehabilitation and re-introduction are discussed by Cheyne (2009), using Gibbons from the Kalaweit Gibbon Rehabilitation Project, Central Kalimantan, Indonesia as an example.

A rehabilitation project viewed as one of the most successful is that of the golden lion tamarind (*Leontopithecus rosalia*) (Beck *et al.*, 1991; Kleiman *et al.*, 1991, Cheyne, 2009). Between 1984 and 1991, 91 animals were reintroduced, of which 33 survived (June 1991 census). During the release stages, only pairs or intact family groups were released. The animals were provided with extensive pre-release training. After release, the animals were under constant observation to monitor behaviour and provide intervention (food) if needed. Observations were gradually reduced and provisional feeding stopped (Beck *et al.*, 1991). After a period of 17 years, the reintroduced population grew to 359 animals in 50 groups (Kierulff *et al.*, 2002).

A few African re-introduction / rehabilitation examples include the re-introductions of chimpanzees (Goossens *et al.*, 2005), lemurs (Wyner *et al.*, 1999) and mandrills (Peignot *et al.*, 2008). Goossens *et al.* (2005) reported on observations made on 37 wild-born, captive chimpanzees (*Pan troglodytes troglodytes*) released in the Republic of Congo. This was done over an eight-year period. It was found that the overall survival rate of the 37 individuals was high, with 62% of the animals remaining in the release zone, and only five (14%) deaths. Some of the females even managed to integrate themselves into wild groups for extensive time periods. Four of the released females gave birth to five offspring collectively. The males, however always had aggressive encounters, which might be the cause of the majority of the fatalities among the males. Almost half of the males would have died if not for veterinary intervention. The data obtained from this study are highly beneficial to planning and executing current and future conservation projects (Goossens *et al.*, 2005).

Wyner *et al.* (1999) used genetic data to assess the suitability of a captive population of black and white ruffed lemurs (*Varecia vareigata variegata*) for reintroduction into wild populations. A founder population, scheduled to join the captive population to supplement the genetic pool, also was tested. A 548 bp segment of the mitochondrial control region was used to evaluate the genetic structure from three lemur populations in Madagascar. It was found that the captive animals more closely resembled the southern populations and that the founder population was more similar to the northern population. With this information in hand, it was possible to provide valuable recommendations for the management of these populations (Wyner *et al.*, 1999), that being, that the introduction of unrelated animals into the inbred population outweighs the risk of merging the different population units.

Peignot *et al.* (2008) reported on the first reintroduction project for mandrills (*Mandrillus sphinx*) in Gabon. The animals originated from a semi-captive ranging breeding colony at the Centre International de Recherches Médicales de Franceville (CIRMF). A total of 36 animals, 16 males and 20 females, were chosen to be released into the Lékédi Park, Gabon. After the first year following release, a

mortality rate of 33% was observed, dependent infants being the most affected. The main causes of the deaths were environmental stress and malnutrition. After eight weeks, food was provided because the animals suffered from malnutrition. Food was provided on a daily basis for one month, following a decrease of provisions over two-and-a-half months until provisioning stopped. One month later, feeding was continued on a twice-weekly basis, because it was observed that the animals started to lose weight. The death rate decreased to 4% during the second year, and reproduction and survival also stabilized at this time. Provisioning ceased during the third year when contact was lost. The group was found one year later, numbering 22 animals, 12 of which were from the original group. All animals were found to be in good condition. Valuable lessons were learned from this project, and the recommendations made will assist in future reintroduction programs for Mandrills as well as the drill (*Mandrillus leucophaeus*) (Peignot *et al.*, 2008).

1.6 Molecular techniques used in primate conservation genetics

Genetics forms an important part of wildlife conservation. Throughout the years many different genetic methods have been used to identify the population structure of various organisms as well as the genetic variation among and between different populations of a specific species. The following few pages provide a short history of some of these methods, with their applications in primate conservation.

Allozymes have been used for almost five decades in population studies, with early reference to this technique by Harris (1966). Subsequent studies included population and conservation biology studies on organisms ranging from mammals (with many focusing on Cercopithecoidea primates: Jolly and Brett, 1973; Turner, 1981; Dracopoli *et al.,* 1983; Melnick and Kidd, 1985; Olivier and Coppenhaver, 1986; Rogers, 1989; Shimada, 1998; Grobler and Matlala, 2002; Li *et al*., 2003), plants (Cruzan, 1998) to microorganisms (Monis *et al*., 1999; Souza *et al*., 1999). These molecular markers are enzymatic proteins, products of coding DNA, which can be viewed through enzyme-specific staining reactions after said proteins are run through an agarose or polyacrilamide gel, a process called gel electrophoresis. The proteins separate through the gel due to the differences in electrical charge ang molecular weight of the different protein molecules (Jarne and Lagoda, 1996; Di Fiore, 2003). Allozymes are known as co-dominant genetic markers with a low mutation rate. The low mutation rate of allozymes influences the effectiveness of this method when working with small populations or sample sizes during conservation genetic studies (Cruzan, 1998; Selkoe and Toonen, 2006). A negative aspect of using allozymes in conservation genetic studies is the requirement of large sample sizes for adequate protein extraction for analysis, which is especially difficult to obtain when working with wild populations (Di Foire, 2003), as some samples (liver, muscle, eye) must be collected invasively or lethally. The redundancy in the genetic code also means that true levels of diversity may be underestimated when using allozymes. Despite the negative points of this method, allozymes were used in a range of genetic studies. This is in part due to the cost-effectiveness of using this method as well as the low level of training needed (Hedrick and Miller, 1992; Cruzan, 1998).

To determine the protein variation of seven vervet monkey (*Cercopithecus* aethiops) populations in the Awash National Park, Ethiopia, Turner (1981) made use of 23 allozyme loci. The low levels of variation detected suggested that these groups function as one genetically interchangeable population. Shimada (1998) used 33 blood protein loci to establish the gene distribution patterns of grivet monkeys (*Cercopithecus aethiops aethiops*) in Ethiopia, and found low levels of variation when their results were compared to those of other cercopithecoid populations.

Dracopoli *et al.* (1983) used 13 serum proteins on samples from 340 vervet monkeys from four localities in central and southern Kenya. The authors found that most of the genetic variation is found within the individual troops, with only a small amount of the genetic variation occurring between the populations from the different trapping sites. Male migration from their natal troops was viewed as the most likely mechanism of gene flow between the various vervet monkey populations, leading to the low levels of overall genetic diversity.

The genetic variability of vervet monkeys (*Chlorocebus aethiops*) was investigated by Grobler and Matlala (2002) using 26 protein loci, in combination with morphological characterisation. Animals from three geographical regions in South Africa were included in the study. The data obtained from the protein analysis indicated low genetic variability. It was concluded that the monkeys form a relatively monotypic unit, but it was also indicated that animals from different geographical origins show slight differences and that further genetic studies are required (Grobler and Matlala, 2002).

Restriction Fragment Length Polymorphisms (RFLP) were first used by Grodzicker *et al.* (1974) to construct a physical map of the locations of the temperature-sensitive mutations found in adenoviruses (Botstein *et al*., 1980). RFLPs are observed through the digestion of DNA with restriction endonucleases. The DNA fragments are then separated through gel electrophoresis. The digested DNA fragments separate according to their individual molecular weight. Various visualization techniques can be used, but the most commonly used is Southern

Blotting (Southern, 1975, Botstein et al., 1980) followed by hybridization of labelled DNA pobes. Before the use of polymerase chain reaction (PCR)-based techniques, large quantities of blood or tissue samples were required to produce high-quality DNA for RFLP studies. This made it difficult to use during studies on wild animals such as primates, which are generally difficult to sample (Di Fiore, 2003; de Ruiter, 2004).

Melnick *et al.* (1992) investigated the mitochondrial genomes of 18 rhesus macaques covering five regions from South-East Asia. These authors used 15 restriction endonucleases for the analysis of these samples. These results were combined with published nuclear genome data to determine the population genetic structure of rhesus macaques. The mtDNA data indicated that the majority of the genetic variation occurs between populations. However, the nuclear genetic variation was predominantly within populations. The different genetic patterns observed from these two genetic markers were attributed to the asymmetrical dispersal patterns of macaque males and females, as well as the maternal inheritance pattern of mtDNA.

Ryder and Chemnick (1993) used restriction endonuclease digestion data from the mitochondrial DNA (mtDNA) obtained from 144 orang-utans, to study the genetic divergence of orang-utan subspecies. These authors concluded that there are two distinct phylogenetic lineages of orang-utans.

Restriction fragment length polymorphism analysis also was used by Shimada (2000a) to study the geographic distribution of the mtDNA variations within grivet monkeys in central Ethiopia. The author studied ten groups of grivets, which totalled to 77 animals. Analysis of the whole mtDNA genome was done using 17 restriction enzymes. Ten haplotypes were identified, which grouped into five clusters.

DNA sequencing, as known today, was introduced by Sanger et al. (1977), based on specific chain-terminating inhibitors of DNA polymerase, or sequencing through the chemical degradation of DNA by Maxam and Gilbert (1977). Mitochondrial DNA (mtDNA) is sometimes preferred over nuclear DNA in population and conservation genetics studies of primates and other vertebrates. This is due to the fast rate of evolution of mtDNA and the fact that it is maternally inherited (Avise et al., 1987; Di

Fiore, 2003; Wilson et al., 1985). Figure 1.3 shows a diagrammatical presentation of the green monkey (*C. a. sabaeus)* mitochondrial genome (Wang, 2006).

A number of mtDNA regions can be used in population-level studies, each with different levels of sensitivity and uses. These regions include two ribosomal RNA genes, 13 protein-encoding genes, 22 mitochondrial tRNA genes and the mitochondrial control region (Grechko, 2002). The 12s rRNA gene is a highly conserved region of the mitochondrion, and is usually used for studies of phyla and subphyla, whereas the 16s rRNA gene is more generally used for research on families and genera.

Mitochondrial protein-encoding genes have faster evolutionary rates than ribosomal RNA genes, and are thus more suited for genetic analysis of families, genera and species. Some of the more commonly used genes include cytochrome b (cyt b), NADH dehydrogenase subunit 5 and cytochrome oxidase I (COI) (Arif and Khan, 2009). COI is an important “barcoding” gene used for the identification of taxa and species, as well as for quality control of samples (Lorenz et al., 2005).

The mitochondrial control region (CR) or D-loop is responsible for the replication and expression of the mitochondrial genome. It is also the most highly variable, non-coding section of the animal mitochondrial genome. It consists of two hypervariable regions (HVR-1 and HVR-2) flanking a central conserved region (Saccone et al. 1991; Sbisà et al., 1997; Avise, 2000). The CR is very useful during species and sub-species level studies (Arif and Khan, 2009).

Collins and Dubach (2000) used sequencing of the CR in conjunction with the mtDNA cytochrome c oxidase subunit II gene to determine the phylogenetic relationships among spider monkeys (*Ateles*) in Central and South America. These authors were able to identify four monophyletic species of spider monkeys, which contradicted previous taxonomic classifications.

Hapke et al. (2001) sequenced the HVR-1 of 74 Eritrean hamadryas baboons (*Papio hamadryas hamadryas*) to establish the influence of dispersal patterns on population

genetic structure. The authors' results pointed toward female dispersal, which supports the behavioural observations done over a broad geographic range.

Shimada et al. (2002) calculated mitochondrial sequence diversity between three subspecies of *C. aethiops* as well as *Cercopithecus mitis* and *Cercopithecus neglectus* using a ± 284 bp section of the mtDNA control region (CR) and the 12S rRNA gene. Analysis of the mtDNA CR data indicated significant mitochondrial clustering within subspecies. This was linked to the occurrence of female philopatry within most species of OWMs, where only males migrate between local populations (Melnick and Hoelzer, 1992, 1996).

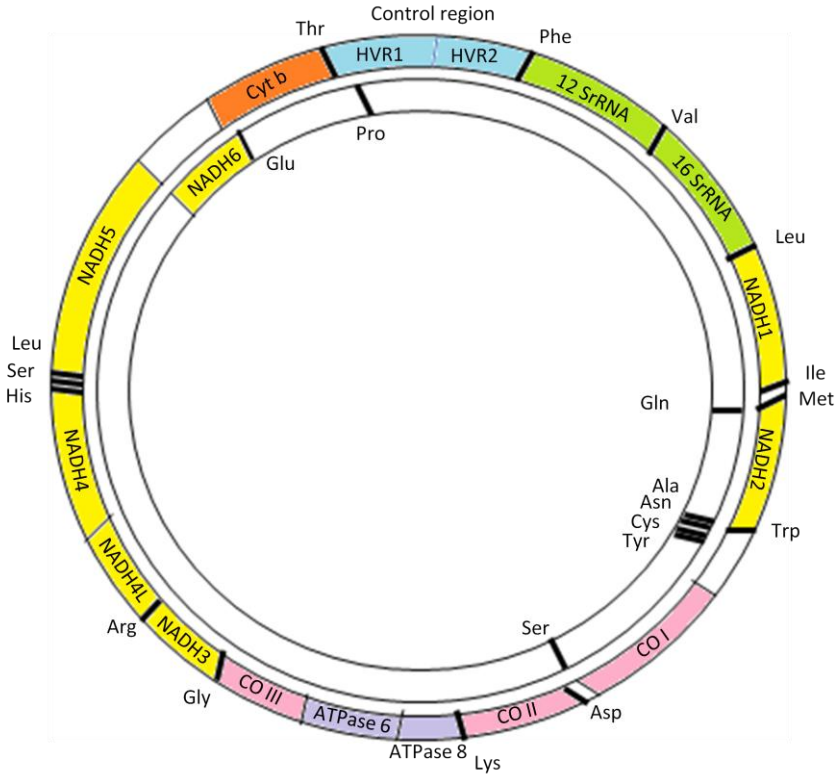


Figure 1.3: Diagrammatic representation of the mitochondrial genome of *C a. sabaeus.* (Derived from Wang, 2006)

Mitochondrial DNA differentiation between three subpopulations of cynomologus macaques (*Macaca fasicularis*) was analysed by Shiina *et al. (*2010). A total of 209 monkeys were sampled for DNA analysis. A fragment of the mtDNA D-loop region was sequenced. The authors were able to identify 87 mtDNA haplotypes. The phylogenetic analysis identified the presence of three distinct lineages (Indochinese, Indonesian and Filipino lineages).

