POPULATION SUBSTRUCTURING IN SCHREIBERS' LONG-FINGERED BAT (*MINIOPTERUS SCHREIBERSII*) IN SOUTH AFRICA

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Thesis presented for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Zoology

Faculty of Science

UNIVERSITY OF CAPE TOWN

October 2001

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ABSTRACT

Population substructuring in Schreibers' long-fingered bat *(Miniopterus schreibersii)* in South Africa

Schreibers' long-fingered bat, *Miniopterus schreibersii* migrates seasonally between winter (hibernacula) and summer (maternity) colonies in South Africa. Previous behavioural studies suggested that roost fidelity is well developed in this species, and that juvenile dispersal may be limited, possibly in both sexes. If males and/or females are strongly philopatric, this may lead to restricted gene flow among colonies, resulting in genetically distinct breeding subpopulations. The population structure of *M. schreibersii* in South Africa was investigated using microsatellites and mitochondrial DNA (mtDNA), with the aim of determining the degree of genetic differentiation among colonies, and the extent and direction of bat movement among the colonies.

A genomic library was constructed for *M. schreibersii,* and was screened for $(CA)_n$ and $(GA)_n$ microsatellite repeats. Five novel, highly polymorphic loci were identified. These five loci, and an existing mammalian microsatellite locus, were amplified in 301 individuals, sampled from ten colonies throughout South Africa. Significant genetic heterogeneity exists within the *M. schreibersii* population, such that the population can be subdivided into three partially discrete breeding subpopulations. Little genetic differentiation exists between colonies within

ACKNOWLEDGEMENTS

First and foremost my thanks must go to my supervisors, Dr David Jacobs and Prof. Eric Harley. Their support and encouragement throughout the last four years have been invaluable, and their assistance and confidence in me is enormously appreciated. Dr Jacobs inspired my passion for bats and bat research, and his enthusiasm and love for his work continue to stimulate me. Professor Harley is the eternal optimist. No matter what complications or challenges arose during the lab work, he always found a positive side to trouble-shooting. Thanks to him, trials and tribulations were never a problem, merely "character-building". Without his continual support, I doubt my character would be as strong as it currently is!

Thanks must also go to our funders, the National Research Foundation of South Africa, and the University Research Committee of the University of Cape Town (UCT). In addition, I would like to thank Ingrid Baumgarten for her enormous assistance with my laboratory work, and Dr Patricia Owen, for her advice and encouragement throughout my degree. I also greatly appreciate the help of Carel van Heerden, and particularly of Di James, with their assistance in sequencing of clones and mitochondrial DNA. Thank you also to my friends, especially Jean Erasmus, Anna Whitehouse, Jacqueline Bishop and Jessica Cunningham, for their unfailing support throughout my degree.

Without the bat specimens, there would be no PhD. For their help in collecting samples I would therefore like to thank Brigitte Eick, Carol Logan, Mark Kirkman, Dr Peter Taylor, Prof. Ric Bernard, Sue Rhind, Robbie Louw, Wolfgang Lück and the UCT Zoology Department Behavioural Ecology students of 1998. I also thank Belinda Cardinal, James Hutcheon, Prof. Brock Fenton, Didi Kaplan and Beni Shalmon for sending me *Miniopterus schreibersii* samples from various international locations. For granting us permission to collect samples on their property, and for their on-site assistance during the collecting, thank you to Mr J. Cornielisen (Brakfontein Farm, Koegelbeen), Prof. Bruce Rubidge (Peppercorn Cave), Mr Ben Swanepoel (De Hoop Guano Cave), Mr Hadrey Honiball (Jozini Dam) and the management of Sudwala Property Holdings (Pty) Ltd.

I would like to thank the UCT Zoology Department and its staff of the last ten years for instilling in me a love for Science. Collectively, staff members made me feel that the Zoology Department was my home from home. One and all, they encouraged my love for animals and my interest in animal biology. Without their encouragement right from the start of my academic career, I may not have embarked on a PhD.

My passion for animals is due primarily to my parents, Tina and Len Miller. Since I was a child they have taught me to love animals and nature in general, to be fascinated by wildlife and the process of evolution. They also taught me to have confidence in my own abilities, and to follow my dreams. They have supported and encouraged me at every juncture, and I cannot find the words to thank them enough for all they have done for me throughout my life.

Last but not least, thank you to my husband, Michael. My parents may have sparked my love for wildlife and conservation, but Mike extended that love into a quest for knowledge. He taught me never simply to accept things, but always to question them. His enquiring mind and passion for Science have inspired me over the years, and will continue to do so.

The Zoology Department inspired me to begin a PhD. But my parents, my husband and my supervisors saw me through it - through the good times and hard ones. I therefore would like to dedicate this thesis to them.

GLOSSARY AND ABBREVIATIONS

Allele	An alternative molecular form of a gene.
Allelic fixation	If all individuals within a population are homozygous for a particular allele, the frequency of that allele equals 1.0, and the population is said to be "fixed" for the allele.
AMOVA	Analysis of molecular variance
amp+ LB plates	Luria Broth plates treated with the antibiotic, ampicillin
ANOVA	Analysis of variance
Aspect ratio	An index of the shape of a bat's wing; the ratio of the square of wingspan to wing area.
Autoradiography	Detection of radioactively labelled products by means of exposure to an X-ray film.
Biome	A broad vegetational zone, influenced by climate, topography and soil composition.
bp	Base pairs
CFU	Colony forming units
Cloning	Incorporation of a DNA fragment into a vector, and the subsequent propagation of the recombinant molecule in a host organism.
Competent	See electro-competent
D-loop	Displacement loop
dNTP	Deoxynucleotide
ddNTP	Dideoxynucleotide
Dinucleotide repeat	A microsatellite containing a repeat unit of two base pairs, *e.g.* $(CA)_n$ or $(GA)_n$.
Dispersal	The permanent movement of an animal away from its natal area, generally leading to the establishment of a new home area in which that animal attempts to breed.
Dropped alleles	Alleles that fail to amplify during PCR due to low concentrations of template DNA.
Electro-competent	Bacteria that have been treated to enhance their ability to take up foreign DNA fragments during electroporation.
Electroporation	A method of transforming bacterial cells with foreign DNA. It involves exposing the cells to brief electrical shock, which creates transient pores in the cell wall, allowing foreign DNA to enter the cell.

ESU	Evolutionarily significant unit
Fixation indices	Statistical coefficients that describe how genetic variation is partitioned within and among different populations or individuals; also referred to as F-statistics.
Founder effect	A small group of individuals from an established population founds a new subpopulation, which experiences high levels of genetic drift.
Gene flow	The spread of genes among populations as a result of successful mating between members of a species, across population boundaries.
Genetic differentiation	Accumulation of differences in allele frequencies between populations that are partially or totally isolated.
Genetic drift	Random changes in allele frequencies over time, due to sampling effects rather than natural selection.
Genomic library	A collection of cloned restriction fragments of DNA, incorporated into cloning vectors (*e.g.* plasmids). In a complete library, these fragments represent virtually the entire genome of an organism. Partial libraries contain only fragments of a certain size range.
Hardy-Weinberg equilibrium	The allele frequencies obtained through random mating, in populations of infinite size, that experience no immigration, emigration, mutation or selection.
Heterozygosity	The frequency of heterozygous loci; a measure of genetic diversity or variation.
H_E	Expected heterozygosity
H_o	Observed heterozygosity
Hibernaculum	A roost utilised by bats in winter for hibernation, and possibly also for mating.
IPTG	isopropyl-thiogalactoside
kb	Kilobases
Ligation	Joining DNA fragments together to form recombinant molecules.
Linkage disequilibrium	When two loci are located close together on the same chromosome, they tend to be inherited together and are only occasionally separated during recombination. Their respective allele frequencies therefore depart from the predicted frequencies for randomly associated alleles of a multiple allele locus.
Maternity roost	A roost occupied in summer, predominantly by female bats, for the purposes of giving birth and raising young.
mtDNA	Mitochondrial DNA
MU	Management unit
NCAM	Neural cell adhesion molecule
N_m	Number of migrants
Null alleles	Alleles that fail to amplify during PCR because a mutation in the DNA sequence flanking a microsatellite prevents optimal binding of one of the primers.
O_H	Origin of replication of the H-strand of mitochondrial DNA

Outgroup	One or more taxa that are assumed to be phylogenetically distinct from the monophyletic group of interest.
^{32}P	Phosphorus-32
PCR	Polymerase chain reaction
Phylogeography	The study of geographic patterns of genetic variation among species or populations.
Plasmid	A circular DNA molecule, generally of bacterial origin, but which is independent of the bacterial chromosome and capable of self-replication.
Population substructure	The spatial variation in distribution and genetic composition of individuals within a species.
Philopatry	The tendency for an animal to remain and breed within its natal or home area, and, in the case of migrants, to return to it in successive years.
Primer	A short (usually less than 30 bp), single-stranded oligonucleotide, which attaches by means of base pairing to a single-stranded template DNA molecule, and acts as the starting point for synthesis of a complementary strand of DNA, *e.g.* during PCR.
^{35}S	Sulphur-35
Subpopulation	One of a number of breeding groups within a larger population, between which movement (or dispersal) of individuals is restricted.
Recombinant plasmid	A plasmid that contains a fragment of foreign DNA.
Restriction fragment analysis	Determination of the number and size of fragments resulting from digestion of a DNA molecule with one or more restriction enzymes.
RFLP	Restriction fragment length polymorphism
UCT	University of Cape Town
Vector	A DNA molecule capable of replication within a host organism.
Wing loading	A measure of the size of a bat's wing; the ratio of mass to wing area.
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ABBREVIATIONS OF COLONY NAMES

Major colonies:

DHL	Die Hel
DHP	De Hoop
G	Grahamstown
JD	Jozini Dam
KB	Koegelbeen
MM	Maitland Mines
PC	Peppercorn
SKK	Steenkampskraal
SHD	Shongweni Dam
SW	Sudwala

Other sample locations in South Africa:

C	Cedarberg
K	Knysna
PRB	Pongola River Bridge

International locations:

A	Australia
I	Israel
M	Madagascar

CHAPTER 1

POPULATION AND BEHAVIOURAL ECOLOGY OF *MINIOPTERUS SCHREIBERSII*:

INTRODUCTION, LITERATURE REVIEW AND STUDY AIMS

1.1. POPULATION SUBSTRUCTURE AND GENE FLOW

1.1.1. What is population substructure?

Population substructure is the spatial variation in distribution and genetic composition of individuals within a species (Hewitt & Butlin 1997). Many species naturally form aggregations such as herds or colonies. Such groups of organisms may separate over time (*e.g.* if they occupy patchy habitats), and may become genetically differentiated because natural selection favours different genotypes in different environments, because of founder effects, or due to random processes such as genetic drift and mutation (Dobson 1998; Hartl & Clark 1997). Genetic drift involves chance fluctuations in allele frequencies over time. In the absence of new alleles resulting from mutation, genetic drift leads to reduction in heterozygosity (especially in small populations) and differentiation of allele frequencies among divergent

populations through the stochastic loss of certain alleles and fixation of others (Hartl & Clark 1997; Hewitt & Butlin 1997; McCracken 1987). Over time, equilibrium is reached between the rate at which new alleles arise through mutation and the rate at which they are lost through genetic drift (Hartl & Clark 1997). The extent of genetic differentiation that develops will therefore depend on the size of the subpopulations, how long they have been separated, and the relative contributions of behavioural processes such as philopatry and dispersal, which limit and enhance gene flow, respectively (Chesser 1991; Theimer & Keim 1994). Knowledge of the underlying genetic structure of populations of a species thus provides information on levels of current and historical gene flow among those populations, enabling one to infer behavioural processes such as colonisation and dispersal events, as well as social organisation (Burland *et al.* 1999; Worthington Wilmer *et al.* 1994).

1.1.2. The effect of gene flow on population substructure

Gene flow results from dispersal coupled with successful mating. It introduces genetic variation to populations, counteracting genetic drift and thus limiting the amount of genetic differentiation that develops between populations (McCracken 1987). In some cases, even very low levels of dispersal (one migrant per generation) can minimise the effects of genetic drift, leading to greater genetic homogeneity among populations (Slatkin 1987). The extent of genetic differentiation (F_{ST}, Chapter 4) may be estimated from the number of migrants, according to the formula (Slatkin 1987):

$$F_{ST} = \frac{1}{1+4Nm}$$ where N is the population size and m is the average migration rate.

However, the effects of dispersal depend not only on the number of dispersers, but also on their genetic similarity to other individuals in the population that they are joining (McCracken 1987).

