POPULATION GENETICS OF THE CAPE SEROTINE BAT (*Neoromicia capensis*) IN SOUTH AFRICA

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ABSTRACT

POPULATION GENETICS OF THE CAPE SEROTINE BAT (*NEOROMICIA CAPENSIS*) IN SOUTH AFRICA

Philopatry to natal roosts is well documented for many bat species and many species will migrate between natal maternity roosts and hibernacula at the onset of winter. These behavioural characteristics will influence the genetic structure of the population by facilitating or inhibiting gene flow. Gene flow within and among populations of mammals in general is often the result of male dispersal and this mode of gene flow is also prevalent in the Order Chiroptera. The Cape serotine bat (Chiroptera: Vespertilionidae) is an endemic species of sub-Saharan Africa and occupies all biomes throughout its distribution. It roosts in anthropogenic structures in small colonies of up to ten individuals. Since its discovery in the early 1800's by Arthur Smith little more than a few aspects of its reproductive biology and diet have been documented. Almost nothing is known about philopatry, migration and dispersal patterns of the Cape serotine bat and therefore nothing is known about its population structure

In this study I use microsatellite and mitochondrial D-loop sequences to determine the genetic structure of the Cape serotine bat population within South Africa. I investigated the degree of genetic differentiation between subpopulations in different biomes, and among colonies within subpopulations.

Three polymorphic microsatellite loci and 850 bp of partial control region sequences revealed genetically distinct subpopulations throughout South Africa. This was also true for colonies within subpopulations. The patterns of divergence in the microsatellite and mitochondrial data were concordant, indicating that both males and females exhibit similar levels of philopatry. Mitochondrial haplotype analysis revealed that the Cape serotine bat population is divided into three major geographic complexes: the Southwestern, Central, and Northeastern. Both males and females from colonies within subpopulations display strong fidelity to natal roosts. However, males sire offspring in other colonies (extracolonial mating). Other roof roosting vesper bats (e.g. *Plecotus auritus*) have a breeding behaviour that conforms to these findings.

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And lastly to you chicco, amore mio. Grazie mile, ti amo tanto, tanto! The answer is "Yes"!

Glossary of Definitions

Autoradiography	A laboratory technique used which allows the visualising of radioactively labelled molecules. This is achieved by exposing x-ray film to a radioactive sample
Base pair	A pair of nitrogenous bases (one purine bonded to a pyrimidine by hydrogen bond) constituting to the double-stranded helix of DNA.
Colony	A group of individuals sharing a habitual space throughout (or for the most part of) their life. These individuals may or may not be related, and is dependant on species behaviour.
Complex	A group of individuals of the same species showing marginal genetic differentiation from a neighbouring group of related individuals. In some cases the ranges of individuals in a complex have been shown to be limited by geographical influences and also may manifest in morphological differences between individuals of opposite complexes. A complex therefore refers to specific ecotypes.
D loop	a displacement loop consisting of a single parental strand of DNA found in both linear and circular DNA. In vertebrate mtDNA this is a non-coding region which does however carry promoters and an origin for replication of the molecule.

Haplotype	An association of individuals by a linked allele within the gene pool of the entire population.
Hardy-Weinberg Law	Gene and genotype frequencies will remain constant between generations in a large, stable interbreeding population where there is no migration, selection or mutation.
Kbp	Base pairs reported as 10^3
Microsatellite	A simple sequence of non-coding DNA consisting of tandemly repeated units randomly dispersed throughout the eukaryotic genome.
Panmictic unit	A population in which the resident individuals mate completely at random (panmixis - as opposed to assortative mating).
PCR	Polymerase Chain Reaction
Philopatry	The tendency of an individual to remain in, or frequently return to its natal range. Philopatry is common in most animal species, and males and females may display philopatry to a differing degree.
Polytocy	The physiological ability of some mammals to give birth to numerous offspring.
Population	A group of individuals of the same species occupying a specific geographical distribution

Population genetics	The study of genetic inheritance, and the process of genetic variation over time, in natural populations.
Population substructure	Where a group of individuals is fractured into many ecotypes which is defined in both genetic and possibly morphological variations
Primer	A synthetic oligonucleotide used for amplification in PCR. These synthetic single strands of DNA are complimentary to the flanking regions of a target allele.
Sequence divergence	Similarly, species divergence. Where environmental circumstances have separated a group of individuals from a population, which have interbred and subsequently show a marked genetic deviation from the gene pool of the original population.
Subpopulation	As a consequence of sequence divergence. Also referred to as an ecotype. It is important to note that the definition of "subpopulation" and "colony" are used somewhat interchangeably in the context of this dissertation. This is because often specific colonies were captured in areas where other individuals where caught in mist nets in random adjacent flight paths. I have tried where possible to make this difference clear.

CHAPTER 1

BEHAVIOURAL ECOLOGY OF THE CAPE SEROTINE BAT, *NEOROMICIA CAPENSIS*

1.1 Classification, Distribution and Ecology of *Neoromicia capensis*

The Cape serotine bat (Fig 1.1), *Neoromicia capensis*, first described by Arthur Smith in 1829, belongs to the subfamily Vespertilioninae which is part of the most diverse family of bats, the Vespertilionidae (Hill & Smith 1984). It was formerly placed in the genus *Eptesicus* (Smith 1829) but was later moved to the genus *Pipistrellus* and subgenus *Neoromicia* by Taylor (2000) on the basis of echolocation characteristics. Recently, chromosome GTG-banding studies and analysis of baculum morphology has prompted the promotion of the sub-genus "*Neoromicia*" to full genus status (Kearney *et al.* 2002) - the genus to which *capensis* is currently associated.

The Cape serotine bat is a common resident of sub-Saharan Africa (Fig 1.2). This species is extremely hardy and is tolerant of a wide range of habitats including those areas that are inhospitable to many other bat species (e.g. deserts; Taylor 2000). *Neoromicia capensis* commonly roost in anthropogenic structures – especially roofs of houses in suburban areas (Taylor 2000). Their colonies consist of between two and ten individuals that roost closely huddled together (Taylor 2000). There have been additional reports of smaller colonies (two to three individuals) roosting under the bark of trees and at the base of aloe leaves (Skinner & Smithers 1990; van der Merwe 1990; Taylor 2000).

FIGURE 1.1 THE CAPE SEROTINE BAT, *NEOROMICIA CAPENSIS*. Two individuals in a rehabilitation institution. Note the prominent pinnae and reduced eyes. A common trait of the Vespertilionidae is a lack of nose leaves. Photograph courtesy of Vivienne Hunter.

These vesper bats become active at dusk where they forage as a group for insects (van der Merwe 1990). They are woodland edge feeders (Taylor 2000) and the bulk of their diet comprises hemipterans, coleopterans, dipterans and to a lesser extent euphemeropterans (Schoeman & Jacobs 2003). Taylor (2000) cautions however, that there is much variation in the diet of these bats throughout their range. Although it is known that *N. capensis* tends to roost in colonies of up to ten individuals (Taylor 2000) it is not known what the sexual composition or demographics of these groups are.

Similarly, little is known of the reproductive biology or mating strategies of this species beyond the timing and length of the gestation period. Females give birth to between one and four altricial young after a twelve-week gestation period (Rautenbach 1982; van der Merwe 1990; Taylor 2000) in late November (van der Merwe 1994). Nothing is known of the mating strategies used by *N. capensis*.

There are three reproductive physiological strategies employed by bats, sperm storage, delayed implantation and arrested embryonic development. After copulation, which typically takes place between late March and early April in the Southern hemisphere, females may store the sperm in the oviduct, uterotubual junction area or the uterus. Females then go into hibernation and the stored sperm is used to fertilise the ovum, e.g. in most vesper and horseshoe bats (Taylor 2000). Copulation in the Cape serotine bat occurs during March and April, and testes are spermatogenically inactive from May and September (van der Merwe 1994). It is unlikely that the Cape serotine bat stores sperm in the uterus because of the presence of leucocytes in the uterine lumen during winter (van der Merwe 1990). Leucocytes maintain sterility by identifying foreign bodies within the lumen and will destroy invasive entities as well as sperm. However sperm may be stored in the uterotubual junction where the incidence of leucocytes is lower during winter (Van Der Merwe 1990). Ovulation occurs in late August and embryogenesis would be well developed by early September (van der Merwe 1994). Sperm storage is synonymous to delayed ovulation.

A second strategy employed by female bats is to fertilise the ovum at copulation but delay implantation of the blastula (Taylor 2000). The blastula remains dormant until early spring when the full term gestation begins after implantation. This phenomenon has only been recorded for bats of the genus Miniopterus, e.g. *Miniopterus natalensis* (Miller-Butterworth et al. 2002). The third strategy is arrested embryonic development after implantation of the fertilised ovum. The embryo remains dormant until females are aroused from hibernation in the spring. Van der Merwe (1990) did not find evidence for this process in the Cape serotine bat, however this strategy has been recorded in *Hipposideros caffer caffer* (Bernard & Meester 1982) and *Scotophilus borbonicus* (Van der Merwe, Rautenbach & Penzhorn 1988). Van der Merwe (1990) reported that female Cape serotine bats could give birth to quadruplets; however singletons and twins are more common. In a polytocous gestation, the full-term weight of the pups (Fig 1.3) can be as much as 43 % of the mother's bodyweight. Parturition occurs around late November, and females are known to carry the non-volant young while feeding, although this is considered to be rare (van der Merwe, 1990).

FIGURE 1.2 GLOBAL DISTRIBUTION OF THE CAPE SEROTINE BAT. Area shaded red indicates the distribution of *N. capensis*.

FIGURE 1.3 NEWBORN CAPE SEROTINE BAT PUP. Newborn pups are born with eyes closed and have no hair. They are suckled for up to three months postpartum (Van der Merwe 1990). Photograph courtesy of Wendy Brodie.

1.2 The effect of mating strategies and gene flow on population genetic structure.

1.2.1 Breeding behavior and population structure

Bats have the widest range of mating behaviours amongst mammals (Clutton-Brock 1989; Altringham 1996). To understand the mating strategies used by bats one must be familiar with the evolutionary history and the selective pressures responsible for these systems. Various authors (Bradbury 1977; Bradbury & Vehrencamp 1977; Clutton-Brock 1989; Altringham 1996) have advanced models in an attempt to explain bat mating systems. Clutton-Brock (1989) proposed a general model for mammalian mating strategies in which he identified the important factors influencing these strategies. He suggested that mating systems represent different forms of mate guarding which is an adaptation to the spatial and temporal distribution of receptive females. The male's mating behaviour is thus related to the ranging behaviour, as well as to the size and stability of the female groups (Clutton-Brock 1989). Altringham (1996) adapted this general model to Chiropteran mating systems and developed a model based largely on the ecological demands and behavioural impulses of the females of each bat genus. The central theme of this model is that the social and behavioural characteristics of females govern the mating strategy in each species. This may be because the females of many bat species are philopatric to natal roosts (also see Taylor 2000 and Miller-Butterworth *et al.* 2003).

In his model, Altringham (1996) identified three possible mating strategies for bats belonging to the Family Vespertilionidae. In all three categories it is assumed that the females rear young within indefensible home ranges, and all groups are unstable in terms of individuals comprising them. The first mating strategy he terms the *variable strategy*. The group is composed of many females that occupy a common range and have distinct migration and hibernation patterns. Individuals converge seasonally in mating aggregations and males hold temporary harems or leks. Altringham (1996) classifies only *Myotis lucifugus, M. sordalis* and *Miniopterus natalensis* (it has been suggested that the latter be classified into its own family: Miniopteridae - Hoofer & Van den Bussche 2003) as species using this mating strategy. Since harems are not permanent (i.e. harem males change from one mating event to another) genetic variation within the colony is high and male alleles are randomly distributed through each generation. Gene flow is also high between spatially separated colonies (i.e. there is high intercolony relatedness) because of seasonal movements and random mating. However, adaptation to local environment or to biomes may cause substructure among larger subunits within the population (Miller-Butterworth *et al.* 2003), where allelic frequency may still be high within these subunits.

The second and third strategies arise when a female group is philopatric to a large home range. Males are then found to defend small groups or small roosts as opposed to large territories. Altringham (1996) terms this resource defence polygyny pertaining to which, two mating strategies are possible. The first is the *female defense strategy*. In this strategy permanent unigroups (harems) are formed in which a small, constant group of females is protected. The second is *roost defense strategy* in which strict mating territories within a roost are defended, and females move, apparently at random, between these territories at different mating events. The effective population size within a roost defense strategy will be larger than in a harem system resulting in the population being closer to Hardy-Weinberg equilibrium. Furthermore, if a single male protects a harem group over a number of generations, allelic frequencies within each group would be biased towards that of the defending male. This results in a decrease in the effective population size and an increase in the coancestry coefficient within such groups (even though the number of alleles in each population may be similar). Altringham (1996) classifies only one miniopterid bat (*Miniopterus australis*) as using the female defense strategy and two vesper bats (*Neoromicia nanus* and *Pipistrellus pipistrellus*) as using the roost defense strategy.

McCracken (1987) lists four features of a social system that determines genetic structuring in a population. These are i) the size of the social unit, ii) stability of adult composition, iii) juvenile dispersal and recruitment and iv) mating strategy, with special attention to the individual reproductive success. Within bat social systems these features are particularly important because they affect three related parameters: i) the effective population size, ii) gene flow and iii) levels of inbreeding. Hence, if the population's genetic structure is affected by the mating system, then effective population sizes and gene flow between subpopulations will vary according to the mating strategy employed by the species (McCracken 1987).

i) Effective population size (N_e).

Effective population size is an estimate of the actual population size that is adjusted for the likely effects of genetic drift on the composition of the population's gene pool. Thus, N_e will be reduced proportionally due to socially restricted mating within a portion of the population. Consequently N_e is almost always less than the number of breeding adults in the population. It is most commonly calculated as:

$$N_e = \frac{4NmNf}{(Nm + Nf)}$$

where Nm and Nf are equal to the number of actively contributing males and females to breeding event. Other factors such as, unequal sex ratios, changes in population size over generations, overlapping generations, and greater than random variation of individual's gametic contribution to the next generation also decrease N_e (Hartl 1980). Especially important in the case of bat populations is the presence of polygyny, which leads to skewed reproductive success in males.

Effective population size is important since stochastic fluctuations in allelic frequencies (genetic drift) can determine the genetic composition of the population, and the smaller the population the more pronounced this effect. The result of genetic drift within populations is that over time some alleles drift toward fixation while other alleles are lost. The implication for the genetic structure of the population is that divergence occurs due to changing allele frequencies.

ii) Inbreeding

Inbreeding (i.e. mating between related individuals) is also a factor that leads to a loss of heterozygosity. In small populations the inbreeding coefficient is high because the probability of encountering and mating with a relative is high. Inbreeding has the same effect that reduced effective population size has, viz. individuals become more and more homozygous. Ultimately this would lead to linkage disequilibrium.

Sugg *et al.* (1996) provide a model that accounts for the impact of coancestry (a measure of kinship of individuals) on the inbreeding coefficient and the resultant genetic variation in subsequent generations. They propose that coancestry develops much quicker than inbreeding within social groups as a result of one sex being philopatric. Hence, genetic variation can be influenced by the social structure of the population. This applies to bats, because the females of many species are apparently philopatric (Taylor 2000; Castella *et al.* 2000; Miller-Butterworth 2002). Males visit the female roosts during mating season. It is, however, not known for some species whether the same male/s return to service the same roosts or whether seasonal fitness governs reproductive success because males may compete for access to females in their roosts.

Wilson *et al.* (1975) and Bush *et al.* (1977) proposed that a small effective population size and local inbreeding promote genetic divergence among social units leading to rapid evolutionary change. Shields (1983) advanced an opposing theory cautioning that small populations (and hence with small effective population size) do not necessarily fix certain alleles leading to speciation. Instead, selection favours some alleles because it leads to a reduction in recombinant genetic load (i.e. reproducing in harems may preserve favourable alleles at different loci). Hence, subpopulations within a larger population should differ in the alleles that become fixed, possibly due to different local selection pressures (on non-neutral alleles) and/or geographic isolation. Although the subpopulations may be predominantly homozygous the population as a whole would still display high allelic variation.

iii) Gene flow

Gene flow through a population is a result of dispersal and the mating strategy employed by the species. This mechanism is one that acts by mixing alleles and hence limits inter-subpopulation genetic variation. In this way the amount of genetic divergence between subpopulations is limited. Very low levels of random migration by only a few individuals can counteract the effects of genetic drift and divergence – even in small populations (Hartl 1980; Slatkin 1987). Since many bat species migrate (Castella *et al.* 2000; McCracken *et al.* 1997; Taylor 2000; Miller-Butterworth *et al.*

2003), the frequency at which individuals do so and the genotypic similarity of the satellite colony will govern the effectiveness of gene flow between subpopulations

Genetic differentiation (F_{ST}, see Chapter 3) between populations can be estimated by the number of migrants using the formula:

$$F_{ST} = \frac{1}{(1 + 4Nm)}$$

where m (for this argument) is the fraction of the population which migrate (per generation). Surprisingly, perhaps, the effect of migrants does not depend on mutation rate or even on population size, since Nm = the effective *number of migrants* per population per generation (M). If M > 1 then there is little substructure, if M < 1 then substructure is significant. This relationship between genetic structure (F_{ST}) and gene flow (Nm) is based on an island model and has assumptions that may not always be met by natural populations (Whitlock & McCauley 1999). This model assumes that i) there is no selection or mutational processes acting ii) that there are an infinite number of individually stable populations, iii) migrations between populations are equally distributed, iv) each individual has the same likelihood of dispersing to any population, and v) that each population is in equilibrium between migration and genetic drift. Should any of these assumptions be violated the relationship between F_{ST} and Nm will be significantly affected, and hence estimates of F_{ST} based on migration should be interpreted with caution (Whitlock & McCauley 1999).

1.2.2 Geographical Population Structure

Phylogeography is the assessment of the patterns and processes that govern the geographic distributions of genealogical lineages, both spatially and temporally (Avise 1994). Many studies of both eusocial and non-eusocial species have revealed that a certain degree of genetic differentiation exists among geographic locales within species (see McCracken *et al.* 1994; McCracken & Gassel 1997; Castella *et al.* 2000; Kerth *et al.* 2000; Kerth *et al.* 2002a; Miller-Butterworth *et al.* 2003). It is postulated that this phenomenon is a result of the tendency of siblings to remain within their natal surrounds (Avise 1994). In species such as *Neoromicia capensis* that are widely distributed, population differentiation is expected to increase due to isolation by distance and habitat structure. However, vagile bat species (e.g. *Miniopterus schreibersii, Tadarida brasiliensis*, and *Myotis myotis* among others) would be expected to display genetic homogeneity over large spatial divides (Miller-Butterworth *et al.* 2003), and hence to have relatively low levels of mean population structure (i.e. one would expect vagile species to approach panmyxia.

Adult vagility and the individual's "perception" of habitat patchiness are forces that influence gene flow and population structure (Avise 1997). However, there are other factors that may influence gene flow in highly vagile species: i) physical barriers, ii) behaviour i.e. philopatry, iii) sex-biased dispersal, iv) selection on genetic markers or v) historical stochastic events influencing genetic composition of a population.

1.2.2.1 Barriers to dispersal

Barriers to dispersal, and hence gene flow, are not always proportionally related to the dispersal ability of the species in question. In a study of the effects on dispersal on gene flow across the Gibraltar Strait, Castella *et al.* (2000) found that the populations of the Greater Mouse-eared bat (*Myotis myotis*) on the Iberian Peninsula were genetically distinct to those found in Morocco. *Myotis myotis* is a migratory species, capable of dispersing on average 500 km at the onset of winter to hibernacula. Their data suggest that these populations have a genetic divergence of several million years in contrast to their potential dispersal ability, even though the Strait is only 14 km wide at its narrowest seaboard.

1.2.2.2 Philopatry to natal site

Site-fidelity and natal philopatric behaviour among many species has an important bearing on population structure. Many highly mobile and volant species, especially bats, display marked population substructure among colonies or subpopulations (Watt & Fenton 1995; Burland *et al.* 2001; Kerth *et al.* 2002b; Heckel & Von Helversen 2003). Many bat species exhibit philopatry to natal roosts (McCracken 1987, Altringham 1994). Burland *et al.* (2001) found that males and females of the brown long-eared bat (*Plecotus auritus*) display roost fidelity. However, the average relatedness between individuals was close to zero. Perhaps the most striking example of roost fidelity is that of the non-migratory Bechstein's bat (*Myotis bechstinii*). Kerth *et al.* (2002a) found that females display extreme (absolute) fidelity to roosts, and that they do not move between roosts despite the close proximity of these roosts to each other (Kerth *et al.* 2000). Again, mean colony relatedness was found to be very low. Burland *et al.* (2001) and Kerth *et al.* (2002a) concluded that extra-colonial mating occurred causing low relatedness, but explained philopatry to be a result of roost familiarity and security among individuals born there. On the other hand, in migratory species, philopatry to natal roosts and hibernacula (typically hundreds of kilometres apart) can cause genetically homogeneous subpopulations within species over large areas (Castella *et al.* 2000; Miller-Butterworth *et al.* 2003). In these examples it is evident that average relatedness within a colony does not necessarily provide a good estimate of social structure of group-living animals, as cautioned by Kerth *et al.* (2002b).