Microsatellites are highly variable, co-dominant markers, consisting of tandem repeat sequences of 2–6 bp. The repeats usually differ in lengths of between 5 and 40 repeats. They are found in most taxa, and are also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR) or short tandem repeats (STR) (Selkoe and Toonen, 2006; Arif and Khan, 2009). These markers are extensively used in population and conservation research. This is because their high mutation rate, which is much higher than that of the rest of the genome (Jarne and Lagoda, 1996), results in highly polymorphic, selectively neutral markers. Table 1.3 presents the microsatellite repeat units most commonly found in primates.

Grobler *et al*. (2006) used four human microsatellite markers to establish the genetic variation of vervet monkeys (*Chlorocebus aethiops*) in the north-eastern parts of South Africa. After analysing data from 36 animals, it was concluded that there is no genetic structuring within this component of the South African vervet monkey population. These authors did, however, suggest that further, more extensive studies should be done to reach a final conclusion.

A vervet monkey genetic linkage map was developed by Jasinska et al. (2007) using over 300 human microsatellite markers. Vervet monkeys are seen as key model animals in biomedical research, and this genetic linkage map was developed to assist in the mapping of complex traits in these animals.

Random Amplified Polymorphic DNA (RAPD) is a PCR-based technique that utilizes a single short primer (usually 10-bp in length) and produces dominant, multi-

locus DNA profiles (Di Fiore, 2003), and was first used in the early 1990s (Williams *et al*., 1990). These markers are known as dominant markers, due to their inability to differentiate between homozygotes and heterozygotes. In other words, RAPDs can only show the presence or absence of an allele (Williams *et al*., 1990; Arif and Khan, 2009). The lack of repeatability of this technique is another disadvantage (Oyler-McCance and Leberg, 2005) which should be considered before implementing RAPD analysis in a study. The upside of using RAPD assays is the requirement of small quantities of DNA, as well as the low cost involved (Di Fiore, 2003) because no prior knowledge of a species genome is required.

Neveu *et al.* (1998) used RAPDs to evaluate the level of genetic diversity between captive and wild mouse lemurs *(Microcebus murinus*) and showed that the captive populations suffered a loss of genetic diversity. There was also variation between captive groups, which was associated with the size of the founder populations, as well as the management of the breeding programmes (Neveu *et al.*, 1998).

Amplified Fragment Length Polymorphism (AFLP) is a DNA fingerprinting technique first described by Vos *et al.* (1995). This method is based on the restriction digestion of whole genomic DNA, ligation of adapters for PCR, and the PCR amplification of the obtained restriction fragments. The amplified fragments are then viewed and scored through gel electrophoresis. AFLP's are dominant markers, producing hundreds of bands per reaction. The main advantages of this technique are its repeatability and the high level of specificity and lack of need of previous knowledge of the genes of the species of interest (Vos *et al.*, 1995; Oyler-McCance and Leberg, 2005; Arif and Khan, 2009).

Tanee *et al.* (2006) used cytogenetics in combination with AFLP markers to evaluate the genetic structuring of five species of macaques from north-eastern and southern Thailand. A combination of seven primer pairs was used. It was found that 50.7% of the obtained bands were polymorphic, and the averages of the inter-specific genetic distance (D) ranged from 0.269 to 0.380. The loss of genetic diversity in Thailand was ascribed to population fragmentation caused by deforestation (Tanee *et al.,* 2006).

Table 1.3: Microsatellite repeat units found in primates. (Derived from Toth *et al.,* 2000)

	Repeat unit length:	Repeat unit:
Mononucleotide	1	A
		C
Dinucleotide	2	AC
		AG
		AT
		CG
Trinucleotide	3	AAC
		AAG
		AAT
		ACC
		ACT
		AGC
		AGG
		ATC
		CCG
Tertanucleotide	4	AAAT
		AAAG
		AAAC
Pentanucleotide	5	AAAAC
		AAAAT
Hexanucleotide	6	AAAAAC
		AAAAAT
		AAAAAG

1.7 Aims of the study

The conservation of individual population groups is a high priority when considering the overall protection and conservation of a species as a whole. Vervet monkeys have been listed by CITES under Schedule 2 as threatened since 1977 (UNEP - WCMC, 2011), but are listed as "Least Concern" on the Red Data list (Kingdon *et al.*, 2008). Despite their CITES status, vervet monkeys are still not protected by any of the nine Provincial Conservation Departments in South Africa. Vervets are seen as problem animals in most agricultural communities (Venter, 2008), reportedly causing substantial damage to crops and thus are persecuted to prevent such damages. Large numbers of vervet monkeys tend to end up in rehabilitation centres due to being injured and / or orphaned during human / non-human primate conflicts. Most rehabilitation centres aim to re-introduce rehabilitated animals back to their natural habitats. The re-introduction of rehabilitated animals should however be done while keeping in mind that the genetic integrity of the natural populations should not be disturbed. It is thus critical to determine whether real genetic differentiation exists among vervet monkeys across South Africa. Thus, the main aim of this project is to identify patterns of genetic differentiation of vervet monkeys across South Africa. If such differentiation does exist, it should also be established in subsequent studies whether the detected differences have real adaptive significance, i.e. the fact that animals from different regions show some differentiation could be a natural consequence of genetic isolation, which does not necessarily preclude mixing of animals. In this regard, Moritz (2002) cautioned that molecular criteria impose arbitrary thresholds and categories on an evolutionary process that is in reality based on a continuum of divergence. Overly zealous assignment of populations as ESUs also ignores natural structure (as caused by isolation) and it has been suggested that incorrect application of the ESU concept could in extreme cases hinder rather than aid in the recognition of biodiversity.

To determine the level of genetic differentiation within and between vervet monkey populations across south-eastern South Africa, a segment of the mitochondrial control region (CR), was sequenced to aid in the identification of patterns of genetic structure.

1.8 Outline of the thesis

Following this introduction to vervet monkeys, the methods and materials used during the field and laboratory phase of the project will be provided in Chapter 2. Chapter 3 will report on the results obtained from the mtDNA analysis of these various conspecific vervet populations. Focus will be given to the control region or D-loop segment of the mitochondrial genome. The results then will be discussed in Chapter 4, with reference to natural patterns of genetic diversity in vervet monkeys, and possible historical routes of migration in the country.

Chapter 2:

Materials and methods

2.1 Material and methods

2.1.1 Sampling locations

Tissue samples from vervet monkeys were collected from 11 localities in three provinces of South Africa, adding up to a total of 140 tissue samples, which were used for DNA analysis (Figure 2.1). Five samples from the Polokwane area, Limpopo, also were included as a reference group to provide a wider perspective of the north-to-south distribution of genetic diversity in vervet monkeys. The sample names and localities of the populations used in the study are listed in Table 2.1. The age and sex of the animals used in this study are listed in Table 2.2.

Soetdoring NR (FS)

Twelve animals were sampled at the Soetdoring Nature Reserve (NR) (28°49'19''S 26°03'34''E). The reserve is situated approximately 25km north-west of Bloemfontein, Free State. All the animals originated from the same troop.

Gariep Dam (FS/EC)

Samples were collected from two locations around the Gariep Dam area. The Gariep Dam is located on the border of the Free State and Eastern Cape. The first location was at the Fish Hatchery (30° 36' 27''S 25° 26' 51''E), located downstream from the dam, where 11 animals were collected. The second sampling location was situated on a local farm (Southey Farm - 30° 36' 24''S 25° 26' 47''E). Four animals were sampled at the farm, adding up to a total of 15 samples from the Gariep Dam area.

Sandveld NR (FS)

The Sandveld NR (27° 40' 33.9''S 25° 40' 54.3''E) near Bloemhof was the most western sampling site, and 13 animals were sampled from two troops. These troops were located at opposite ends of the reserve. The first troop was located near the office of the reserve, and six animals were trapped and sampled from this location. The second troop was situated on to south-eastern edge of the reserve, with seven animals sampled from this troop.

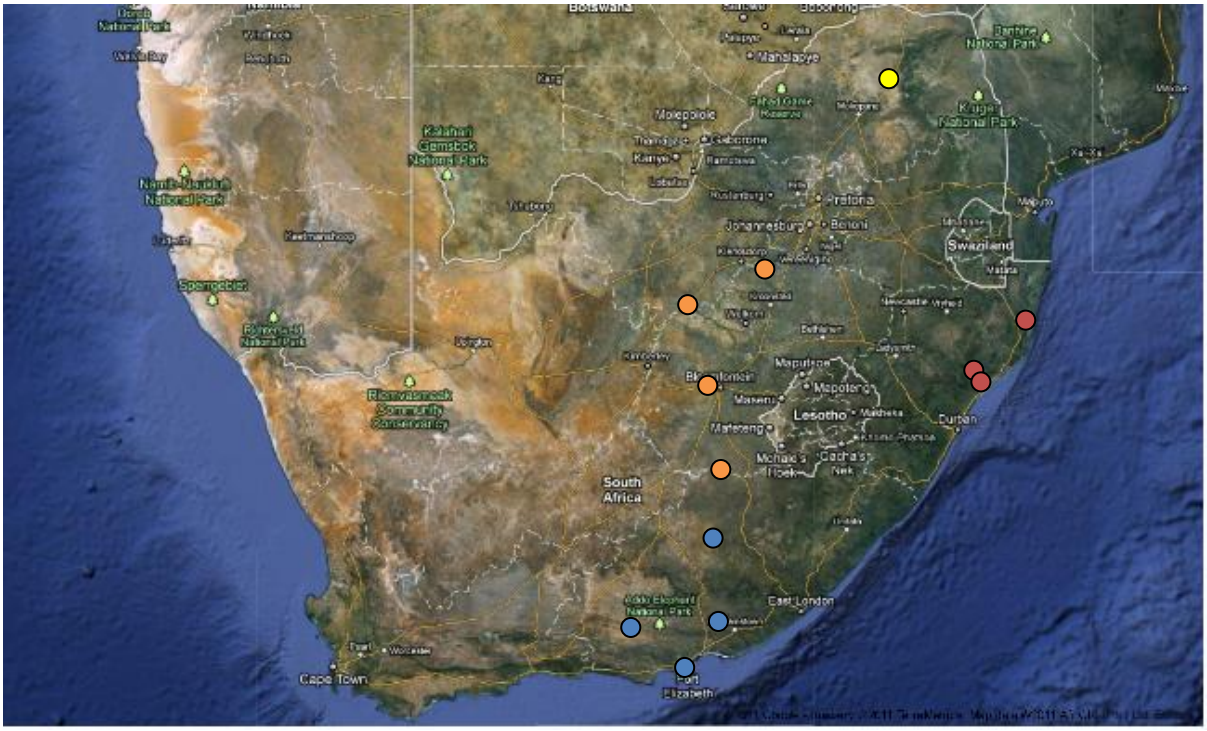


Figure 2.1: Sampling locations across the Eastern Cape (blue dots), Free State (orange dots), Kwa-Zulu Natal (red dots) and Polokwane (yellow dot).
[**Image:** http://maps.google.com]

Parys (FS)

The Parys area was the most northern sampling locality in the Free State province. The sampling was carried out at the Parys Golf Estate (26° 53' 37.9''S 27° 27' 30.3''E), where 11 animals were sampled from one troop.

Tsolwana NR (EC)

Tsolwana NR was the most northern site of the Eastern Cape sampling localities. This reserve (32°08'41.8''S 26°26'38.22''E) is located ± 40 km south-east of Tarkastad and ± 45 km south-west of Queenstown. A total of 13 animals were sampled from two trapping locations. It was unclear whether all samples were from one population or two, since the animals were never trapped at both locations at the same time, and both trapping locations were within a reasonable distance to be in the home-range of a single troop.

Baviaanskloof (EC)

Two sampling locations were chosen in the Baviaanskloof area. Five animals were trapped and sampled in the Geelhoudbos (33° 39' 59.82"S 24° 14' 38.22"E) area and six animals were sampled at the Rooiplaat farm (33° 36' 22.80"S 24° 11' 45.36"E), for a total of 11 animals.

Shamwari PGR (EC)

The third sample group from the EC were trapped and sampled at Shamwari Private Game Reserve (PGR). The reserve is situated ± 70 km north-east of Port Elizabeth. A total of 19 animals were trapped at two localities. Seven animals were trapped at the Shamwari garbage dump (33° 28’ 41.9’’S 26° 01’ 46.8’’E) and 13 at the Harden lodge (33° 28’ 25.0’’S 26° 02’ 32.2’’E).

NMMU (EC)

The Nelson Mandela Metropolitan University (NMMU) (30° 00’ 34’’S 25° 40’ 10’’E) is located in the Summerstrand area of Port Elizabeth, and includes a conservation area along the coast. Several monkey troops have been reported in this area, as well as on the NMMU campus. A total of 11 monkeys were trapped on the campus, in the gardens of a local hostel.

St. Lucia area (KZN)

Two localities were sampled in the St. Lucia area, Kwa-Zulu Natal, namely Futululu Park (32°16’55.26’’S 28°26’21.06’’E) situated 17 km west of St. Lucia and the Maurann Farm (32°17’16.14’’S 28°26’51.72’’E) located ± 20 km west of St. Lucia. In total, 20 monkeys were trapped in this area. Nine animals were trapped at Futululu and 11 animals were trapped at the Maurann Farm.

Blythedale Beach (KZN)

A number of animals were sampled at the Alize Beach Cottage, Blythedale Beach, Kwa-Zulu Natal (29°22’’28.8’S 31°20’56.4’’E). Only four of these samples were viable for DNA extraction. This was due to technical difficulties in the field.