It should also be noted that the above relationship between genetic structure (F_{ST}) and gene flow (N_m) is based on an island model of population structure, which makes a number of assumptions that may not always be met in natural populations (Whitlock and McCauley, 1999). The model assumes (i) there is no selection or mutation, (ii) that there is an infinite number of populations, all with an equal number of individuals, (iii) that each population gives and receives migrants to the other populations at the same rate, (iv) that each migrant has an equal probability of dispersing to any other population, and (v) that each population has reached an equilibrium state between migration and genetic drift (Whitlock and McCauley, 1999). If one or more of these assumptions is violated, the mathematical relationship between F_{ST} and N_m will be affected significantly, and thus estimates of migration rate based on F_{ST} (or *vice versa*) should be interpreted with caution (Whitlock and McCauley, 1999).

Differences in population substructure may be examined at different hierarchical levels, including the individual, groups of individuals (*e.g.* families or colonies) and subpopulations, as well as at the level of the total population (Hartl & Clark 1997; Hewitt & Butlin 1997). For example, differences in male and female breeding behaviour and levels of dispersal may influence the patterns of genetic subdivision that develop within or among populations, *i.e.* at different levels of the hierarchy (Pope 1992). If females remain within their natal home range while males disperse, females within a particular population may be more closely related to one another than they are to males of that population (Dobson 1998). Furthermore, if individual males in a polygynous society try to monopolise matings, females in local breeding groups may be more likely to mate with the same male. Offspring in these social breeding groups may therefore have a high degree of kinship because their mothers are closely related and they have the same father. Kinship within a particular breeding group would therefore be high, but that group should differ genetically from other breeding groups (Dobson 1998).

Strong female philopatry coupled with male dispersal may also influence patterns of genetic substructuring at other levels of the population structure hierarchy. For example, while female

philopatry may lead to high levels of female kinship within populations, it may result in significant genetic differentiation among maternal lineages occurring in different populations (as detected, for example, by mitochondrial DNA markers, Chapter 5). Male dispersal and associated gene flow, on the other hand, may counteract the effects of genetic drift and mutation at the nuclear level, reducing any genetic differentiation that may be detected by nuclear markers (*e.g.* microsatellites, Chapter 4).

The geographical distance between the populations may also affect the extent of genetic differentiation because, in general, more gene flow occurs between populations that are located close to each other than between populations that are far apart. This phenomenon is referred to as "isolation by distance" (Dallas *et al.* 1995; Hewitt & Butlin 1997). The degree to which distant populations are genetically isolated from each other will therefore depend largely on the dispersal habits of the species involved.

1.1.3. Methods of estimating gene flow

Levels of gene flow may be estimated directly by observational methods, or indirectly through genetic analysis. Direct methods involve estimates of dispersal distances and breeding success through direct observation or, for example, mark-release-recapture studies. Such observational studies are fraught with problems, and are limited in both space and time. Because they provide a "snap-shot" representation of dispersal rates during a limited time frame only, they may not reflect levels of gene flow on historical time scales (Bohonak 1999; Slatkin 1987). Such studies also rely on monitoring or recapturing all marked individuals within a restricted area, which may be extremely difficult when dealing with highly migratory, volant and/or gregarious species.

Furthermore, it is frequently difficult to assess why marked individuals are not recovered. For example, low recovery levels of marked individuals (less than 1% for some migratory bat

species; Griffin 1970) may be due to chance, loss of tags (*e.g.* they may fall off during grooming or moulting), or mortality of marked individuals (due to natural processes or as a result of the marking process itself). In the case of large populations, it is usually not possible to mark all individuals within a colony, therefore when unmarked individuals are captured, one cannot distinguish between immigrants and those that were not previously marked. Accurate levels of true dispersal rates may thus be difficult to determine.

High capacity for dispersal (*e.g.* volant and/or migratory species) does not necessarily indicate how much gene flow actually occurs (Slatkin 1987). The potential ability of a species to disperse long distances does not necessarily imply that individuals will in fact disperse (discussed in Chapter 8), or that they will breed successfully if they do disperse. Therefore, assessment of levels of gene flow and identification of genetically distinct subpopulations may not always be possible using direct observational methods alone (Burland *et al.* 1999).

An alternative, indirect measure of gene flow (and thus the extent of genetic differentiation between subpopulations) is genetic analysis. This involves examination of varying allele frequencies among populations, and subsequent application of genetic models of gene flow and population differentiation such as fixation indices (Chapter 4). These indices estimate the relative proportions of genetic variation within and among the different hierarchical levels of population substructure (Chesser 1991; Slatkin 1987). Examination of genetic variation provides an estimate of historical levels of gene flow among subpopulations, averaged over many generations (Bohonak 1999), thus making an invaluable contribution to the understanding and management of species (Worthington Wilmer *et al.* 1994). Ideally, genetic analysis should be combined with direct estimates of dispersal from behavioural and ecological studies. Furthermore, it is preferable to compare results from different molecular markers (*e.g.* microsatellites and mitochondrial DNA) to obtain a more detailed and accurate picture of the effects of dispersal and philopatry, particularly if these behavioural processes differ between males and females, as discussed above (Bohonak 1999).

1.2. CLASSIFICATION AND DISTRIBUTION OF *MINIOPTERUS SCHREIBERSII*

Schreibers' long-fingered bat, *Miniopterus schreibersii* (Kuhl) is a small (10 – 13 g), highly gregarious, insectivorous microchiropteran (Figure 1.1). It is a member of the subfamily Miniopterinae (Figure 1.2), within the most diverse chiropteran family, Vespertilionidae (Nowak 1991). Also known as bent-winged or long-winged bats, the Miniopterinae are characterised by an elongated third finger, the second phalanx of which is about three times longer than the first (Nowak 1991; Taylor 2000). The terminal part of the wing therefore folds back on itself when the bat is at rest.

There are eleven *Miniopterus* species, two of which occur in South Africa (Nowak 1991), namely *M. schreibersii* and *M. fraterculus* (the Lesser long-fingered bat). *Miniopterus schreibersii*, represented in South Africa by *M. s. natalensis* (Smith), is by far the more common and widely distributed of the two species (Figure 1.3). A third southern African species, *M. inflatus* (the Greater long-fingered bat) occurs in only a few isolated localities in Namibia and Zimbabwe (Taylor 2000). The remaining eight *Miniopterus* species are found in Madagascar, East Africa, India, Asia and Australia (Nowak 1991; Strahan 1998).

Miniopterus schreibersii is the most widely naturally distributed mammal species in the world (Richarz & Limbrunner 1993). It roosts in large colonies throughout most of Africa, as well as Madagascar, the Middle-East, southern Europe, Russia, Australia, India, Japan and the Philippines (Gazaryan 1999; Mills & Hess 1997; Nowak 1991; Strahan 1998). This species usually roosts in caves, but also occupies man-made structures, such as abandoned mines, water supply tunnels and dam wall tunnels.

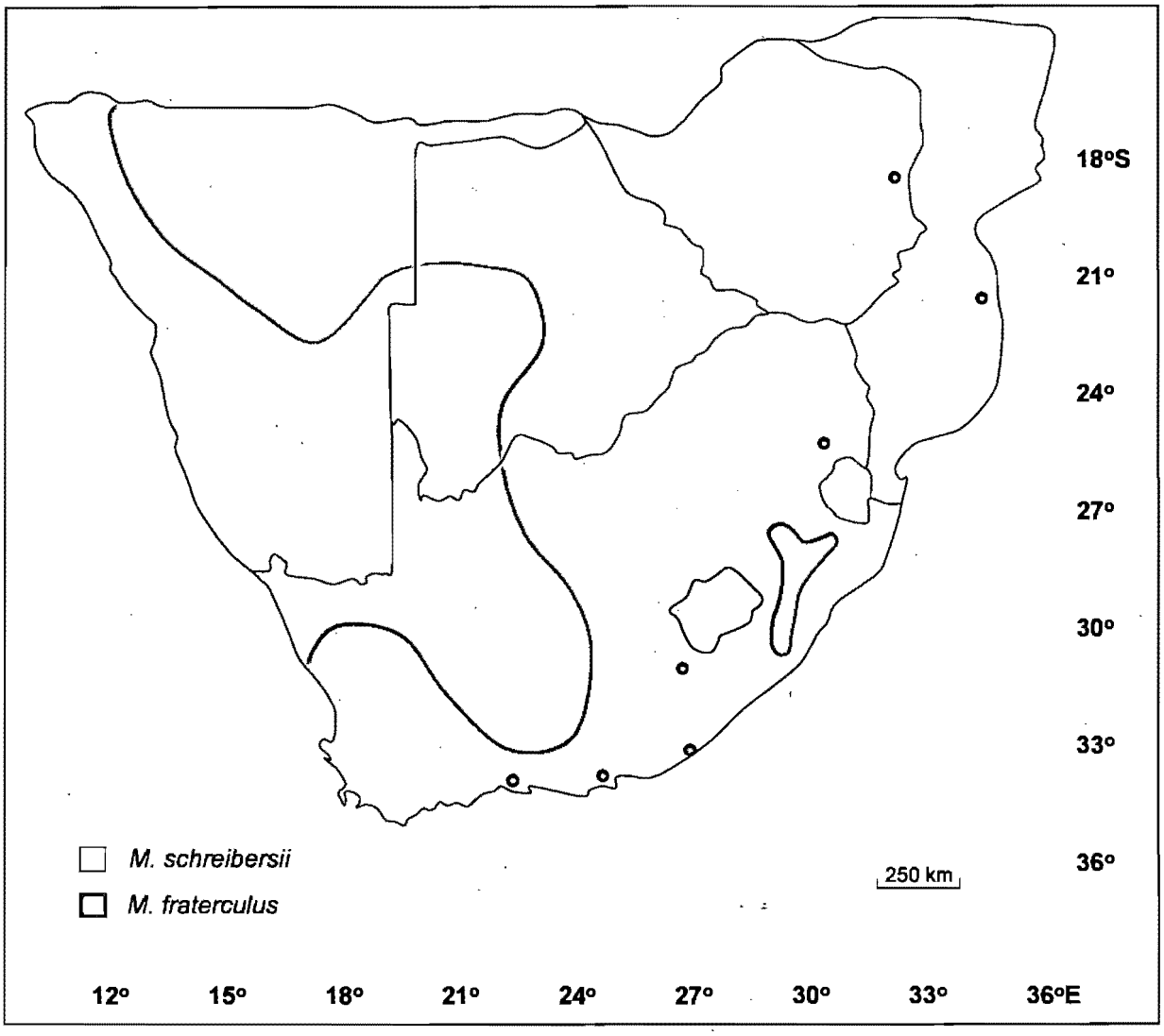

Figure 1.3. Distribution of *Miniopterus schreibersii* and *M. fraterculus* in southern Africa. Modified from Taylor (2000).

Despite its widespread distribution, *M. schreibersii* is classified as Near Threatened in South Africa, according to the International Union for Conservation of Nature (IUCN) 2000 Red List of Threatened Animals, and is regarded as endangered in western Europe (Nowak 1991). The species is restricted to areas that offer suitable roosting sites (Mills & Hess 1997), particularly breeding roosts (Section 1.3), and any threat to these sites may jeopardise the survival of widespread populations (Strahan 1998). Furthermore, these bats are highly sensitive to environmental disruption and human disturbance, and several European colonies that previously contained thousands of individuals have declined or disappeared (Nowak 1991; Richarz & Limbrunner 1993).

The classification of the Miniopterinae within the family Vespertilionidae has recently been called into question. It has been suggested that the subfamily should be raised to the familial rank of Miniopteridae, and that it is perhaps more closely related to the free-tailed bats, the Molossidae than to the Vespertilionidae (J. Hutcheon, pers. comm.). This is supported by genetic evidence (J. Hutcheon pers. comm.; Hoofer & Van den Bussche 1999), as well as unique dental (Mein & Tupinier 1977), embryological (Gopalakrishna & Chari 1983) and morphological features. For example, the miniopterines possess a well-developed vomeronasal organ (VNO), which is absent or rudimentary in other vespertilionids (Bhatnagar 1980; Cooper & Bhatnagar 1976; Wible & Bhatnagar 1996).

The VNO is believed to be involved in the detection of odour and flavour of food, and may possibly also have a sexual function, playing a role in the detection of pheromones (Cooper & Bhatnagar 1976). In general, bats with a well-developed VNO (considered the primitive condition in chiropterans) are frugivores, nectivores or sanguivores: species that are highly dependent on a well-developed olfactory sense. Insectivorous species, which are more dependent on echolocation than olfaction for detection of prey, generally show the derived condition: a reduced or absent VNO (Cooper & Bhatnagar 1976). *Miniopterus sp.* appear to

be the exception, which may suggest this olfactory organ plays a role in another, unknown behavioural pattern (Cooper & Bhatnagar 1976).