1.2.2.3 Sex-biased dispersal

In general, but not without exception, mammalian species exhibit male-biased dispersal when asymmetric philopatry is inherent (Greenwood 1980). Sex-biased dispersal is selected for when the cost of inbreeding is high and the cost of dispersal is low (Motro 1994). Consequently, when the costs (or benefits) of dispersal are different for either sex, gender biased dispersal is able to evolve (Vitalis 2002). In polygamous behaviour, male biased dispersal is expected to evolve when local resource competition (in females) is exceeded by local mate competition if males. An obvious consequence of asymmetric dispersal is that a population will show different geographic gene structure at a karyotypic level in contrast to genes transmitted clonally [i.e. mitochondrial DNA and the Y chromosome (Avise 1994)]. Kerth *et al.* (2002a) recently documented an account of extreme gender-biased dispersal in the communally breeding Bechstein's bat (*Myotis bechstinii*). It was found that absolute female philopatry (96% mtDNA differentiation) was opposed by strong (and possibly complete) male dispersal. Kerth *et al.* suggest further that inbreeding avoidance is the crucial factor driving male dispersal. Additionally, competition for mates may be a cause of sex-biased dispersal (Vitalis 2002).

1.2.3 Estimation of Gene Flow in Natural Populations

There are two methods of estimating the rate at which genes flow through a population. An indirect estimate can be obtained through direct observation of the breeding successes of individuals, or, over a larger scale, by determining the dispersal distances of individuals. However the ability of an individual to migrate over large distances does not indicate the "actual" gene flow in a population. Mark-recapture studies can provide insight into which individuals return to the site of capture in successive years, but all individuals must be accounted for to provide reliable conclusions. These methods fail to provide accurate data in terms of long term (historical) gene flow within populations (Slatkin 1987).

A direct method of determining gene flow within a population is through molecular analysis. Evaluation of genetic variation provides a historical perspective of gene transmission over generations (Slatkin 1987). Four techniques have been used in the past decade, each with varying levels of success. Hedrick & Miller (1992) list these as i) protein electrophoresis, ii) analysis of highly variable nuclear DNA sequences, iii) mitochondrial DNA analysis and iv) DNA sequence amplification by PCR (i.e. microsatellites). Protein electrophoresis may not be efficient in estimating gene flow since protein loci may be monomorphic in populations. Both mitochondrial DNA and microsatellite analysis provide sufficient resolution to determine local and widespread population structure. Due to its mode of transmission and rate of evolution, mtDNA analysis can be used to determine the geographic structure of, and the individuals' relatedness within, a population (Hedrick & Miller 1992). The most robust method of estimating dispersal and philopatry in natural populations is a combination of both direct observational studies, and analyses of mtDNA sequences and autosomal microsatellite distributions (Bohonak 1999).

1.3 Site attachment in *Neoromicia capensis*

Roost fidelity in the Cape serotine bat has not been documented. Some studies suggest that groups of individuals live together in anthropogenic structures as well as under the bark of trees (Rautenbach 1982; Taylor 2000). However, there is no empirical data available to suggest that individuals return to the same locales across seasons (i.e. strong site attachment), or whether migratory routes exist between natal roosts and hibernacula – if hibernation occurs in this species. It has been found that other vesper bats display either site fidelity (*Myotis bechsteinii*) or roost philopatry as Thompson (1992) found in *Pipistrellus pipistrellus.*

1.4 Study Aims

Although the Cape serotine bat is a common resident throughout Africa, no quantitative genetic studies have been conducted on this species. The most intensive studies to date have been conducted on the reproductive physiology, however very little is known about its ecology and behaviour. Current knowledge of roost fidelity in this species is based on single observations, and no data are available on the genetic structure of colonies or on gene flow within and between subpopulations of *N. capensis*.

In this study we therefore aim to:

1. provide an estimate of genetic relatedness within and between subpopulations in different regions in Southern Africa
2. determine if philopatry in one or both sexes has caused genetic structuring in populations of this species.

CHAPTER 2

STUDY SITE AND SAMPLE ACQUISITION

2.1 Location of study sites

2.1.1 Specimens collected from the field

Field trips within the Western Cape Province of South Africa were carried out throughout 2003/2004. Eight sites were sampled, and included nine subpopulations or specific colonies (Table 2.1). The number of individuals that were captured for analysis totalled 105.

TABLE 2.1 LIST OF ALL SITES SAMPLED. M : F refers to the ratio of males and females captured at each site. "C" refers to captured individuals known to have come from a specific roost (i.e. colony). "SP" refers to individuals whose specific roost is not known (referred to as subpopulations).

LOCATION	STATUS	M : F	TOTAL
Knysna (CXK)	SP	0 : 16	16
Plettenberg Bay (CXP)	SP	2 : 18	20
De Hoop N. R. (DH)	SP	2 : 23	25
Die Hel (DA)	SP	2: 0	2
Cedarberg (CA)	SP	2 : 1	3
Ceres (CT)	SP	3 : 4	7
Vrolijkheid (House; VRH)	C	1 : 5	6
Vrolijkheid (Office; VRO)	C	3 : 2	5
Wellington (CN)	C	1 : 20	21

2.1.2 Transvaal Museum accessioned specimens

We included museum specimens (site abbreviations and numbers of individuals in parentheses) collected from six locations from the Kuruman, Northern Cape (KM - 12 bats), Mpumalanga (MP - 1 bat), Hluhluwe, Kwa-Zulu Natal (HH - 1 bat), Pafuri, North Limpopo (PP - 5 bats), Timbavati Nature Reserve (TN - 12 bats) and Malawi (MW - 1 bat) collectively. The Museum Accession codes are listed for each individual in Appendix II.

2.2 Field data collection procedure

2.2.1 Bat capture

The Cape serotine bat roosts predominantly in roofs of houses and these roosts are easily found by the presence of scat on the walls. The entrance to the roost is identified by the pelt-oil stain around the entrance hole which is usually a gap between the rafters supporting the roof and the wall of the building. Mist nets were placed in front of roost entrances in an attempt to capture entire colonies. We also placed nets in flight paths or adjacent to buildings where no roost entrance could be identified. Bats captured using this netting method were considered representatives of a subpopulation and not allocated to a specific known colony. Nets were monitored continually throughout the netting session, and bats were removed from the nets immediately to minimize stress. Bats were placed individually into soft cloth bags and left in a quiet dark room overnight.

Bats were processed early in the morning while they were still in torpor as this minimized stress while processing.

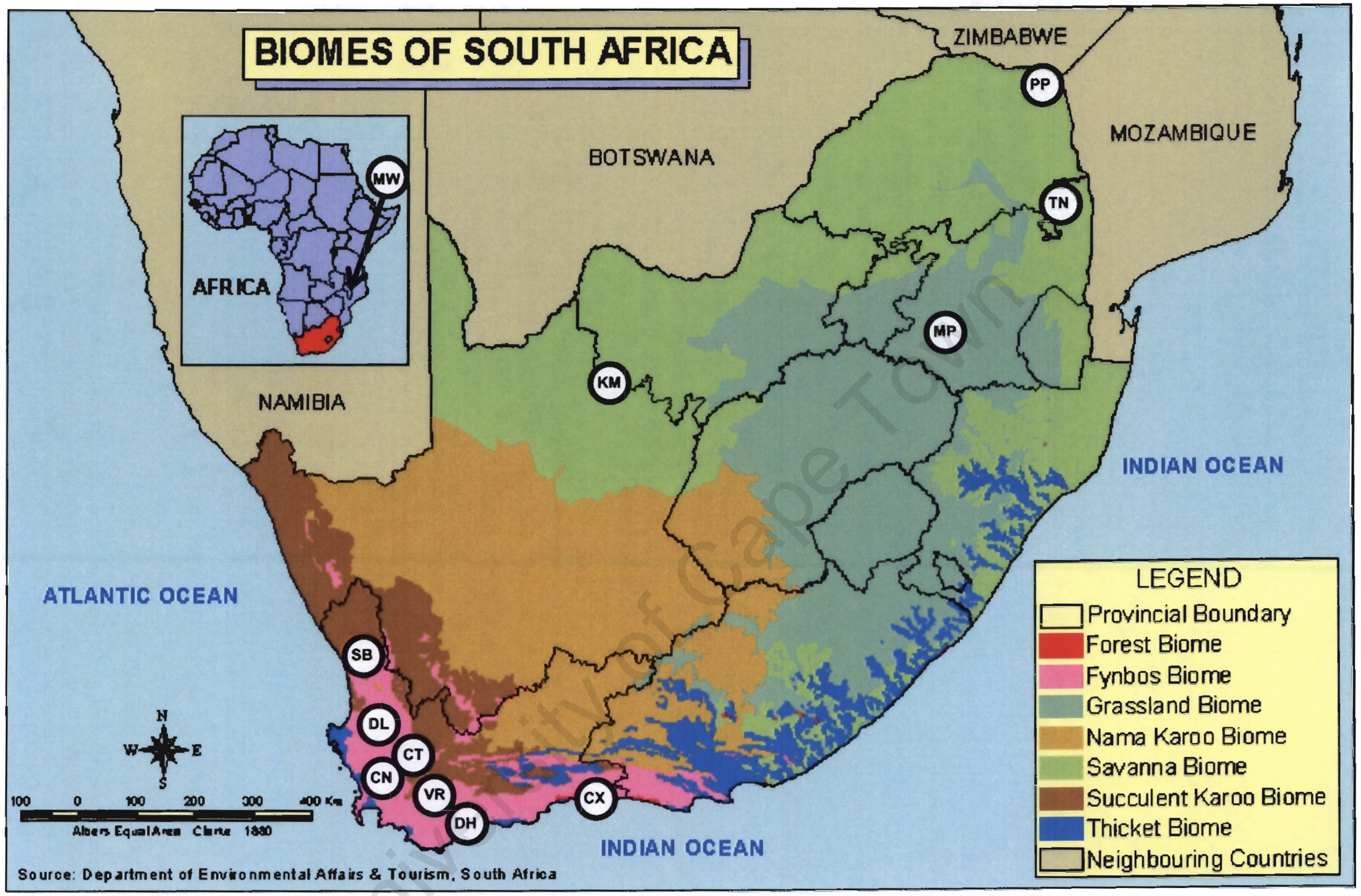
Bulk DNA was obtained from the heart of a male bat captured at Zuurvlei Reserve, Western Cape. The individual was euthanased using Halothane B.P. This DNA was used to optimise the PCR protocol where bulk DNA is required, as a skin biopsy would not provide sufficient DNA for these pilot tests. This individual was not included in genetic analyses because it was the only individual caught in the area in a random flight path.

2.2.3 Field identification of specimens

Voucher specimens were not collected routinely from the field. Bats were identified in the field by morphological and echolocation data using the taxonomic key of Taylor (2000) and Skinner & Smithers (1990). Samples provided by the Transvaal Museum, which were correctly identified as isotypes of *Neoromicia capensis*, were used to test for genetic similarity of the specimens collected from the field. In this way we were able to ensure that the specimens were correctly identified.

2.2.4 Biological and Morphological Data

Each individual was assigned a field code that identifies the date of capture and the location of capture (for laboratory use only) and was tagged using alpha-numerical imprinted, aluminium armbands (see 2.2.5 - data presented in Appendix II). The weight



Source: Department of Environmental Affairs & Tourism, South Africa

FIGURE 2.1 GEOGRAPHIC DISTRIBUTION OF SUBPOPULATIONS THROUGHOUT THE BIOMES OF SOUTH AFRICA (map and biome data courtesy of the Department of Environmental Affairs & Tourism, South Africa). SB = Cedarberg (mountain fynbos) & DL = Die Hel, Groot Winterhoekberge NR [(fynbos), individuals referred to as samples "DA" in analysis]; CT = Ceres (fynbos / orchard); CN = Wellington (forest karoo); VR = Vrolijkheid NR (fynbos / succulent karoo) consists of two colonies: i) VRH = House and ii) VRO = Office; DH = De Hoop NR (fynbos); CX consists of two colonies: i) CXK = Knysna (thicket / forest) & ii) CXP = Plettenberg Bay (forest); KM = Kuruman (savanna); MP = Mpumalanga (grassland savanna); TN = Timbavati Nature Reserve; PP = Pafuri Research Station (woodland savanna); MW = Malawi of each individual at capture is also listed in Appendix II. Forearm length was measured, and a digital photograph was taken of the extended right wing (as per Saunders & Barclay 1992) using an Olympus digital camera. Care was taken to position the camera at 90^0 above the wing. Each specimen was photographed on standard graph paper to allow calibration of measurements in the computer software programme SigmaScan Pro 5.0 (SPSS LTD) used to measure the digital images. Body length, wing span, and wing area were measured from the wing images and these measurements were used to calculate wing loading and aspect ratio (Norberg & Rayner 1987). Again, this data was captured for identification purposes only, and is not presented in this dissertation.

2.2.5 Skin Biopsies

A small piece of the uropatagium (tail membrane) of each bat was taken from either side of the tail using a sterile 3 mm biopsy punch (Worthington, Wilmer & Barratt 1996). Two biopsies were necessary since the amount of DNA extracted from each sample was not always sufficient for comprehensive analysis. The procedure was carried out on a soft white plastic cutting board, allowing the veins to be visible, to ensure that the veins were not punctured during the procedure. After the biopsies were taken Cicatrin antiseptic powder was applied to the tail membrane. Biopsy samples were immediately placed and stored in 70 % ethanol.

Prior to release each individual was given an aluminium armband with individual identification codes engraved on each. Two individuals recaptured in De Hoop Nature

Reserve a year later provided an opportunity to investigate the effects of the biopsy procedure. Both individuals had shown full recovery from the wounds, and both showed no signs of adverse health conditions. The uropatagium plays a vital role in prey acquisition and it was decided to continue taking biopsies from the pteroptagium (arm wing membrane), on either side of, and as close to the body without puncturing any blood vessels.

2.3 DNA Extraction

2.3.1 Protocol

DNA was extracted from one of the biopsy punches, leaving a reserve cache of tissue. Extraction of DNA was performed using a simple salt extraction protocol. Biopsy samples were incubated at 56 °C overnight in 1000 µl of 0.05 M solution of proteinase kinase. After incubation 400 µl of 6 M NaCl was added to the digestion and spun at 10 000 $U.min^{-1}$ for 10 minutes. The supernatant was pipetted off and added to a fresh 1.5 ml eppendorf tube, and 1000 µl of isopropyl alcohol was added to precipitate the DNA from the solution. This solution was left in a –20 °C freezer for 60 min to maximize precipitation. The suspension was centrifuged at 30 000 U.min-1, and the supernatant poured off. 1000 µl of 70 % Ethanol was added to the DNA pellet and spun for 20 min at 13 000 $U.min^{-1}$. This purification stage was repeated twice to ensure that all salt was removed. The DNA pellet was left to dry overnight and resuspended in 100 µl of 1 x Tris EDTA buffer (pH 7.5). The samples were stored at –20 °C until use.

2.3.2 Quantification of DNA

Three methods were used to quantify DNA: i) Diode Array spectrophotometry, ii) micro-capillary spectrophotometry and iii) agarose gel electrophoresis (AGE). The first two methods proved unsuccessful in providing reliable quantitative data due to very low concentrations of DNA in the samples. Products were electrophoresed through a 1% agarose gel stained with ethidium bromide. 10µl of each sample were run with a standard DNA marker of a known concentration, in this case human DNA with a concentration of 100ng.µl^{-1}. As the DNA migrates through the matrix of the gel, EtBr (ethidium bromide) is incorporated into the helical structure of DNA. When analysed under ultraviolet light, the EtBr fluoresces proportionally to the amount of the DNA in the sample. In comparison to the human DNA control, this enables estimation of the amount of DNA per sample. Faint bands in the region of 40 kbp were visualized and concentration approximations of 10 to 20ng.µl^{-1} recorded for all samples.

In spectrophotometric quantification, each sample is diluted one hundred-fold, and assayed in a diode-array spectrophotometer at absorbance wavelengths of 260nm and 280nm. The ratio of these two readings (λ260nm / λ280nm) provides an estimate of purity of the sample. The concentration of DNA (ng.µl^{-1}) in each sample was calculated according to the following equation:

$$[DNA] = \lambda 260nm\left(5 \times 10^{3}\right)$$

All DNA was quantified using AGE estimates because of the low, and highly variable results (possibly resulting from a combination of low yield and high dilution factor) obtained using this method.

Details pertaining to the genetic analysis of samples are given in Chapters 3 and 4.

CHAPTER 3

AN INVESTIGATION OF POPULATION SUBSTRUCTURE IN *NEOROMICIA CAPENSIS* USING MICROSATELLITE MARKERS

***AIM:** To determine the population structure of Neoromicia capensis in South Africa*

3.1 Introduction

3.1.1 What are microsatellites markers?

Microsatellites are simple sequences of genomic DNA consisting of tandemly repeated nucleotides (Hearne *et al.* 1992, Queller *et al.* 1993, Jarne & Lagoda 1996 & Li *et al.* 2002). They occur frequently throughout eukaryotic genomes, apparently distributed at random, and are considered to be selectively neutral genomic markers (Schlötterer 2000). Most frequently used in population genetics are those with mono-, di-, tri-, or tetra-nucleotide repeats. Weber (1990) classifies three families of microsatellites – pure, compound and interrupted. Pure microsatellites consist of a single unit repeated a number of times e.g. $(GA)_n$, where '*n*' represents the number of times the unit is repeated. Compound microsatellites consist of two or more different units repeated a number of times, e.g., $(CA)_n(AT)_n$. Interrupted, or impure, repeats are those that incorporate an additional sequence of repeats nested within a repeated unit, e.g., $(CA)_n(GA)_n(CA)_n$.

Repeats can be made up of any combination of these families (Jarne & Lagoda 1996). For example, a sixteen-repeat pure dinucleotide array, $(GT)_{16}$, would be sequenced as: GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT (Fig 3.1).

Tautz (1989) suggests that approximately 5% of the mammalian genome consists of microsatellites. It is estimated that the human genome may have up to 500 000 microsatellites of any type or combination (Ashley & Dow 1994). The most common microsatellites in the human genome are those with $(CA)_n$ repeats (van den Bussche *et al.* 1995), with a lower (yet significant) incidence of tri- and tetra- nucleotide repeats (Hamada *et al.* 1982). Mono-, di- and tetra- nucleotide repeats are found in the non-coding regions of the genome (introns), whereas trinucleotide repeats are found in both introns and exons (coding regions).

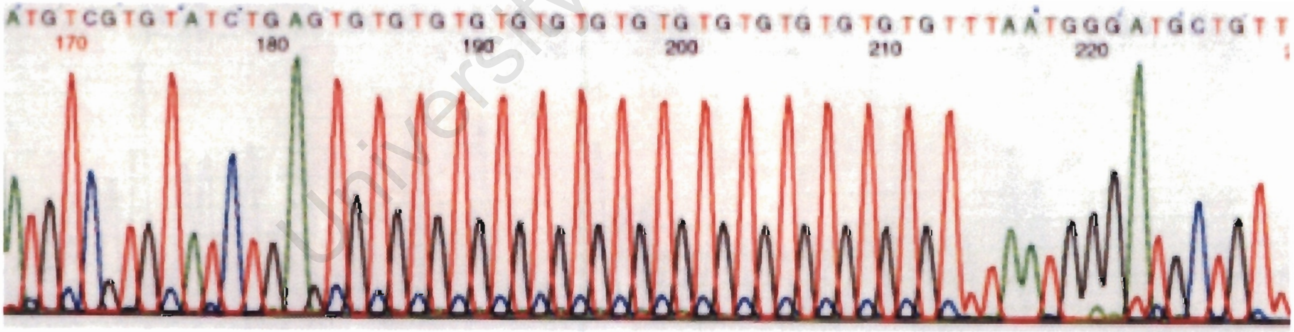


FIGURE 3.1 SEQUENCE OF A TYPICAL MICROSATELLITE LOCUS. The GT repeat has characteristic flanking regions that allow for the design of specific primers. These flanking regions are usually conserved and can allow genetic comparisons not only between individuals of a species but often also between individuals in another genus within the same Family.