Thorny Park Estate, Zinkwazi (KZN)

The Thorny Park Estate is located near Zinkwazi, Kwa-Zulu Natal (29°11’09.1’’S 31°26’30.1’’E). Only five of the tissue samples taken were viable for DNA analysis. This was also due to technical difficulties.

Polokwane (Lim)

Five samples of animals trapped and sampled at the Bird Sanctuary just outside Polokwane were also included in this study. These samples represent the most northern group of the sample populations, and were included in the study to provide an indication of the genetic association between vervet monkeys in the northern parts of South Africa and in sample regions chosen for the current study.

Table 2.1: A listing of the localities at which vervet monkeys were sampled in this study. The total number of samples from each province is indicated at the bottom of the table. A total number of 140 samples were used for genetic analysis.

Area of Origin:			
Eastern Cape	**Free State**	**Kwa-Zulu Natal**	**Limpopo**
Baviaanskloof	**Soetdoring**	**St. Lucia**	**Polokwane**
BKL 01	SO 01	SL 01	PK 01
BKL 02	SO 02	SL 02	PK 02
BKL 03	SO 03	SL 03	PK 03
BKL 04	SO 04	SL 04	PK 04
BKL 05	SO 05	SL 05	PK 05
BKL 06	SO 06	SL 06	
BKL 07	A 07	SL 07	
BKL 08	A 08	SL 08	
BKL 09	A 09	SL 09	
BKL 10	A 10	SL 10	
BKL 11	A 11	SL 11	
	A 12	SL 12	
Port Elizabeth		SL 13	
PE 01	**Parys**	SL 14	
PE 02	P 01	SL 15	
PE 03	P 02	SL 16	
PE 04	P 03	SL 17	
PE 05	P 04	SL 18	
PE 06	P 05	SL 19	
PE 07	P 06	SL 20	
PE 08	P 07		
PE 09	P 08		
PE 10	P 09		
PE 11	P 10		
	P 11		

**** Table continues on the next page***

Table 2.1 (Continued):

Area of Origin:			
Eastern Cape	**Free State**	**Kwa-Zulu Natal**	**Limpopo**
Tsolwana	**Bloemhof**	**Blythedale Beach**	
T 01	SNR 01	BB 01	
T 02	SNR 02	BB 02	
T 03	SNR 03	BB 03	
T 04	SNR 04	BB 04	
T 05	SNR 05		
T 06	SNR 06	**Thorny Park Estate**	
T 07	SNR 07	TP 01	
T 08	SNR 08	TP 02	
T 09	SNR 09	TP 03	
T 10	SNR 10	TP 04	
T 11	SNR 11	TP 05	
T 12	SNR 12		
T 13	SNR 13		
	SNR 14		
Shamwari			
SW 01	**Gariep**		
SW 02	G 01		
SW 03	G 02		
SW 04	G 03		
SW 05	G 04		
SW 06	G 05		
SW 07	G 06		
SW 08	G 07		
SW 09	G 08		
SW 10	G 09		
SW 11	G 10		
SW 12	G 11		
SW 13	G 12		
SW 14	G 13		
SW 15	G 14		
SW 16	G 15		
SW 17			
SW 18			
SW 19			
54	52	29	5

Table 2.2: The age and sex of vervet monkeys trapped at each site, with the number of individuals within each category. [BKL – Baviaanskloof; PE – Port Elizabeth; SW – Shamwari PGR; T NR – Tsolwana NR; SO NR – Soetdoring NR; S NR – Sandveld NR; P - Parys; G - Gariep; BB – Blythedale Beach; TP Estate – Thorny Park Estate; St. L – St. Lucia; PK – Polokwane]

Animal age:	Animal sex:	BKL	PE	SW	T NR	SO NR	S NR	P	G	BB	TP Estate	St. L	PK
Infant	M		2					2			1	1	
	F											2	
Young Juvenile	M	1	3	2	1	2		1	2			1	
	F	1	2	1	1		3	3	1		2	1	1
Juvenile	M		1	2	1	5	1	1	2			2	1
	F	2	1			1	1	1		1		3	1
Sub-adult	M			1						1		1	
	F		2	3	2	1	1	1	7	1		2	1
Adult	M	3		3	7		1	1					
	F	4		4	1	3	2	1	3	1	2	4	
Old	M												
	F			1			3					2	1

2.1.2 Sampling methods

Animals were sampled using a trap-and-release method, using basic drop traps derived from an old African bird trap design (Figure 2.2) described by Grobler and Turner (2010). The trap is activated when the animal touches a set trigger stick attached to suitable bait item. The trapped animals then were sedated by a licensed veterinarian using Zolatil 100 or Ketamine. The animal remained sedated while samples were taken. An ear biopsy of approximately 5x3 mm was taken from each individual and then stored in absolute ethanol. All samples were then stored at 4°C until DNA extraction. After sampling, the animals were placed in a recovery area where they were protected from predators as well as conspecific rivals. The animals gradually recovered from the anaesthetics within a period of approximately 1 hour, though usually much less. All techniques for trapping, sedation and sampling were approved by the Interfaculty Animal Ethics Committee of UFS, and carried out under permits issued by the relevant provincial conservation authorities.

Figure 2.2: The drop traps used during the trapping and sampling procedures. [**Photo** by J.P. Grobler]

2.2 DNA extraction

Tissue samples obtained from the ear biopsies were used for DNA extraction. Two DNA extraction kits were used interchangeably, namely the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics) and the Qiagen QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) using the manufacturer's instructions, as follows:

Roche High Pure PCR Template Preparation Kit:

Approximately 50 mg of tissue was finely cut and placed in a 1.5-ml microcentrifuge tube, containing 200 μl tissue lysis buffer and 40 μl proteinase K. The mixture was then vortexed for ± 20 seconds, and incubated overnight at 55°C. After digestion, 200 μl binding buffer was added. The mixture was then vortexed for ± 20 seconds and incubated at 70°C for 10 min. After incubation, 100 μl isopropanol was added and the tube contents mixed. The liquid was then added to the upper reservoir of a High Filter tube assembly and centrifuged for 1 min at 8 000 x g. After centrifugation, the filter tube was placed in a new collection tube and the flow-through liquid was discarded with the used collection tube. Next, 500 μl inhibitor removal buffer was added to the upper reservoir of the filter tube and it was centrifuged for 1 min at 8 00 x g. The filter tube was again placed in a new collection tube, and 500 μl wash buffer was added to the upper reservoir of the filter tube and centrifuged for 1 in at 8 000 x g. This step was done twice. After discarding the flow-through liquid the second time, the filter tube was placed in the same collection tube and centrifuged for an additional 10 s at full speed. This was to ensure the removal of all residual wash buffer. To elute the DNA, the filter tube was placed in a clean 1.5-ml microcentrifuge tube and 200 μl pre-warmed elution buffer added to the upper reservoir of the filter tube. The assembly was the centrifuged for 1 min at 8 000 x g.

Qiagen QIAamp DNA Mini Kit:

Approximately 25 mg of tissue sample was cut into small pieces. The sample was added to a 1.5-ml microcentrifuge tube containing 180 μl of buffer ATL, after which 20 μl proteinase K was added and the contents mixed by vortexing. The mixture was then incubated at 56°C until the tissue samples were completely lysed. The tubes were occasionally mixed by vortexing to ensure the dispersal of the samples. After incubation, the tubes were briefly centrifuged, to ensure the removal of any water drops from the inside of the lid. 200 μl buffer AL was added to the sample mixture. The mixture was then mixed by vortexing and incubated at 70°C for 10 min. The tubes were again briefly centrifuged after incubation, to ensure the removal of any water drops from the inside of the lid. 200 μl ethanol (96-100 %) was added to the sample mixture and mixed by vortexing. The mixture was then carefully added to the QIAamp mini spin column without wetting the rim. The assembly was centrifuged at 6 000 x g (8 000 rpm) for 1 min. The QIAamp mini spin column with its contents was then moved to a clean 2-ml collection tube, while discarding the tube containing the filtrate. 500 μl buffer AW1 was added without wetting the rim. The assembly was then centrifuged at 6 000 x g (8 000 rpm) for 1 min. The QIAamp mini spin column was next placed in a clean 2 ml collection tube, while discarding the collection tube containing the filtrate. 500 μl Buffer AW2 was added without wetting the rim and the tube was centrifuged at full speed (20 000 x g; 14 000 rpm) for 3 min. The flow-through was discarded and the QIAamp Mini spin column was placed in the same collection tube and centrifuged for an additional 1 min at full speed. This was to ensure that all residual wash buffer was removed. For DNA elution, the QIAamp mini spin column was placed in a clean 1.5-ml microcentrifuge tube. The collection tube containing the filtrate was discarded. 200 μl buffer AE was added to the QIAamp mini spin column. The assembly was incubated at room temperature for 1 min, and then centrifuged at 6 000 x g (8 000 rpm) for 1 min.

DNA quantification was done with a Nanodrop® ND-1000 Spectrophotometer v3.7 to evaluate the success of the extraction procedures.

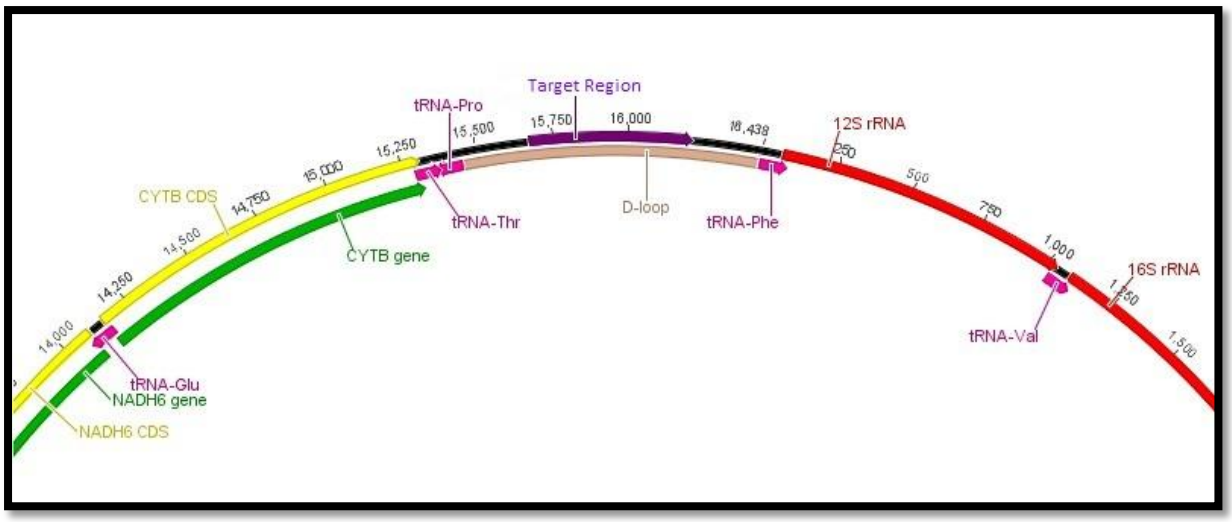


Figure 2.3: The target region of the mitochondrial control region, covering sections of both hypervariable regions and the central conserved region. (Image generated with Geneious v5.4 software)

2.3 Gene Sequencing

Following DNA extraction and quantification, a ± 700 bp region of the mitochondrial control region (CR or D-Loop), which included part of hypervariable region 1 (HVR-1), the central conserved region and part of hyper variableregion 2 (HVR-2), was amplified (Figure 2.3). The primers Vervet-HVR Fw and Vervet-HVR Rev were designed in collaboration with Inqaba Biotec. The primers were designed with the following sequences:

Vervet-HVR Fw – 5'-CGT GCA TTA CTG CTA GC-3', and

Vervet-HVR Rev – 5'-GTG TTG TGG GTT GGT TG-3'.

The minimum / maximum melting temperature (Tm) for both primers was 57°C. PCR reaction mixtures consisted of 25-100ng DNA, 1x buffer containing 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 1.5 U Super Therm GOLD Taq polymerase, 5 μM of each primer and 9.3 μl dH_2O to make up a 25-μl reaction mixture. The cycle parameters were: 10 minutes at 95°C for initial denaturation, amplification for 40 cycles of 30 seconds at 94°C, 30 seconds at 55-63°C and 1 minute at 72°C, with a final elongation step of 10 minutes at 72°C, and with a 4°C hold. The amplified products were viewed with a

known size-standard ladder on a 1% agarosegel stained with GelRed™ (Biotium Inc., Hayward, CA, USA). Visualization was done under a UV light.

PCR products of adequate quality were purified using the BioSpin PCR Purification Kit (BioFlux, Tokyo, Japan) to remove any unused primers, dNTPs and denatured DNA. The purified products then were labelled using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Division, Perkin-Elmer, Foster, CA, USA). The labelling reaction mixture consisted of 2.5 μl BigDye Terminator v3.1 sequencing buffer (X5), 0.5 μl BigDye® Terminator mix, 1 μl of 2 mM primer, 4 μl dH_2O and 2 μl DNA template. The cycle parameters were: 2 minutes at 94°C for initial denaturation, amplification for 35 cycles of 15 seconds at 94°C, 10 seconds at 53°C and 3 minutes at 60 min, with a 4°C hold. The labelled products were purified using the ZR DNA Sequencing Clean-up™ Kit (Zymo Research, Orange, CA, USA) and sequenced on an ABI 3130 Genetic Analyzer.

2.4 Statistical Analysis

2.4.1 Sequence alignment

Raw gene sequences were viewed and assembled using the software Geneious Pro 5.4 (Drummond *et al.,* 2011). This programme is an integrated, cross-platform bioinformatics software package. The programme can be used to perform various tasks, which include the manipulation, identification and exploring of biological data. DNA as well as protein sequences can be analysed. The main features of this programme include sequence alignment, phylogenetic analysis, contig assembly, primer design and cloning (About – Geneious, 2012). Table 2.3 lists the parameters used for sequence assembly during the current study.