Miniopterus sp. are also unique among vespertilionids in that hibernating bats of this genus synchronise male and female gamete production (Bernard *et al.* 1996). Sperm production and insemination coincide with ovulation and conception, which is followed by a period of delayed implantation of the blastocyst until after the hibernation period (Krutzsch & Crichton 1990; van der Merwe 1979). Delayed implantation has been recorded in *M. schreibersii* (Bernard *et al.* 1996; Dwyer 1963; Richardson 1977; Taylor 2000; van der Merwe 1979), *M. fraterculus* (Taylor 2000), *M. minor* (McWilliam 1988) and *M. australis* (Richardson 1977), but not in any non-miniopterine, hibernating species (Richardson 1977; Strahan 1998). All other hibernating vespertilionids have dysynchronous male and female gametic cycles, during which sperm production and insemination generally occur in late summer, females store sperm during the winter hibernation period, and ovulation and conception occur the following spring (Krutzsch & Crichton 1990; Richardson 1977).

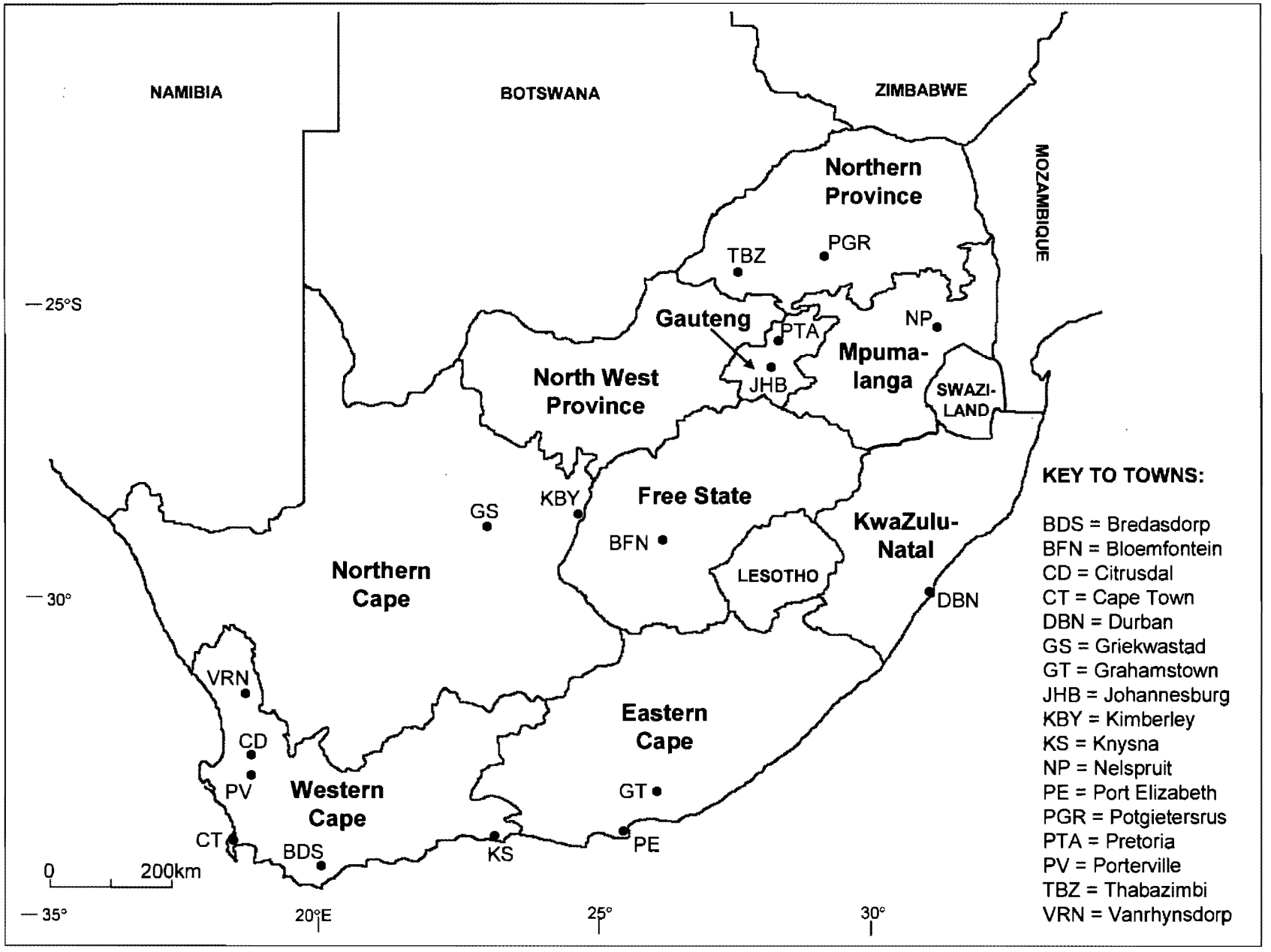

Figure 1.4. Political map of South Africa showing provincial borders and locations of major cities and towns

1.3. MIGRATION PATTERNS AND REPRODUCTIVE CYCLE OF *M. SCHREIBERSII*

1.3.1. Types of population movement

Hill & Smith (1984) recognise two kinds of population movements in bats. The first involves daily, short distance movements between roosts and foraging areas, or movements within and between local roosts. The second type of population movement is a moderate (300 – 500 km) to long-distance (1000 – 1500 km) seasonal migration, undertaken in winter and spring in temperate regions, or in the wet and dry seasons in the tropics and subtropics. Many bat species undertake these seasonal migrations, including members of the genera *Tadarida* (McCracken *et al.* 1994; Svoboda *et al.* 1985), *Pipistrellus* (Webb *et al.* 1996), *Lasiurus* and *Lasionycteris* (Hill & Smith 1984), *Leptonycteris* (Wilkinson & Fleming 1996) and *Nyctalus* (Petit & Mayer 2000). In South Africa, *M. schreibersii* undertake both short-distance, local movements between roosts, as well as moderate to long-distance seasonal migrations between summer and winter roosts (van der Merwe 1975).

1.3.2. Roost selection

Bats select particular roosts for protection against predators, light intensity, varying temperature and humidity (Hill & Smith 1984), the latter two factors being of particular importance (Churchill *et al.* 1997). By selecting appropriate sites, bats are able to decrease the energetic costs of diurnal roosting, as well as satisfy the physiological requirements of both adults and young

(Findley 1993; Hill & Smith 1984). The availability of suitable roosts determines the geographic distribution of bat species. For example, in forested areas of northern Europe and temperate regions of North America, the location of summer bat communities is constrained by the proximity of suitable wintering sites (Findley 1993). Similarly, in South Africa and Australia, *M. schreibersii* are limited to areas that have summer and winter roosts offering suitable microclimates (Mills & Hess 1997; Strahan 1998; Baudinette *et al.* 1994). The selection of suitable roosts, particularly in winter, may significantly affect survival rates (Brown 1999).

i. Maternity roosts

Roosting requirements frequently differ seasonally. During the summer breeding season, *M. schreibersii* females require warm, humid sites (known as maternity roosts) in which to give birth and raise their young. High temperatures reduce the energetic costs of thermoregulation, increasing rates of both pre- and postnatal development (Bernard & Bester 1988; Dwyer 1963; McNab 1982), as well as reducing heat loss from the young while their mothers are absent at night (Dwyer & Harris 1972; McNab 1982).

Maternity roosts are established only at sites with suitably high temperatures (> 20°C), or where the activity of the bats themselves can raise the ambient temperature to levels suitable for juvenile development (Dwyer 1963). Endothermic activity by large numbers of these bats is believed to be the primary factor involved in creating a suitable microclimate for maternity roosts, and has been found to raise the ambient temperature of the roosts by up to 10°C (Baudinette *et al.* 1994; Bernard & Bester 1988; Dwyer 1964; Dwyer & Harris 1972; McDonald *et al.* 1990a). Furthermore, *M. schreibersii* may select maternity roost sites whose geological structure facilitates such modification of the microclimate (Baudinette *et al.* 1994; Dwyer & Harris 1972). Major maternity roosts, such as De Hoop Guano Cave in South Africa and Bat Cave in Australia, are frequently located in enclosed caves or chambers with high domed ceilings, which facilitates heat retention (Baudinette *et al.* 1994; Dwyer & Harris 1972; McDonald *et al.* 1990a).

ii. Hibernacula

Bats need to consume larger quantities of food than terrestrial animals of comparable size, due to their high metabolic rate, high levels of heat loss (because of their small size and highly vascularised wings), and low capacity for fat storage (Strahan 1998). Temperate species are therefore unable to meet the energetic costs of homeothermy during winter, when insect prey abundance is reduced. They therefore either need to migrate to more favourable climates, or temporarily abandon homeothermy and hibernate (Brown & Bernard 1994). Hibernation involves a drop in body temperature to within 1°– 2°C of ambient, as well as decreased oxygen consumption and metabolic rate (Hill & Smith 1984). This results in more than a 90% energy saving compared to homeothermy (Brown 1999). The lower the ambient (and thus body) temperature, the longer hibernation can be continued, and the greater the energy saving (Nagel & Nagel 1991). Hibernating bats therefore select winter roosting sites (known as hibernacula) that have constant low temperatures, as well as high humidity to reduce evaporative water loss (Brown & Bernard 1994; Norton & van der Merwe 1978; van der Merwe 1973a). In addition, hibernacula must offer protection from predators, weather, noise and light (Hill & Smith 1984). Suitable hibernacula may not always be available in the immediate vicinity of maternity roosts. Therefore, even those bats that hibernate may need to migrate to find winter roosts suitable for hibernation.

1.3.3. Mating and hibernation

In South Africa, male and female *M. schreibersii* congregate in wintering colonies or hibernacula between March and May, *i.e.* in late summer and autumn (van der Merwe 1973a, 1973b, 1975). It is not known if male *M. schreibersii* defend territories, but no harem formation has been reported for this species, and it is assumed they follow a typical polygynous mating system.

As has been noted in other temperate bat species, the timing of the *M. schreibersii* reproductive cycle depends on latitude and environmental conditions (Bernard 1980; Happold & Happold 1990; McWilliam 1988; Medway 1971). This species is able to alter the time of copulation, length of gestation and timing of parturition according to seasonal variation in rainfall, temperature and food supply, displaying an adaptive response to climatic change (Bernard 1994; Richardson 1977). Copulation is believed to occur at the wintering colonies during April and May (Bernard *et al.* 1996; van der Merwe 1986). Mating generally occurs earlier at higher latitudes to allow fertilisation to occur before the onset of winter (Bernard 1980; Strahan 1998), but this pattern may be altered by climate. For instance, *M. schreibersii* from the Gauteng and Northern Provinces (~25°S; Figure 1.4) mate one month earlier than those from the more southerly (~29°S) KwaZulu-Natal province (Bernard 1980). This is probably largely due to differences in monthly temperature. KwaZulu-Natal has a subtropical climate, and winter effectively arrives one month later than in the more temperate Gauteng and Northern Provinces (Bernard 1980). Similar variation in the timing of mating according to climatic differences has also been noted in Australia (Dwyer 1963; Richardson 1977; Strahan 1998).

In Australia, *M. schreibersii* have been reported to visit transient mating colonies while *en route* to the hibernacula (Dwyer 1966). These mating colonies form between April and mid-June, when spermatogenesis is at its peak. They consist of large numbers of sexually mature males, who mate with large numbers of transient females. The mating colonies disband after the copulatory period, and both sexes migrate to common hibernacula (Dwyer 1966).

In temperate environments, there is insufficient time during spring and summer for females to initiate and complete a full reproductive cycle (Hill & Smith 1984). Gestation and postnatal development must be completed before autumn to give adult females and juveniles sufficient time to build up resources for the winter hibernation (McNab 1982). Furthermore, birth and lactation must coincide with the limited period of maximum insect abundance (Bernard & Davison 1996; Bernard & Tsita 1995; Happold & Happold 1990; Racey 1982). Temperate bat

species adapt to irregular food supplies by extending their gestation period (Racey 1982) so that each reproductive cycle begins in late summer or autumn, and is temporarily arrested during the hibernation period (Hill & Smith 1984). Most hibernating species accomplish this by means of sperm storage in the female reproductive tract.

Miniopterus sp. are unique among hibernating bats in having a period of delayed implantation rather than sperm storage (Bernard *et al.* 1996; Strahan 1998). Fertilisation immediately follows copulation, and the embryo develops until the blastocyst stage. Thereafter, further development is arrested, and the blastocyst floats freely in the uterine horn for the duration of the hibernation period (Bernard *et al.* 1996). In *M. schreibersii*, the length of the delay increases with latitude, lasting about five months in France (~45°N, the northern limit of this species' range), two to three months at 28°S in Australia and southern Africa, and does not occur at all in the tropics (Bernard *et al.* 1996). This flexibility in the occurrence and length of the delay period has enabled *M. schreibersii* to exploit both temperate (where hibernation is required) and tropical environments (Happold & Happold 1990).

In South Africa, *M. schreibersii* generally begin their hibernation at the beginning of May, and remain in hibernation throughout winter until the end of July or early August (van der Merwe 1973a, 1973b). Implantation occurs shortly before the females emerge from hibernation (Strahan 1998). In both northern and southern latitudes, implantation is believed to be initiated by increasing day length (Racey 1982). It occurs at the same time throughout a particular colony, thus synchronising reproduction (Bernard 1994). Post-implantation development depends on ambient temperature and food availability, the latter being linked to rainfall (Bernard 1994). The period of active foetal growth in *M. schreibersii* is approximately four months (Bernard 1980; Richardson 1977).