Equilibrium between mechanisms of amplification and amplification inhibitors result in a reduced quantity of repetitive DNA in the genome (Baker *et al.* 1992; van den Bussche *et al.* 1995). In the absence of this equilibrium repetitive sequences of DNA tend to accumulate. This appears to be a common trend throughout karyotypic megaevolution (Bradley & Wichman 1994). The density of microsatellite loci in eukaryotic genomes is dependent on the total size (i.e. number of chromosomes and base pairs) of the genome. Bats and birds have the smallest eukaryotic genomes (Baker *et al.* 1992 and Primmer *et al.* 1997, respectively). Bats have the smallest eutherian genome comprising at least 50% of the size of the genome of other animals (Bradley and Wichman 1994). Baker *et al.* (1992) reported that there was a four-fold decrease in the incidence of repeated ribosomal DNA sites in the bat genome compared to those in rodents. Van den Bussche *et al.* (1995) found that dinucleotide repeats occur less frequently in bats (ca. 20 000 $(CA)_n$ repeats) compared to *Mus* spp. and humans, that have ca. 100 000 and 50 000 $(CA)_n$ repeats throughout their genomes, respectively. Primmer *et al.* (1997) and Baker *et al.* (1992) suggest that animals with a high metabolic rate, such as bats and birds, would be physiologically compromised should they have a large genome. Consequentially, these genomes would have fewer and shorter introns and proportionally fewer microsatellites allowing transcription to be optimal under heightened physiological demand.

3.1.2 Functions of microsatellites

Although microsatellites are found in relatively high abundance throughout the eukaryotic genome, their function remains elusive. Since most microsatellite markers are found in the non-coding regions of the genome, they impart no obvious functional evolutionary importance to the fitness of the organism (Avise 1994). Some authors have speculated that the main functions of these selectively neutral markers are related to genetic recombination and they may play a role in the evolution of genetic variation across taxa (Tautz & Renz 1984; Weber *et al.* 1993). This is consistent with the postulate that high frequencies of repetitive units in rapidly evolving genomes are directly responsible for genomic evolution (Bradley and Wichman 1994).

Tautz & Renz (1984) and Li *et al.* (2002) propose further that microsatellites may have a functional importance in chromosomal behaviour. They suggest that these sites may play a role in chromatin folding and in the formation of telomeres. Microsatellites may also have a functional role in the regulation of gene expression (Tautz & Renz 1984; Moxon & Willis 1999). Li *et al.* (2002) propose further that microsatellites regulate DNA metabolic processes as well as gene activity. Despite limited understanding of the function of microsatellites, they are nevertheless excellent tools for the quantitative genetic analysis of natural populations (Moore & Kukuk 2002).

3.1.3 Applications in evolutionary and conservation biology

The lack of suitable segregating genetic markers has limited evolutionary studies especially in the evaluation of kinship (Queller *et al.* 1993). Although, many molecular tools such as analysis of allozymes, RFLP's (restriction fragment length polymorphisms), multi- and single- locus minisatellites and RAPD's [(randomly amplified polymorphic DNA), Queller *et al.* 1993; Jarne & Lagoda 1996], have been employed in the analysis of descent and kinship, none of these applications have provided the analytical detail required in population genetic studies. Often there is too little variation within a population to estimate relatedness by these methods, and consequentially an understanding of inclusive fitness strategies is lacking (Queller et al. 1993). The ideal marker would be codominant, and have high variation at defined loci. Microsatellites fulfil these requirements and are widely used in the field of quantitative population genetics. They have been used in a variety of analyses, including the assessment of population size and inbreeding (Hedrick & Miller 1992), examination of social structure (Burland *et al.* 2002), reproductive behaviour (Heckel & von Helversen 2003), migratory patterns and philopatry (McCracken *et al.* 1997; Miller-Butterworth *et al.* 2003).

3.1.3.1 Polymorphic loci and heterozygosity

Microsatellites are distributed predominantly in the non-coding regions of the genomes of eukaryotes. They are selectively neutral markers (i.e. different alleles have no apparent effect on the fitness of the individual), and the alleles are therefore functionally equivalent

(Avise 1994). Since they are not acted upon by selection, these loci display a high level of variation within and between populations of species (hypervariable loci). This is in contrast to allozyme loci, which are likely to have more constraints with respect to amino acid composition, and hence show less variation within species (loci display low polymorphism). Consequentially, microsatellite loci display a higher level of heterozygosity than allozymes (Queller *et al.* 1993).

The variation in the length of microsatellite loci is believed to be a result of misalignment of strands during the process of DNA replication. Tautz and Schlötterer (1994) recognized this phenomenon as "slipped-strand mispairing". It is assumed that the variation in number of repeats in microsatellites occurs by displacement of the nascent strand and preceded by out-of-register pairing (Levinson & Gutman 1987; Bachtrog *et al.* 2000). If the mismatch repair system fails to identify the incorrect alignment a repeat will be added to, or be deleted from the new strand. Mutations will thus accumulate. Jarne and Lagoda (1996) identify this mutational process as a stepwise mutation (i.e. follows the stepwise mutation model). Bruford and Wayne (1993) have estimated that pure microsatellite loci mutate at a rate of between 5 x 10^{-5} and 10^{-4} per generation.

3.1.3.2 *In vitro* amplification

Minisatellites (tandem repeats often ranging up to kbp length) have also been useful in conservation and forensic applications, but the allelic size can inhibit clear amplification. The efficacy of the polymerase chain reaction or PCR (Saiki *et al.* 1988) is best applied to short lengths of DNA, i.e. microsatellites < 500 bp in size. Microsatellites can therefore be amplified *in vitro* with ease. If the sequences of the flanking regions are known, primers can be designed to amplify the target sequence. A disadvantage of microsatellites, however, is that if no primers exist for the species in question, a genomic library must be constructed. This is an extremely time-consuming and laborious task.

A benefit of using PCR for these applications is that picogram quantities of DNA can be successfully amplified and analysed (Taberlet *et al.* 1996). The exponential amplification of target alleles by PCR, and the need for only minute amounts of starting template DNA has proven especially beneficial in analysis of ancient and forensic samples. It has also allowed tissue sampling for DNA to become less invasive, benefiting conservation genetics because animals need not be euthanased for collection of tissue samples. Only a small quantity of blood, or a small biopsy or tissue scraping supplies sufficient material.

3.1.3.3 Allele scoring

After PCR amplification the target locus can be electrophoresed through a vertical 6% polyacrylamide gel. The matrix of these gels has sufficient resolution to separate alleles that differ by as little as one base pair. If radioactive primers are used (as in this study) an autoradiograph is used for visualisation. All samples are run together with a DNA marker ladder, and alleles can therefore be scored visually with ease (Fig. 3.2). Shorter alleles will migrate through the matrix faster than longer strands. If an individual inherits the same allele from both parents only a single band will appear on the gel – both alleles are present but they migrate at the same speed through the matrix. Such an individual will be scored as a homozygote, whereas if two bands are present the individual is scored as being heterozygous for the target locus (see Fig 3.2). This clear banding makes it easy to score the alleles.

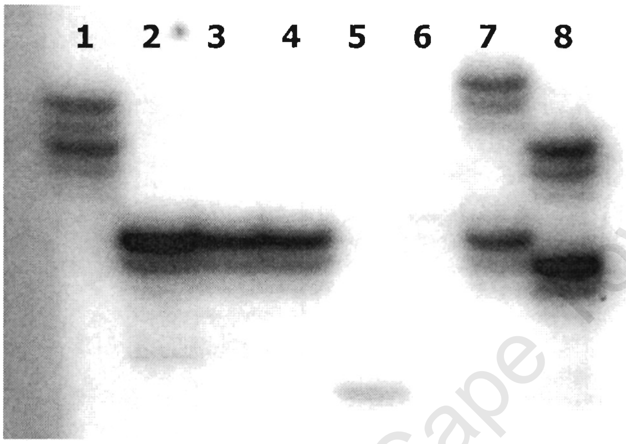


FIGURE 3.2 AUTORADIOGRAPH OF THE GENOTYPES OF LOCUS EF6 OF EIGHT *NEOROMICIA CAPENSIS* INDIVIDUALS FROM DE HOOP NATURE RESERVE. Lanes 1, 6, 7, and 8 are heterozygotes, while lanes 2, 3, 4, & 5 are homozygotes. The dark bands are the alleles, and the lighter bands immediately below are stutter bands – characteristic of microsatellite alleles.

3.1 4 Limitation of microsatellite applications

Perhaps the most vexing issue of microsatellite applications is when suitable markers have not been identified for the species in question. Under these circumstances, as in many studies, it is necessary to construct a gene library and screen the genome for microsatellites. Once these have been identified, suitable primers complimentary to the flanking regions must be designed. Further complications arise in the analysis of products. The incidence of null-alleles and stutter bands, characteristic of microsatellites (Fig. 3.2), can lead to confusion as to what the actual allele is in the analysis. Null-alleles are usually identifiable by homozygote excess in the sample. A mutation in the flanking region of the microsatellite prevents optimal binding of the primer and results in what seem to be an excess of single alleles, leading to the mistyping of potentially heterozygous individuals (Jarne & Lagoda 1996). A deviation from Hardy-Weinberg equilibrium is a common result of the presence of null-alleles in the population sample. Another cause of homozygote excess is the random non-amplification of alleles, especially likely to occur if the amount of genomic DNA available is low. When a primer fails to amplify an allele, it is said to be a “dropped allele”, and will cause deviation from Hardy-Weinberg equilibrium in the same way.

Stutter bands may also cause problems in the analysis. The PCR reaction is a fast acting “evolutionary chamber”. The continual recombination and dissociation of primers or partially extended amplicons can cause slippage (stepwise mutation model – at super speed) due to misalignment of the two strands. Normal *Taq* polymerase is unable to identify the error (it lacks an endonuclease function – i.e. no proof reading ability) and as a result an accumulation of PCR artifacts occurs. These “mutated” alleles are usually less prevalent than the full sized product, so they generally appear less intense on autoradiography and are easy to identify as such (see Fig 3.2).

Finally, microsatellite typing is visually determined as a size variation, as opposed to direct sequencing of the product. Any variation in length of the product is attributed to a mutation in the repeat sequence itself, and not the flanking regions (Levinson & Gutman (1987). Schlötterer and Pemberton (1994) caution that observations such as these will increase the variation based on the genetic distances between individuals and populations. Fortunately mutations in the more conserved flanking regions occur less frequently than within the repeat region. Mutations in the flanking regions can sometimes be detected on an autoradiograph through an inappropriate base pair size change (i.e. deletion or addition of a single base as opposed to a double base pair).

In this study microsatellite analysis will be used to investigate population substructure in *N. capensis* in South Africa

3.2 Methods & Materials

3.2.1 Identification of suitable primers

Vonhoff *et al.* 2002 provided six primer set sequences that successfully amplified polymorphic products in *Eptesicus fuscus* and five other genera in the Vespertilionidae. On initial optimization of these oligonucleotides using bulk *Neoromicia capensis* DNA, all primers yielded satisfactory results. In addition, Petri *et al.* (1997) provided sequences used for amplification of PCR products in *Nyctalus noctula* and *Myotis myotis* (NN8 and MM5 primers respectively). These primer sequences were redesigned by Castella *et al.*

(2001), and I was able to obtain reliable and reproducible products for *N. capensis* using them. Appendix IV lists the primer sequences I used for each locus.

3.2.2 Polymerase Chain Reactions

3.2.2.1 End-labeling of reverse primers

a) γ ^{32}P ATP

The reverse primer of each oligonucleotide set was end-labelled using γ ^{32}P ATP. The stock γ ^{32}P ATP was supplied by Amersham Pharmacia Biotech in 150mCi.µl^{-1} aliquots, which was diluted to a working solution of 20µCi.µl^{-1} prior to use. End-labelling was carried out by incubating the reaction with T4 polynucleotide kinase (PNK, New England Biolabs, Cape Town) for 90min at 37°C, and terminated by a final incubation at 80°C. PNK catalyses the transfer of the terminal phosphate of ATP to the 5' hydroxyl terminus of the DNA strand. The protocol used for endlabelling was as follows.

Sterile deionised water = 6.0 µl
50 pmol.µl^{-1} primer = 1.5 µl
10X PNK Buffer = 1.0 µl
10U.µl^{-1} T4 PNK = 0.5 µl
20 µCi.µl^{-1} γ ^{32}P ATP = 1.0 µl
REACTION VOLUME = 10 µl

b) Fluorescence

End-labelling of primers using $\gamma\ ^{32}P$ ATP is a process that requires many hours to run to completion. Two additional approaches were attempted to maximize efficiency in terms of data output. These protocols involved the use of fluorescent labeling techniques. Inazuka *et al.* (1996) designed a methodology catalised by the Klenow Fragment of DNA polymerase in which the 3' terminal residue of the PCR-amplified product is exchanged for a fluorescent nucleotide (fluorescein-dUTPs). Initially PCR conditions were performed using the standard protocol (see below) and the fdUTPs added to the PCR products. We were unable to incorporate the fdUTP into the products. In a second trial we attempted to substitute dTTP for fdUTP of the standard protocol, however the fluorescent product was too bright to be able to score alleles accurately. Finally 0.01mM dilutions of both nucleotides were added to a reaction volume of 10 µl to allow competition between them to reduce fluorescent intensity. In this way, we were able to score alleles successfully, but when compared to an autoradiograph of the same samples there were discrepancies in the allelic distribution. Due to time constraints and dubious results, the optimising of these reactions was not completed and all consequent analyses were performed by autoradiography.

3.2.2.2 PCR master mixes and cycling

Master mixes were prepared to facilitate the analysis of a full population in a single PCR reaction depending on its size. Master mixes were prepared in series, each mix being sufficient for 10 individuals once the endlabelled reaction was added to the final cocktail.

Taq DNA polymerase and its buffer (Promega, 10X magnesium-free reaction buffer and 25mM MgCl$_2$) were used in each application, where MgCl$_2$ concentration varied between reactions. Deoxynucleotides were supplied individually in 10mM solutions (Sigma), and each was added separately to the master mix. The master mix was prepared according the following protocol:

sdH$_2$O = 51µl
Reaction buffer = 10µl [1X]
MgCl$_2$ (25mM) = 8µl [2 mM]
F primer (50pmol.µl) = 1.5µl [1 pmol.µl^{-1}]
DNTPs (2.5mM) = 8µl [0.2 mM]
End-lablled rxn. = 10µl [1.1 pmol.µl^{-1}]
Taq polymerase = 1.5µl [*ca.* 0.3U activity]
FINAL VOLUME= 90µl

The magnesium chloride concentration for each reaction varies according to optimization for the primer annealing (details listed in Table 3.1). Figures listed in parentheses are the final concentration in the total reaction volume.

All PCR reactions contained 9µl of the master-mix and 1µl of template DNA (ca. 10 – 20ng.µl^{-1}) in 0.2ml thin-wall eppendorf tubes. All PCRs were performed in a Perkin-Elmer thermocycler. Thermal profiles started with an initial denaturation at 95°C for 2min, followed by 35 cycles of 95°C for 50sec, primer-specific annealing temperature for 50sec and 72°C for 1min. Cycles were terminated with a final extension of 72°C for 10min, and 4µl of formamide loading dye was added to each sample. PCR products were kept refrigerated at 4°C until electrophoresis.

TABLE 3.1 SUMMARY OF PCR CONDITIONS FOR EACH PRIMER SET. Those loci listed with an asterisk did not produce repeatable results and were excluded from analyses. Conditions shown are those that provided the most results in one run. T_A is the annealing temperature.

LOCUS	[$MgCl_2$] (mM)	T_A (°C)	OPTIMISED
EF1	2	50	NO*
EF4	2	53	NO*
EF6	2	48	YES
EF14	1.5	45	YES
EF15	2	55	NO*
EF20	1.5	50	NO*
MM5'	1.5	55	YES
NN8'	1.5	50	NO*

3.2.2.3 Electrophoresis and scoring of alleles

All products were denatured at 95°C for 3min prior to electrophoresis. Microsatellite alleles were separated through a 6% denaturing polyacrylamide gel for 120min at 65 Watts. DNA strands are negatively charged, and so they migrate through the matrix towards the anode. Larger fragments meet with greater resistance to the polymer-matrix and consequently migrate slower. After electrophoresis the gel is vacuum dried and left to expose on Agfa CP-BU X-Ray film overnight.

Microsatellites were scored manually according to an M13mp18 AT sequencing ladder containing standard marker positions. This marker is prepared by a dideoxynucleotide chain-termination protocol (developed by Stanger *et al.* 1977) using the -40 universal primer (5' -GTT TTC CCA GTC ACG AC- 3'). Instead of one of the deoxynucleotides being radioactive, the universal primer is endlabelled, and two dideoxynucleotides are used: ddATP and ddTTP. The sequencing was performed using Sequenase Sequencing Kit, version 2 (USB Corporation, supplied by Separations Scientific) according to the manufacturer's protocol.

3.2.2.4 Problems encountered

Four primers, viz., EF 1, 4, 15 and 20 were not optimized (Appendix IV). Irreproducible amplification of products and failure of many samples to amplify led to the disqualification of these loci from the analysis due to time constraints. Furthermore, DNA extraction of samples was not always optimal. The standard salt precipitation method of DNA extraction could well have been the cause of poor quality or inadequate DNA in some samples, ultimately causing failed amplification of products. Lastly, continuous efforts to optimize these primers, as well as those that amplified depleted the DNA stock. In this analysis, therefore, only data for four subpopulations (De Hoop, Wellington, Kuruman and Timbavati) over three loci are presented (Section 3.2.3.1.).

3.2.3 Statistical analyses

3.2.3.1 Locus Characteristics

The classification of the Cape serotine bat has been controversial ever since its description by Arthur Smith in 1829. It is possible that the genus *Neoromicia* is sufficiently different from the other vespertilionids so that primers that work for other vespertilionids do not work optimally for bats in this genus. In this study, eight vesper primers were utilised: six specific to *Eptesicus fuscus* (Vonhoff *et al.* 2002), one specific to *Myotis myotis* and one designed for *Nyctalus noctula* (both modified primers from Castella *et al.* 2001 – see Appendix IV). Four of the eptesicoid primers did not amplify any PCR products in all populations and were excluded from the analysis. Consequentially, only data for three primers are presented (Table 3.2). Data for four populations are presented, in which some individuals had to be disqualified from analysis due to failed amplification of products. In total 23 individuals were excluded from these populations (8 from De Hoop; 9 from Wellington; 3 from Kuruman; 3 from Timbavati). Ultimately only 47 individuals of a total capture of 139 (i.e. 34%) were successfully genotyped. Under these circumstances, it may be necessary to construct a genomic library for *Neoromicia capensis* to screen for specific microsatellite loci. Time constraints did not allow me to construct such a library.

The total number of alleles at each locus, the allele frequency, as well as the average genetic variability (observed versus expected heterozygosity – H_O vs. H_E) was calculated for each locus with the aid of the software package AGARst (Harley 2001). The frequency of alleles at each locus was tested for compliance with Hardy-Weinberg expectations by examining whether a change in sampling effects attributed to a change in the allele frequency (Fischer's exact test). Critical significance levels for deviation from equilibrium were calculated using sequential Bonferroni corrections (Rice 1989). The Hardy-Weinberg Equilibrium Principle states that the genotypic frequencies in a population are distributed according to the binomial expansion of allele frequencies - $(p+q)^2$ for a two allele locus, where p = the frequency of allele 1, and q, the frequency of allele 2, giving: $p^2+2pq+q^2$. This principle holds true in a randomly mating population where there is no selection, or migration. If gene frequencies are not found to be in Hardy-Weinberg equilibrium, then a number of theoretical explanations may be proposed. i) There is assortative mating within subpopulations, ii) there is a high level of philopatry or fidelity (possible inbreeding), iii) population structuring (Wahlund effect), iv) selection between formation of zygotes and/or v) genotypes having different likelihoods of being included in the sample (Murphy *et al.* 1996).

Linkage disequilibrium was calculated for each locus to ascertain whether all loci are independent of the others. A Fischer's exact test (using the Markov Chain method) was used to test whether there was significant deviation from independence (Raymond & Rousset 1995), by analyzing contingency tables of each pair of loci. Dememorisation steps were defined so that each iteration began at a random point (to simulate independent loci – Schneider *et al.* 2000) for each contingency table generated.

3.2.3.2 Population assignment tests

Paetkau *et al.* (1995) described the method of population assignment, which analyses the relatedness of an individual's multi-locus genotype to that of the subpopulation in which it was captured. In these tests, each individual's genotype of the query subpopulation is compared to the pooled sample of genotypes of all other individuals in the analysis to determine which subpopulation has the highest frequency of the individual's genotype in question. The individual is then assigned to that or another population in which its genotype appears more frequently. In the same way, individuals of unknown colonial / subpopulation affiliation can be assigned to a group of genotypes (subpopulation sample) that they are most likely to be affiliated to genotypically. The resultant likelihood percentage is therefore a measure of the probability of the individuals from a sample (subpopulation or colony) that have a characteristic genotype compliment of the sample group in question. Individuals' genotypes from De Hoop, Wellington, Timbavati and Kuruman subpopulations were analysed using this assignment method.