The assembled sequences were aligned using ClustalW as implemented in MEGA v5 (Tamura *et al.,* 2011). The sequences were trimmed to 417 bp to ensure uniform sequence lengths during analysis. MEGA v5 was developed to provide the biologist with an integrated suite of tools for statistical analyses of DNA and protein sequence data from an evolutionary perspective. Such tools include sequence alignment, phylogenetic tree construction and visualization, as well as the testing of an array of evolutionary hypotheses and determining sequence divergences (Tamura *et al.*, 2011). Table 2.4 indicates the parameters used for the sequence alignments. One published sequence, AY863426 for *Cercopithecus aethiops,* was used as an out-group.

Table 2.3: Parameters used for DNA sequence assemble using Geneious Pro 5.4 (Drummond *et al.,* 2011).

Assembly Parameters:	
Assembly Options:	
Minimum Overlap:	25
Overlap Identity %:	80
Alignment Options:	
Gap Open / Extend Penalty:	18
Mismatch Score:	-9
Match Score	5

Table 2.4: Parameters used for DNA alignment using ClustalW in Mega v5 (Tamura *et al.,* 2011).

Alignment parameters:	
Pairwise Alignment:	
Gap Opening Penalty	15
Gap Extension Penalty	6.66
Multiple Alignment:	
Gap Opening Penalty	15
Gap Extention Penalty	6.66
DNA Weight Matrix	IUB
Transition Weight	0.5
Use Negative Matrix	OFF
Delay Divergent Cutoff (%)	30

2.4.2 Genetic diversity

Haplotype frequencies

The haplotype frequency distribution among samples was calculated using the program Arlequin v3.1 (Excoffier *et al.* 2005). It was chosen to estimate the haplotype frequencies by counting and to present them as haplotype counts. A search for shared haplotypes was conducted during the analysis, thereby identifying shared haplotypes within and among samples.

Nucleotide diversity

Intrapopulation nucleotide diversity analysis was done for each sampled population. This is the measure of the nucleotide diversity among several sequences in a given region of the genome within a particular population, and is then given as an average over all loci within that population. Arlequin v3.1 software was used for these calculations.

2.4.3 Genetic differentiation and drift

Analyses of Molecular Variance (AMOVA) and population pairwise F_{ST} values

An Analyses of Molecular Variance (AMOVA) and population pairwise F_{ST} values were calculated using Arlequin v3.1. The F_{ST} values were calculated using Kimura's (1980) two-parameter model (K2P). Arlequin v3.1 consists of several basic and advanced methods for population genetics analysis. Some of the features include the computation of standard genetic diversity indices, allele and haplotype frequency estimation, as well as analyses of population subdivision using the AMOVA framework (Excoffier *et al.*, 2005). F-statistics were introduced by Sewall Wright (1951) and Gustave Malécot (1948), who worked independently during the 1940s and 1950s. This measure is used to evaluate the levels of genetic diversity among populations due to allele frequency differentiation among said populations. The F_{ST} values were calculated at a significance level of 0.05 with 10,000 permutations.

2.4.4 Molecular Phylogenetic Analysis

Maximum likelihood analysis

A phylogenetic tree was constructed using maximum likelihood procedures in Mega v.5 using the Tamura - Nei (1993) method to identify the relationships between the individual samples and sample sites. Phylogenetic testing was done using the bootstrap method, with 1,000 bootstrap replications.

jModelTest v0.1.1 (Guidon and Gascuel, 2003; Posada, 2008) was used to identify the most appropriate nucleotide substitution model to use for the calculation of an evolutionary tree. The haplotype data obtained from Arlequin v3.1 were used for these calculations. It was established that Kimura's (1980) two-parameter model (K80 or K2P), with a gamma (Γ) distribution for rate variation across sites, would best fit the haplotype data set at hand for the construction of a ML phylogenetic tree. Mr Bayes (Ronquist et al., 2012) software then was used for the construction of a ML phylogenetic tree using a K80 + Γ statistical model and the Markov Chain Monte Carlo (MCMC) technique. The Mr Bayes v3.2 software is used for Bayesian inference and model choice across a vast array of phylogenetic and evolutionary models.

Haplotype networks

A phylogenetic network was constructed with the software Network v4.6.1 (Fluxus Engineering, 2011) using the haplotype data obtained. The number of mutational steps which most probably might have occurred between the linked haplotypes was calculated and indicated on the network. Network v4.6.1 is used for the construction of phylogenetic networks and trees, to infer ancestral types and potential types, determine evolutionary branching and variants, and to estimate datings. Data that can be used include mtDNA, Y-STR, amino acid, RNA, viral DNA, bacterial DNA, some effectively non-recombining autosomal DNA, as well as non-biomolecular data such as linguistic data (Fluxus Engineering, 2011).

Chapter 3:

Results

3.1 Gene sequences of the mitochondrial control region

A sequence of 550–700 bp was amplified from each vervet monkey sample using the primer pair Vervet-HVR Fw and Vervet-HVR Rev. The amplified product contained a section of the mitochondrial control region (CR or D-loop), which overlaps hypervariable region 1 (HVR-1), the central conserved region (CCR) and hypervariable region 2 (HVR-2). The length of trimmed assemblies obtained from Geneious Pro v4.7.4 ranged from 550-680 bp. A total of 140 assemblies were obtained for analysis. The final alignments acquired from Mega v5 (Tamura *et al.*, 2011) were then further trimmed to 417 bp to ensure equal sequence lengths for all individuals throughout the analysis phase. The trimmed sequences can be viewed in Appendix A.

3.2 Genetic diversity

Haplotype frequencies

Twelve distinct haplotypes were identified. Haplotype frequencies obtained using the software package Arlequin v3.1 are shown in Table 3.1. Out of the 12 haplotypes, five were observed in the Free State Province (FS_Hap 01-05), four in the Eastern Cape Province (EC_Hap 01-04), two haplotypes in Kwa-Zulu Natal and one in Limpopo Province. These haplotype sequences are currently being submitted to the GenBank database.

Only one or two haplotype were exhibited by each locality. Haplotype FS_Hap 01 was exhibited by individuals from the Soetdoring NR, Parys and Sandveld NR populations. Haplotype FS_Hap 02 was exhibited by animals from Soetdoring NR and Parys. Haplotypes FS_Hap 03 and 04 originated in the Sandveld NR sample group. Haplotype FS_Hap 05 comprise of all the animals from the Gariep sample group. All the animals from the Baviaanskloof sample group form part of haplotype EC_Hap 01, with haplotype EC_Hap 02 consisting of all the individuals from the Port Elizabeth and Shamwari NR sample groups and haplotypes EC_Hap 03-04 comprising of the animals from the Tsolwana NR sample group.

Table 3.1: The 12 haplotypes identified among South African vervet monkeys. The relative haplotype frequencies within each population are provided in each respective column, with the number of individuals exhibiting each haplotype. [BKL – Baviaanskloof; PE – Port Elizabeth; SW – Shamwari PGR; T NR – Tsolwana NR; SO NR – Soetdoring NR; S NR – Sandveld NR; P - Parys; G - Gariep; BB – Blythedale Beach; TP Estate – Thorny Park Estate; St. L – St. Lucia; PK – Polokwane]

Haplotype Name:	**SO NR (12)**	**G (15)**	**P (11)**	**S NR (14)**	**BKL (11)**	**PE (11)**	**SW (15)**	**T NR (13)**	**BB (4)**	**TP Estate (5)**	**St L (20)**	**PK (5)**
FS_Hap 01	8	0	8	10	0	0	0	0	0	0	0	0
	0.667	0	0.727	0.714	0	0	0	0	0	0	0	0
FS_Hap 02	4	0	3	0	0	0	0	0	0	0	0	0
	0.333	0	0.273	0	0	0	0	0	0	0	0	0
FS_Hap 03	0	0	0	3	0	0	0	0	0	0	0	0
	0	0	0	0.214	0	0	0	0	0	0	0	0
FS_Hap 04	0	0	0	1	0	0	0	0	0	0	0	0
	0	0	0	0.0714	0	0	0	0	0	0	0	0
FS_Hap 05	0	15	0	0	0	0	0	0	0	0	0	0
	0	1.0	0	0	0	0	0	0	0	0	0	0
EC_Hap 01	0	0	0	0	11	0	0	0	0	0	0	0
	0	0	0	0	1.0	0	0	0	0	0	0	0
EC_Hap 02	0	0	0	0	0	11	15	0	0	0	0	0
	0	0	0	0	0	1.0	1.0	0	0	0	0	0
EC_Hap 03	0	0	0	0	0	0	0	10	0	0	0	0
	0	0	0	0	0	0	0	0.769	0	0	0	0
EC_Hap 04	0	0	0	0	0	0	0	3	0	0	0	0
	0	0	0	0	0	0	0	0.231	0	0	0	0
KZN_Hap 01	0	0	0	0	0	0	0	0	4	5	0	0
	0	0	0	0	0	0	0	0	1.0	1.0	0	0
KZN_Hap 02	0	0	0	0	0	0	0	0	0	0	20	0
	0	0	0	0	0	0	0	0	0	0	1.0	0
Lim_Hap 01	0	0	0	0	0	0	0	0	0	0	0	5
	0	0	0	0	0	0	0	0	0	0	0	1.0

All the Blythedale Beach and the Thorny Park Estate animals exibiting haplotype KZN_Hap 01, with all of the St. Lucia animals forming part of the KZN_Hap 02 haplotype. Haplotype Lim_Hap 01 comprises of all the Polokwane animals.

Nucleotide diversity

The level of nucleotide diversity (π) for each population (Table 3.2) ranged from 0-0.038%. The Sandveld NR population showed the highest levels of nucleotide diversity, with the Gariep, Baviaanskloof, Port Elizabeth, Shamwari PGR, St. Lucia, Blythedale Beach, Thorny Park Estate and Polokwane populations showing no nucleotide diversity.

Table 3.2: Nucleotide diversity levels for each individual population of vervet monkeys.

Population:	Nucleotide diversity (π):
Soetdoring NR	0.001 ±0.001
Sandveld NR	0.038 ±0.02
Parys	0.001 ±0.001
Gariep	0
Baviaanskloof	0
Port Elizabeth	0
Shamwari PGR	0
Tsolwana NR	0.003 ±0.002
Blythedale Beach	0
Thorny Park Estate	0
St. Lucia	0
Polokwane	0

3.3 Genetic differentiation and drift

Analysis of Molecular Variance (AMOVA)

Analysis of molecular variance was done after grouping the populations according to general geographical origins. These regions consisted of the central plateau region of South Africa, which comprised most of the Free State province, the eastern coastal region, which form part of the Kwa-Zulu Natal province, and the south – eastern coastal region, which form part of the Eastern Cape province. The results from the AMOVA test are shown in Table 3.3. It was observed that the majority of the genetic variation is found between the different groups (i.e. between Free State, Eastern Cape and Kwazulu-Natal), with only 4.87% of the variation occurring within the populations. Results of this test show that there is some degree of genetic structuring within the overall vervet monkey sample population.

Table 3.3: The AMOVA test results for vervet monkey populations grouped by province of origin. Eastern Cape group – Baviaanskloof, Port Elizabeth, Samwari PGR and Tsolwana NR; Free State group – Soetdoring NR, Sandveld NR, Gariep and Parys; Kwa-Zulu Natal group – St. Lucia; Limpopo group – Polokwane.

Source of variation	Percentage of variation
Among groups	52.47
Among populations within groups	42.66
Within populations	4.87

A second AMOVA test was done with data from the two Kwa-Zulu Natal groups; St. Lucia forming one grouping and Blythedale Beach and Thorny Park Estate forming the other. The basis for this test was based on the results from the Haplotype Network analysis (see page 55), which indicated a significant difference between these two groupings. The results from this test are shown in Table 3.4.

Table 3.4: The second AMOVA test results represented as percentage of variation. The populations were grouped according to the clustering observed with the Haplotype Network results. Eastern Cape group – Baviaanskloof, Port Elizabeth, Samwari PGR and Tsolwana NR; Free State group – Soetdoring NR, Sandveld NR, Gariep and Parys; Kwa-Zulu Natal group – St. Lucia; Limpopo group – Polokwane.

Source of variation	Percentage of variation
Among groups	64.1
Among populations within groups	31.12
Within populations	4.78

F_{ST} values

The F_{ST} values and associated p-values among population pairs are listed in Table 3.5. In the Free State Province (cell in green), all pair-wise combinations of the Soetdoring NR, Sandveld NR and Parys population show low F_{ST} values and p-values that do not support the hypothesis of significant differentiation. The F_{ST} and p-values between these three populations and the animals from Gariep, further to the south, do however show significant differentiation for this gene region. In the Eastern Cape, there is significant genetic differentiation between most population pairs (cells with orange tone), with the exceptions of the Port Elizabeth and Shamwari PGR populations, which show insignificant levels of genetic differentiation.

Table 3.5: F_{ST} values among pairs of vervet monkey populations. Population designations: (1) Baviaanskloof, (2) Port Elizabeth, (3) Shamwari PGR, (4) Tsolwana NR, (5) Sandveld NR, (6) Gariep, (7) Parys, (8) Soetdoring NR, (9) St. Lucia, (10) Blythedale Beach, (11) Thorny Park Estate and (12) Polokwane. Green cells – Free State group; Orange cells – Eastern Cape group; Blue cells – Kwa-Zulu Natal group; Yellow toned cells – Intergroup F_{ST} values.