1.3.4. Migration to maternity roosts

In late winter or spring the bats leave the hibernacula, and migrate to different roosts located in warmer areas, with an abundance of insects (Mills & Hess 1997; van der Merwe 1973c, 1975). Pregnant females congregate at maternity roosts, where they each give birth to a single altricial young (Dwyer 1963). Births generally occur within a period of one month, between October and December, depending on the location of the colony. Colonies in Gauteng and the Western Cape province of South Africa (Figure 1.4), as well as in Zimbabwe (~18°S), give birth from late October to mid-November (Bernard *et al.* 1996; Herselman & Norton 1985; van der Merwe 1975), while those in the Eastern Cape province of South Africa give birth in late November and early December (Bernard 1994). The difference in timing of births could be due to variation in rainfall pattern, which affects insect abundance (Bernard *et al.* 1996). Food availability in turn influences maternal body temperature, which determines the rate of post-implantation development and thus timing of the births (Bernard 1994). This was illustrated in 1990 when the November rains failed in the Eastern Cape, and the bats gave birth a month later than usual, possibly due to a reduction in foetal growth rate and concomitant extension of the gestation period (Bernard 1994). Plasticity in timing of parturition has also been noted for *M. schreibersii* in Australia. These bats synchronise births to occur within a month, generally in December (Dwyer 1963), although at certain colonies parturition time varies between October and December (Richardson 1977).

Maternity roosts may be located 250 km or more from the hibernacula (Mills & Hess 1997; Herselman & Norton 1985). Banding and recapture studies conducted in South Africa (van der Merwe 1973b, 1975) have shown that female *M. schreibersii* that hibernate near Pretoria in the cool, dry Gauteng province (Figure 1.4) migrate to summer maternity roosts located near Thabazimbi and Potgietersrus in the warmer, wetter Northern Province. These migrations may be made via transient pre-maternity caves. Seasonal migrations of up to 250 km have also been

reported in other regions of South Africa, such as to and from the maternity roost at De Hoop Guano Cave, near Bredasdorp (Figures 1.4 and 2.1, Chapter 2) in the Western Cape province (Herselman & Norton 1985; Taylor 2000). In the Eastern Cape province, however, movements between winter and summer roosts occur on a much smaller scale. At Maitland Mines near Port Elizabeth (Figures 1.4 and 2.1, Chapter 2), the bats undertake short movements (<1 km) between three adjacent mine tunnels (Bernard & Bester 1988). At the end of summer (April – May), one tunnel acts as a nursery roost for juveniles, while adults use another as a mating roost. In late May, the bats move to the third tunnel, which is used as a hibernaculum. Because the microclimate within this third tunnel differs in summer and winter, the bats remain in this roost after hibernation and also use it as a maternity roost in summer (December – February).

Migration patterns followed by male *M. schreibersii* are less clear than females. At the end of winter, most males that hibernate with the females in the Gauteng hibernacula, for example, depart with the females. Some migrate to the same colonies the females use as maternity roosts in the Northern Province, but the majority depart before the females give birth, and their destinations thereafter are unknown (van der Merwe 1975). It is presumed they stay at other unknown caves in this region. Other males do not migrate, but remain at the hibernacula, awaiting the return of the females in autumn (van der Merwe 1975). In most cases, therefore maternity colonies are characterised by a predominance of pregnant or lactating females, and few males and non-pregnant females. At Sandspruit Cave in the Northern Province, for example, van der Merwe (1973b) estimated there were about 110 000 females at the height of the breeding season, 95% of which were reproductive. Only ~1.5% of the adult bats in this maternity cave were males. However, this is not true of all maternity roosts. Palmeirim & Rodrigues (1995), for instance, recorded significant numbers of males in *M. schreibersii* maternity colonies in Portugal. Several of the maternity colonies visited in the present study also contained large numbers of males (Chapter 2).

Hill & Smith (1984) suggest that, when it occurs, segregation of the sexes during pregnancy, birth and rearing of young may reduce competition for available food resources, giving nursing females exclusive access to foraging areas near the maternity colony. This may be of particular importance for *M. schreibersii,* as the number of females at maternity roosts is generally much higher than at the hibernacula. Van der Merwe (1973b, 1973c), for example, reported that while 1000 to 6000 individuals roost at hibernacula in Gauteng, between 50 000 and 100 000 individuals (predominantly females) may be found at the maternity caves in the Northern Province. Similarly, up to 300 000 *M. schreibersii* have been recorded during summer at De Hoop Guano Cave in the Western Cape, while numbers drop to ~20 000 in winter (McDonald *et al.* 1990a, 1990b; Mills & Hess 1997). Although maternity colonies are located in areas with higher insect abundance than the hibernacula (van der Merwe 1973c), and so can support much larger numbers of individuals, the additional presence of males may lead to excessively high levels of competition.

Maternity colonies are equally large elsewhere in this species' distribution. Up to 200 000 *M. schreibersii* congregate at Bat Cave near Naracoorte, South Australia during the breeding season (Baudinette *et al.* 1994), and average densities of 2000 bats per square metre of cave surface have been reported at maternity roosts in New Guinea (Hill & Smith 1984). Sizes of maternity colonies in Portugal range between 1000 and 20 000 individuals (Palmeirim & Rodrigues 1995). The gregarious nature of these bats may reflect a high degree of sociality and sophisticated communication. Alternatively, there may be no underlying social structure, and gregariousness may simply be the result of limited roosting sites (Fenton 1985), or an adaptation to allow thermal modification of the maternity cave, as discussed above.

Most temperate and a few tropical bat species congregate in unisexual maternity roosts to give birth and raise their young. Maternity colonies numbering in the thousands to hundreds of thousands are also common among other bat species, including members of the genera *Rhinolophus*, *Pipistrellus* and *Myotis*, and particularly among members of the family Molossidae

(Nowak 1991). The Mexican free-tailed bat, *Tadarida brasiliensis mexicana* holds the record for the largest mammalian aggregations in the world. An estimated 20 million of these bats congregate at Bracken Cave in Texas, United States of America each summer (Dwyer 1966; Nowak 1991).

One reason *M. schreibersii* maternity roosts are so large is that they frequently act as focal points for several different hibernacula. Van der Merwe (1973b, 1975) found that the majority of females that over-wintered at three separate hibernacula in Gauteng (Figure 1.4) migrated to a common maternity roost in the Northern Province in summer. The maternity colony in the Maitland Mines system, in the Eastern Cape is also believed to act as a central roost for a number of satellite colonies in the region (Bernard & Bester 1988). Dwyer (1966, 1969) similarly reported that *M. schreibersii* maternity caves in Australia (*e.g.* Bat Cave) act as focal points for particular sets of non-maternity colonies within a region. The same is true in other parts of the *M. schreibersii* range: a maternity colony at Mahalleshwar, India, for example, is believed to service an area of ~15 000 km^2 (Hill & Smith 1984).

Maternity caves generally start to break up towards the end of summer. In the Northern Province of South Africa, adult females begin leaving the maternity caves in February and migrate back to the Gauteng hibernacula, where they rejoin the males (van der Merwe 1975). Maternity colonies in Australia also disband between February and March, with adults generally departing before the juveniles (Dwyer 1963; Strahan 1998).

1.4. SITE ATTACHMENT IN *M. SCHREIBERSII*

1.4.1. Banding and recapture studies

Roost fidelity is well developed in *M. schreibersii.* Banding studies conducted by van der Merwe (1973b, 1975) in South Africa indicated a strong tendency for males and females to return in successive years (1967 – 1974) to the hibernaculum or maternity roost here they were originally banded. For example, between 40% and 96% of females recaptured each year at Peppercorn Cave (one of the sample sites used in the present study; Figure 2.1, Chapter 2) were originally banded at this maternity cave (van der Merwe 1975). Laycock (1976) conducted a homing study on *M. schreibersii* during which she captured individuals at maternity caves in KwaZulu-Natal (Figure 1.4) and released them from the nearby town, Pietermaritzburg. She found that over 70% of recaptured females returned to the cave from which they were originally captured.

Similarly high levels of site attachment have been found for this species in Australia. Dwyer (1966) found that colonies of *M. schreibersii* in New South Wales were also relatively permanent in terms of the individuals comprising them (specific roosts served specific social groups), and that this site attachment persisted from year to year. He found that females from particular hibernacula migrated to specific maternity colonies each year to give birth and rear their young, and concluded that the social organisation of this species consists of relatively stable groups attached to a fixed roosting site.

1.4.2. Roost fidelity or natal philopatry?

It is not known whether the strong site attachment displayed by *M. schreibersii* in South Africa (van der Merwe 1973b, 1975; Laycock 1976) represents natal philopatry in one or both sexes, or merely a high level of fidelity to a new roost, developed, for example, after juvenile dispersal from the natal colony. Philopatry may be defined as the tendency for an animal to remain and breed within its natal or home area, and, in the case of migrants, to return to it in successive years (Thompson 1992). Dispersal, on the other hand, involves the permanent movement of an animal away from its natal area, generally leading to the establishment of a new home area in which that animal attempts to breed (Dobson 1982; Palmeirim & Rodrigues 1995). Dwyer (1966) recorded long-distance dispersal by *M. schreibersii* juveniles (up to 300 km), and found a greater tendency for young bats to move between the subpopulation ranges adhered to by adults. He found no differences in site attachment between females of different ages, but noted that roost fidelity in males tended to increase with age.

However, Dwyer (1966) also noted that although juveniles initially appeared to disperse by temporarily visiting a variety of roosts during their first year, they frequently failed to establish themselves in a new adult colony, and returned to their natal colonies as yearlings. Furthermore, Palmeirim & Rodrigues (1995) recorded very low rates of juvenile *M. schreibersii* dispersal in Portugal, and found that all banded and recaptured females returned to their natal maternity colony to give birth. As described above, males also occurred in the Portugese maternity colonies, and more than 90% of ringed males were recaptured in the colony where they were born. Dwyer (1966) and in particular Palmeirim & Rodrigues' (1995) studies suggest that the strong site attachment displayed by *M. schreibersii* may represent natal philopatry (possibly in both sexes), and not merely fidelity to a new roost, developed after juvenile dispersal.

As described above, it is not possible to determine from existing banding studies (van der Merwe 1973b, 1975; Laycock 1976) whether the high levels of site attachment displayed by *M. schreibersii* in South Africa are similarly indicative of natal philopatry or merely adult roost fidelity. However, this may be assessed by means of genetic analysis. Restricted movement as a result of natal philopatry should limit gene flow between roosts, which could lead to the establishment of discrete breeding subpopulations with marked genetic differences. On the other hand, dispersal, if coupled with successful breeding in new areas, is a major source of gene flow between populations, and may reduce genetic differentiation between those populations (Greenwood 1980; Hewitt & Butlin 1997). Examination of the genetic structure of the South African *M. schreibersii* population should therefore provide an indication of historical levels of gene flow within and between *M. schreibersii* colonies, which may be used to assess levels of philopatry and/or dispersal.

1.5. STUDY AIMS

To date, no genetic studies have been conducted on *M. schreibersii* in South Africa, and those conducted elsewhere in the species range (*e.g.* Australia; Cardinal & Christidis 2000) have focussed on taxonomic issues rather than on population structure or gene flow. As discussed above, although behavioural and ecological studies indicate that site attachment is well developed in this species, the degree of natal philopatry and dispersal displayed by either sex in South Africa is unknown. Furthermore, almost nothing is known about the genetic structure of the South African *M. schreibersii* population, or about the genetic relatedness and levels of gene flow within and between the major colonies.

The primary aims of this study, therefore, were:

1. to determine whether the high levels of site attachment displayed by *M. schreibersii* in South Africa have led to significant genetic substructuring of the population;
2. if significant population subdivision is found, to establish whether this is the result of natal philopatry in one or both sexes;
3. if significant population substructure is found, to use this information to determine the extent and direction of bat movement around the country.

This information is essential to the formulation of an effective conservation plan to halt the marked decline in populations of this bat.

CHAPTER 2

SAMPLING METHODS

2.1. LOCATION OF SAMPLE SITES

2.1.1. *Miniopterus schreibersii* sample sites within South Africa

In total, 316 *Miniopterus schreibersii* were sampled in South Africa. The majority of these samples (n = 307) were collected from ten major colonies (Figure 2.1 and Table 2.1). Die Hel (DHL), Jozini Dam (JD), Peppercorn (PC) and Sudwala (SW) are used exclusively as maternity roosts. Shongweni Dam (SHD) is used as a summer roost by males, and as a transient, pre-maternity roost by pregnant females *en route* to an as yet unidentified maternity site (P. Taylor, pers. comm.). Steenkampskraal (SKK) is used exclusively as a hibernaculum. The remaining colonies, De Hoop (DHP), Grahamstown (G), Koegelbeen (KB) and Maitland Mines (MM), are occupied throughout the year, and are used as both maternity roosts and hibernacula.