3.2.3.3 Fixation indices

In addition to population assignment tests, standard fixation indices (F-statistics – Wright 1951) were calculated to determine subpopulation differentiation. These analyses compartmentalise genotypes and reflect the structure of the population as a whole. Subpopulations, therefore, are screened for inbreeding and differentiation between them. F_{ST} is calculated to assess the reduction in heterozygosity due to non-random mating in a subpopulation, between individuals within the entire population, and between subpopulations and the population as a whole (Sugg *et al.* 1996). F-statistic values close to or equal to one suggest an inbred, or highly structured subpopulation (i.e. alleles have become fixed) and those closer to zero suggest a panmitic population (Long *et al.* 1998; Pope 1998).

An equivalent measure of population genetic variability, G_{ST} (gene differentiation coefficient), was developed by Nei (1973). This coefficient defines the extent of inter- and intra- population genetic differentiation. Similar to the assessment of F_{ST}, G_{ST} values larger than about 0.05 indicate a structured population (Nei 1973). The drawback of using both F_{ST} and G_{ST} analyses of population structure is that these values assume an infinite allele model and a low mutation rate. These clearly conflict with the mutation model proposed by Slatkin (1995) for microsatellite evolution, i.e. stepwise mutation with high incidence of mutation. Slatkin (1995), thus, proposed a coefficient, R_{ST}, based on the variation in allele size (as opposed to variation of allele frequency for F_{ST} and G_{ST}) that avoids the assumption of the former coefficient models. This coefficient is robust with respect to the effects of new mutations and assumes a stepwise mutation model as opposed to an infinite allele model, and quantifies gene flow through populations (Valsecchi *et al.* 1997). Values for R_{ST}, however, have a higher variance than F_{ST} and G_{ST} so are less reliable if the sample size (i.e. number of alleles) is small. R_{ST} values of zero would identify a random mating strategy with maximum gene flow between subpopulations. A value of one would identify extreme fidelity or philopatry. In comparisons of subpopulation structure, the R_{ST} value must be corrected for varying population size, as a smaller population will contribute less to the final variation than would a larger sample. Allele sizes are thus expressed as standard deviations from a global mean, and not the number of repeat units. Global and pairwise estimates of *Rho* (R_{ST} corrected for varying sample size) and G_{ST} were calculated for the *N. capensis* subpopulations in South Africa, using AGARst (Harley 2001). Arlequin was used to implement permutation tests to determine whether R_{ST} (*Rho*) was significantly different from zero. Critical significance estimates were computed with the implementation of sequential Bonferroni corrections (Rice 1989).

3.3 Results

3.3.1 Locus characteristics of subpopulations

There was an average of between 3.6 and 7 alleles per locus in the *N. capensis* subpopulations of De Hoop, Wellington, Kuruman and Timbavati after Jacknife corrections. Correction for the difference in population sizes (using 1000 jacknife permutations) revealed that the Wellington and Timbavati subpopulations were out of Hardy-Weinberg equilibrium ($p \leq 0.05$), and that the De Hoop and Kuruman subpopulation showed no significant deviation from H-W expectations (Table 3.2). The sample size from the Timbavati subpopulation was, however, small.

3.3.2 Population assignment in South Africa

Only individuals from De Hoop, Wellington, Kuruman and Timbavati were analysed in the population assignment tests. Fig 3.3a, b, c, & d are graphical representations of the assignment of each individual to their respective subpopulations. Data points falling above the diagonal indicate correct assignment of the individual to the subpopulation in question. Almost all individuals were correctly assigned to their native subpopulations with high probability (global mean = 87% - Table 3.3).

TABLE 3.2 SUMMARY OF LOCUS CHARACTERISTICS OF FOUR POPULATIONS OF *N. CAPENSIS.* "N" is the number of individuals in the analysis. (A/L) is the average number of alleles per locus. Corrected numbers of alleles per locus are calculated over 1000 jacknife permutations. H_O and H_E are the observed and expected heterozygosity values, respectively.

POPULATION	N	ALLELES	(A/L)	JACKNIFE CORRECTION	H_O	H_E
DH	23	20	6.67	3.80	0.7544	0.7525
CN	16	21	7.00	3.08	0.7083	0.7847
KM	12	15	5.00	3.27	0.6400	0.6725
TN	4	11	3.67	3.67	0.6000	0.6875

TABLE 3.3 LIKELIHOOD RATIOS OF INDIVIDUALS CORRECTLY ASSIGNED TO THEIR SUBPOPULATION. "Ratio" refers to the median value of the likelihood ratios of the correct assignments. DH = De Hoop (FIG 3.3a), CN = Wellington (Fig 3.3b); KM = Kuruman (Fig 3.3c) & TN = Timbavati (Fig 3.3d). *Last column is % correctly assigned.

POPULATION	RATIO	RANGE	LIKELIHOOD (%)*
DH	2.95×10^1	1.04 to 1.62×10^4	87
CN	5.06	1.17 to 4.07×10^1	94
KM	2.43×10^1	2.12 to 1.10×10^3	67
TN	4.36×10^2	7.88 to 4.52×10^3	100
		GLOBAL MEAN	87

Three individuals captured in the De Hoop subpopulation were considered to be genetically more similar to individuals captured in the Kuruman subpopulation. Two of these individuals were females caught in the KH colony in De Hoop Nature Reserve, and the other was a male from the GH colony approximately 3km away from the latter (See Appendix II). One female from the Wellington colony (CN) was found to have a genotype more similar to the Kuruman subpopulation. The low percentage of individuals belonging to the Kuruman subpopulation (67%) may be resultant of a male biased sample. These samples were provided by the Transvaal Museum and were collected for a study on baculum morphology. Consequentially most of the individuals were males. In further analysis it was found that three of the males from the Kuruman subpopulation were assigned as more genetically similar to the De Hoop, Wellington and

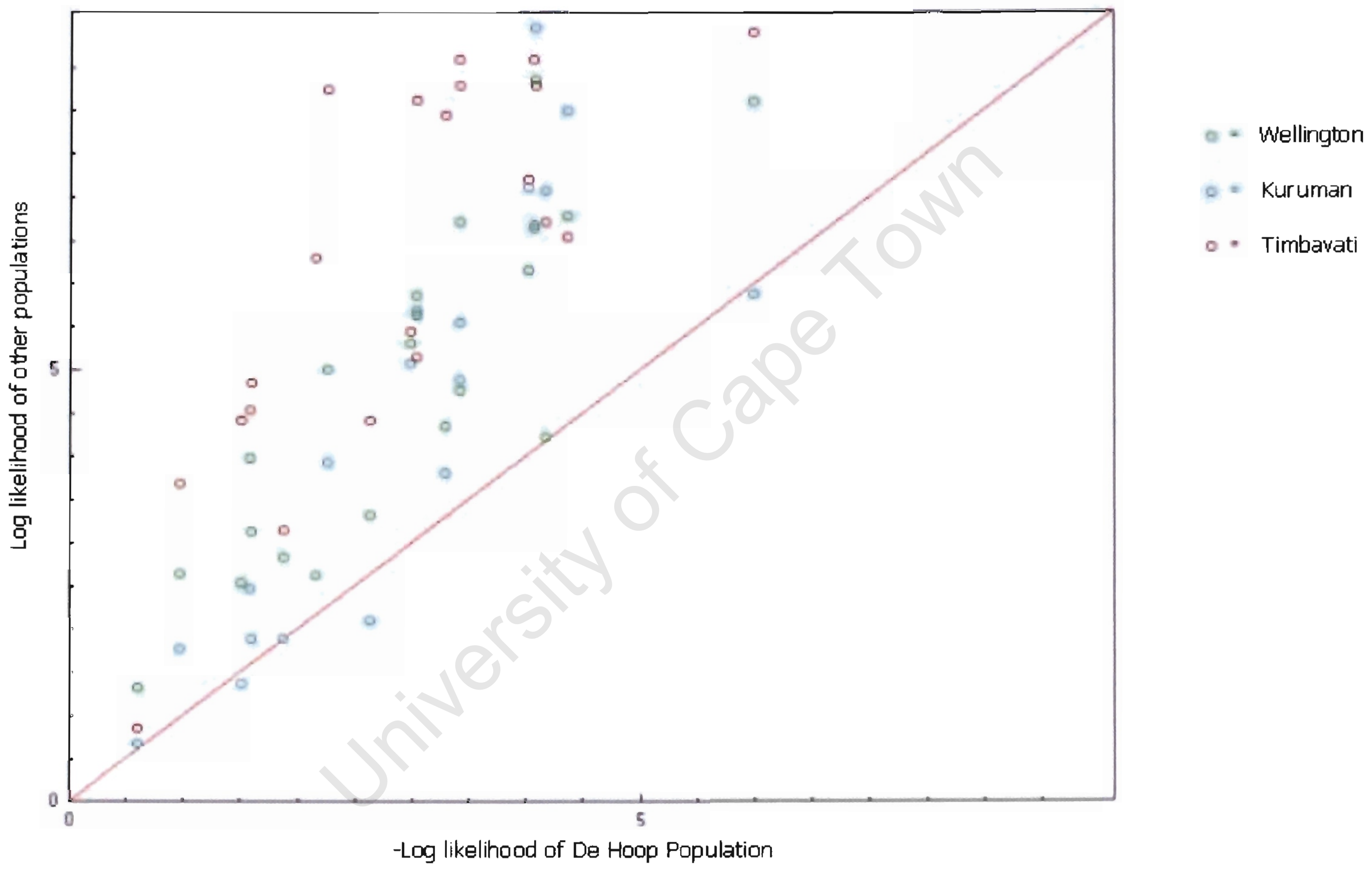


FIGURE 3.3a PERCENTAGE OF THE DE HOOP INDIVIDUALS BEING CORRECTLY ASSIGNED TO THE DE HOOP POPULATION. Points on or above the diagonal indicate that the De Hoop individuals have been correctly assigned to the De hoop subpopulation

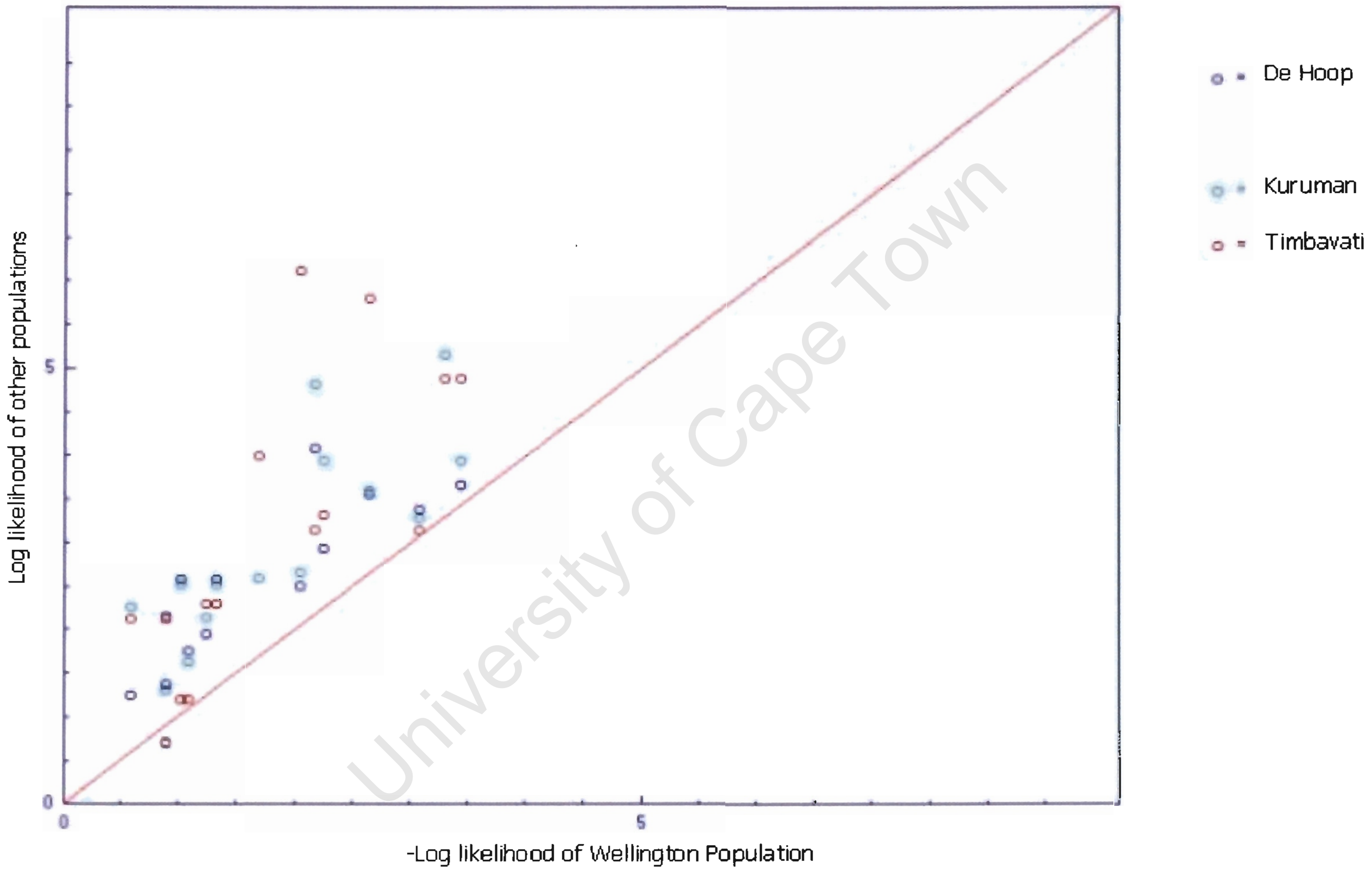


FIGURE 3.3b PERCENTAGE OF THE WELLINGTON INDIVIDUALS CORRECTLY ASSIGNED TO THE WELLINGTON POPULATION. Values on or above the diagonal indicate that the Wellington individuals have been correctly assigned to the Wellington subpopulation

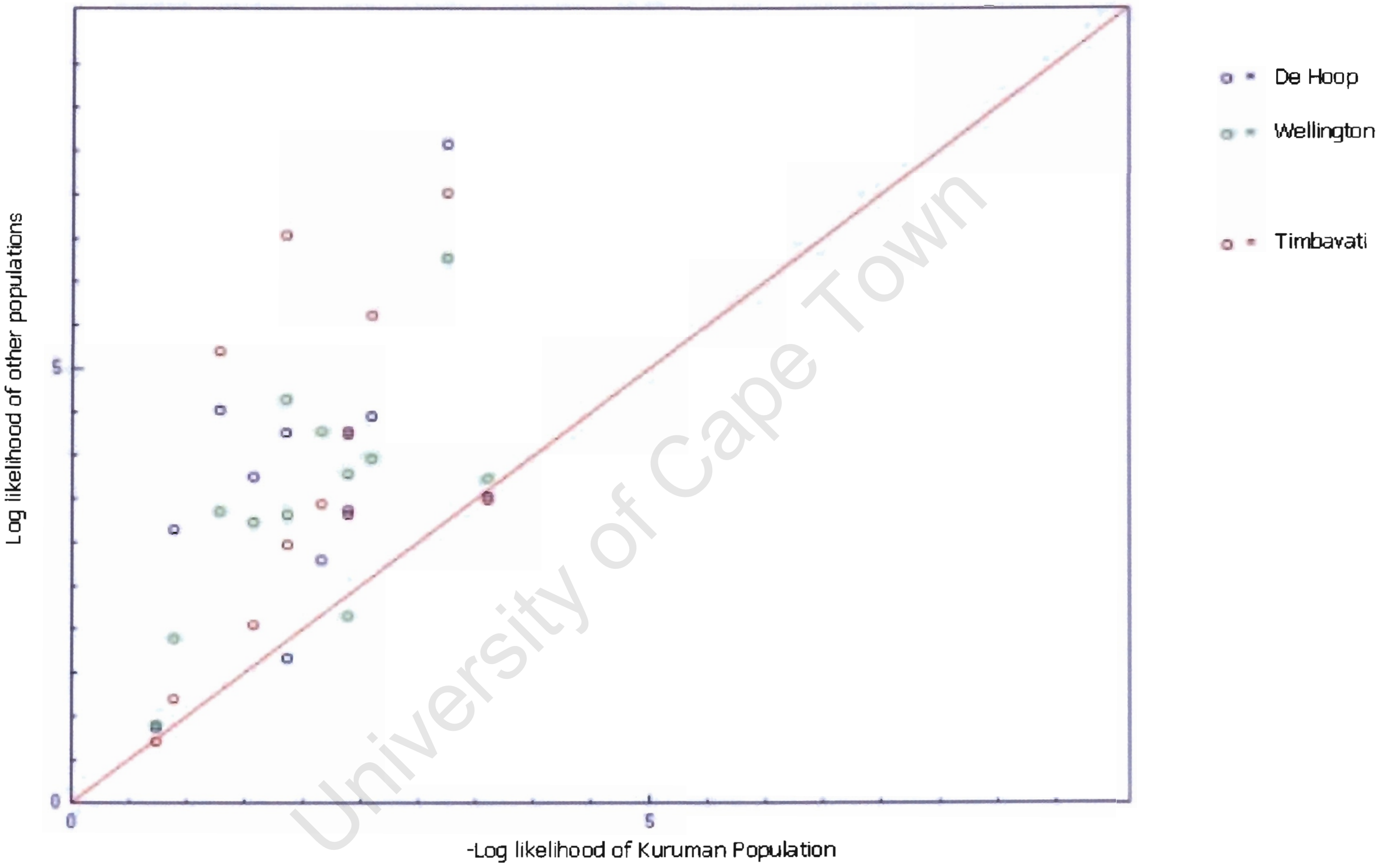


FIGURE 3.3c PERCENTAGE OF THE KURUMAN INDIVIDUALS CORRECTLY ASSIGNED TO THE KURUMAN SUBPOPULATION. Values on or above the diagonal indicate the correct assignment of the Kuruman individuals to that subpopulation

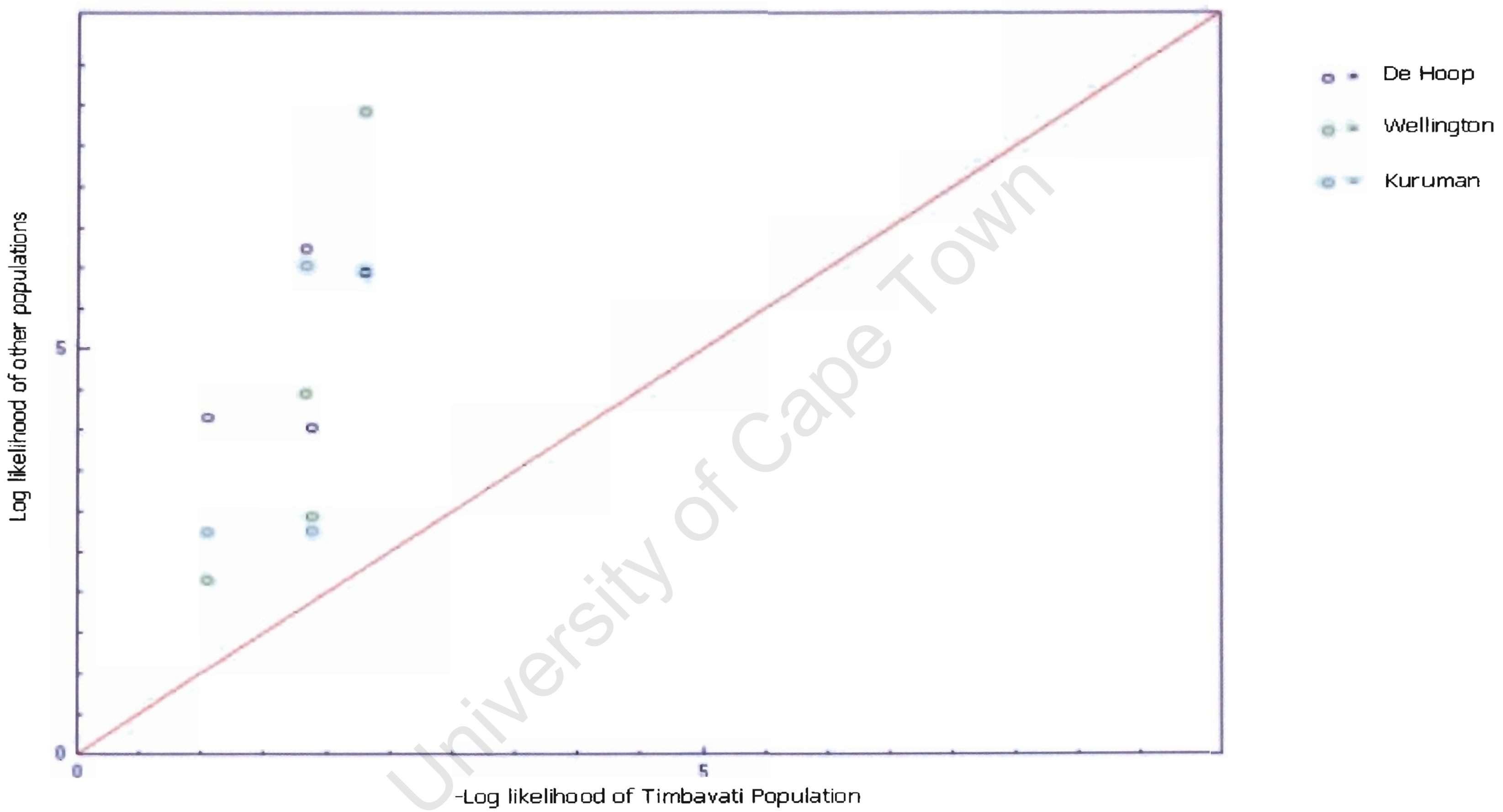


FIGURE 3.3d PERCENTAGE OF TIMBAVATI INDIVIDUALS CORRECTLY ASSIGNED TO THE TIMBAVATI POPULATION. Values on or above the diagonal represent the correct assignment of the Timbavati individuals to that population

Timbavati subpopulations, respectively. One of the Kuruman females had a genotype similar to those from Timbavati. 100% of all individuals from Timbavati subpopulation had alleles associated with those genotypes representative of only that population (i.e. private alleles).