	1	2	3	4	5	6	7	8	9	10	11	12
1	*											
2	1; p = 0	*										
3	1; p = 0	0; p = 0.991	*									
4	0.883; p = 0	0.856; p = 0	0.891; p = 0	*								
5	0.808; p = 0	0.803; p = 0	0.847; p = 0	0.803; p = 0	*							
6	1; p = 0	1; p = 0	1; p = 0	0.982; p = 0	0.822; p = 0	*						
7	0.996; p = 0	0.996; p = 0	0.997; p = 0	0.983; p = 0	0.114; p = 0.207	0.996; p = 0	*					
8	0.995; p = 0	0.995; p = 0	0.996; p = 0	0.983; p = 0	0.124; p = 0.09	0.995; p = 0	-0.086; p = 0.991	*				
9	1; p = 0	1; p = 0	1; p = 0	0.987; p = 0	0.869; p = 0	1; p = 0	0.997; p = 0	0.996; p = 0	*			
10	1; p = 0	1; p = 0	1; p = 0	0.798; p = 0	0.735; p = 0	1; p = 0	0.993; p = 0	0.992; p = 0	1; p = 0	*		
11	1; p = 0	1; p = 0	1; p = 0	0.809; p = 0	0.749; p = 0	1; p = 0	0.994; p = 0	0.993; p = 0.009	1; p = 0	0; p = 0.991	*	
12	1; p = 0	1; p = 0	1; p = 0	0.974; p = 0	0.738; p = 0	1; p = 0	0.993; p = 0	0.991; p = 0.009	1; p = 0	1; p = 0	1; p = 0.018	*

Significant levels of differentiation are observed between the St. Lucia population and Blythedale beach and Thorny Park Estate populations (cells with blue tone). There is however no significant differentiation between the Blythedale beach population and the Thorny Park Estate population. All comparisons between regional groups (Free State, Eastern Cape, KwaZulu-Natal and Limpopo) show significant differentiation (average F_{ST} value = 0.851).

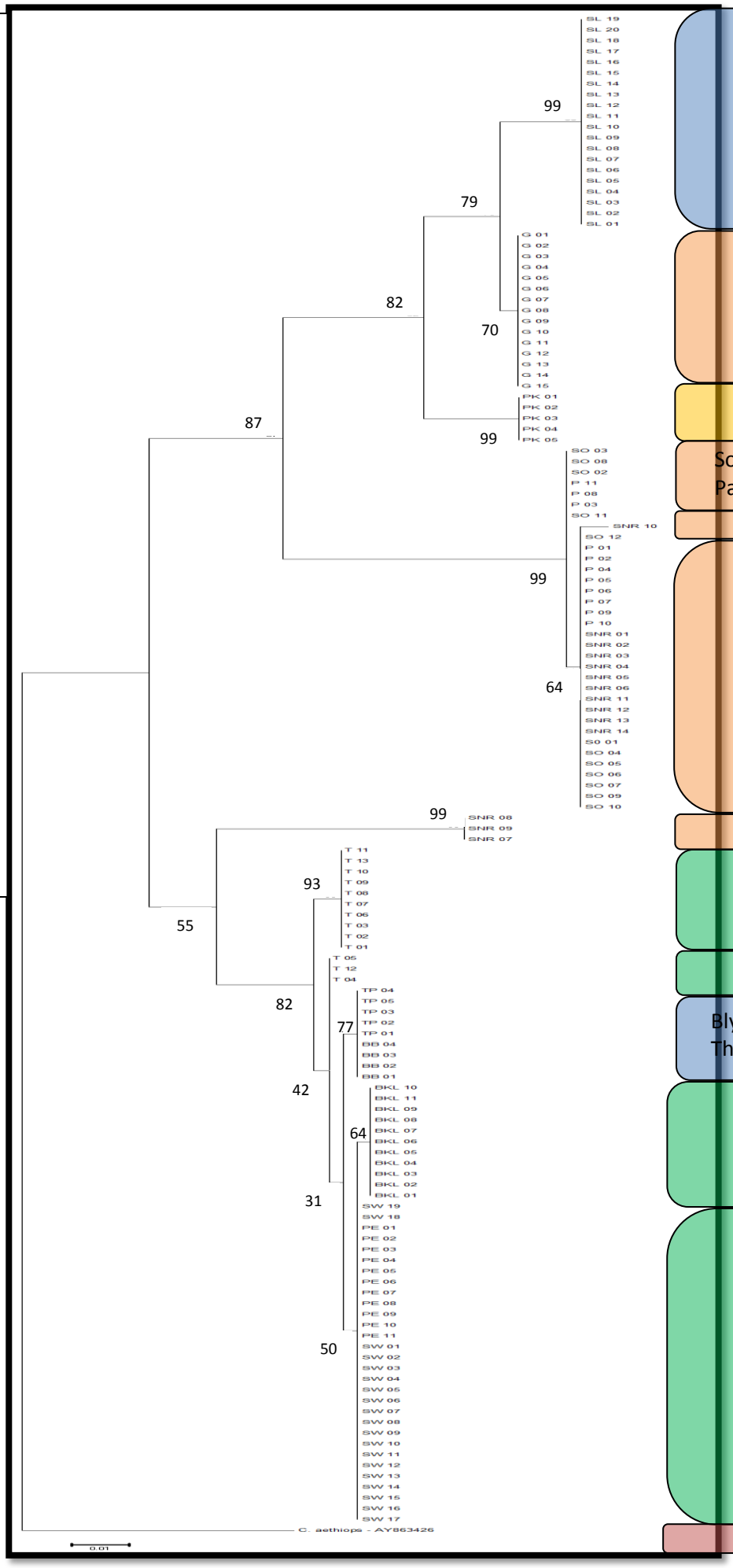
3.4 Molecular Phylogenetic Analysis

Maximum likelihood analysis

Molecular phylogenetic analysis of mtDNA control region sequences was done using the maximum likelihood (ML) approach. The sequence groupings observed in the subsequent ML tree (Figure 3.1) coincides with results of the haplotype analysis done with the software Arlequin v3.1: analysis of the phylogram showed two major branches. One branch contained all the Eastern Cape sequences with three sequences from the Sandveld NR and all the Blythedale Beach and Thorny Park Estate sequences. The three Sandveld NR sequences show a high level of differentiation when compared to the other Free State samples. The other branch includes the rest of the Free State sequences as well as the St. Lucia and Polokwane sequences. This second branch has another split, with the Soetdoring NR, Parys and Sandveld NR sequences diverging to one branch and the Gariep, Polokwane and the St. Lucia sequences to the other.

The phylogenetic tree constructed with Mr Bayes v3.2 using the calculated haplotype data is presented in Figure 3.2. Topology of this tree is largely congruent with that of the ML tree. Haplotypes EC_Hap 01 – 02 and FS_Hap 03 clustered to their respective locations within the phylogenetic tree with a high certainty (posterior probability = 100%). The Kwa-Zulu Natal haplotype KZN_Hap 01 was grouped with Eastern Cape haplotypes EC_Hap 03-04 with 51% posterior probability.

Figure 3.1: Molecular Phylogenetic analysis of vervet monkey mtDNA control region sequences by Maximum Likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches, based on 1,000 bootstrap replications. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 141 individual mtDNA sequences. All positions containing gaps and missing data were eliminated. There were a total of 406-bp positions in the final dataset. Evolutionary analyses were conducted in MEGA v5 (Tamura *et al.*, 2011).



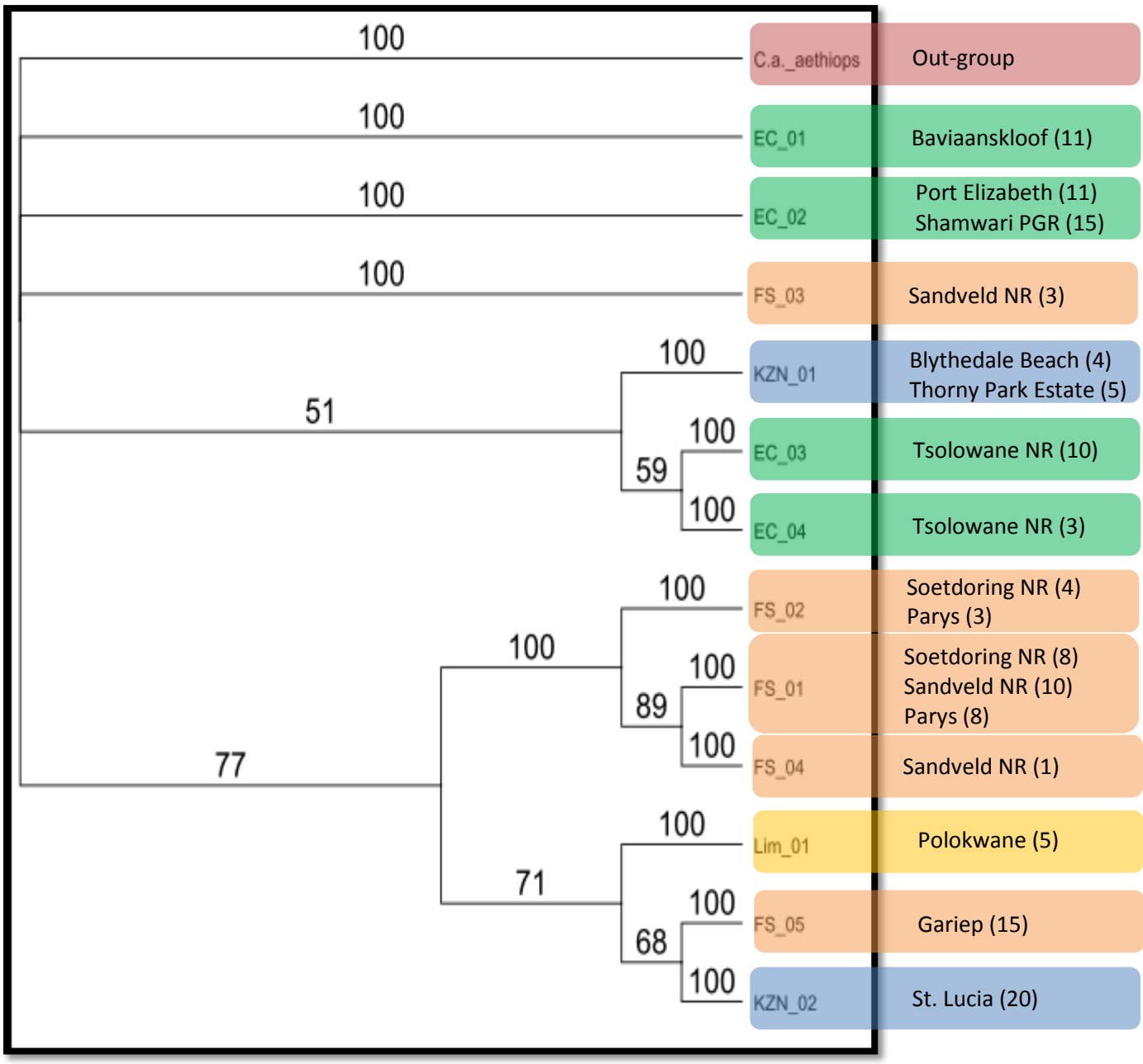


Figure 3.2: Phylogenetic tree based on a 417 bp fragment of the mitochondrial control region (CR), of the 12 vervet monkey haplotypes identified with Arlequin v3.1. One published sequence (*C. aethiops*, AY863426) was used as an out-group. The posterior probabilities for each split are provided next to every branch. The number in brackets next to each location name is an indication of the number of individuals in that particular haplotype.

Haplotype networks

The phylogenetic network for mitochondrial haplotypes constructed with the software Network v4.6.1 (Fluxus Engineering, 2011) is depicted in Figure 3.3. All the sample populations found in the Free State region show significant identity, with the exception of the Gariep population. The Eastern Cape sample populations also showed significant levels of identity with each other. Haplotypes within both of these groups were separated by 1-2 mutational steps. Haplotype KZN_Hap 01 is also rather closely linked with haplotypes in the Eastern Cape sample populations, with between 2-5 mutational steps between them. When the Network layout in Figure 3.3 is superimposed on a geographical map of South Africa (Figure 3.4), it can be seen that mitochondrial diversity corresponds closely to the geographical origin of the sample populations, the exception being the one Eastern Cape haplotype observed in the Free State.

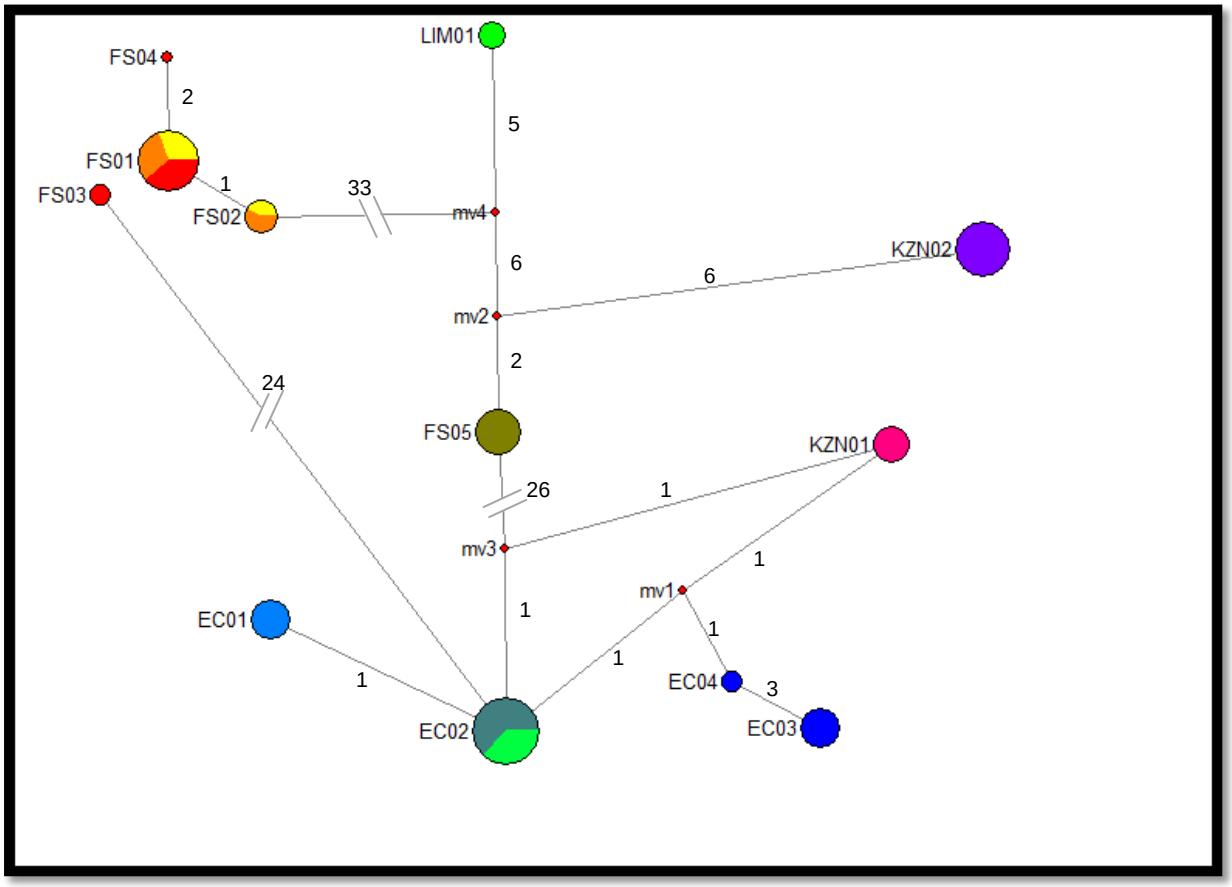


Figure 3.3: A visualisation of the phylogenetic network for mitochondrial control region sequences for vervet monkeys. The numbering between the various nodes is an indication of the number of mutational steps between each haplotype observed. The size of the circles is indicative of the number of individuals associated with that specific haplotype.