In addition to the ten major colonies listed above, a small number of *M. schreibersii* samples were also obtained from the following locations in South Africa (abbreviations and geographical locations are given in brackets):

1. Pongola River Bridge (PRB; 27°1'S; 32°16'E) in the Pongola Biosphere Reserve. Skin biopsies were taken on-site from a female found underneath this bridge. Dr Peter Taylor collected an additional five specimens (four females; 1 male) from this site for the Durban Museum (collection numbers DM5726 – DM5730), and kindly supplied muscle biopsies from these individuals.

2. Bedingo Mine, Knysna (K; 34°02'S; 23°03'E). Two males were collected by Mr Wolfgang Lück from Stellenbosch University, and were donated to Dr David Jacobs.

3. Algeria Forestry Station, near Citrusdal in the Cedarberg mountain range (C; 32°22'S; 19°03'E). A single female was captured in a mist net by Dr David Jacobs.

2.1.2. *Miniopterus schreibersii* samples from international locations

Thanks to the generosity of several overseas workers, fourteen *Miniopterus schreibersii* samples were obtained from locations outside of South Africa (Table 2.2). These locations were Madagascar (four *M. s. manavi*), Australia (three *M. s. bassanii* and three *M. s. orianae*) and Israel (four *M. s. pallidus).*

2.1.3. *Miniopterus fraterculus* samples

One female *Miniopterus fraterculus* sample was collected from Shongweni Dam. An additional 13 *M. fraterculus* were identified by means of genetic analysis (Chapter 7) from the samples collected from Die Hel (n = 1), Maitland Mines (n = 3), Peppercorn (n = 1) and Sudwala (n = 8).

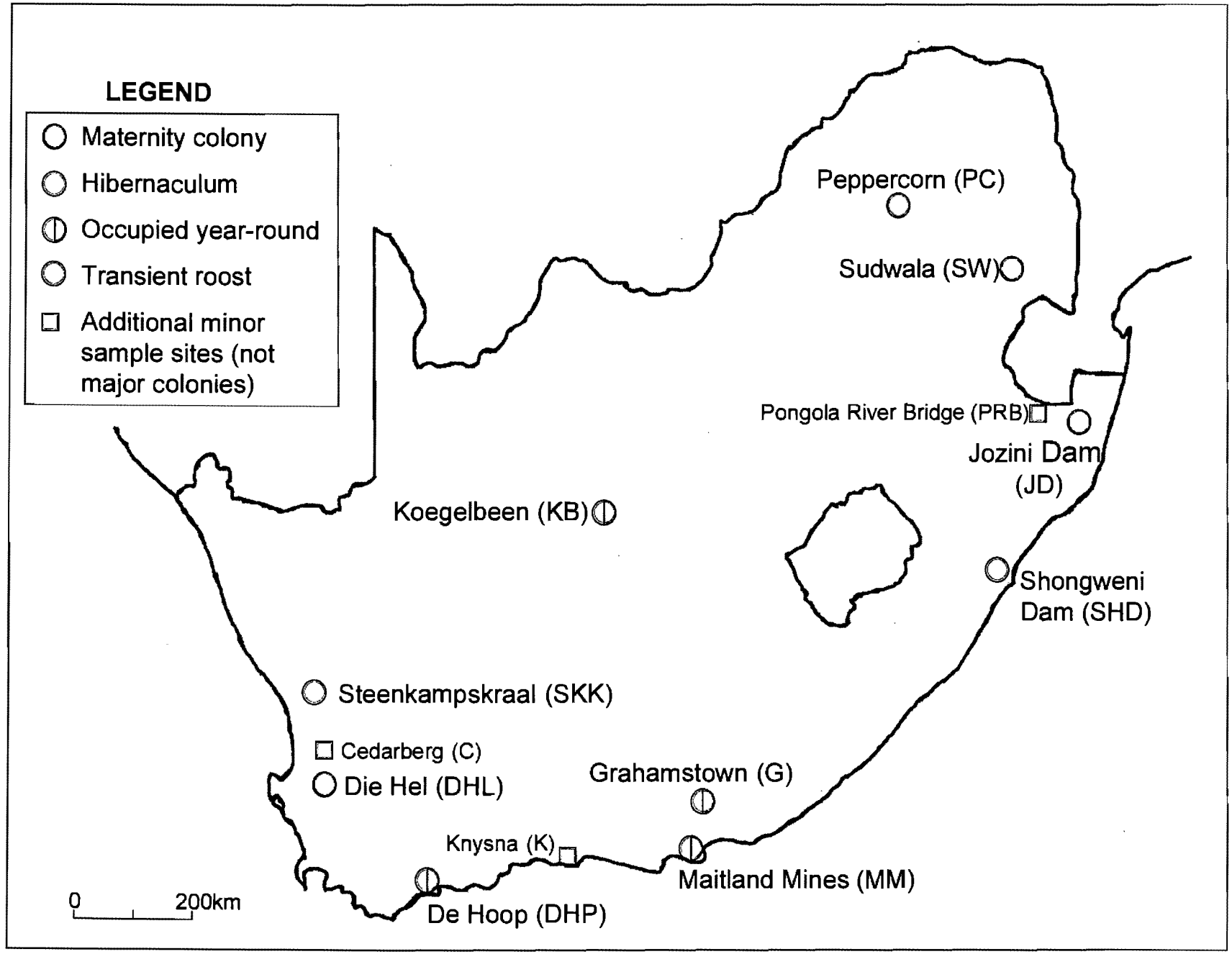

Figure 2.1. Locations of major *M. schreibersii* colonies sampled during this study, and of additional minor sample sites in South Africa.

Table 2.1. *Miniopterus schreibersii* colonies sampled in South Africa from 1997-1999. Major towns near the sample sites are shown on Figure 1.4, Chapter 1.

Colony name (Abbreviation)	Location	Description of colony site	Type of colony	Nº sampled (♀= females; ♂ = males)	Estimated total sex ratio (♀ : ♂)	Date sampled
De Hoop (DHP)	34°25′S; 20°21′E	"De Hoop Guano Cave" in De Hoop Nature Reserve, near Bredasdorp	Maternity roost & hibernaculum	44 (24 ♀; 20 ♂)	Unknown	January 1998
Die Hel (DHL)	33°05′S; 19°05′E	"Die Hel Cave", near Porterville	Maternity roost	19 (4 ♀; 15 ♂)	Unknown	November 1997 (collected by Dr D. Jacobs)
Grahamstown (G)	33°17′S; 26°31′E	70 m long water supply tunnel (1 x 1.5 m) on Table Farm, ~10 km outside Grahamstown	Maternity roost & hibernaculum	37 (17 ♀; 20 ♂)	1.5 : 1	September 1998
Jozini Dam (JD)	27°25′S; 32°4′E	Disused inspection tunnel in the Jozini Dam (also known as the Pongolapoort Dam)	Maternity roost	29 (20 ♀: 9 ♂)	3 : 1	November 1997
Koegelbeen (KB)	28°39′S; 23°20′E	Sinkhole located ~25 km from Griquastad, near Kimberley	Maternity roost & hibernaculum	40 (20 ♀; 20 ♂)	1 : 2.5	November 1998
Maitland Mines (MM)	33°59′S; 25°17′E	Abandoned copper and lead mine near Port Elizabeth	Maternity roost & hibernaculum	37 (20 ♀; 17 ♂)	1.5 : 1	September 1998
Peppercorn (PC)	24°08′S; 29°12′E	"Peppercorn Cave", Makapansgat, near Potgietersrus	Maternity roost	19 (10 ♀; 9 ♂)	1.5 : 1	November 1999
Shongweni Dam (SHD)	29°52′S; 30°43′E	Overflow tunnel of Shongweni Dam, near Durban	Male summer roost & pre-maternity roost for pregnant females *en route* to unknown maternity site	29 (14 ♀; 15 ♂)	1 : 3	November 1997
Steenkampskraal (SKK)	31°36′S 18°45′E	Abandoned monosite mine, near Vanrhynsdorp	Hibernaculum	20 (8 ♀; 12 ♂)	Unknown	July 1997 (collected by Mr R. Louw)
Sudwala (SW)	25°22′S; 30°42′E	"Miggiesgat cave" located adjacent to Sudwala Caves, near Nelspruit	Maternity roost	33 (15 ♀; 18 ♂)	2.5 : 1	November 1999

Table 2.2. *Miniopterus schreibersii* samples from outside South Africa

Species	Country of origin	Location of colony	Nº of samples	Collector
M. s. manavi	Madagascar (south)	Ranomafama	2 (sex unknown)	James Hutcheon
M. s. manavi	Madagascar (north)	Ankarana	2 (sex unknown)	James Hutcheon
M. s. pallidus	Israel	Alma Cave, Northern Galilee	4 ♀	Beni Shalmon, Didi Kaplan & Brock Fenton
M. s. bassanii	Australia (south)	Naracoorte	2 ♀; 1 ♂	Belinda Cardinal
M. s. orianae	Australia (Northern Territories)	• Darwin • Kakadu National Park • Batchelor	1 ♂ 1 ♂ 1 ♂	Belinda Cardinal

2.2. SAMPLING PROCEDURE

(South African specimens)

2.2.1. Capture of bats

In most cases, colonies were entered during the day, and roosting bats were captured by means of a large hand-net. At De Hoop, Koegelbeen and Sudwala, bats were also captured at night with Tuttle traps (Tuttle 1974) erected at the entrance to the caves. With the exception of those collected from Steenkampskraal, bats were placed temporarily in individual cloth bags, and were released back into the colony as soon after processing as possible.

Bats from Steenkampskraal were euthenased with Halothane. These bats were collected from the mine by Mr R. Louw and were delivered to the Zoology Department at the University of Cape Town. Construction of a genomic library for *M. schreibersii* requires a starting quantity of at least 50 μg DNA, which was not obtainable from skin biopsies (see below). Heart and abdominal muscle were therefore dissected out of the euthenased bats from Steenkampskraal, chopped finely, and stored in 1 ml extraction buffer (Appendix 1) prior to DNA extraction. The genomic library was subsequently constructed using DNA from these bats as the starting material (Chapter 3). After dissection, whole bat specimens were stored at -70°C.

2.2.2. Biological data and morphological measurements

Age (adult or juvenile), sex and reproductive status (*i.e.* whether or not females were pregnant) were determined for each bat, and the total sex ratio in the colony was estimated (Table 2.1). Mass (in grams) and forearm length (in millimetres) were also measured. Wing tracings were obtained for each bat by tracing the outline of the extended right wing, according to the method described in Saunders & Barclay (1992). Alternatively, the wing was photographed with a digital camera (Olympus, model C-800L). Wing tracings were obtained from the Steenkampskraal bats prior to euthanasia, as the extended wing may easily be distorted after death. Wing tracings and digital photographs were analysed as described in Chapter 6, Section 6.2.2.

2.2.3. Skin biopsies

All bats, other than those collected from Steenkampskraal, were sampled non-destructively. Two 3 mm diameter biopsies were taken from the tail membrane (uropatagium) of each bat using a sterile medical biopsy punch (Figure 2.2), according to the method described by Worthington Wilmer & Barratt (1996). This technique does not appear to have any adverse effects on the health of the bats, as the holes heal quickly and do not impair flight ability (Petri *et al.* 1996; Worthington Wilmer & Barratt 1996). Each bat was held firmly against a cork clipboard, and the tail membrane was spread out flat, to minimise the area to be biopsied, and to ensure blood vessels were as visible as possible. A biopsy was taken on each side of the tailbone, in areas with no visible blood vessels. The skin was swabbed with 70% ethanol before the biopsy was taken, and *Cicatrin* antiseptic powder was applied to the biopsy area afterwards. Skin biopsies were immediately placed in 500 µl extraction buffer (Appendix 1).

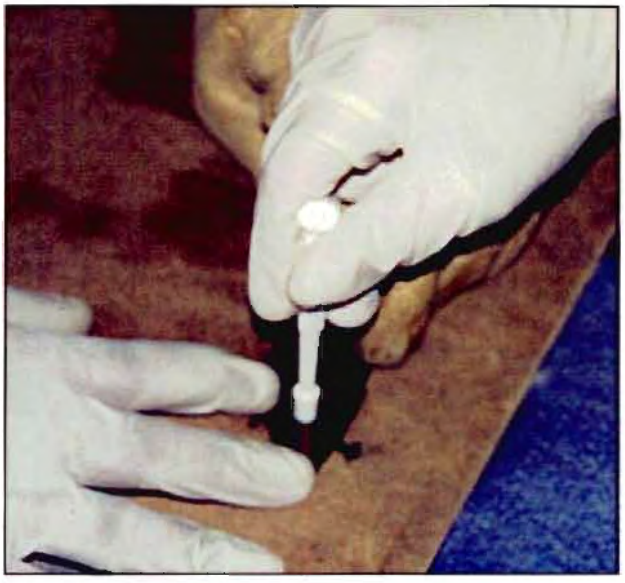

Figure 2.2. (Taking a skin biopsy from the uropatagium of a *Miniopterus schreibersii* specimen.