3.3.3 Subpopulation differentiation

Global averages of R_{ST} (*Rho*) and G_{ST} suggest that there is significant differentiation between subpopulations of *N. capensis* at three loci (Table 3.5). G_{ST} values are higher than *Rho* values, but the mean across all loci gives broadly similar results for both estimators. The differences at individual loci reflect the differences in the way each estimator is calculated hence the requirement to average over as many loci as possible. Since microsatellite loci mutate in a stepwise manner, the *Rho* values are considered a more reliable reflection of genetic substructure in the population. When analysed separately, loci have low levels of differentiation. Locus EF14, however, indicates that the population is highly structured for that locus, and was responsible for the high global mean. These figures should be interpreted cautiously however, because of the small number of loci. It is necessary to increase the number of microsatellite loci in this analysis before reliance can be placed on these estimates.

Table 3.6 provides data for the pattern of differentiation between subpopulations through comparisons of pairwise differences for *Rho* and G_{ST}. Values falling below 0.05 for both R_{ST} and G_{ST} are generally taken as indicative of low genetic divergence between populations, and hence frequent gene flow. There is evidence of marginal gene flow between De Hoop and Wellington subpopulations (R_{ST} = 0.059), and extensive gene flow between Timbavati and Kuruman (R_{ST} = 0) - essentially panmictic. Given the results of the assignment tests there is also strong support for restricted gene flow between Kuruman and De Hoop subpopulations (Rst = 0.13; Gst = 0.07). All other subpopulations are considered to be moderately structured since all values of G_{ST} and R_{ST} fall above 0.07.

TABLE 3.5 MEAN *Rho* AND G_{ST} VALUES FOR EACH LOCUS, AND GLOBAL MEAN ACROSS ALL LOCI.

LOCUS	R_{ST} (*Rho*)	G_{ST}
EF6	0.0000	0.0193
EF14	0.2374	0.1200
MM5'	0.0168	0.1445
GLOBAL MEAN	0.0847	0.0946
VARIANCE	0.0176	0.0044
SEM	0.0765	0.0383

TABLE 3.6 MATRIX OF PAIRWISE COMPARISON OF SUBPOPULATION DIFFERENTIATION. Pairwise G_{ST} values are given above the diagonal, and pairwise R_{ST} (Rho) values are given below the diagonal. DH = De Hoop; CN = Wellington; KM = Kuruman & TN = Timbavati

	DH	CN	KM	TN
DH		0.0348	0.0746	0.0710
CN	0.0594		0.0790	0.0726
KM	0.1335	0.0733		0.0893
TN	0.1352	0.1793	0.0000	

3.4 Discussion

Three separate analyses were conducted to test the hypothesis that the South African population of *Neoromicia capensis* is genetically substructured. Locus characteristics, population assignment tests and measures of population differentiation (G_{ST} and Rho analyses) suggest that the South African population of *N. capensis* has a moderate to high spatial structure.

Characteristics of the three loci of each subpopulation show that there are low numbers of alleles per locus in all subpopulations. In this analysis the subpopulations have 3.5 alleles per locus (after jacknife corrections for varying population size). A low number of alleles per locus is indicative of some degree of local inbreeding (Clutton-Brock 1989, Avise 1994). When tested for compliance with Hardy-Weinberg equilibrium, both Wellington (CN) and Timbavati (TN) subpopulations show significant deviation from the model's expectations. This finding would be expected since CN subpopulation consisted entirely of females, and the sample size for the TN subpopulation was small. According to the expectations of the H-W principle, one could assume that the individuals captured at the Wellington site are closely related. *Neoromicia capensis* females are therefore thought to remain in closely related matrilineal cohorts.

Population assignment tests support the existence of subpopulation structure. All four subpopulations were correctly assigned to the characteristic gene pool from which they were sampled with varying likelihood, however. TN individuals did not share alleles with other subpopulations. These individuals were vouchered specimens of *Neoromicia capensis* specimens from the Transvaal Museum, Pretoria (see accession numbers Appendix III). It is evident from these data that certain subpopulations bear no relation to other subpopulations. Two plausible explanations arise: i) these individuals were incorrectly identified or, ii) subpopulations in different biomes are genetically isolated from other subpopulations. The former argument is not considered viable, since comparative chromosomal and morphological data have been published for these specimens (see Kearney *et al.* 2003).

Individuals from the KM subpopulation had low probabilities of being correctly assigned to this subpopulation. 23% of the genotypes present at this location were accounted for in other subpopulations. Individuals from this group were predominantly males, however, and the subpopulation is in H-W equilibrium, suggesting random mating and therefore low genetic relatedness. Thus the genotypes of these males are characteristic of a wider population and suggest gene flow via male dispersal. On the other hand, the CN population, which was predominantly female, was out of H-W equilibrium suggesting non-random mating. Females also have a very high percentage of belonging to this subpopulation. Thus females have a high probability of being related and suggests that females are philopatric. Such sex biased dispersal/philopatry could have caused the substructure revealed by the microsatellite data.

CN and DH have a low variation in allele frequency (R_{ST} = 0.06) and extremely moderate variation in allele size (*Rho* = 0.03). This suggests that there is gene flow, albeit low (even historical), between the two populations, and although they may be related the individual subpopulations show marked structure. TN and KM populations display a random flow of genes between the two (*Rho* = 0). KM individuals are all males and display a random sample of genotypes from surrounding colonies. This provides additional evidence for male dispersal. It is concluded that the low *Rho* value is indicative of unrelated individuals within the KM subpopulation. These two subpopulations are genetically distinct to the CN and DH colonies as is suggested by the variation in allele frequencies (G_{ST} = 0.07 – TN, and G_{ST}= 0.076 – KM). There is a north-south divide of genotype frequencies and allele size variations between subpopulations situated on the plateau and those located below the escarpment of South Africa.

In conclusion, the population of *Neoromicia capensis* in South Africa is structured at a geographic level. This appears largely due to female philopatry and male dispersal. The numbers of comparable loci in these analyses are low and these findings should be interpreted with caution. However, a more substantial analysis of partial sequences of the mitochondrial control region of the individuals from these subpopulations (Chapter 4) has revealed a similar outcome that would, in concert, help to justify the conclusions drawn here.

CHAPTER 4

ANALYSIS OF THE MITOCHONDRIAL CONTROL REGION

AIMS: ***a) To determine whether male and female Neoromicia capensis are philopatric***

b) To determine the mode of gene flow between populations.

4.1 Introduction

4.1.1 Structure of the mammalian mitochondrial genome

The mammalian mitochondrial genome has very little genetic redundancy and is highly conserved across vertebrate taxa. It is a homologous circular molecule of 16 to 30 kbp (Figure 4.1) containing the control region, 22 transfer RNAs (tRNA), 2 ribosomal RNAs (rRNA) and 13 genes that code for proteins used in oxidative metabolism (Brown 1985, Harrison 1989, Wilkinson *et al.* 1991). The control region (*v. inf.*) functions in control of transcription and replication. Although mitochondrial gene order is highly conserved in vertebrates, major rearrangements are evident across phyla. In contrast to the nuclear genome, there are no introns (with the exception of the control region) and insertions, transpositions and occurrences of intergenic sequences are rare (Brown 1985, Harrison 1989).

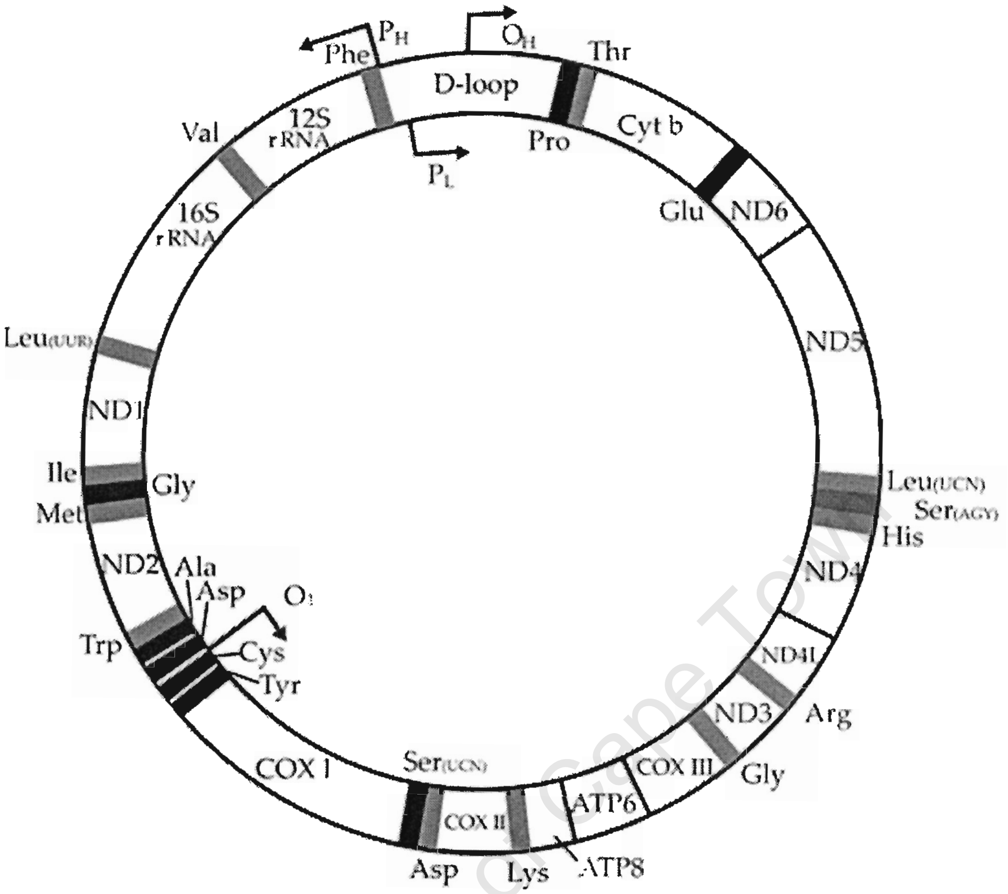


FIGURE 4.1. SCHEMATIC REPRESENTATION OF THE MAMMALIAN MITOCHONDRIAL GENOME. Abbreviations: CR, control region; ND 1-6 (incl. 4L), NADH Dehydrogenase subunits; COX I-III, cytochrome oxidase subunits; ATP8 & ATP6, Adenosine triphosphate synthetase subunits; cyt b, cytochrome b. RNA subunits are represented as the shaded areas. Image courtesy of the Department of Clinical Laboratory Science, University of Cape Town.

4.1.2 The Control Region

4.1.2.1 Structure

The control region (Fig 4.2) is approximately 1000 bp in length and is seated between tRNA^{Pro} and tRNA^{Phe} genes (Wilkinson *et al.* 1997; Schleffer 2000). It consists of three domains, each characteristically identifiable by the content of specific regions involved in transcription, gene expression and mtDNA replication (Harrison 1989, Wilkinson *et al.* 1991). Domain I is high in A-T content, and is highly variable in sequence configuration and length due to the presence of the R1 repeat region. It also contains the Termination Associated Sequence (TAS) which terminates the replication of the H-strand. Domain II is rich in G & C nucleotides and consists of five conserved sequence blocks. Domain III, flanked by $tRNA^{Phe}$, consists of the Origin of Heavy strand replication (O_H), the R2 repeat sequences and the light and heavy strand promoters (LST and HST respectively), involved in gene expression. The Displacement loop (D-Loop) spans across the three Domains from the O_H to the TAS. In H-strand replication, a 7s DNA strand is formed that remains associated with the L-strand, displacing the original H-strand to form a triple stranded structure (Doda *et al.* 1991). Tandem repeat sequences, (found in the R1 repeats) containing TAS elements, will govern D-loop size depending on which of them are used to terminate replication (Wilkinson *et al.* 1997).

4.1.2.2 Repeat Sequences

Domains I and III of the control region are characteristic of highly variable repetitive sequences. R1 tandem repeat sequences (Fig 4.2) are ca. 80 bp in length consistent across many species including shrews, sheep, frogs and vesper bats. The function of these repeats is unclear, however Wilkinson *et al.* (1997) argue that since Domain I has binding sites for regulatory proteins, multiple repeats act as a buffer to overcome mutational events that could hinder effective binding of these proteins. They further suggest that repeat duplications or deletions occur through competitive displacement among the three D-loop strands thereby eliminating damaged repeat sequences. In this way replication and transcription efficiency is maintained and metabolism remains optimal. In contrast, the shorter R2 repeats (ca. 6 – 30 bp) exhibit length polymorphisms similar to that of microsatellite loci (Chapter 3). However, due to the location of these repeats (i.e. upstream of the O_H) it is projected that they bear no influence on the length of the D-loop (Wilkinson *et al.* 1997).

It is clear then that the central conserved area of the control region would be appropriate for phylogenetic analysis among species. However, for intraspecific comparisons, sequences in Domains I and III would provide the best resolution for phylogeny reconstruction due to the highly polymorphic nature of these sites.

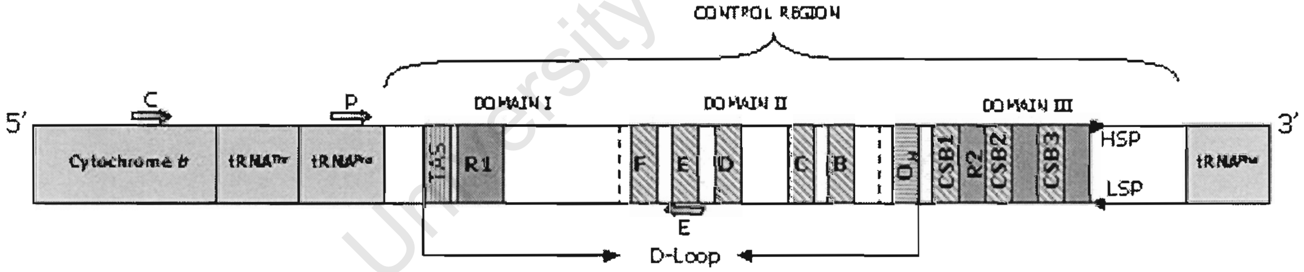


FIGURE 4.2 SCHEMATIC REPRESENTATION OF THE MAMMALIAN CONTROL REGION (modified from Taberlet 1996 and Wilkinson *et al.* 1997). The control region is situated between proline and phenylalanine tRNAs and has three domains (I – III). The displacement loop (D-loop) stretches across all domains beginning at the origin of heavy strand replication (O_H) to the termination associated sequence (TAS). Abbreviations: $tRNA^{Thr}$, $tRNA^{Pro}$ & $tRNA^{Phe}$ = threonine, proline and phenlyalanine tRNAs; R1 & 2 = repeat sequences; F, E, D, C, B = conserved sequences; CSB1 – 3 = conserved sequence blocks; LSP & HSP = light and heavy strand promoters. Binding sites and direction of amplification of Wilkinson & Chapmans' (1991) primers C, P & E are illustrated.

4.1.3 Characteristics of Mammalian Mitochondria

4.1.3.1 Transmission genetics

Animal mitochondrial DNA is clonally inherited with no (or little) male contribution because the cytoplasm of sperm does not enter the egg (Barton & Jones 1983; Taberlet 1996). Due to this strict maternal transmission, mtDNA is effectively haploid (Harrison 1989; Taberlet 1996). Maternal inheritance, consequently, leads to a much smaller mtDNA effective population size about four times smaller than that of nuclear genes (Randi et al. 1994). Stochastic events are likely to have important impacts on the frequency of mtDNA haplotypes in populations because only a portion of the population is responsible for mtDNA turnover. Nevertheless, analyses of haplotypes would reflect patterns of colonization and even founder events because mtDNA affinities between molecules reflect maternal phylogenies (Harrison 1989). These linked markers (inheritance of a single molecule in the absence of recombination) not only allow for the definition of female genealogies, but also provide a clear distinction between common ancestry and convergence (Harrison 1989; Wilkinson *et al.* 1997).

Genetic drift occurs faster in small populations and is emphasized by clonal inheritance of genetic material. In the case of mitochondrial haplotypes the resolution of the marker is greatly increased, and is observed as highly subdivided (structured) over geographical regions (in contrast to nuclear genes – Barton & Jones 1983). This in itself highlights an advantage with respect to analysis of population differentiation. In addition mtDNA estimates of population differentiation are based purely on female dispersal patterns, and therefore provide limited insight as to male movement throughout the population (since males are most commonly responsible for gene flow in natural populations). In this way, sequence divergence estimates provide an over-estimate of population sub division, and comparative studies (viz. nuclear markers) are necessary in order to fully understand male and female contributions to gene flow within populations (Barton & Jones 1983; Taberlet 1996).

4.1.3.2 Sequence evolution

The mitochondrion is essential for life in all animals because the mitochondrion is the repository of the enzymes for oxidative phosphorylation as well as for a number of other metabolic functions. It is therefore expected that mtDNA sequences would be highly conserved within species. However, this is not the case since comparative studies have revealed that there are extensive differences between haplotypes of different species (Brown *et al.* 1979). In primates, for example, it has been reported that sequence divergence occurs quickly – up to 1% per lineage per million years (Brown *et al.* 1979; Harrison 1989), the equivalent of five to ten times faster than coding nuclear genes. Variable blocks within the control region (R1 & R2 repeats – Fig 4.2) evolve five times faster still (Taberlet 1996). In their study of evening bats, Wilkinson & Chapman (1991) proposed a mutation rate per generation of 10^{-2} for haplotype length polymorphisms in this species, two to four orders of magnitude higher than values known for microsatellites (see Chapter 3) and allozymes (Bruford & Wayne 1993; Jarne & Lagoda 1996).

There are two reasons for the extremely high rate of mutation in mtDNA. Firstly, the turnover of mtDNA in somatic tissues is higher than that of nuclear DNA. This increases the probability of the accumulation of mutations. Secondly, mtDNA lacks a repair mechanism as observed for nuclear DNA. In addition, the editing function of polymerases of replication is poorly developed or lacking (Barton & Jones 1983; Avise 1994) in the mitochondrion. The control region is mostly selectively neutral and is non-coding, and therefore mutation events will often have no effect on fitness. Mutations are thus not removed nor corrected. This phenomenon causes an accumulation of independent point mutations and length polymorphisms, as well as high variation in nucleotide arrangement in the control region (Harrison 1989; Wilkinson & Chapman 1991; Taberlet 1996), making it ideal for phylogenetic inference (Taberlet 1996).

4.2 Methods and Materials

4.2.1 Identification of suitable primers

Wilkinson and Chapman (1991) have developed primer sequences that amplify partial control region loci in at least five families of bats. A section of the control region was amplified successfully in 20 Vespertilionid species using nested primer P in the sequencing reaction (Wilkinson *et al.* 1997). In this analysis nested primer P was used together with primers C and E (sequences available in Wilkinson & Chapman 1991) to construct partial control region sequences from PCR products. The DNA Synthesis Laboratory in the Department of Molecular and Cellular Biology, University of Cape Town synthesized oligonucleotides.