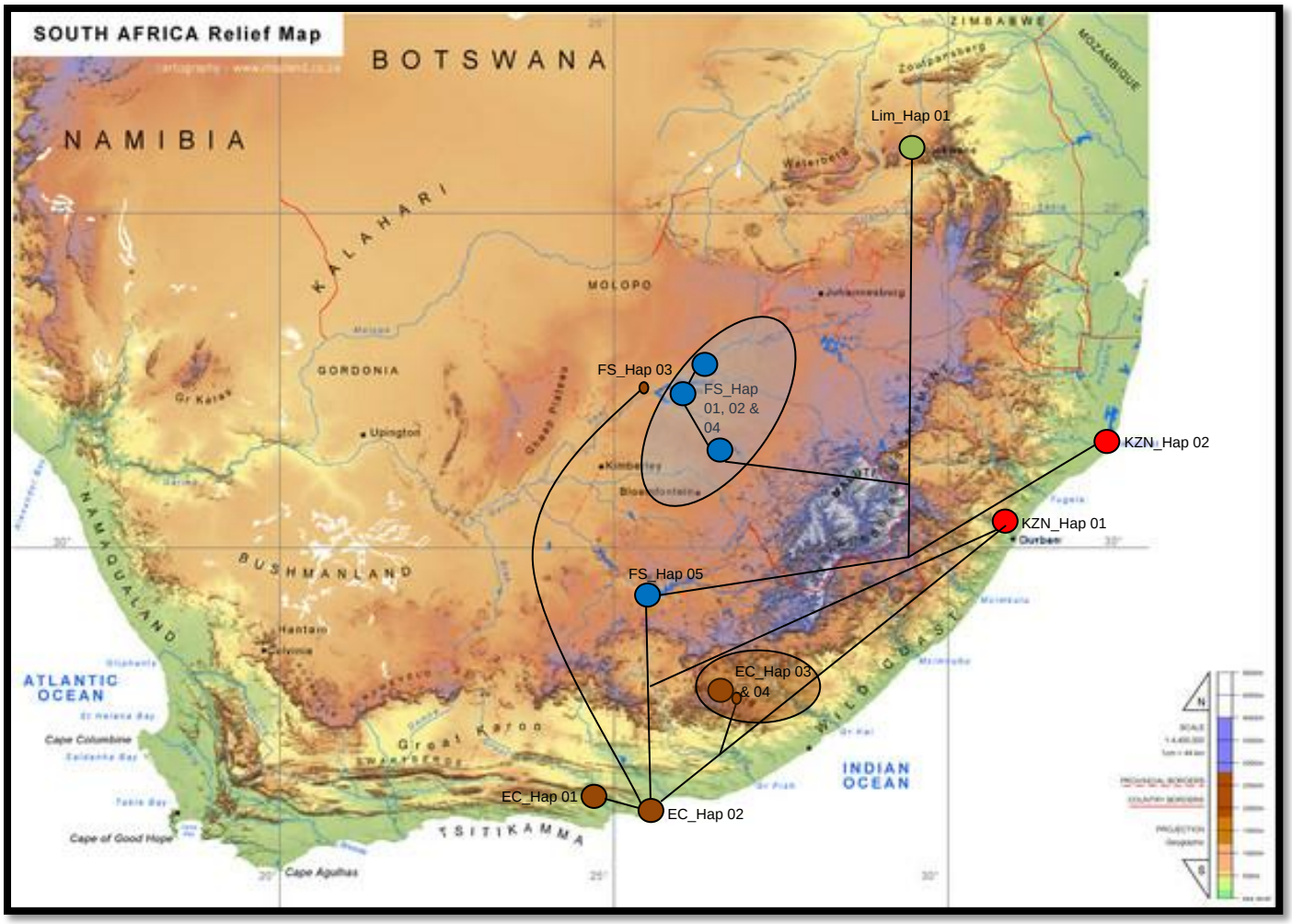


Figure 3.4: Relatedness between the populations / haplotypes and their geographical locations. The blue nodes are representative of the Free State haplotypes; the brown nodes are representative of the Eastern Cape haplotypes; the red nodes are representative of the Kwa-Zulu Natal haplotype; the green node is representative of the Limpopo haplotype [**Image:** Online at http://mappery.com/maps/South-Africa-Physical-Map.mediumthumb.jpg]

Chapter 4:

Discussion

4.1 Population genetic structuring

Analysis of the data obtained from the analysis of the mitochondrial DNA region sequences of 140 individual vervet monkeys show structuring of the populations that correspond to their geographical areas of origin. The observed F_{ST} values indicate significant levels of genetic differentiation among the majority of the population pairs. Shimada *et al.* (2002) asserted that there is significant genetic diversity within *C. aethiops* subspecies. This assertion was based on the apparent clustering structure found within the subspecies *C.a. aethiops* from Ethiopia (Shimada *et al.,* 2002). The geographical clustering pattern observed in the current study was supported by all tests conducted.

The Kwa-Zulu Natal haplotype KZN_Hap 01 was exhibited all the Blythedale Beach and Thorny Park Estate individuals and KZN_Hap 02 by all the St. Lucia samples. The Blythedale Beach and Thorny Park Estate populations show but one haplotype reflected by the maximum likelihood tree constructed with MEGA v5. The pairwise differentiation analysis also showed insignificant levels of variation between these two populations. A significant level of genetic variation was, however, identified between these two populations and the St. Lucia population, which exhibited KZN_Hap 02.

All the Polokwane samples form part of the Limpopo haplotype, Lim_Hap 01. This was also shown by the phylogenetic analysis done with MEGA v5.

Individuals sampled in the Free State area collectively exhibited five haplotypes. Haplotype FS_Hap 01-04 was shown by all vervet monkeys sampled in the central (Soetdoring NR) and north-western regions (Sandveld NR and Parys) of the Free State plains, with FS_Hap 05 exhibited by animals from the southern region of the Free State (Gariep). This was reflected in the maximum likelihood analysis, since the ML tree constructed with MEGA v5 and the ML tree constructed with Mr Bayes v3.2 both reflect these findings. Exceptions among the Free State populations are the clustering of haplotype FS_Hap 03 (from the Sandveld NR area) with sequences from the Eastern Cape and the grouping of haplotype FS_Hap 05 (Gariep) with one of the Kwa-Zulu Natal haplotypes and the Polokwane haplotype, Lim_Hap 01

(supported by 71% posterior probability). The clustering of these last three haplotypes was further supported by the haplotype Network analysis. It was inferred that there were between 8-17 mutational events between any of these three haplotypes from the Free State, Kwa-Zulu Natal and Limpopo Provinces. There are 41 inferred mutational steps between the Gariep haplotype and the nearest other Free State haplotype and 41 inferred mutational steps between the two Kwa-Zulu Natal haplotypes. Haplotypes FS_Hap 01, 02 and 04 form a distinct interior grouping when mapped on a geographical map of South Africa (Figure 3.4). This is supported by the close linkage between these haplotypes, as seen from the network analysis. There are only 1-3 mutational events between these haplotypes. The pairwise differentiation between these three localities indicates little genetic variation, and is in support of the inferred clustering pattern. Further support was the grouping observed with the Mr Bayes v3.2 constructed ML tree, with 100% posterior probability. It also can be seen that the Gariep population showed significant differentiation compared to the other Free State samples.

The Eastern Cape haplotypes form well-defined clusters. Haplotype EC_Hap 01 consists of all the animals from the Baviaanskloof area, with EC_Hap 02 consisting of all the Port Elizabeth and Shamwari PGR samples further to the east and Haplotypes EC_Hap 03-04 comprising of all the Tsolwana NR samples (further to the north-east). This population structure was strongly supported by the phylogenetic analysis. The Network analysis indicated that there are between 1-6 mutational events between any of the Eastern Cape haplotypes, which is further support for the distinct clustering patterns which are observed in this region. The pairwise differentiation between these populations showed that there are insignificant levels of genetic differentiation between the Port Elizabeth and Shamwari PGR populations, and this finding is in line with the haplotype and phylogenetic analysis of this region. Phylogenetic analysis showed that the Kwa-Zulu Natal haplotype KZN_Hap 01 is linked, to the Tsolwana haplotypes, EC_Hap 03–04 (51% posterior probability). This was supported by the Network analysis, which indicated that there are only two mutational events between this Kwa-Zulu Natal haplotype and the nearest Eastern Cape haplotypes, EC_Hap 04 and 02, which includes the Port Elizabeth / Shamwari PGR haplotype. The F_{ST} values showed significant levels of

genetic differentiation between these Kwa-Zulu Natal populations and the Eastern Cape populations.

The results obtained from the AMOVA test is in full support of the above-mentioned inferences. The greatest variation was observed between the provinces (52.5% of variation); with moderate variation occurring between populations within provinces (42.7% of variation). The within population variation was the least, contributing only 4.9% to total variation. This shows significant levels of genetic structuring among the vervet monkey populations studied. This is contradictory to results that were found by Grobler *et al.* (2006), who used four microsatellite loci to estimate the level of genetic differentiation among regional vervet monkey populations in South Africa. It was established that the most variation occurred within the individual troops, and that only 1.1% of the variation occurred among the regional populations. These contrasting inferences can be attributed to female philopatry and the maternal mode of inheritance of mtDNA (as discussed more fully later in this chapter).

The results from the current study follow the same trend as those found by Rosenblum *et al.* (1997). These authors analysed the mtDNA data obtained from 107 pigtail macaques (*Macaca* nemestrina), covering all the recognized subspecies. A significant amount of variation was found among regions (63% of variation), with low levels of variation within populations. This was explained by the occurrence of both a historical geographical event (the formation and dissolution of land bridges between islands) and a more recent geographical event which influenced the evolution of pigtail macaques.

The results from the second AMOVA test, with two Kwa-Zulu Natal groups, showed higher levels of variation between groups, with 64.1% of variation. The variation between populations within groups was lower, explaining 31.1% of variation. The within-population variation stayed approximately the same. This support the conclusion that there are significant levels of genetic variation between the St. Lucia and the Blythedale Beach and the Thorny Park Estate populations.

The nucleotide diversity (π) results indicate relatively low levels of nucleotide diversity within each population. The Free State sample group showed the highest levels of nucleotide diversity. The Sandveld NR samples showed the highest levels of nucleotide diversity within the Free State province (π = 0.038%± 0.02), with the Soetdoring NR and Parys populations having the second highest (π = 0.001% ± 0.001). This noticeable difference can be attributed to the three 'outlier' samples which were identified within the Sandveld NR sample population. The Gariep population shows no nucleotide diversity. The only Eastern Cape population showing any nucleotide diversity is the Tsolwana NR population, with π = 0.003% ± 0.002, with no nucleotide diversity within any of the other Eastern Cape populations. Similarly, none of the Kwa-Zulu Natal populations or the Polokwane populations showed any nucleotide diversity.

Figure 4.1: Geographical map of South Africa, depicting the major mountain ranges, with possible vervet monkey migratory routes. [Green dot – Polokwane (Lim_Hap 01); Orange dots – central / north-western Free State sample areas); Blue dot – St. Lucia (KZN_Hap 01); Pink dot – Blythedale Beach and Thorny Park Estate; Red dot – Gariep dam (FS_Hap 05); Purple dots – Eastern Cape sample areas)] (**Image:** http://www.rocksport.co.za/Maps/sapanlrg.jpg)

4.2 Geographical and social factors as mechanisms for current patterns of genetic differentiation

All of the regional groupings observed for vervet monkey populations are separated from each other by either major mountain ranges, rivers and / or absolute geographical distance.

The major factors which can influence migratory patterns between the Free State and Polokwane populations are geographical distance and various river systems, in particular the Vaal River. The river systems of the Free State province could have influenced the haplotype structuring observed within this region. It was seen in other primate species that rivers may influence gene flow among populations. Eriksson *et al.* (2004) found that the population genetic structure of bonobos (*Pan paniscus*) is significantly influenced by impassable rivers.

The distinct lack of population structuring found among most of the Free State samples could be attributed to a lack of major geographical barriers such as mountain ranges, with the only barriers which could have an influence on migration being the river systems of this province. However, many of the rivers in the Free State are seasonal rivers (River Health Programme, 2003). For the various permanent rivers, such as the Orange River, the Vaal River, the Modder River and the Vet River, there are also sections which are narrow enough for vervet monkeys to cross. I personally observed that a troop of vervet monkeys crossed the Modder River at Soetdoring NR by simply swimming across. It is thus clear that these rivers do not form significant barriers to migration in this area, making it highly likely that at least occasional migrations will occur. These rivers also provide natural resources to vervet monkeys, and can thus in fact stimulate migratory events. The Soetdoring NR, Sandveld NR and Parys populations constitute haplotypes FS_Hap 01-04, which fall in the central and north – western Free State plains region and further support the conclusion that river systems do not present absolute barriers to migration. When considering the geographical distances between these localities, it can be hypothesized that it is possible that these populations might have some common ancestral relation, as the distances between these localities are not significant in terms of the dispersal ability of vervets. This hypothesis is supported

by the close linkage between these haplotypes evident from the network analysis. In contrast to the closely related central populations, the Gariep population does show significant variation when compared to the rest of the Free State samples, and this can probably be explained as a result of geographical distance. Another explanation could be that the Gariep region was colonized through a different migratory event than the central and north–western regions.