2.3. EXTRACTION AND QUANTIFICATION OF DNA

2.3.1. Extraction of DNA

Skin or tissue samples stored in extraction buffer were incubated overnight at 56°C with 60 µl proteinase-K (20 mg/ml; Roche Diagnostics, Cape Town) per 500 µl extraction buffer, to digest proteinaceous material. Deoxyribonucleic acid (DNA) was extracted by a standard phenol-chloroform extraction protocol, as described in Ausubel *et al.* (1994) and Sambrook *et al.* (1989). After ethanol-precipitation, DNA extracted from skin biopsies and muscle dissections was resuspended in 100 µl or 200 µl respectively 1x Tris-EDTA (TE) buffer pH 7.4 (Appendix 1) and stored at -20°C.

2.3.2. Quantification of DNA

The concentration of DNA in the extracted samples was estimated either through agarose gel electrophoresis, or by spectrophotometry.

i. Agarose gel electrophoresis

Five to ten microlitres of the extracted DNA sample were electrophoresed through a 1% agarose gel containing 0.5 µg/ml ethidium bromide. Ethidium bromide binds to DNA and fluoresces under UV light, and the intensity of fluorescence is proportional to the total mass of DNA (Ausubel *et al.* 1994). The amount of DNA in the sample was estimated by comparing the intensity of the sample fluorescence to that of a marker of known concentration, namely Lambda DNA, digested with either *Dra*I or *Hind*III.

ii. Spectrophotometry

Absorbance readings (λ) of 1 in 20 dilutions of each extracted DNA sample were measured with a diode-array spectrophotometer, at wavelengths of 260 nm and 280 nm. An estimate of the purity of DNA was obtained by calculating the ratio of the two absorbance readings (λ280 nm / λ260 nm): pure preparations of DNA should have λ280 nm / λ260 nm readings of ≥ 1.8 (Ausubel *et al.* 1994). Ratios obtained from extracted skin biopsies ranged from 1.3 to 2.3. The concentration of DNA (in ng/µl) was calculated from the absorbance reading at 260 nm according to the equation:

Concentration of DNA (ng/µl) = λ260 nm x 50 x dilution factor

The concentration of DNA in the extracted skin biopsy samples ranged from 10 – 50 ng/µl. To prevent contamination of the original samples, and to avoid numerous cycles of defrosting and refreezing, which could damage the DNA, 15 µl diluted (1 in 5) aliquots of each sample were prepared and stored at 4°C for regular use. These aliquots were replenished as required.

3.2.2. Cutting the DNA with restriction enzymes

Aliquots of DNA from the eight Steenkampskraal bats were pooled and partially digested with three restriction enzymes, *Alu*I, *Rsa*I and *Hae*III (Figure 3.1, Step 2), manufactured by Amersham Life Science or Promega (supplied by Amersham Pharmacia Biotech, Cape Town or Whitehead Scientific, Cape Town respectively). These enzymes recognise and cleave DNA at specific nucleotide sequences that display two-fold symmetry (palindromic sequences, *e.g.* GGCC or AGCT). They cut both DNA strands at the same point, at the axis of symmetry, yielding restriction fragments that are blunt-ended. Other restriction enzymes cut each strand at similar positions on opposite sides of the axis of symmetry, producing a staggered cut with either a 3' or 5' overhang. The resulting restriction fragments are said to have cohesive or "sticky" ends. *Alu*I cuts between the G and C in the sequence AGCT, *Rsa*I between the T and A in the sequence GTAC and *Hae*III between the G and C in GGCC (Brown 1995). Digests were performed in 100 µl reactions, containing 80 µl pooled bat DNA (~80 µg), 1 µl each of *Alu*I, *Rsa*I and *Hae*III (each at 10 U/µl), 10 µl 10x enzyme buffer (supplied with the enzymes by the manufacturer) and 7 µl sterile, deionised water. The digest was incubated at 37°C until more than 90% of the restriction fragments were less than 1000 bp in length.

The optimal duration of the digest was initially determined by removing 2 µl samples from the digest every five to ten minutes. These samples were electrophoresed through a 1% agarose gel against a size marker. Two size ladders were used: (1) Bacteriophage Lambda (λ) DNA (Amersham Life Science) digested with *Hind*III (λ*Hind*III), which recognises the restriction site AAGCTT, and produces fragments of 23 130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bp; (2) Lambda DNA digested with *Dra*I (λ*Dra*I), which recognises the restriction site TTTAAA and produces fragments of sizes 8596, 8360, 7834, 6816, 6038, 3599, 2303, 2152, 1075, 696, 533, 174 and 90 bp. Cleavage for approximately 30 minutes was found to be optimal, at which time digestion was stopped by incubation at 65°C for 15 minutes.

3.2.3 Selection of fragments of the required size (size selection)

Restriction fragments were electrophoresed at 100 V through a 1% agarose gel containing ethidium bromide (1 µl per 100 ml agarose), against a λ*Hind*III marker, and visualised under ultraviolet (UV) light (Figure 3.1, Step 3). Fragments of between 200 bp and 600 bp in length were extracted from the gel (see below). Care was taken to minimize exposure of the DNA to UV radiation, to avoid damage to the DNA strands. The concentration of the size-selected DNA was estimated by comparing the intensity of its fluorescence under UV light to that of the 564 bp band of the λ*Hind*III size marker (when 6 µl of 0.3 µg/µl λ*Hind*III is loaded onto the gel, the 564 bp band contains ~21 ng DNA).

Fragments of the required size were extracted from the gel by means of electro-elution onto dialysis tubing. Two small slits, each 1 – 2 mm in diameter, were cut into the gel across the lane containing digested DNA. The slits were positioned at approximately 200 bp and 600 bp, as judged by the λ*Hind*III marker. Dialysis tubing of diameter 10 mm was cut to size (~1 cm x 2 cm), inserted into each slit, and gentle pressure applied to ensure contact was made between the gel and the tubing. Electrophoresis at 100 V was continued for approximately 15 minutes, until all visible DNA in the required size range had migrated onto the dialysis tubing at the 200 bp position. This piece of tubing was then immediately placed in 100 µl 1x TE buffer (Appendix 1), and left at 4°C overnight to allow the DNA to elute off the membrane into the solution. Prior to electro-elution, the dialysis tubing was sterilised by boiling in 1x TE buffer for 10 minutes. The electro-elution procedure is described in more detail in Sambrook *et al.* (1989).

To ensure that the molecular grade agarose (Whitehead Scientific) used in electro-elution of the insert DNA did not contain any inhibitors which might affect future ligations, the 6557 bp and 9416 bp fragments of a λ*Hind*III ladder were individually electro-eluted out of the gel, pooled and ligated with bacteriophage T4 DNA ligase (Promega). The ligation of these fragments was

successful: a band larger than the original fragments was visible after electrophoresis through a 1% agarose gel. This confirmed that the agarose did not contain any ligation inhibitors.

3.2.4. Cloning the DNA using the pUC18 cloning vector

The bacterial plasmid, pUC18 (Roche Diagnostics, Cape Town) was used as a cloning vector in this study (Figure 3.2). Plasmids are circular DNA molecules, which are independent of the bacterial chromosome, and are capable of self-replication. In nature, plasmids frequently carry genes for resistance to antibiotics, heavy metals or bacteriophages, or genes for production of restriction enzymes, rare amino acids etc. (Ausubel *et al.* 1994). Many plasmids have been engineered for use as cloning vectors. These vectors have three major features: a cloning site, a replicator and a selectable marker (Ausubel *et al.* 1994). The cloning site is the site into which foreign DNA can be inserted. It contains multiple recognition sites for restriction enzymes, and hence is also referred to as the restriction site bank or polycloning site. The polycloning site in pUC18 (Figure 3.2) contains cleavage sites for 13 restriction enzymes (Sambrook *et al.* 1989).

Self-replication is enabled by a replicator, which is a stretch of DNA containing the origin of replication site (or *ori*) and other genes that code for the RNAs and proteins required for replication. The selectable marker is usually a dominant gene that encodes resistance to an antibiotic (Ausubel *et al.* 1994). The pUC18 plasmid contains the selectable marker gene, amp^r, which confers ampicillin resistance upon bacteria. It codes for the enzyme β-lactamase, which inactivates ampicillin (Sambrook *et al.* 1989), allowing bacteria that contain pUC18 to grow on Luria Broth plates treated with this antibiotic (hereafter referred to as amp+ LB plates). Plasmids such as pUC18 also carry a gene known as *lacZ'*, derived from the *lac* operon of *Escherichia coli* (*E. coli*). This gene can be utilised in screening procedures, as described in Section 3.2.8 below (Sambrook *et al.* 1989).

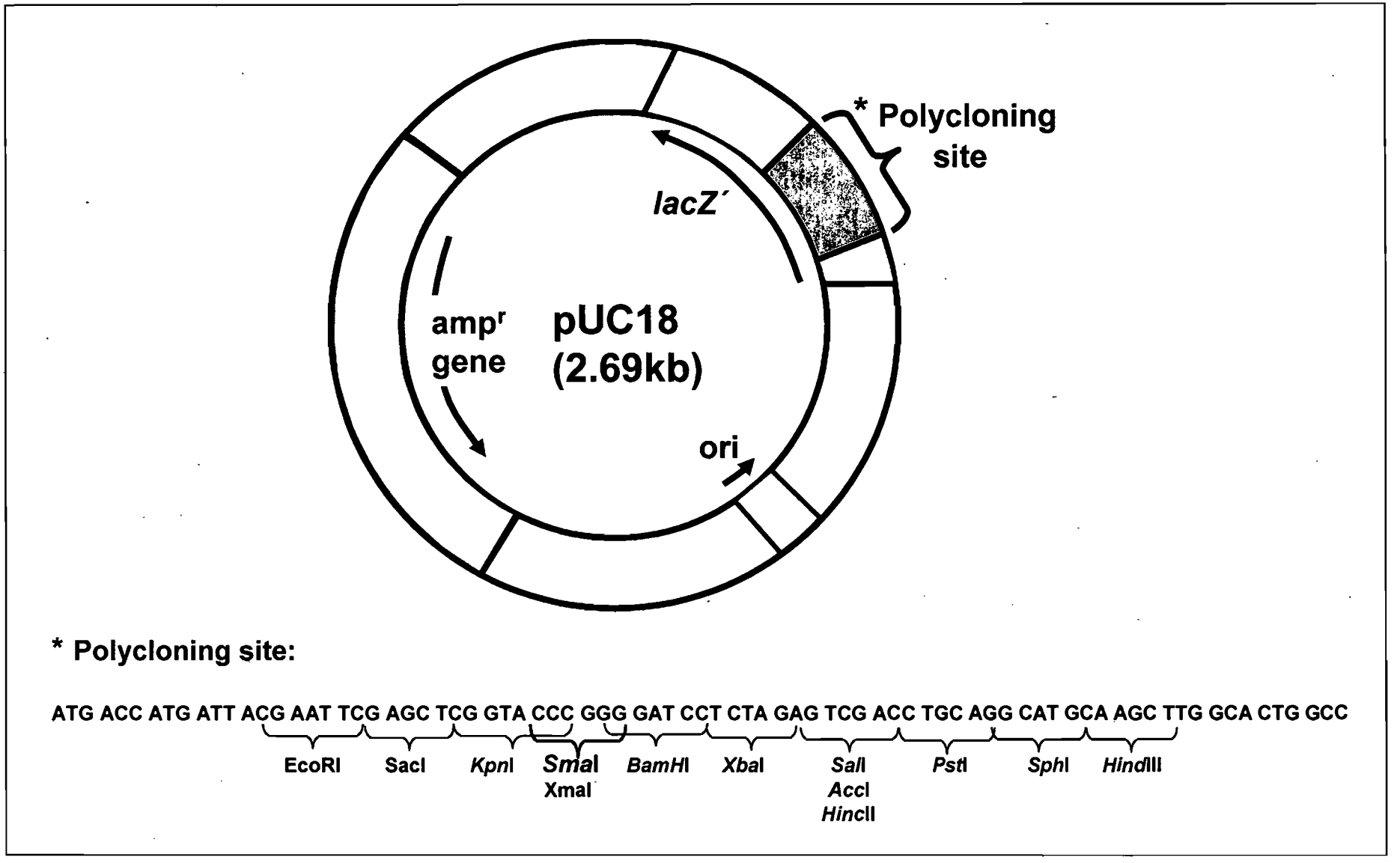

Figure 3.2. Schematic diagram of the plasmid vector, pUC18. Modified from Ausubel *et al.* (1994) and Sambrook *et al.*(1989) .