4.2.2 Amplification of partial control region

Initial PCRs were performed using the Roche FastStart Taq DNA Polymerase kit (Roche Molecular Systems, Inc. US). Initial PCRs using Taq Polymerase (Promega) proved unsuccessful in amplifying PCR products. "Hot Start" PCR performed better, and provided constant results for all individuals in this analysis. Hot Start Taq is a temperature sensitive polymerase that is activated only at 94 °C, which increases the sensitivity of the PCR reaction.

All reagents were thawed, vortexed and stored on ice before setting up the reactions. PCRs were performed in 50 µl reactions containing 1X PCR buffer (magnesium free), 2mM $MgCl_2$ (25mM stock), 0.2mM of each deoxynucleotide (supplied individually as 10mM dNTP's), 1pmol up- and down- stream primers (Wilkinson & Chapman's (1991) primers C and E respectively), 2U Taq Polymerase (Promega), and 4µl of template DNA irrespective of stock concentration. PCR reactions were carried out in a Hybaid Sprint Thermocycler using the conditions specified by Wilkinson & Chapman (1991). PCR conditions consisted of an initial denaturation / activation cycle of 2min at 95°C, followed by 35 cycles of 50sec at 95°C, 50sec at 55°C and 1min at 72°C, and a final extension of 10 min at 72°C.

A 10µl aliquot of each product was electrophoresed through a 2%, EtBr stained agarose gel to assess the specificity of the reaction. All samples were run against a standard 100bp DNA Ladder (Promega) with known concentration. When visualised under ultraviolet light, the concentration of each sample was estimated according to the intensity of the band in comparison to the size marker. Only samples with a single ca. 800 – 850 bp band were used, and these were either excised from the gel and purified with a QIAquick Gel extraction kit, or the remainder of the PCR product was purified with a QIAquick PCR purification Kit (both kits manufactured by QIAGEN, supplied by Southern Cross Biotechnology, Cape Town).

4.2.3 Sequencing of partial control region

Sequencing reactions were carried out on purified PCR products using an ABI PRISM® BigDye® Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Cape Town) according to manufacturer's guidelines. Each reaction contained only one of the primers – i.e. two separate reactions for each sample. A typical reaction consisted of the following components:

Sterile distilled H_2O = 4µl
3.2pmol Primer = 1µl
BigDye® readymix = 4µl
MtDNA template = 1µl
Total = 10µl

All cycle-sequencing reactions were carried out on a Hybaid Sprint Thermalator, and then sent to the Department of Human Genetics, Sequencing Unit, University of Cape Town, where they were run on an ABI PRISM® 3100 Genetic DNA Analyser (Applied Biosystems, Cape Town). Electropherograms were obtained digitally.

4.2.4 Statistical analysis

4.2.4.1 Sequence editing and alignment

Sequences acquired from the sequencing reactions had a substantial overlap region. Wilkinson & Chapman's (1991) primer P amplified sequences of approximately 500 bp on average, and primer E amplified an average of 650 bp sequences in the reverse reaction. After calculating the reverse compliment of the E sequences, both primer-generated sequences could be aligned. This allowed a robust method of editing a single sequence as follows:

Firstly, electropherograms (see Appendix VI) were edited manually, correcting any miscalls prior to electronic editing. Text sequences, obtained from the electropherograms in the chromatogram editor programme, Chromas, version 1.43 (McCarthy 1997), were imported into the computer software package DAPSA, version 6.31 (Harley 2004), where the reverse-compliment of the E primer-derived sequences were computed for all samples. Primer sequences were identified within the sample, and were trimmed at these sites to ensure that all sequences were representative of comparable loci. The sequences were edited manually, referring dubious base-pair calls to the original printout. Once all sequences were edited, they were combined to obtain one sequence per individual. All the sequences were aligned manually in DAPSA 6.31 (Harley 2004), followed by a final automatic alignment to rectify questionable alignments.

4.2.4.2 Haplotype Analyses

A matrix of the genetic distances between haplotypes was constructed, and values were corrected for multiple hits using the Kimura two-parameter model (Kimura 1980). The corrected sequence divergence estimated using this model are values that account for differing rates at which transitions and transversions take place. The software package Arlequin version 2.0 (Schneider *et al.* 2000) was used to generate estimates of gene diversity. This is a measure used for haploid data, assessing the probability that two haplotypes drawn, at random, from the pool of samples, will be different (Nei 1987).

Analysis of molecular variance (AMOVA) was calculated to determine inter- and intra-colony relatedness, with the aid of Arlequin. In this statistical procedure, information on haplotypic divergence is derived from a matrix of squared Euclidean distances, based on a hierarchical analysis of variance. Additionally, fixation indices are calculated based on haploid transmission of mtDNA. This index is analogous to F-statistics (diploid transmission, Chapter 3) and is called Φ-statistic (Excoffier *et al.* 1992). Two components are analysed by AMOVA: i) variation within populations and ii) variation between populations. The significance of each Φ-statistic is evaluated by 1000 randomised pseudoreplications. Pairwise colony comparisons were not calculated since five colonies only had a single representative. Φ_{ST} was calculated to provide an estimate of population subdivision in South African subpopulations.

A minimum spanning tree was constructed to indicate the extent of sequence divergence between each haplotype. The tree provides a visual representation of the degree of evolutionary divergence between haplotypes (Excoffier *et al.* 1992). The numbers of mutational steps are generated by the squared Euclidean distances determined by AMOVA with the aid of *Arlequin.* It is important to note that the connection lengths (evolutionary divergences) on the minimum spanning tree may not equal those of phenetic distances, because the number of mutational events may not be linear, or because of the possible incidence of homoplasy (Excoffier *et al.* 1992).

In addition, phylogenetic reconstruction was performed on the colonies of South Africa using the neighbour-joining and maximum likelihood methods. *Neoromicia capensis* from Malawi was used as the out-group to root the trees. The software package PAUP* version 4.Ob10 (Swofford 2001) was used to construct both trees. The neighbour-joining tree was constructed with settings as per software default configuration, and with the implementation of 100 bootstrap pseudoreplications. The maximum likelihood tree was constructed using 1000 quartet puzzling steps.

4.3 Results

4.3.1 Indices of molecular diversity

Thirty partial sequences of the control region of *Neoromicia capensis* were successfully amplified. From these, twenty-eight haplotypes were identified (Table 4.1). Two pairs of individuals shared haplotypes. One pair consisted of a male caught at Die Hel Bridge (DA2) and another male caught at Ceres (CT4 - ca. 120 km apart). The other pair consisted of two females captured at Knysna (individuals CX20 & CX26). However, the observed gene diversity between subpopulations was high (0.99 ± 0.01), indicating that the majority of all haplotypes are distinct. Sequence alignment (Fig 4.3) yielded 514 polymorphic sites of which 97 were parsimony informative and 12 were informative indels. The mean number of pairwise differences between individuals was 52.29 ± 23.29. Comparisons of pairwise differences between haplotypes of subpopulations were not calculated due to the presence of only one specimen per site in some samples (viz. CN, Die Hel, Cedarberg, MP and PP). Pairwise genetic distances between twenty eight haplotypes were calculated and corrected for multiple hits using the Kimura two-parameter method (Table 4.2).

TABLE 4.1 Molecular diversity indices for control region haplotypes of *Neoromicia capensis* populations in South Africa

OBSERVATION	VALUE
NUMBER OF SAMPLES	30 bats
NUMBER OF HAPLOTYPES	28
USABLE LOCI	859
POLYMORPHIC SITES	514
PARSIMONY INFORMATIVE SITES	97
TRANSITION:TRANSVERSION	123:109
INFORMATIVE INDELS	12
GENE DIVERSITY ± SD	0.99 ± 0.01

Sequence divergence estimates (Table 4.3) were calculated from the corrected genetic distances (Table 4.2). These estimates reveal that there are three distinct complexes of haplotypes in South Africa: i) the Southwestern Complex (SWC), ii) the Central Complex (CTC), and iii) the Northeastern Complex (NEC). Sequence divergences between respective complexes are between 6 and 8 percent (Table 4.3). Evolutionary divergences within the SW and CT Complexes are 1.5% and 2% respectively, where divergence estimates of subpopulations within the NEC are over 6% (Table 4.3).

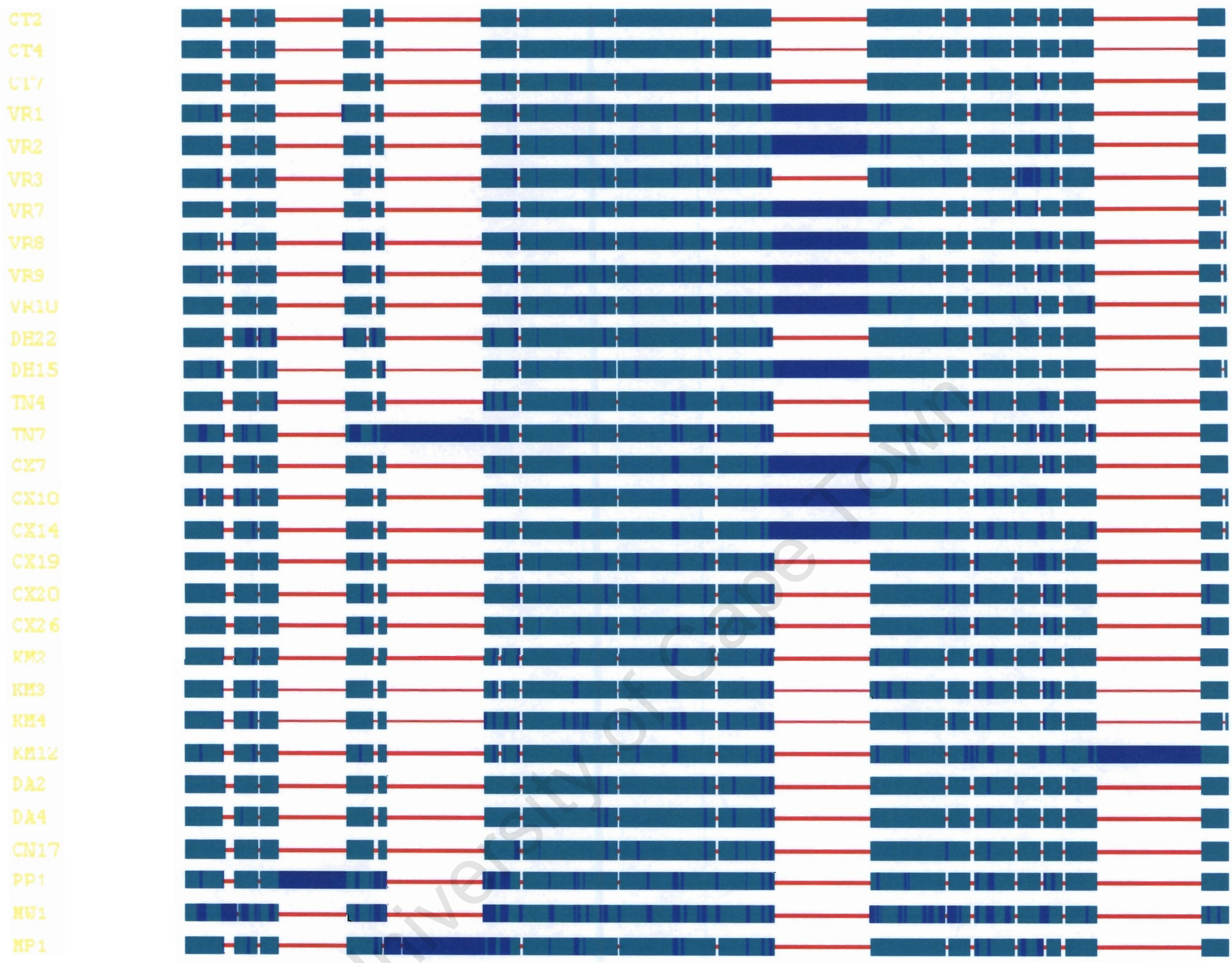


FIGURE 4.3 SUMMARY OF HAPLOTYPE DISTRIBUTION OF *NEOROMICIA CAPENSIS* SPECIMENS OF SOUTH AFRICA. Individual CT2 was arbitrarily chosen as the standard and all other haplotypes are shown relative to that sequence. Areas represented in turquoise are sites that are in complete alignment, dark blue areas identify the position of polymorphic sites, and red dashes represent gaps relative to other sequences. Site codes are listed in Appendix I, and haplotype affiliations are defined in Appendix IV.

TABLE 4.2 Matrix of genetic distances between individuals based on the partial control region haplotypes of Neoromicia capensis. Figures above the diagonal are uncorrected p- values associated with genetic distances between individuals. Values corrected for multiple hits (Kimura two-parameter model) are shown below the diagonal. Haplotype classifications are listed in Appendix V.

	CT2	CT4	CT7	VR1	VR2	VR3	VR7	VR8	VR9	VR10	DH15	DH22	TN4	TN7	CX7	CX10	CX14	CX19	CX20	KM2	KM3	KM4	KM12	DA4	CN17	PP1	MP1	MW1
CT2		0.0053	0.0072	0.0073	0.0066	0.0094	0.0073	0.0077	0.0075	0.0075	0.0069	0.0089	0.0095	0.0127	0.0082	0.0089	0.0089	0.0086	0.0080	0.0084	0.0089	0.0107	0.0091	0.0057	0.0063	0.0113	0.0106	0.0212
CT4	0.0150		0.0060	0.0077	0.0070	0.0094	0.0073	0.0081	0.0075	0.0075	0.0073	0.0089	0.0099	0.0130	0.0091	0.0094	0.0094	0.0086	0.0079	0.0091	0.0096	0.0113	0.0096	0.0019	0.0056	0.0118	0.0109	0.0208
CT7	0.0265	0.0188		0.0092	0.0086	0.0109	0.0089	0.0096	0.0091	0.0090	0.0088	0.0106	0.0092	0.0125	0.0093	0.0096	0.0096	0.0098	0.0094	0.0093	0.0098	0.0107	0.0098	0.0063	0.0079	0.0104	0.0103	0.0210
VR1	0.0313	0.0346	0.0485		0.0028	0.0061	0.0066	0.0066	0.0068	0.0068	0.0076	0.0077	0.0110	0.0128	0.0111	0.0108	0.0111	0.0066	0.0062	0.0096	0.0103	0.0113	0.0096	0.0079	0.0062	0.0119	0.0111	0.0185
VR2	0.0262	0.0296	0.0433	0.0048		0.0054	0.0059	0.0068	0.0070	0.0061	0.0074	0.0073	0.0105	0.0125	0.0107	0.0107	0.0107	0.0059	0.0054	0.0091	0.0098	0.0109	0.0092	0.0073	0.0054	0.0115	0.0107	0.0183
VR3	0.0456	0.0456	0.0597	0.0228	0.0178		0.0063	0.0074	0.0076	0.0070	0.0078	0.0097	0.0130	0.0150	0.0107	0.0111	0.0111	0.0071	0.0078	0.0118	0.0126	0.0138	0.0114	0.0096	0.0078	0.0142	0.0132	0.0229
VR7	0.0315	0.0315	0.0453	0.0262	0.0212	0.0245		0.0060	0.0057	0.0028	0.0081	0.0079	0.0105	0.0127	0.0112	0.0112	0.0112	0.0069	0.0067	0.0094	0.0101	0.0112	0.0093	0.0075	0.0057	0.0117	0.0110	0.0185
VR8	0.0348	0.0382	0.0523	0.0262	0.0279	0.0328	0.0214		0.0028	0.0062	0.0079	0.0081	0.0112	0.0129	0.0110	0.0114	0.0117	0.0073	0.0071	0.0101	0.0104	0.0118	0.0099	0.0083	0.0066	0.0120	0.0117	0.0192
VR9	0.0332	0.0331	0.0470	0.0279	0.0295	0.0345	0.0197	0.0049		0.0057	0.0077	0.0079	0.0107	0.0124	0.0109	0.0113	0.0116	0.0074	0.0069	0.0095	0.0099	0.0113	0.0095	0.0077	0.0059	0.0116	0.0113	0.0188
VR10	0.0331	0.0330	0.0469	0.0278	0.0228	0.0294	0.0048	0.0229	0.0196		0.0082	0.0081	0.0106	0.0123	0.0113	0.0113	0.0110	0.0070	0.0068	0.0095	0.0102	0.0112	0.0092	0.0077	0.0059	0.0118	0.0110	0.0186
DH15	0.0280	0.0314	0.0452	0.0345	0.0329	0.0361	0.0383	0.0365	0.0348	0.0399		0.0075	0.0101	0.0122	0.0113	0.0117	0.0118	0.0072	0.0066	0.0090	0.0097	0.0107	0.0090	0.0075	0.0057	0.0114	0.0108	0.0189
DH22	0.0416	0.0415	0.0574	0.0360	0.0327	0.0492	0.0378	0.0394	0.0378	0.0394	0.0344		0.0123	0.0148	0.0106	0.0112	0.0112	0.0093	0.0087	0.0110	0.0118	0.0129	0.0107	0.0091	0.0073	0.0138	0.0130	0.0229
TN4	0.0458	0.0498	0.0438	0.0677	0.0624	0.0822	0.0625	0.0697	0.0643	0.0642	0.0588	0.0754		0.0121	0.0085	0.0090	0.0087	0.0122	0.0117	0.0083	0.0088	0.0078	0.0087	0.0099	0.0102	0.0101	0.0100	0.0206
TN7	0.0905	0.0942	0.0886	0.1022	0.0973	0.1218	0.0993	0.1026	0.0961	0.0943	0.0942	0.1196	0.0845		0.0106	0.0108	0.0108	0.0139	0.0137	0.0121	0.0115	0.0124	0.0110	0.0131	0.0131	0.0102	0.0108	0.0200
CX7	0.0397	0.0484	0.0501	0.0695	0.0642	0.0655	0.0698	0.0678	0.0660	0.0714	0.0713	0.0653	0.0431	0.0729		0.0043	0.0046	0.0098	0.0093	0.0060	0.0049	0.0089	0.0070	0.0092	0.0093	0.0094	0.0095	0.0175
CX10	0.0465	0.0516	0.0534	0.0660	0.0643	0.0709	0.0697	0.0732	0.0713	0.0713	0.0766	0.0723	0.0481	0.0761	0.0113		0.0040	0.0102	0.0099	0.0064	0.0059	0.0092	0.0071	0.0094	0.0096	0.0098	0.0092	0.0173
CX14	0.0464	0.0516	0.0533	0.0695	0.0642	0.0707	0.0696	0.0767	0.0748	0.0678	0.0783	0.0721	0.0447	0.0760	0.0129	0.0097		0.0102	0.0099	0.0064	0.0059	0.0092	0.0066	0.0094	0.0096	0.0100	0.0089	0.0178
CX19	0.0380	0.0379	0.0479	0.0262	0.0212	0.0262	0.0279	0.0313	0.0329	0.0295	0.0312	0.0453	0.0721	0.1069	0.0552	0.0604	0.0603		0.0032	0.0108	0.0116	0.0124	0.0105	0.0088	0.0074	0.0133	0.0124	0.0215
CX20	0.0323	0.0323	0.0442	0.0229	0.0179	0.0320	0.0263	0.0296	0.0280	0.0279	0.0262	0.0396	0.0662	0.1033	0.0502	0.0570	0.0569	0.0055		0.0102	0.0111	0.0119	0.0101	0.0082	0.0066	0.0130	0.0121	0.0209
KM2	0.0362	0.0421	0.0441	0.0536	0.0484	0.0697	0.0504	0.0574	0.0521	0.0520	0.0467	0.0613	0.0359	0.0832	0.0213	0.0245	0.0245	0.0578	0.0520		0.0046	0.0083	0.0054	0.0091	0.0093	0.0093	0.0103	0.0207
KM3	0.0401	0.0461	0.0481	0.0607	0.0554	0.0780	0.0575	0.0610	0.0557	0.0591	0.0538	0.0694	0.0398	0.0759	0.0146	0.0212	0.0212	0.0660	0.0602	0.0113		0.0093	0.0067	0.0096	0.0098	0.0097	0.0100	0.0210
KM4	0.0563	0.0624	0.0562	0.0715	0.0662	0.0908	0.0685	0.0757	0.0702	0.0701	0.0646	0.0819	0.0320	0.0868	0.0453	0.0486	0.0485	0.0743	0.0685	0.0345	0.0425		0.0090	0.0113	0.0115	0.0111	0.0110	0.0218
KM12	0.0494	0.0545	0.0563	0.0603	0.0557	0.0751	0.0574	0.0636	0.0589	0.0557	0.0542	0.0677	0.0458	0.0787	0.0331	0.0346	0.0301	0.0647	0.0596	0.0177	0.0276	0.0479		0.0096	0.0094	0.0094	0.0101	0.0186
DA4	0.0169	0.0019	0.0208	0.0364	0.0313	0.0476	0.0332	0.0400	0.0348	0.0348	0.0331	0.0435	0.0498	0.0960	0.0484	0.0517	0.0516	0.0399	0.0342	0.0421	0.0461	0.0625	0.0546		0.0060	0.0118	0.0109	0.0208
CN17	0.0207	0.0168	0.0323	0.0229	0.0179	0.0320	0.0196	0.0262	0.0212	0.0212	0.0195	0.0280	0.0518	0.0958	0.0502	0.0535	0.0534	0.0282	0.0226	0.0440	0.0480	0.0643	0.0527	0.0187		0.0119	0.0114	0.0211
PP1	0.0688	0.0744	0.0594	0.0848	0.0798	0.1044	0.0820	0.0852	0.0801	0.0834	0.0783	0.1004	0.0573	0.0661	0.0552	0.0599	0.0630	0.0931	0.0877	0.0486	0.0522	0.0671	0.0561	0.0745	0.0762		0.0090	0.0173
MP1	0.0655	0.0690	0.0619	0.0781	0.0734	0.0976	0.0768	0.0864	0.0816	0.0768	0.0750	0.0956	0.0584	0.0671	0.0591	0.0561	0.0529	0.0868	0.0832	0.0620	0.0585	0.0692	0.0678	0.0690	0.0742	0.0519		0.0185
MW1	0.2000	0.1952	0.1976	0.1790	0.1770	0.2279	0.1796	0.1898	0.1837	0.1812	0.1856	0.2278	0.1920	0.2040	0.1646	0.1610	0.1686	0.2062	0.1968	0.1928	0.1975	0.2095	0.1829	0.1952	0.1992	0.1547	0.1798	

TABLE 4.3 MEAN CORRECTED SEQUENCE DIVERGENCE ESTIMATES OF *N. CAPENSIS* HAPLOTYPES IN SOUTH AFRICA.