The distinctive clustering patterns observed within the Eastern Cape samples can be as a result of the various mountain ranges found in this region, which can act as physical barriers to migration and gene flow. In this regard, the barrier presented by mountain ranges is mostly attributable to a temperature effect rather than the physical presence of the mountains. The low levels of nucleotide diversity found within each of the Eastern Cape populations, with significant genetic structuring among populations, is further support to the hypothesis that there is difficulty of migration between populations due to the occurrence of the vast mountain ranges found in this region. For example, the Baviaanskloof Mountain range and the Groot Winterhoek Mountain range could have a major effect on migration between the Baviaanskloof population and the rest of the Eastern Cape populations. The Groot Winterhoek mountain range, with its highest peak at 1,758 meters above sea level (Map Studio, 2007), forms the main physical barrier between the Baviaanskloof sample population and the Port Elizabeth and Shamwari PGR populations. The occurrence of two separate haplotypes within the Tsolwana NR sample population can be an indication of a recent migratory event, as all of the individuals from the EC_Hap 04 haplotype are males. These male animals could thus be immigrants from another troop through male dispersal. The most significant barrier between Tsolwana NR and Shamwari PGR / Port Elizabeth, other than distance, is the Winterberg mountain range, with its highest peak rising to 2,369 meters above sea level (Map Studio, 2007). This mountain range, in conjunction with various anthropogenic barriers, could have a significant influence on the occurrence of migration, as well as the rate of migration between these areas. The Port Elizabeth and Shamwari PGR populations show insignificant levels of differentiation among one other, and this observation can be explained by the short distance between these areas and a lack of significant physical barriers. The only permanent river

system between these groups is the Bushmans River, but as previously mentioned it seems that rivers do not present a significant barrier to vervet monkey movements. This is also in support of the grouping of these two populations into one haplotype.

Both the phylogenetic trees and the network analysis show that the Eastern Cape haplotype EC_Hap 02 is grouped with the Free State 'outlier' (FS_Hap 03) and the Kwa-Zulu Natal haplotype KZN_Hap 01, consisting of the Blythedale Beach and Thorny Park Estate populations. KZN_Hap 01 is also closely linked with EC_Hap 03-04 (Tsolwana NR). The grouping of the FS_Hap 03 'outlier' can be explained by a north–south migratory event (white arrow in Figure 4.1) which might have happened along the western limits of the vervet monkey's distribution range. There is however no additional data to support this hypothesis and further data from this region are needed to confirm this notion.

The grouping of the Kwa-Zulu Natal haplotype KZN_Hap 01 with the Eastern Cape haplotypes is strongly supported by the network analysis. This might be the result of a migratory event occurring from a south–western direction moving northward, up the eastern coast. Further analysis of vervet monkey populations between these Eastern Cape and Kwa-Zulu Natal populations is needed to obtain a better understanding of the possible cause of this pattern of genetic structuring.

The apparent link between the FS_Hap 05 (Gariep) and KZN_Hap 02 (St. Lucia) groupings is another interesting result. The geographical distance between these two localities is great, and with a significant geographical barrier between them, in the form of the Drakensberg Mountain range. This part of the mountain range can reach heights of up to 3,300 meters (Drakensberg, 2012). These two haplotypes are also grouped with haplotype Lim_Hap 01 (Polokwane). The Polokwane sample group is the most northern group sampled. Polokwane is also separated from St. Lucia by the northern stretches of the Drakensberg mountain range. This northern part of the mountain range can reach heights of up to 3,000 meters (Drakensberg, 2012). The haplotypes in this grouping are also separated from each other by a reasonable geographic distance, especially when compared to the populations in the other groupings. They are thus clearly divided by physical barriers, yet they still group together. This could be explained by an ancient migration from the north

down to the south. There are various corridors along the Drakensberg Mountain range, like that seen in the Ladysmith area (blue arrow in Figure 4.1), which can be used as passageways to the eastern coast. It is possible that groups of vervet monkeys could have moved east through these corridors toward the East coast, while other groups moved South toward the Eastern Cape region.

It can be inferred from these results that physical barriers in combination with geographical distance played an important role in gene flow and population segregation in vervet monkeys. These clustering patterns can, however, also be explained by the sex-linked nature of migratory patterns found in many Old World monkeys (OWM). This dispersal pattern is common for most mammals (Greenwood, 1980). Cercopithecine primates (including vervet monkeys), are known to show strong female philopatry, with dispersal patterns primarily based on the movements of males (Melnick and Pearl, 1987; Pusey and Packer, 1987 cited in Di Fiore, 2003). Such biased dispersal will result in the relatively even distribution of nuclear DNA throughout the populations, but with mtDNA diversity showing strong homogeneity on the population level and well structured inter-population variation. It is thus expected that more distinct clustering patterns will be observed when analysing mtDNA compared to work based on nuclear DNA. Cheney and Seyfarth (1983) showed that East African male vervet monkeys follow a non-random dispersal pattern from their natal troops as soon as they reach sexual maturity, with the females not migrating from their natal troops. By contrast, the younger males tend to migrate with siblings or peers to minimize the risks of predation and attack from conspecific rivals. This dispersal pattern decrease the likelihood of mating with related females, but continual non-random movement of males can increase the chance of the occurrence of inbreeding depression as males from the same natal troop tend to migrate to the same neighbouring troop. These primates also demonstrated, to a lesser extent, a random dispersal pattern in the sense that some males migrate to non-adjacent troops (Cheney and Seyfarth, 1983). It was suggested that the male dispersal patterns of these animals follow the stepping-stone model of migration (Kimura and Weis, 1964). Thus, groups transferring males at high rates will act as both source and recipient populations of migrants, and will be able to keep inbreeding coefficients sufficiently low (Bodmer and Cavalli-Sforza,

1968 cited by Cheney and Seyfarth, 1983) and within population nuclear gene diversity high.

The difference in rate of evolution for mtDNA versus nuclear DNA also can have an effect on the observed differences between the results from these two analytic methods. It is known that vertebrate mtDNA evolves 5-10 times more rapidly than nuclear DNA (Brown, 1979). However, microsatellite DNA has a higher rate of mutation than coding regions due to replication slippage. Another possible factor is the difference in effective population size for nuclear DNA and mtDNA. The haploid nature of mtDNA, as well as being maternally inherited, causes the effective population size to be four times less than that for autosomal nuclear DNA. The influences of genetic drift and population bottlenecks will thus be more clearly expressed, with large inter-population differentiation, when working with mtDNA (Birky *et al.,* 1983; Karl *et al.,* 1992).

4.3 Conclusion

It is clear that, when looking at results from mtDNA, that there is detectable genetic structuring among the vervet monkeys of South Africa. It was shown that the haplotypes roughly group together according to their geographical area of origin. This clustering effect was supported by all statistical analytic approaches applied. The observed level of genetic structuring can be ascribed to various factors and the effects of these factors on migration events. The main features which could play a role in this case are female philopatry (Melnick and Hoelzer, 1992; Shimada, 2000b), geographic barriers (such as mountains and rivers) (Eriksson *et al.,* 2004), and absolute geographic distance. Nevertheless, vervet monkeys could find ways to traverse some of the mountain ranges, especially those at lower elevations and with suitable corridors, and to cross rivers. Mountains and rivers are thus not absolute barriers to migration, but mountains at very high elevations will be more difficult to cross because of the lower temperatures and lack of resources.

It is known that studies based on mtDNA and nuclear DNA markers respectively can result in conflicting outcomes when studying genetic structure (Melnick and Hoelzer, 1992; Shimada, 2000b; Tosi *et al.*, 2003b). This is especially true of Cercopithecine

primates. For example, Shimada (2000b) showed this to be true of Ethiopian Grivet monkeys (*C.a. aethiops*). Shimada found that the same population showed different gene dispersal patterns when comparing mtDNA and nuclear DNA data. This pattern was attributed to male migration, due to female philopatry. Various South African studies showed low levels of genetic diversity at the nuclear DNA level of vervet monkeys (Grobler *et al.,* 2006), and it is thus crucial that data from mtDNA should be supplemented by results from nuclear markers to obtain the true pattern of genetic structure in vervet monkeys across South Africa.

The clustering of the Free State haplotype FS_Hap 03 and the Kwa-Zulu Natal haplotype KZN_Hap 01 with the Eastern Cape haplotypes, is a strong indication that vervet monkey migration was not only a north to south event. It is thus possible that the eastern coast was colonized through various migratory events, which took place all along the eastern escarpment and possibly included migration back to the north from the south through the Eastern Cape region. Further data, however, are needed to support these hypotheses and to gain a better picture of how these primates came to be in their current distribution ranges.

The conservation of all genetic variability is important, whether it is mtDNA or nuclear DNA variation. The main reason for the different genetic differentiation patterns between nuclear DNA and mtDNA is the occurrence of female philopatry among vervet monkeys. The higher mutation rate of mtDNA compared to nuclear DNA (Brown, 1979; Arif and Khan, 2009), as well as the lower effective population size of mtDNA compared to nuclear DNA (Birky *et al.,* 1983; Karl *et al.,* 1992) should also be considered when designing and execution population genetic studies. These factors all can influence the genetic patterns observed with the different genetic markers. It is thus important to acknowledge these differences and to be aware of the possibility of different genetic patterns and the reasons for these. It would thus be extremely beneficial to combine mtDNA data with nuclear DNA data in follow-up work (Roos *et al.,* 2011). This would make it possible to obtain a better overall assessment of the genetic structure of vervet monkeys in South Africa. An increase in the sample size, as well as better coverage of the distribution range of vervet

monkeys, will also help to give a better picture of the genetic status of these non-human primates.

At this point, no recommendations can be made regarding the release of rehabilitated sanctuary animals. A better understanding of the population genetic structure of these primates is needed before any recommendations can be made toward such actions.

In addition to further work on patterns of genetic variation, the adaptive significance of observed genetic differences should be investigated. In this regard, it would be dangerous to assume that genetic structure based on differences at specific genes, specifically mtDNA genes, is necessarily correlated to adaptations which will make vervet monkeys able to survive in narrow habitat ranges only.

SUMMARY

Vervet monkeys (*Chlorocebus aethiops*) are one of the most widely distributed primate species in Africa. The aim of this study was to determine the level of genetic differentiation among conspecific vervet monkey populations in the south-eastern regions of South Africa, as part of a bigger project to determine levels of differentiation across South Africa. For this purpose, samples were taken from four localities in the Free State Province (Soetdoring Nature Reserve (NR), Gariep Dam NR, Sandveld NR and the Parys area), four Eastern Cape locations (Tsolwana NR, Baviaanskloof NR, Shamwari Private Game Reserve (PGR) and the Nelson Mandela Metropolitan University (NMMU) campus, Port Elizabeth), three Kwa-Zulu Natal location (St. Lucia area) and one Limpopo Province locality. Genetic differentiation was quantified using sequence data from a portion of the mtDNA control region. Twelve Haplotypes were identified within the total sample group. The nucleotide diversity for each grouping was calculated over all loci. Nucleotide diversity ranged from 0 to 0.038% ±0.02. Haplotype frequencies distribution among samples was calculated. An analysis of Molecular Variance (AMOVA) test was conducted and population pairwise F_{ST} values were estimated. The AMOVA test revealed that the majority of the genetic diversity occurred among the different groups (52.5%), with only 4.9% of the variation found within populations. The populations were assigned to groups according to geographic origins. The pairwise analysis identified significant levels of genetic variation among populations, with an average F_{ST} value of 0.851. These haplotypes were found to coincide with the geographical borders of Provinces. A ML tree was constructed using the haplotype data, and results showed clustering corresponding to geographical borders. A phylogenetic network was constructed, and this showed clustering similar to that found with the ML tree analysis. According to these results it is clear that there is genetic structuring among vervet monkey populations in South Africa. This clustering of populations can be potentially explained by female philopatry and geographical barriers. Female philopatry is a well known occurrence amongst Cercopithecine primates. The occurrence of geographical barriers, such as rivers and mountains had influence on migration rates and genetic structuring. This clustering pattern observed with mtDNA analysis contradicts results from previous studies working with nuclear DNA

markers. This can be caused by various factors. Except for female philopatry having an effect on mtDNA differentiation patterns, it should be noted that the faster evolutionary rate of mtDNA vs. nuclear DNA can also cause different genetic patterns. The effective population size of mtDNA is also four-fold smaller than that of nuclear genes, and will also cause skewed results when comparing mtDNA data with nuclear DNA data. No reliable recommendations can be made toward the release of rehabilitated vervet monkeys, as further analysis is needed. It is thus suggested to use both genetic markers in follow-up studies. An increase in sample size from a broader geographical range is also recommended. In addition to further work on patterns of genetic variation, the adaptive significance of observed genetic differences should also be investigated.

Key words: Vervet monkey, *Chlorocebus aethiops*, genetic differentiation, mtDNA, control region, AMOVA, female philopatry, haplotype frequencies, clustering.