3.2.5. Digestion of pUC18 with *Sma*I

Prior to ligation with size-selected DNA, pUC18 was digested with the restriction enzyme, *Sma*I, manufactured by either Amersham Life Science or Promega (Figure 3.1, Step 4). Like the three restriction enzymes used to digest the bat DNA, *Sma*I also yields blunt ends, and cuts the plasmid at a single recognition site (between the C and G in the sequence CCCGGG; Figure 3.2) within the polycloning site (Sambrook *et al.* 1989). Digests of pUC18 were carried out in 12 µl reactions containing 10 µl undigested pUC18 (0.25 µg/µl), 1 µl 10x enzyme buffer (supplied by the enzyme manufacturer) and 1 µl *Sma*I (8 U/µl). Digests were incubated at 37°C for one hour, and then stopped by incubation at 70°C for 15 minutes.

To determine the efficiency of the digestion, pUC18 that had been digested with *Sma*I (hereafter referred to as *Sma*I-pUC18) was electroporated into *E. coli* (please refer to Section 3.2.7 below for a detailed description of electroporation and the protocol followed). The efficiency of this electroporation was compared to that of:

i. *Sma*I-pUC18 incubated with 1 U T4 DNA ligase (3 U/µl), which allows the plasmids to re-circularise (hereafter referred to as ligated *Sma*I-pUC18)
ii. undigested pUC18: the control

In circumstances (i) and (ii), the plasmids taken up by the bacteria would be intact, and should therefore supply the cells with the amp^r genes necessary for growth on amp+ LB plates. Thus a high concentration of bacterial colonies should be found on both these plates. On the other hand, *E. coli* that are transformed with *Sma*I-pUC18 (without the presence of ligase) should not be able to grow on amp+ LB plates because the plasmid is cut open by *Sma*I, *i.e.* linearised, and therefore non-functional. Very few colonies would therefore be expected to grow on these plates if the linearisation (*i.e.* digestion by *Sma*I) is complete. The efficiency of the electroporation, in terms of the number of colony forming units (CFU) able to grow on amp+ LB plates, can therefore be used

as an indication of the efficiency of the digestion of pUC18 by *Sma*I (Table 3.1). The electroporation efficiency (also known as the competency of the electroporated cells) was calculated according to the following equation:

$$Colony\ forming\ units\ (CFU)\ /\ \mu g\ DNA = \frac{\left(\frac{No\ of\ colonies\ on\ plate}{pg\ DNA\ transformed}\right) \times 10^6}{Percent\ of\ total\ transformation\ plated}$$

In all cases, 50 µl of a 1 ml solution of electroporated *E. coli* was grown overnight at 37°C on amp+ LB plates.

Table 3.1. Efficiency of digestion of pUC18 by *Sma*I.

Type of plasmid used	Amount of plasmid DNA used in electroporation	Number of colonies on plate	Electroporation efficiency (CFU)
*Sma*I-pUC18	20 ng	~ 850	~ 8.5 x 10^5
Ligated *Sma*I-pUC18	8 ng	~1400	~ 3.5 x 10^6
Undigested pUC18	5 ng	Lawn (uncountable)	–

Although the transformation efficiency of *Sma*I-pUC18 was considerably lower than that of ligated *Sma*I-pUC18 or of undigested pUC18, it was still high, suggesting that the enzyme had not digested all the plasmid. The presence of any uncut vector would increase the background of colonies that lack insert DNA (Sections 3.2.6 and 3.2.8 below). Although it is possible that the enzyme was inhibited by high glycerol concentration (because the volume of enzyme, which is supplied in a glycerol solution, was greater than 5% of the total reaction volume), an additional 1 µl *Sma*I (8 U/µl) was added to the existing pUC18 digest, and incubated for one hour at 37°C. The digested product was electrophoresed through a 1% agarose gel with a λ*Hind*III marker. Linear (digested) and circular (undigested) DNA molecules migrate at different speeds during

electrophoresis, and one can therefore distinguish one from the other by their position on the gel (Harley & White 1973; Sambrook *et al.* 1989). Circularised plasmids exist in two forms: superhelical and nicked-open circular. The superhelical form (also known as covalently closed) is completely double stranded, has no nicks or discontinuities (Brown 1995), and migrates fastest through a 1% agarose gel (Harley & White 1973). The nicked-open circular form migrates slowest (Harley & White 1973), and as its name suggests, contains nicks or single-stranded breaks due to the absence of one or more nucleotides (Brown 1995). Linear plasmids migrate to a position in between the two circular forms (Harley & White 1973). The band corresponding to the linear plasmid was excised and extracted from the gel by electro-elution onto dialysis tubing, as described in Section 3.2.3 above. Any remaining circular plasmid, which migrated to different positions on the gel, was therefore eliminated. Approximately 5 ng electro-eluted plasmid DNA was electroporated into *E. coli* cells. Fifty microlitres of the electroporated solution was grown overnight at 37°C on amp+ LB plates, and the electroporation efficiency determined (Table 3.2).

Table 3.2. Efficiency of electroporation with electro-eluted *Sma*I-pUC18.

	Quantity and type of plasmid used	Number of colonies on amp+ LB plate		Electroporation efficiency (CFU)	
		Replicate 1	Replicate 2	Replicate 1	Replicate 2
1	20 ng *Sma*I-pUC18, electro-eluted from gel	1	0	0	0
2	Control: 20 ng original *Sma*I-pUC18 (2 µl was removed before more *Sma*I was added)	~ 240	~ 240	~ 2.4×10^5	~ 2.4×10^5
3	5 ng electro-eluted *Sma*I-pUC18, religated with 1 U T4 DNA ligase	~ 580	~ 120	~ 2×10^6	~ 4.8×10^5
4	5 ng religated electro-eluted *Sma*I-pUC18 (as for (3) above, but ligation performed in presence of *Sma*I)	12	73	~ 4.8×10^4	~2.9×10^5
5	5 ng undigested pUC18	Lawn (uncountable)	Lawn (uncountable)	–	–

After a second incubation with *Sma*I, the plasmid was digested almost to completion (Table 3.2). Furthermore, these results indicated that the *Sma*I-pUC18 was extracted successfully from the agarose gel, and that any residual undigested pUC18 was eliminated. If the *Sma*I-pUC18 had not been electro-eluted successfully, then Electroporation 3 (with ligated electro-eluted *Sma*I-pUC18) would not have worked, as no plasmid would be present.

Electroporation 4 (Table 3.2) indicated that performing the ligation in the presence of *Sma*I decreased ligation (and therefore electroporation) efficiency when no insert DNA was present, because the enzyme cleaved any plasmids that were ligated closed. In the presence of insert DNA, however, this would increase ligation efficiency. Plasmids that re-circularise cannot ligate with bat insert DNA, because the *Sma*I cleavage site is restored. The presence of *Sma*I in ligation reactions reopens re-circularised plasmids, and therefore reduces the incidence of colonies containing only re-circularised vector. Electro-eluted *Sma*I-pUC18 was diluted to 20 ng/µl for use in ligation reactions.

3.2.6. Ligation of bat insert DNA into plasmid vectors

i. Optimisation of ligations

Size-selected bat DNA (insert DNA) was ligated into electro-eluted *Sma*I-pUC18 with the enzyme T4 DNA ligase (Promega), to produce recombinant plasmids (Figure 3.1, Step 5). Unlike other enzymes, such as *E. coli* DNA ligase, T4 DNA ligase is able to catalyse the joining of blunt-ended DNA fragments efficiently at room temperature (Sambrook *et al.* 1989). Ligations were carried out overnight at room temperature, followed by inactivation at 65°C for 1 hour. They were performed in 10 µl reactions containing 1 µl electro-eluted *Sma*I-pUC18 (20 ng/µl), 0.5 µl T4 DNA ligase (3 U/µl), 1 µl 10x ligase buffer (supplied with the enzyme by the manufacturer), 1 µl *Sma*I (8 U/µl),

and either 3 µl or 6 µl insert DNA (~1 ng/µl). Ligations were performed in the presence of *Sma*I, in order to restore the linear form of self-ligated plasmids (Section 3.2.5 above), and were stored at -20°C until electroporation (see below).

Ligations of inserts and vectors with blunt ends (such as those produced by the restriction enzymes used here) are less efficient than those with cohesive (or "sticky") ends (Sambrook *et al.* 1989). They require high concentrations of ligase, as well as high ratios of insert DNA : vector to increase the number of recombinants produced. This ratio must be optimised because excess re-circularisation of the plasmid may occur if the concentration of insert DNA is too low. On the other hand, too high an insert DNA concentration may favour formation of recombinants containing multiple inserts (Dugaiczyk *et al.* 1975; Sambrook *et al.* 1989;).

Three different molar ratios of insert DNA to plasmid DNA were attempted (1:1; 2:1 and 3:1). Ligations containing insert : vector ratios of 1:1 and 2:1 (3 ng and 6 ng of insert DNA respectively) were found to be most efficient in terms of producing the greatest number of white *E. coli* colonies (explained in Section 3.2.8 below) after electroporation. The amount of insert DNA required to achieve these ligation ratios was calculated according to the following formula:

$$\textit{Amount of insert (ng)} = \frac{\textit{ng vector} \times \textit{size of insert (kb)}}{\textit{size of vector (kb)}} \times \textit{molar ratio of insert / vector}$$

For example, for an insert : vector ratio of 2:1 and 20 ng vector, where pUC18 is 2.686 kb long, and the size-selected fragments range from 200 bp to 600 bp (average of 400 bp):

$$\text{Amount of insert DNA required} = \frac{\text{20 ng pUC18} \times \text{0.4 kb insert}}{\text{2.686 kb}} \times \frac{2}{1}$$

$$\approx 6 \text{ ng insert}$$

ii. Trouble-shooting ligations

Initially, ligations were performed with a Fast-Link Ligation and Screening Kit (Epicentre Technologies, Cape Town). These kits have the advantage that at room temperature, blunt-end ligations can be performed within five minutes, rather than overnight. However, although the first few ligations performed were successful, they were not optimal, due to the relatively low number of white colonies obtained after electroporation (Section 3.2.8), and soon ceased working altogether (for a summary of possible explanations for this, as well as the methods used to test these explanations, please see Appendix 2). It was established that the Fast-Link Kit was probably too old, and that the ligase and possibly the ATP were no longer active. Instead of using a new Fast-Link Kit, it was decided to obtain standard bacteriophage T4 DNA ligase (Promega), and all subsequent ligations were performed with this enzyme, as described in Section 3.2.6 above.

3.2.7. Cloning of recombinant pUC18 vectors

Recombinant plasmids were incorporated into competent bacteria (*E. coli*) and cloned (Figure 3.1, Steps 6 and 7). The incorporation of foreign, plasmid DNA into bacterial cells is known as transformation (Griffiths *et al.* 1993). This was achieved through electroporation, which involves exposing the bacterial cells to brief (*i.e.* 5 – 10 msec) electrical shock. This creates transient pores in the cell wall, allowing the plasmids to enter the bacterial cells (Ausubel *et al.* 1994; Brown 1995).

i. Preparation of electro-competent cells

Electro-competent cells (*E. coli* strain XL-1 Blue) were prepared as described in Ausubel *et al* (1994). Electroporation efficiency or competency was tested by electroporating 40 µl *E. coli* with 5 ng undigested pUC18. Competency levels of 1×10^8 to 1×10^9 colony forming units (CFU) per

microgram transformed plasmid DNA were attained. Aliquots containing 40 µl of freshly made competent cells were snap-frozen in liquid nitrogen and stored at -70°C until electroporation.

ii. Transformation of *E. coli*

All electroporations were performed on an Invitrogen Electroporator II. In each reaction, 1 µl of thoroughly defrosted ligation mix (containing either 3 ng or 6 ng insert DNA) was mixed with 40 µl ice-cold electro-competent *E. coli* and transferred to an ice-cold *E. coli* Pulser Cuvette (Bio-Rad Laboratories) with a 0.2 cm electrode gap. The untransformed bacterial cells were previously stored at -70°C, and were retrieved, on ice, from the freezer immediately before transformation. The ice-cold cells were electroporated at a constant voltage of 1500 V and capacitance of 50 µF. A load resistance of 150 Ω was found to be optimal. Immediately after electroporation, 1 ml of warm (37°C) SOC medium (see Appendix 1) was added to the cuvette and mixed with the cells. The sample was transferred to a sterile culture tube, and allowed to recover in a Gallenkamp Orbital Incubator at 200 rev/min for 1 hour at 37°C. Thereafter, the cultures were stored at 4°C. Each time an electroporation with ligation products was performed, 5 ng undigested pUC18 and ~10 ng *Sma*I-pUC18 were each electroporated with 40 µl electro-competent *E. coli.* These served as positive and negative controls respectively.