COMPARISON	CORRECTED SEQUENCE DIVERGENCE ESTIMATE
WITHIN COMPLEX	
SWC	0.015
CTC	0.020
NEC	0.064
AMONG COMPLEXES	
SWC vs. CTC	0.059
CTC vs. NEC	0.059
SWC vs. NEC	0.079

4.3.2 Analysis of Molecular Variance

Results of the Analysis of Molecular Variance suggest significant sequence heterogeneity between samples (Table 4.4). The high fixation index (Φ_{ST} = 0.637) is indicative of a highly structured population of the Cape serotine bat, a figure indicative of very low female dispersal (Kerth *et al.* 2000). In fourteen subpopulations of *N. capensis* in South Africa (Appendix V), almost two thirds (63.73%) of the genetic variation was found among subpopulations, where all had relatively low variation internally (36.27%). When analysed individually, all subpopulations had a fixation index of one (data not presented). Again, these values should be treated with some caution since five subpopulations had only one representative.

TABLE 4.4 AMOVA RESULTS COMPARING INTER- AND INTRA- SPECIFIC MOLECULAR VARIATION OF THE HAPLOTYPES OF FIFTEEN SUBPOPULATIONS OF *NEOROMICIA CAPENSIS* IN SOUTH AFRICA.

SOURCE OF VARIATION	df	VARIATION COMPONENTS	PERCENTAGE OF VARIATION	FIXATION INDEX
AMONG POPULATIONS	14	31.72	63.73	
WITHIN POPULATIONS	14	18.05	36.27	$\Phi_{ST} = 0.637$ $p \ll 0.0001$

4.3.3 Evolutionary relationships between haplotypes

A minimum spanning tree (MST, Fig 4.4) was redrawn from the Euclidean square distance matrix to reflect the evolutionary divergence between haplotypes. In this analysis the data supports the idea of three complexes in South Africa. The NEC shows an unresolved evolutionary relationship because of the high evolutionary divergence between subpopulations within the complex. This is possibly due to poor sample representation of the region. Furthermore, the grouping of these individuals into complexes is supported by the neighbour-joining and maximum likelihood trees (Figs 4.5 & 4.6).

The haplotypes of the Southwestern complex (SWC) have a connection length of 31 (synonymous to almost 6% divergence estimate) with those grouped in the Central

Complex (CTC) whereas the internal branch connection lengths of the SWC range from 2 to only 17 (approximately 1.5% sequence divergence) – Fig 4.4; Table 4.2. The division of these two complexes is therefore based on this high evolutionary divergence estimate between the two Complexes. The haplotypes of the SWC include the individuals from De Hoop (DH 15 & DH 22), Cedarberg (DA4), Knysna (CX19, CX20 & CX 26), Ceres (CT 2 & CT4) with the latter sharing a haplotype with the Die Hel Bridge (DA2) individual, Wellington (CN17), and both Vrolijkheid colonies, Office (VR1, VR2 & VR3) and House (VR7, VR8, VR9 & VR10). Demographic details are available in Appendix V. The most striking observation within this Complex is that all individuals are grouped correctly with their colony mates (where sample size permits comparison), i.e. Vrolijkheid House, Vrolijkheid Office and Knysna Colonies have relatively high values dividing each from the Wellington sample, and low internal values within their colony (Fig 4.4). Both Neighbour Joining and Maximum likelihood trees (figs 4.5 & 4.6) support this grouping. Furthermore, both Vrolijkheid colonies are shown to be distantly related, yet are situated only about 70m away from each other (approximately 3% divergence estimate – Table 4.2), and each colony has a sequence divergence of under 1.5% respectively. This finding suggests that females are extremely philopatric. The Knysna individuals of the SWC provide further evidence for female philopatry. Two females within this colony share a haplotype (haplotype CX20/CX26 – Fig 4.4) and provide evidence that females born of the same colony remain together. The colonies of the CTC reflect similar structuring to the SWC – low divergence estimates between individuals within a colony and marginally higher values separating neighbouring colonies from each other.

The shared haplotype DA2/CT4 may provide an indication as to the mode of gene flow in the Cape serotine bat. Both of these individuals were adult males (epiphyseal plates completely fused), and their capture sites were approximately 120km apart. This indicates that either of the males had moved at least 120km away from their natal roost, which would ultimately lead to gene flow between colonies and subpopulations. However, in both Vrolijkheid colonies and the Knysna colony there is evidence for male philopatry to natal roosts. Alternatively, it should not be overlooked that these males may have been juvenile and had not yet dispersed. In these colonies the males are more closely related to at least one female than are females to each other. It is imperative to note that this finding is based on a single observation, and though not conclusive, it does provide insight into the behaviour of males.

The CTC includes the Plettenberg individuals (CX7, CX10 & CX14), Kuruman haplotypes (KM2, KM3, KM4 & KM12), and an individual from Timbavati Nature Reserve (TN4). However, both KM4 and TN4 are considered to be unresolved based on the very high divergence values in the MST (Fig 4.4), but clarity on their affiliation to CTC is nevertheless evident in both NJ and ML results. Since there is evidence for male biased gene dispersal in the SWC, it is possible that the high divergence between these individuals (both adult males) and their subpopulations indicate that they dispersed to these areas from elsewhere. In support of this argument, the corrected sequence divergence estimate between TN4 and TN7 (the latter is a female) is 8.45% (Table 4.2) indicating a substantial haplotype divergence, but both individuals are resident in the same subpopulation.

The NEC subpopulations are considered to be unresolved by MST analysis. There are only single representatives of each site, and extremely high divergence values between each. Additional individuals from each colony are necessary to be able to make a definitive judgment of the structure in this Complex. PP1 (individual from Pafuri, Appendix V) is an adult male, and due to evidence founded for male biased dispersal in both the SWC and the CTC, females would have to be sequenced to confirm the colony structure in this region. However, the NJ and ML trees (Figures 4.5 & 4.6, respectively) support the idea that these individuals form a separate complex from the other two complexes. In both analyses individuals making up the NEC form a separate (albeit weakly supported) clade.

Finally, MW1 (outgroup from Malawi) is separated from PP1 by a divergence value of 133, and has a mean divergence estimate of 19% (Table 4.2) when compared to all other individuals.

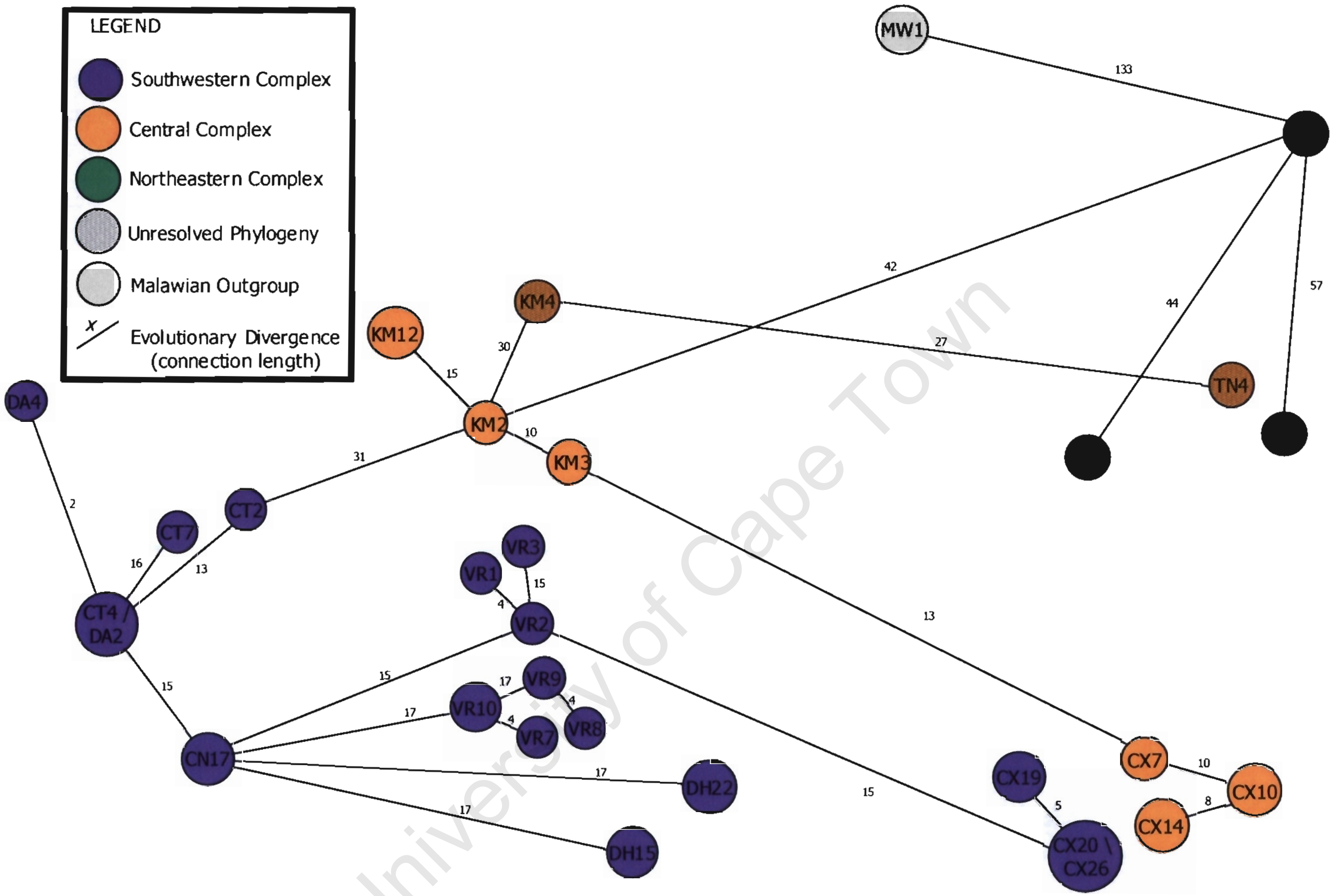


FIGURE 4.4 MINIMUM SPANNING TREE OF *N. CAPENSIS* HAPLOTYPES. A schematic representation of the evolutionary divergence of haplotypes. The tree has been rearranged to reflect the relative location of the subpopulations (not to scale). Haplotype abbreviations are listed in Fig 2.3 and appendix IV provides subpopulation affiliations of sequenced haplotypes. The Knysna/Plet subpopulation seems to be where the SWC and CTC subpopulations meet but do not mix, possibly as a result of female philopatry. The NE Complex seems unresolved possibly as a result of inadequate sampling. Compare haplotype distributions to colony and subpopulation geographic distribution in Fig 2.1.

4.3.4 Phylogenetic reconstruction of haplotypes

Both neighbour-joining (NJ) and maximum likelihood trees (ML, Figs 4.5 & 4.6) produced similar results and are comparable with the findings generated in the MST. The CTC has strong internal support for the hypothesis that females are philopatric. The separation of the SWC from the CTC is supported by 89% bootstrap support and a likelihood value of 96. High internal support values for the colonies of the SWC and the CTC confirm the notion of extreme philopatry in the Cape serotine bat. These trees represent only a portion of the mitochondrial genome (i.e. partial control region sequences), so it is important to note that they are gene trees as opposed to species trees. However, even though a single region of the mitochondrial genome is used to reflect phylogeny, a direct inference is made to the evolutionary relationships between the subpopulations (Moritz & Hillis 1996) of *N. capensis* throughout South Africa.

There is low internal bootstrap (57%) and likelihood (47) support for the grouping of the NEC individuals as is suggested by the MST. However, support for both MP1 and TN7 groupings have high likelihood values (91) although somewhat lower bootstrap (61%) support. The NJ and ML trees provide evidence that TN4 and KM4 are closely related (Figs 4.4 & 4.5) and the separation of these two individuals supports male biased gene dispersal.

The ML tree has high support for grouping the DA2/CT4, DA4, CT7 and CT2 haplotypes together (70), but the internal support values for these individuals are low (<50). This could be indicative of the close relatedness between these individuals that prevents a well resolved phylogeny between them. Indeed, the likelihood support for grouping DA2 and CT4 together is 24 (these are shared haplotypes). The NJ tree provides a somewhat clearer result of the phylogenetic relationships of these individuals, with higher internal support.

4.4 Discussion

Sequence divergence estimates support the hypothesis that the subpopulations of *Neoromicia capensis* are genetically structured. Microsatellite analysis of three loci revealed that there is a North-South genetic divide between subpopulations of the Cape serotine bat in South Africa. Mitochondrial analysis of partial control region haplotypes not only support this divide, but suggest that there are three genetically distinct complexes of subpopulations in this range. This finding is not unique to the Cape serotine bat. Miller-Butterworth (2003) reported that *Miniopterus natalensis* (formerly *schreibersii*) form genetically homogeneous geographical groups of colonies (i.e. significant population substructure within this species, with substantial heterogeneity across subpopulations). This species is known to migrate to hibernacula at the onset of winter, and this behaviour facilitates panmictic geographically divided groups. Notably the findings of *N. capensis* forming three genetic complexes within the political boundaries of South Africa, has a striking resemblance to the findings of Miller-Butterworth et al. (2003) for *M. natalensis*. Although the findings in this analysis do not support a migration hypothesis for *N. capensis*, individuals are nonetheless historically related over specific geographical divides.

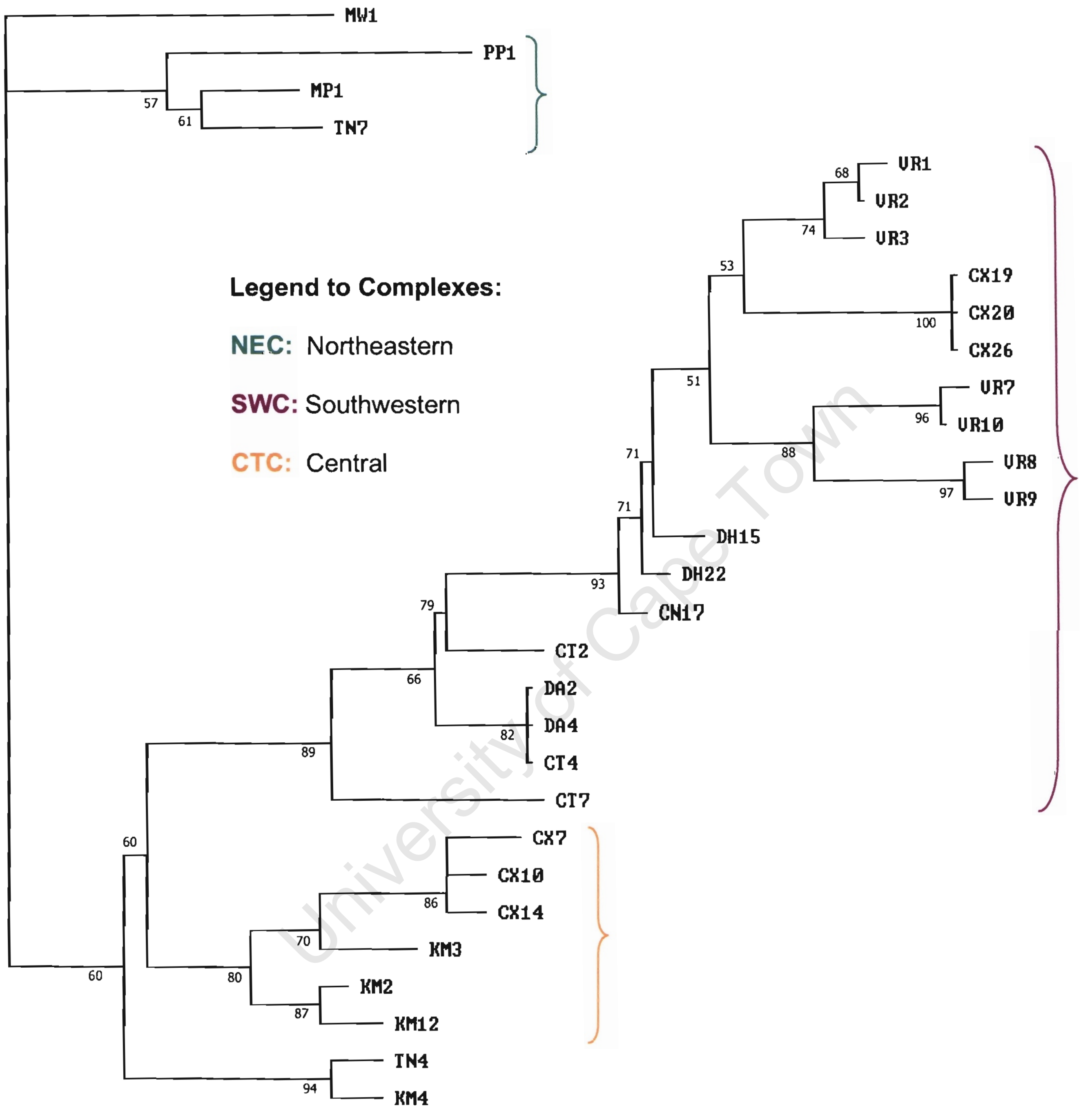


FIGURE 4.5 NEIGHBOUR-JOINING TREE OF THE PHYLOGENETIC RELATIONSHIPS BETWEEN INDIVIDUALS OF *N. CAPENSIS*. The specimen from Malawi had sufficient sequence divergence (19%) to warrant its use as the outgroup to root the topology. The values on the internal nodes are bootstrap support values.