OPSOMMING

Blouape (*Chlorocebus aethiops*) is een van die mees wyd-verspeide primaat spesies in Afrika. Die doel van hierdie studie was om die vlak van genetiese differensiasie tussen blouaap bevolkings in die suidoostelike gebiede van Suid-Afrika te identifiseer. Hierdie studie vorm deel van ‘n groter projek wat gefokus is op die genetiese variasie van blouape regoor Suid-Afrika. Biologiese monsters was versamel vanaf vier areas in die Vrystaat (Soetdoring Natuurreservaat (NR), die Gariep dam area, Sandveld NR en die Parys area), vier areas in die Oos-Kaap (Tsolwana NR, die Baviaanskloof area, Shamwarie Privaat Wildreservaat en die NMMU kampus, Port Elizabeth) en drie Kwa-Zulu Natal areas (Blythedale Beach, Thorny Park Estate en St. Lucia area). ‘n Gedeelte van die mtDNS kontrole gebied was gebruik vir die analiese van genetiese variasie deur middel van DNS volgorde bepaling. ‘n Analiese van die Molekulêre Variasie (AMOVA) toets was gedoen en tussen-bevolking F_{ST} waardes was bepaal. Die AMOVA toets het gewys dat die meerderheid van die genetiese diversiteit tussen die verskillende groepe voorkom (52.47%) en dat net 4.87% van die diversiteit binne elke populasie voorkom. Die groepe was volgens hul geografiese oorsprong (provinsies) gegroepeer. Betekenisvolle variasie was tussen die bvolkings gevind, met ‘n gemiddelde F_{ST} waarde van 0.851. Die nukleotied diversiteit was vir elke populasie bepaal en was tussen 0 tot 0.038% ±0.02. Haplotiepe frekwensie verspreiding was ook bepaal. Twaalf haplotiepes was tussen die totale proef groep gevind. Die haplotiepe groepering was in lyn met die geografiese grense van Suid-Afrika. ‘n ML filogenetiese boom was opgestel deur gebruik te maak van die bepaalde haplotiepe data. Hierdie resultate was meer en deels in lyn met die bogenoemde haplotiepe frekwensie data. Die konstruksie van ‘n haplotiep netwerk het die ML boom resultate ondersteun. Hierdie resultate wys na defnitiewe genetiese strukturering tussen die blouaap populasies in Suid-Afrika. Hierdie groeperings patrone kan deur twee hoof meganismes beskryf word, naamlik vroulike trop-gebondenheid (philopatry) en geografiese skeidings. Cercopithecine primate is bekend daarvoor dat vroulike individue gebondenheid tot hul trop van oorsprong toon. Die tempo van migrasie en genetiese strukturering kan ook deur geografiese skeidings, soos berge en riviere, beïnvloed word. Die groeperings patrone wat deur mtDNS analiese waargeneem

was is teenstrydig met vorige werk wat op kern DNA gefokus het. Die vinniger evolusionêre tempo van mtDNS teenoor kern DNS kan ook ‘n invloed op genetiese patrone hê. Die effektiewe bevolkingsgrootte van mtDNS is ook vier keer kleiner as dié van kern DNS, en kan oneweredige resultate verskaf indien mtDNS data met kern DNS data vergelyk word. Tot op datum kan geen betroubare aanbevelings ten opsige van die vrylating van gerehabiliteerde blouape dus gemaak word nie, aangesien verdere inligting benodig word. Dit word dus voorgestel dat altwee tipes genetiese merkers vir toekomstige studies gebruik word, asook ‘n groter proef groep wat ‘n groter geografiese gebied dek. Behalwe vir die addisionele werk wat op die patrone van genetiese variasie gedoen moet word, moet die adaptiewe betekenis van die waargenome genetiese variasie ook ondersoek word.

Sleutelwoorde: Blouape, *Chlorocebus aethiops*, genetiese differensiasie, mtDNS, kontrole area, AMOVA, “vroulike trop-gebondenheid”, haplotiep frekwensies, groepering.

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APPENDIX A:

Haplotype names and sequences

Haplotype Name:	**Haplotype sequence:**
FS_Hap 01	CAACCGAACAAGCACCTGTTAGCCGCACATAACACATCACGTTATTTACCGGACAT AGCACATACTCATTATAAACCTTTCTCCTCACCACGGATGACCCCCCTCACTTAGG AATCCCTTACTCACCATCCTCCGTGAAATCAATATCCCGCACAAGAGTGCTACTCT CCTCGCTCCGGGCCCATAACCTGTGGGGGTAGCTAGGAATGAGCTGTATCCGGC ATCTGGTTCTTACCTCAGGGCCATAGCAACCAAAATCGCCCACACGTTCCCCTTAA ATAAGACATCTCGATGGATCACGGGTCTATCACCCTATTAACCAGTCACTGGAGCT CTCCATGCATTTGGTATCTTTTATCTCTGGTCTGCACGCAACACCATCGCAGCATG CTGACTGCCACCACATCCCGTCCTGAA
FS_Hap 02	CAACCGAACAAGCACCTGTTAGCCGCACATAACACATCACGTTATTTACCGGACAT AGCACATACTCATTATAAACCTTTCTCCTCACCACGGATGACCCCCCTCACTTAGG AATCCCTTACTCACCATCCTCCGTGAAATCAATATCCCGCACAAGAGTGCTACTCT CCTCGCTCCGGGCCCATAACCTGTGGGGGTAGCTAGGAATGAGCTGTATCCGGC ATCTGGTTCTTACCTCAGGGCCATAGCAACCAAAATCGCCCACACGTTCCCCTTAA ATAAGACATCTCGATGGATCACGGGTCTATCACCCTATTAACCAGTCACTGGAGCT CTCCATGCATTTGGTATCTTTTATCTCTGGTCTGCACGCAACACCATCGCAGCATG CTGACTCCCACCACATCCCGTCCTGAA
FS_Hap 03	?????????CCCACCCAAAC-- CCGCACATAGTACATCTCTTTATTTACCGTACATGGCACATATCTATTACGAATTCT CCTCCTCACCACGGATGCCCCCCCTCACTTAGGAATCCCTTATTCACCATCCTCCG TGAAATCAATATCCCGCACAAGAGTACTACTCTCCTCGCTCCGGGCCCATAATCCG TGGGGGTAGCTAAAAATGAACTGTATCCGACATCTGGTTCTTACCTCAGGGCCATA ACAATCAAAACCGCTCACACGTTCCCCTTAAATAAGACATCTCGATGGATCACGGG TCTATCACCCTATTAACCAGTCACTGGAGCTCTCCATGCATTTGGTATCTTTTATCT CTGGTCTGCACGCAACACCATCGCAACATGCTGACTCCCACCACATCCCGTCCTG AA

FS_Hap 04	CAACCGAACAAGCACCTGTTAGCCGCACATAACACATCACGTTATTTACCGGACAT AGCACATACTCATTATAAACCTTTCTCCTCACCACGGATGACCCCCCTCACTTAGG AATCCCTTACTCACCATCCTCCGTGAAATCAATATCCTGCACAAGAGTGCTACTCT CCTCGCTCCGGGCCCATAACCTGTGGGGGTAGCTAGGAATGAGCTGTATCCGGC ATCTGGTTCTTACCTCAGGGCCACAGCAACCAAAATCGCCCACACGTTCCCCTTAA ATAAGACATCTCGATGGATCACGGGTCTATCACCCTATTAACCAGTCACTGGAGCT CTCCATGCATTTGGTATCTTTTATCTCTGGTCTGCACGCAACACCATCGCAGCATG CTGACTGCCACCACATCCCGTCCTGAA
FS_Hap 05	CCACAAGTACTAAACTCATCACCCGTACATAACACATCCTCTTATCTACCGTACATG GCACATATCTATTAAAAACCCTCCTCCTCACCATGGATGCCCCCCCTCACTTAGGG GTCCCTTACTCACCATCCTCCGTGAAATCAATATCCCGCACAAGAGTGCTACTCTC CTCGCTCCGGGCCCATAACCTGTGGGGGTAACTAGGAATGAACTGTATCCGGCAT CTGGTTCTTACTTCAGGGCCATAGCAGCCAAGATCGCCCACACGTTCCCCTTAAAT AAGACATCTCGATGGATCACGGGTCTATCACCCTATTAACCAGTCACTGGAGCTTT CCATGCATTTGGTATCTTTTATCTCTGGTCTGCACGCAACACCATCGCAACATGCT GACTCCCACCACATCCCGTCCTGAA
EC_Hap 01	CCACAAGTACTAAACTCACTACCCGTACATAGTACATTCCTTCATTTACCGTACATA GCACATATCTATTACGAACTCTCCTCCTCACCATGGATGCCCCCCCTCACTTGGGA ATCCCTTATTCACCATCCTCCGTGAAACCAACATCCCGCACAAGAGTACTACTCTC CTCGCTCCGGGCCCATAACCCGTGGGGGTAGCTAGAAATGAACTGTATCCGGCAT CTGGTTCTTACCTCAGGGCCATAACAATCAAGATCGCCCACACGTTCCCCTTAAAT AAGACATCTCGATGGATCACGGGTCTATCACCCTATTAACCAGTCACTGGAGCTCT CCATGCATTTGGTATCTTTTATCTCTGGTCTGCACGCAACACCATCGCAACATGCT GACTCCCACCACATCCCGTCCTGAA

EC_Hap 02	CCACAAGTACTAAACTCACTACCCGTACATAGTACATTCCTTCATTTACCGTACATA GCACATATCTATTACGAACTCTCCTCCTCACCATGGATGCCCCCCCTCACTTGGGA ATCCCTTATTCACCATCCTCCGTGAAATCAACATCCCGCACAAGAGTACTACTCTC CTCGCTCCGGGCCCATAACCCGTGGGGGTAGCTAGAAATGAACTGTATCCGGCAT CTGGTTCTTACCTCAGGGCCATAACAATCAAGATCGCCCACACGTTCCCCTTAAAT AAGACATCTCGATGGATCACGGGTCTATCACCCTATTAACCAGTCACTGGAGCTCT CCATGCATTTGGTATCTTTTATCTCTGGTCTGCACGCAACACCATCGCAACATGCT GACTCCCACCACATCCCGTCCTGAA
EC_Hap 03	CCACAAGTACTAAACTCACTACCCGTACATAGTACATTCCTTCATTTACCGTACATA GCACATACCTATTACGAACTCTCCTCCCCACCATGGATGCCCCCCCTCACTTGGGA ATCCCTTATTCACCATCCTCCGTGAAATCAATATCCCGCACAAGAGTACTACTCTCC TCGCTCCGGGCCCATAACCCGTGGGGGTAGCTAGGAATGAACTGTATCCGGCATC TGGTTCTTACCTCAGGGCCATAACAATCAAGATCGCCCACACGTTCCCCTTAAATA AGACATCTCGATGGATCACGGGTCTATCACCCTATTAACCAGTCACTAGAGCTCTC CATGCATTTGGTATCTTTTATCTCTGGTCTGCACGCAACACCATCGCAACATGCTG ACTCCCACCACATCCCGTCCTGAA
EC_Hap 04	CCACAAGTACTAAACTCACTACCCGTACATAGTACATTCCTTCATTTACCGTACATA GCACATACCTATTACGAACTCTCCTCCTCACCATGGATGCCCCCCCTCACTTGGGA ATCCCTTATTCACCATCCTCCGTGAAATCAACATCCCGCACAAGAGTACTACTCTC CTCGCTCCGGGCCCATAACCCGTGGGGGTAGCTAGAAATGAACTGTATCCGGCAT CTGGTTCTTACCTCAGGGCCATAACAATCAAGATCGCCCACACGTTCCCCTTAAAT AAGACATCTCGATGGATCACGGGTCTATCACCCTATTAACCAGTCACTAGAGCTCT CCATGCATTTGGTATCTTTTATCTCTGGTCTGCACGCAACACCATCGCAACATGCT GACTCCCACCACATCCCGTCCTGAA

KZN_Hap 01	CCACAAGTACTAAACTCACTACCCGTACATAGTACATTCCTTCATTTACCGTACATA GCACATATCTATTACGAACTCTCCTCCTCACCATGGATGCCCCCCCTCACTTGGGA ATCCCTTATTCACCATCCTCCGTGAAATCAACATCCCGCACAAGAGTACTACTCTC CTCGCTCCGGGCCCATAACCTGTGGGGGTAGCTAGAAATGAACTGTATCCGGCAT CTGGTTCTTACCTCAGGGCCATAACAATCAAGATCGCCCACACGTTCCCCTTAAAT AAGACATCTCGATGGATCACGGGTCTATCACCCTATTAACCAGTCACTAGAGCTCT CCATGCATTTGGTATCTTTTATCTCTGGTCTGCACGCAACACCATCGCAACATGCT GACTCCCACCACATCCCGTCCTGAA
Lim_Hap 02	CCACAAGTACTAAACTCATCGCCCGTACATAACACATCCTCTTATCTACCGTACATG GCACATACCTATTAAAAACCCTCCTTCCCACCATGGATGCCCCCCCTCACTTAGGG GTCCCTTACTCACCATCCTCCGTGAAATCAATATCCCGCACAAGAGTGCTACTCTC CTCGCTCCGGGCCCATAACTTGTGGGGGTAACTAGGAATGAACTGTATCCGACAT CTGGTTCCTACTTCAGGGCCATAGTAGCCAAGATCGCCCACACGTTCCCCTTAAAT AAGACATCTCGATGGATCACGGGTCTATCACCCTATTAACCAGTCACTGGAGCTTT CCATGCATTTGGTATCTTTTATCTCTGGTCTGCACGCAACACCATCGCAACATGCT GACTCCCACCACATCCCGTCCTGAA
Lim_Hap 01	CCACAAGTACTAAACCCACCACCTGTACACAACACATCATCTTATCTACCGTACAT GGCACATACCTATTAAAAACCCTTCTTCTCACCATGGATGCCCCCCCTCACTTAGG AATCCCTTACCCACCATCCTCCGTGAAATCAATATCCCGCACAAGAGTGTTACTCT CCTCGCTCCGGGCCCATAACCTGTGGGGGTAACTAGGAATGAACTGTATCCGGCA TCTGGTTCTTACCTCAGGGCCATAGCAGCCAAGATCGCCCACACGTTCCCCTTAAA TAAGACATCTCGATGGATCACGGGTCTATCACCCTATTAACCAGTCACTGGAGCTT TCCATGCATTTGGTATCTTTTATCTCTGGTCTGCACGCAACACCATCGCAACATGCT GACTCCCACCACATCCCGTCCTGAA