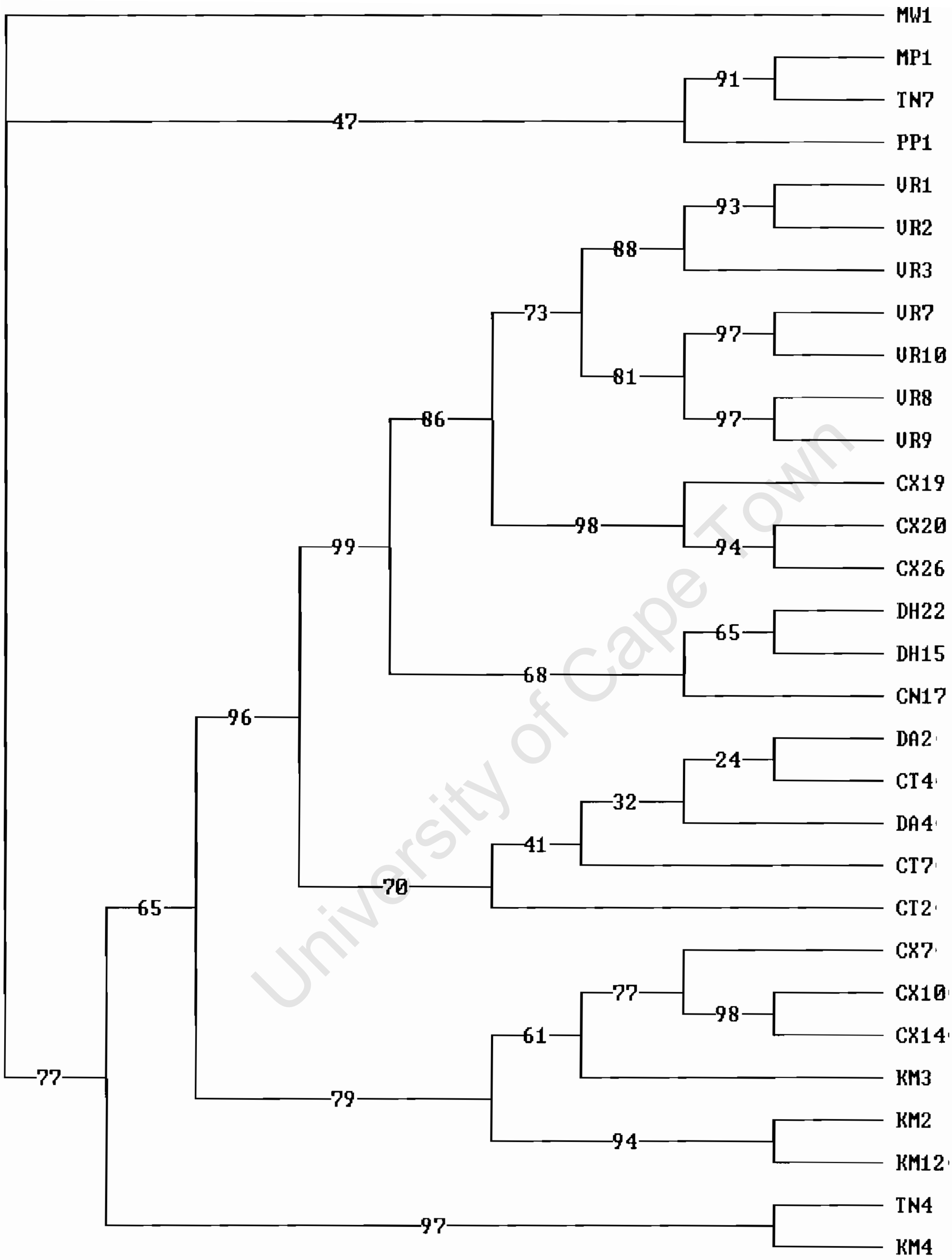


FIGURE 4.6 MAXIMUM LIKELIHOOD TREE OF THE PHYLOGENETIC RELATIONSHIPS BETWEEN HAPLOTYPES OF *N. CAPENSIS* IN SOUTH AFRICA. Values on the internal branches are likelihood support values after 1000 quartet puzzling steps.

Minimum spanning tree (MST), Maximum Likelihood tree (MLT) and Neighbour Joining tree (NJ) analyses support the division of the South African *N. capensis* population into three Complexes: the Southwestern, Central and Northeastern – each displaying genetic divergence estimates of at least 6% between each Complex. Subpopulations within each Complex have a low mean genetic divergence which is further supported by topological analyses (strong internal branch support).

The mean genetic divergences between subpopulations within a Complex are higher than 3%. This suggests that the individual subpopulations or colonies are structured and have limited gene flow between them. The genetic divergence between individuals of known colonies within Complexes are 0.05%, 1.1% and 1.5% (CX – Knysna, CX – Plettenberg and both Vrolijkheid colonies), respectively. This suggests that all individuals in a colony are highly related, and are philopatric to natal roosts. In those areas where sampling was random and not targeted towards a specific colony, genetic divergence could be as high as 8.5% (NEC only). This indicates that individuals live within small colonies where females are philopatric to natal roosts and males disperse. . Hence high relatedness within colonies, and close relatedness within complexes. This finding is further supported by a high fixation index of mitochondrial genetic variation between subpopulations (Φ_{ST} = 0.637, p « 0.0001). Population assignment tests performed on microsatellite loci support this view, since most individuals were correctly assigned to their own populations with high likelihood. *Rho* and G_{ST} values, although representative of only three loci, display moderate genetic differentiation between subpopulations. Natal philopatry to roosts and home ranges is a common phenomenon and has been reported in other bat species, viz.

the Ghost bat, *Macroderma gigas* (Worthington Wilmer *et al.* 1994), and the Greater mouse-eared bat, *Myotis myotis* (Castella *et al.* 2001). Apart from extreme female philopatry, only one other vespertilionid displays a dual-sex philopatric social organisation. Burland *et al.* (2001) provided evidence that the brown long-eared bat, *Plecotus auritus*, displays natal philopatry in both sexes. In that study, as in this, there was however considerable gene flow between colonies. In the latter study it was concluded that males remain faithful to their natal colonies and father offspring in neighbouring colonies (extra-colonial matings). This conclusion is consistent with the findings of this study.

The Malawian individual had a sequence divergence of 19% in comparison to other individuals found within South Africa. Immediately this calls to question the authenticity of the individual's identity. The Transvaal Museum has confirmed the identity of this individual as male *Neoromicia capensis* by comparative scull and baculum morphology (*pers. comm.* Teresa Kearney, Transvaal Museum). This suggests a substantial population divergence, and is therefore a suitable candidate for out grouping the topology.

CHAPTER 5

OVERVIEW

5.1 Phylogeography in *N. capensis*

Bats display a wide variety of mating systems (Clutton-Brock 1986; Altringham 1996) and each method has the potential to influence the rate at which genes flow through and between populations. Thus even in highly vagile organisms, and in the absence of physical barriers, mating systems have the potential of structuring a population. Here I report on the substructure in the South African population of the Cape serotine bat.

Microsatellite and mitochondrial analyses indicate that individuals of subpopulations (and even at colony level) of *Neoromicia capensis* display strong genetic affiliations to each other. Individuals of each subpopulation were assigned correctly to their natal areas with high probability and likelihood values in excess of 85%. Mitochondrial haplotypes show distinctive phylogeographic substructure. Haplotype analysis supports the division of the South African population into three subpopulations with substantial connection lengths between haplotypes. Genetic divergence among these subpopulations was high, while divergence within them was low. The three subpopulations identified were: the Southwestern (including DH, CN, SB, DL, CT, CXK and VR), Central (KM, CXP), and

Northeastern (PP, MP, and TN). These subpopulations correspond very broadly to a distribution over specific biomes.

The Northeastern Subpopulation: Savanna and Grassland Biomes.

The Northeastern subpopulation appears to be restricted to the savanna biome in South Africa. This biome is dominated by grasses and small shrubs with scattered trees. Rainfall is highly seasonal, falling predominantly in the summer (ca. 1000mm) and the remainder of the year is warm and dry. The average temperature is ca. 30°C. Grasslands tend to be milder in winter with a high incidence of frost.

The Central Subpopulation: Savanna/Grassland and Fynbos/thicket/ Forest Biomes and proportional karoo.

The Central subpopulation appears to represent an overlap or transition zone of the Northeastern and Southwestern complexes. It may also include localities that fall within the karoo, although no subpopulations in this study were captured within strictly defined karoo habitats. This biome is a semi-arid region characterised by sparse shrubland. The Succulent-Karoo has limited winter rainfall and mild winters (ca. 4°C) and very hot, dry summers (ca. 30°C). The Succulent Karoo has a high incidence of succulents. The Nama-Karoo is differentiated from the Succulent-Karoo by the proportions of grass and succulent composition. Summers are hot with seasonal rain, and winters are cold (0°C). These Biomes are heavily affected by rainfall and the boundaries are not distinct (Cowling et al. 1997).

Southwestern Subpopulation: Fynbos Biome.

The Southwestern Subpopulation is demarcated by the fynbos biome. This region is primarily a winter rainfall region, with nutrient-starved, duplex soils giving rise to characteristic dwarfed shrubs. Dominant vegetation include Proteaceae, Ericaceae, Restionaceae, with a high inclusion of Rhenosterveld (Cowling *et al.* 1997). Mild coastal temperatures of ca. 20 °C are common.

The observed population substructure may in effect be attributed to physical barriers or isolation by distance. The first obvious geographic deterrent would be the escarpment, but isolation may also be attributed to specific biome adaptation. On the other hand, colonies that are only 70 m apart display high genetic divergence while males from colonies 120 km apart share haplotypes. Given the association of the subpopulations with different biomes, it is likely that the substructure is caused by bats restricting their ranges to the biomes to which they have become locally adapted.

Upon closer inspection of the haplotype distribution, caution has to be drawn to the classification of the Central complex. Firstly, the distribution of individuals is extremely low, and demographic data limited. Secondly, both subpopulations are found in completely different biomes. It would seem that the Central complex represents a transition zone between two peripheral “super complexes”. Due to the individuals originating from only two major biomes is difficult to draw conclusions in relation to local adaptation. It is therefore essential for further extensive sampling to be conducted within the central parts of South Africa (e.g. the karoo) before a definitive distribution can be argued for.

5.2 Philopatry, dispersal and mating strategy in *N. capensis*

Genetic substructure can illuminate the extent to which one or both sexes disperse or are philopatric to a natal roost. Many of the sister species of the Vespertilionidae, and other families of the Microchiroptera display roost fidelity and, or philopatry to a greater or lesser extent. Although genealogies may be similar over vast areas due to extensive migration patterns, and complex mating strategies, bats generally have a closed breeding pattern within specific boundaries that are often correlated to biome adaptation (Barratt *et al.* 1997; Aspetsberger *et al.* 2003; Miller-Butterworth *et al.* 2003). It has been observed that *Myotis myotis* will migrate long distances to maternity hibernacula, but the gene flow between a neighbouring subpopulation is restricted resulting in genetically distinct subpopulations within close proximity (Castella *et al.* 2000).

Partial control region sequences of the mitochondrial DNA of the Cape serotine bat were aligned and compared to determine the extent of dispersal and philopatry. A total of thirty individuals were sequenced representing all, but two, of the biomes in South Africa. Mitochondrial haplotypes from each colony were representative of only those groups of individuals, indicating extreme philopatry to those sites. In the Plettenberg colony, for example, two females shared a haplotype, providing evidence that females tend to remain as a group within natal roost sites. Similarly, although the two colonies in Vrolijkheid were only 70m apart the mean divergence between them was almost double the mean within colony divergence. The situation with males is less clear probably because of the small sample sizes collected. Microsatellite data for subpopulations (Kuruman and Timbavati) dominated by males yielded low *Rho* values suggesting a low level of relatedness amongst males. This is probably because they have dispersed from elsewhere. Male dispersal is also supported by the mitochondrial data. For example, two males captured 120 km apart shared a haplotype. However, in two colonies (Vrolijkheid and Knysna) males were more closely related to at least one female than females were related to each other. This does not necessarily contradict the view that males disperse because these two males could have been captured prior to dispersal. Alternatively, male decisions to disperse or not may be based on the availability of roosts and mates. In this case males would only disperse if suitable roosts and mates were not available in their natal area.

The dispersal patterns elucidated here and the low numbers of males in each colony, despite efforts to capture entire colonies, is a suggestive of a harem mating system. It would seem that Altringham's 'female defense strategy' would best diagnose the mating strategy of the Cape serotine bat. However it should be noted that although (in agreement with this model) female occupancy within a colony seems to remain constant, male occupants are thought to disperse to peripheral colonies prior to mating (extracolonial mating). Thus resident males are not defending a cohort of copulatory partners; instead roost male occupancy will change at the onset of the breeding season. It is not conclusively clear from the results of this study as to whether the same males return to the same colony at each breeding event. The pattern of genetic substructure resulting from this behaviour resembles that found by Miller-Butterworth *et al.* (2002) for *Miniopterus natalensis*.

In conclusion the Cape serotine bat displays population substructure with marginal evidence that dispersal is associated with adaptation to local conditions and/or biome boundaries. This structure is achieved by females remaining faithful to natal roosts and male dispersal – though restricted to specific regions/biomes.

CHAPTER 6

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APPENDICES

APPENDIX I

Classification of sample sites throughout South Africa. Those sites marked with an asterisk were sampled by the Transvaal Museum.

SITE CODE	DEFINITION	VEGETATION TYPE
CN	WELLINGTON BLOCKHOUSE	FOREST / KAROO
CT	CERES FRUIT FARM	FYNBOS / ORCHARD
CXK	KNYSNA	THICKET
CXP	PLETTENBERG BAY	FOREST
DA	CEDARBERG FORESTRY STATION	FYNBOS / ARTIFICIAL FOREST
DH	DE HOOP NATURE RESERVE	FYNBOS
DL	DIE HEL	FYNBOS
HH*	HLUHLUWE/UMFOLOZI NATIONAL PARK	SAVANNA
KM*	KURUMAN	SAVANNA
MP*	MPUMALANGA	GRASSLAND
MW*	MALAWI	MOUNTAIN SCRUB
PP*	PAFURI RESEARCH STATION	WOODLAND SAVANNA
TN*	TIMBAVATI NATURE RESERVE	SAVANNA
VRH	VROLIJKHEID HOUSE	FYNBOS / KAROO
VRO	VROLIJKHEID OFFICE	FYNBOS / KAROO

APPENDIX II

LIST OF INDIVIDUALS CAPTURED AND BANDED FOR RECAPTURE STUDIES

SITE	INDIVIDUAL	BAND CODE	SEX	DATE BANDED	CAPTURE WEIGHT (g)
WELLINGTON	CN1	TM0457	F	28.10.02	8.0
	CN2	TM0458	F	28.10.02	9.0
	CN3	TM0459	F	28.10.02	9.0
	CN4	TM0460	F	28.10.02	9.0
	CN5	TM0461	F	28.10.02	8.0
	CN6	TM0462	F	28.10.02	7.0
	CN7	TM0463	F	28.10.02	8.0
	CN8	TM0465	F	28.10.02	8.0
	CN9	TM0466	F	28.10.02	9.0
	CN10	TM0467	F	28.10.02	9.0
	CN11	TM0468	F	28.10.02	9.0
	CN12	TM0469	F	28.10.02	10.0
	CN13	TM0470	F	28.10.02	9.0
	CN14	TM0471	F	28.10.02	9.0
	CN15	TM0472	F	28.10.02	9.0
	CN16	TM0473	F	28.10.02	9.0
	CN17	TM0474	F	28.10.02	6.0
	CN18	TM0475	F	28.10.02	8.0
	CN19	TM0476	F	28.10.02	8.0
	CN20	TM0477	F	28.10.02	9.0
	CN21	TM0478	M	28.10.02	9.0
CERES	CT1	DB 0201	F	15.03.03	10.0
	CT2	DB 0202	M	15.03.03	8.0
	CT3	DB 0203	F	15.03.03	7.0
	CT4	DB 0204	M	15.03.03	6.5
	CT5	DB 0205	F	15.03.03	8.0
	CT6	DB 0206	F	15.03.03	8.0
	CT7	DB 0227	M	15.03.03	7.0
CEDARBERG	DA3	TM 0453	M	6.10.02	6.5
	DA4	TM 0454	M	5.10.02	5.5
	DA5	TM 0455	F	8.10.02	6.0
DE HOOP	DH11	TM0479	F	27.01.03	8.0
	DH20	TM0485	F	29.01.03	10.0
	DH33	TM0481	M	28.01.03	10.0
	DH34	TM0484	F	28.01.03	8.0
	DH35	TM0483	M	28.01.03	7.0
	DH36	TM0482	F	28.01.03	8.0
	DH37	TM0480	F	28.01.03	11.0
DIE HEL	DA1	TM 0451	M	29.9.02	6.5
	DA2	TM 0452	M	3.10.02	6.0

APPENDIX II (ctd.)

VROLIJKHEID	VR1	TM0486	F	22.02.03	7.5
	VR2	TM0487	M	22.02.03	6.0
	VR3	TM0488	M	22.02.03	4.5
	VR4	TM0489	F	22.02.03	7.0
	VR5	TM0490	M	22.02.03	6.0
	VR6	TM0491	F	22.02.03	6.0
	VR7	TM0492	M	22.02.03	6.0
	VR8	TM0493	F	22.02.03	7.0
	VR9	TM0494	F	22.02.03	7.0
	VR10	TM0495	F	22.02.03	8.0
	VR11	TM0496	F	22.02.03	7.0

APPENDIX III

LIST OF ACCESSIONED SAMPLES SUPPLIED BY THE TRANSVAAL MUSEUM, PRETORIA. ** INDICATES UNAVAILABLE DATA

SITE	INDIVIDUAL	ACCESSION CODE	SEX	DATE	CAPURE WEIGHT (g)
HLUHLUWE	HH1	TM44392	F	23.07.93	7.0
PAFURI	PP01	TM41732	M	07.03.90	**
	PP02	TM41733	F	07.03.90	**
	PP03	TM41734	M	07.03.90	**
	PP04	TM41735	M	07.03.90	**
	PP05	TM41736	F	21.05.90	**
MPUMALANGA	MP1	TM46476	F	**	6.7
	MP2	TM46477	M	**	5.8
	MP4	TM46630	F	**	8.5
KURUMAN	KM1	TM44445	M	04.08.93	6.8
	KM2	TM44448	M	03.08.93	5.0
	KM3	TM44450	F	03.08.93	6.4
	KM4	TM44451	M	03.08.93	5.4
	KM5	TM44452	F	03.08.93	7.4
	KM6	TM44453	F	03.08.93	6.6
	KM7	TM44454	M	03.08.93	5.2
	KM8	TM44455	F	03.08.93	7.6

APPENDIX III ctd.

	KM9	TM44473	M	06.08.93	5.0
	KM10	TM44474	M	06.08.93	5.7
	KM11	TM44475	M	06.08.93	**
	KM12	TM44476	M	06.08.93	5.5
MALAWI	MW01	TM41796	M	21.05.90	**

APPENDIX IV

LIST OF PRIMER SEQUENCES USED IN PCR

LOCUS	SEQUENCE	REPEAT MOTIF	AUTHOR
EF1	5' –ATC TGG GCA ATG ATA CCT TT- 3'	$(GT)_{22}CT(GT)_{17}$	Vonhoff *et al.*
	5' –GCA GGC TGG GCT GAG- 3'		2002
EF4	5' –ATA GGC TCC CAG AAA TAG C- 3'	$(CT)_4(GT)_{17}$	Vonhoff *et al*
	5' –GAT CAC CAC AAA ATG TGC- 3'		2002
EF6	5' –ATC ACA TTT TTG AAG CAT- 3'	$(GT)_{20}$	Vonhoff *et al*
	5 -ATC TGT TTT TCT CTC CTT AT- 3'		2002
EF14	5' –ATC ATA TAT TTG TGT TCT GG- 3'	$(GT)_{19}$	Vonhoff *et al*
	5' –AAA ATC AGC TAT TGA GCA C- 3'		2002
EF15	5' –AGC AGC AAA GGG GAC TCA GA- 3'	$(CA)_3GA(CA)_{20}$	Vonhoff *et al*
	5' –GAG AAG CAG GGA GGG CAT TT- 3'		2002
EF20	5' –TTA TCT TTG CCG TGG TT- 3'	$(GT)_{18}$	Vonhoff *et al*
	5' –CCC CAC AAT GCC ATT A- 3'		2002
NN8'	5' -GGT GAT TTC CAT TCC CAG C- 3'	*	Castella *et al.*
	5' -TTG TGT TTT AAA GAA AAT CCG- 3'		2001
MM5'	5' -AGG GTT GAT TTA ACA TGC- 3'	*	Castella *et al.*
	5' -CTT TCA TCC AGT TCT GG- 3'		2001

APPENDIX V

LIST OF INDIVIDUALS USED IN MITOCHONDRIAL ANALYSIS

INDIVIDUAL	SITE	SEX	INDIVIDUAL	SITE	SEX
CT2	CERES	M	KM3	KURUMAN	F
CT4	CERES	M	KM4	KURUMAN	M
CT7	CERES	M	KM12	KURUMAN	M
CN17	WELLINGTON	F	MP1	MPUMALANGA	F
CX7	PLETTENBERG	F	MW1	MALAWI	M
CX10	PLETTENBERG	F	PP1	PAFURI	M
CX14	PLETTENBERG	F	TN1	TIMBAVATI	M
CX19	KNYSNA	M	TN4	TIMBAVATI	M
CX20	KNYSNA	F	VR1	VROLIJKHEID OFFICE	F
CX26	KNYSNA	F	VR2	VROLIJKHEID OFFICE	M
DA2	DIE HEL	M	VR3	VROLIJKHEID OFFICE	M
DA4	CEDARBERG	M	VR7	VROLIJKHEID HOUSE	M
DH15	DE HOOP	M	VR8	VROLIJKHEID HOUSE	F
DH20	DE HOOP	F	VR9	VROLIJKHEID HOUSE	F
KM2	KURUMAN	M	VR10	VROLIJKHEID HOUSE	F

APPENDIX VI

An electropherogram of an individual's D-loop sequence amplified using Wilkinson & Chapman's "P" primer – amplification of the light strand.