After recovery at 37°C for 1hour, 50 µl of the transformed culture were plated onto ~10 cm diameter amp+ LB plates, which had been treated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl-thiogalactoside (IPTG; Section 3.2.8 below), and were grown overnight at 37°C. The amp^r gene of pUC18 confers ampicillin resistance on transformed *E. coli* cells (Sambrook *et al.* 1989). Therefore, when electroporated *E. coli* are plated on amp+ LB plates, only those cells that have taken up the plasmid (and therefore contain the amp^r gene) will be able to grow and multiply, thereby cloning the plasmid. This collection of cloned recombinant DNA fragments, propagated in bacteria, is known as a genomic library (Watson *et al.* 1992).

The efficiency of the electroporation procedure (as determined by CFU/µg DNA, Section 3.2.5 above) was found to depend not only on the efficiency of the ligation reaction, but also on the freshness of the ligation product. It was found that electroporation efficiency decreased significantly once the ligation product was more than one or two months old. Therefore, to avoid continually having to perform fresh ligations and electroporations, once a good transformation was achieved, glycerol stocks (Appendix 1) of the transformed cultures were prepared and stored at -70°C. These stocks were used as source material for all subsequent screening procedures.

3.2.8. Lac selection

Not all transformed *E. coli* cells contained bat insert DNA. This is because during the ligation procedure, some plasmids self-ligated (re-circularised or formed plasmid dimers) rather than ligating with insert DNA (the re-circularisation process can be limited by retaining the enzyme, *Sma*I in the ligation reaction, as described above). Self-ligated plasmids can be incorporated into *E. coli* during electroporation, and must be distinguished from the recombinant plasmids, which contain insert DNA of interest.

The following procedure, known as Lac selection, was used to distinguish between *E. coli* colonies containing recombinant plasmids and those containing non-recombinants. The procedure involves the insertional inactivation of the *lacZ'* gene, which codes for the alpha (α) subunit of the enzyme β-galactosidase (Ausubel *et al.* 1994; Brown 1995). This enzyme is involved in lactose metabolism, and is normally coded for by the *lacZ* gene on the *E. coli* chromosome. Certain strains of *E. coli*, such as XL-1 Blue, have a modified *lacZ* gene, which lacks a segment of the gene, known as *lacZ'* (Sambrook *et al.* 1989). As a result, the enzyme produced by these cells lacks the α-subunit and is therefore non-functional (Sambrook *et al.* 1989). When these modified bacterial strains are transformed with plasmids such as pUC18, which express the *lacZ'* gene

(Figure 3.2), the plasmid is able to supply the missing α-fragment, and thus functional β-galactosidase can be produced. This process is known as α-complementation. However, the polycloning site of pUC18 (and hence the *Sma*I recognition site) lies within the *lacZ'* gene (Sambrook *et al.* 1989). Therefore, when insert DNA is ligated into the plasmid at the *Sma*I site, it is inserted into and inactivates the *lacZ'* gene, preventing synthesis of the α-fragment of β-galactosidase.

During lac selection, amp+ LB plates are treated with the enzyme inducer, isopropyl-thiogalactoside (IPTG), and a lactose analogue, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), which acts as a chromogenic substrate. In the presence of IPTG, X-gal is broken down by β-galactosidase to produce a blue-coloured product. When grown on X-gal/IPTG/amp+ LB plates, any bacterial colonies that contain self-ligated (non-recombinant) plasmids will produce blue colonies, because the *lacZ'* gene is intact and β-galactisidase is synthesised. However, colonies containing recombinant plasmids do not produce β-galactosidase because the *lacZ'* gene has been inactivated. Consequently, X-gal is not broken down, and the colonies that grow on X-gal/IPTG/amp+ LB plates are uncoloured (white).

As described previously, 50 µl transformed culture were spread onto amp+ LB plates, treated with 40 µl X-gal (20 mg/ml) and 4 µl IPTG (100 mM), and grown overnight at 37°C (Figure 3.3, Step 1). The following morning, the ratio of white : blue colonies was inspected, and the number of colony forming units (CFU) / µg DNA transformed (Section 3.2.5) was determined, to give an indication of the efficiency of both the ligation and electroporation procedures. Approximately 3×10^6 CFU/µg DNA was achieved for pUC18 ligated with insert DNA, and fewer than 5% of the colonies were blue.

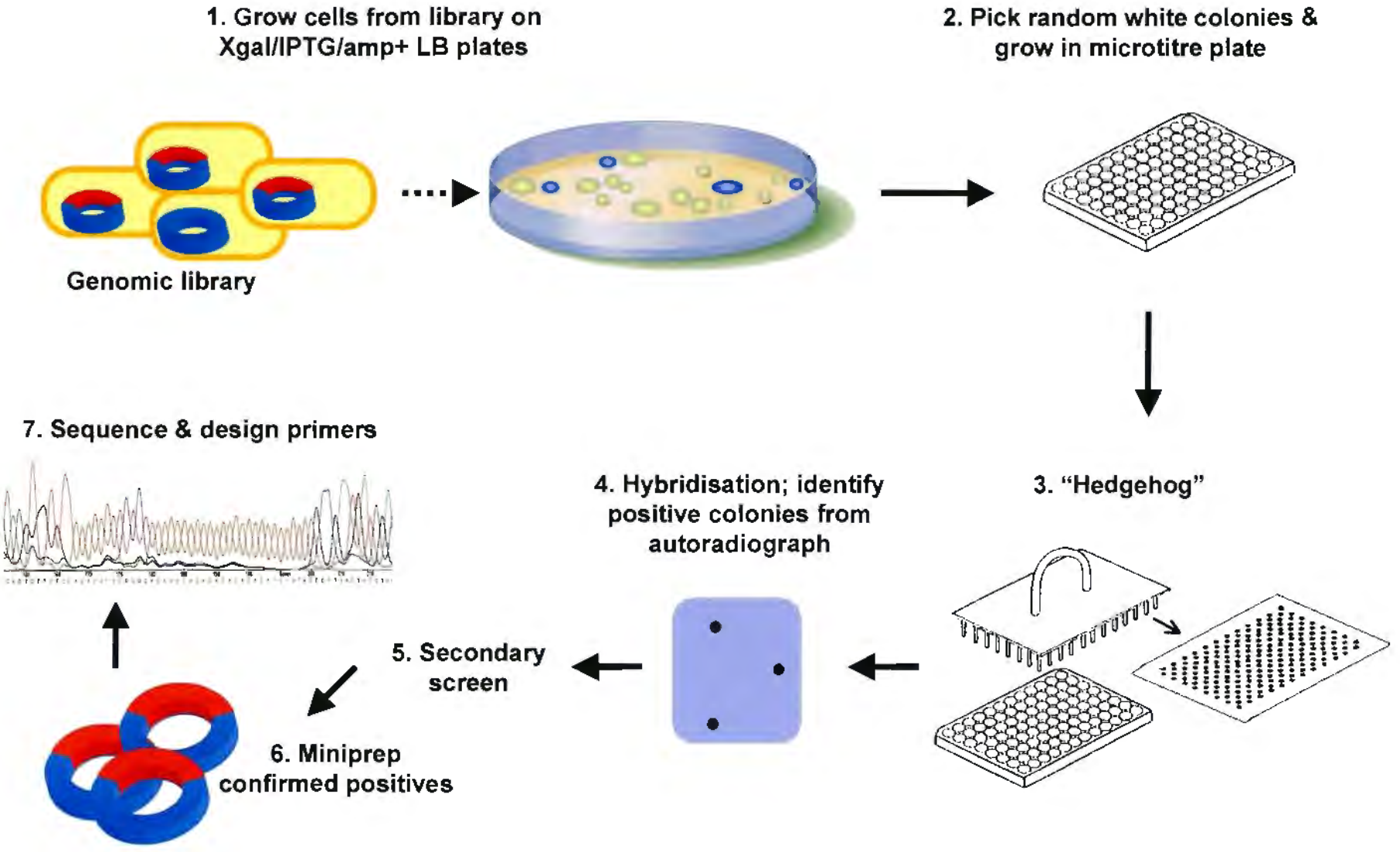

Figure 3.3. The major steps involved in screening a genomic library for microsatellites.

3.2.9. Screening the library for microsatellites

As described above, once a good electroporation efficiency and high ratio of white : blue colonies had been achieved, glycerol stocks (Appendix 1) of this electroporation mix were made and stored at -70°C. These stocks were used to inoculate 5 ml amp+ LB medium, which were grown for several hours at 37°C in an orbital shaker. Dilutions (between 1 in 1000 and 1 in 100 000) of these cultures were prepared, and 20 – 50 µl of these dilutions were plated onto ~10 cm diameter Xgal/IPTG/amp+ LB plates and grown overnight in an incubator at 37°C (Figure 3.3, Step 1). The volume plated varied slightly depending on the efficiency of the electroporation. After overnight growth, an even spread of well-spaced colonies was required.

i. Selecting colonies

White colonies were picked at random and placed in individual wells of a 96-well microtitre plate containing amp+ LB medium (Figure 3.3, Step 2). One well of the microtitre dish was inoculated with a clone known to contain a $(CA)_n$ repeat, which served as a positive control. Colonies were allowed to grow for a minimum of four hours at 37°C, after which they were transferred to a sheet of nylon Hybond-XL membrane (Amersham Pharmacia Biotech), placed on a 25 cm x 25 cm amp+ LB plate. The transfer was achieved by means of a "hedgehog" (Figure 3.3, Step 3). This is a transfer device consisting of a metal plate with a wooden handle attached to one side, and 96 metal spikes welded to the other side. The spacing of these spikes corresponds to that of a 96-well microtitre dish. The transferred colonies were allowed to grow overnight on the membrane at 37°C.

The positively charged Hybond-XL membrane covalently binds DNA at high pH (Ausubel *et al.* 1994). Accordingly, after overnight incubation, the membrane was peeled off the LB plate and placed, colony-side up, on Whatman 3 MM paper saturated with an alkaline denaturation buffer

(1.5 M NaCl and 0.5 M NaOH). This buffer promotes lysis of the bacterial cells and denaturation of the plasmid DNA. After two to five minutes, the membrane was transferred for a further five minutes to 3 MM paper saturated with neutralisation buffer (1.5 M NaCl and 0.5 M Tris pH 7.5), and then washed vigorously with 2x SSC (Appendix 1) to remove proteinaceous debris. The washed membrane was either heated in the microwave for three minutes or incubated at 80°C for two hours, to fix the denatured plasmid DNA to the membrane.

ii. Preparation of hybridisation probe

The membranes were hybridised with radioactively-labelled oligonucleotide probes, poly(dC-dA).(dG-dT) and poly(dG-dA).(dC-dT). These oligonucleotides were supplied unlabelled by Amersham Pharmacia Biotech at a concentration of 1330 µg/ml. Prior to hybridisation, each probe was diluted with 1x TE to a working concentration of 25 µg/ml, and random-primed with Redivue α^{32}P-dATP (250 µCi; supplied by Amersham Pharmacia Biotech at 3000 Ci/mmol) using the Roche Molecular Biochemicals Random Primed DNA Labelling Kit. Each oligonucleotide was denatured at 100°C for five to ten minutes, and then placed immediately on ice. Random-priming was performed in 20 µl reactions containing 2 µl (50 ng) denatured poly(dC-dA).(dG-dT) or poly(dG-dA).(dC-dT), 1 µl each dCTP, dGTP and dTTP, 2 µl 10x hexanucleotide mix (supplied by the manufacturer), 5 µl α^{32}P-dATP and 1µl Klenow enzyme (2 U/µl). This was incubated at 37°C for 45 minutes, and then stopped with 4 µl 0.1 M EDTA. The solution was made up to 200 µl and then loaded onto a Sephadex-G50 column prepared with 1x TE. All radioactivity passing through the column was collected in microcentrifuge tubes (four drops per tube), and the relative radioactivity of each aliquot estimated with a Geiger counter. Two peaks in radioactivity were obtained. The aliquots corresponding to the first peak, containing probe that had incorporated radioactivity, were pooled and used in the hybridisation procedure. Unincorporated radioactivity (in the second peak) was eliminated. The individually purified poly(dC-dA).(dG-dT) and poly(dG-dA).(dC-dT) probes were pooled, and denatured at 100°C for ten minutes immediately prior to use.

iii. Hybridisation

Up to four colony-lift membranes were stacked on top of each other, interleaved with nylon mesh, rolled up and placed in a glass hybridisation tube. The membranes were initially incubated with continuous rotation in ~60 ml prehybridisation buffer (Appendix 1) for 90 minutes at 65°C. The non-fat milk powder in this buffer acts as a blocking agent. It binds to the positively charged membrane where DNA is not present, preventing large-scale random binding of the oligonucleotide probes (Ausubel *et al.* 1994). After 90 minutes, denatured oligonucleotide probes were added to the prehybridisation buffer (now referred to as hybridisation buffer). The membranes were hybridised in this buffer overnight at 65°C (Figure 3.3, Step 4). Both prehybridisation and hybridisation were performed in a Hybaid hybridisation oven. After overnight hybridisation, the membranes were washed three times for 15 minutes each wash, with medium stringency wash buffer (Appendix 1). These washes ensured that only probe hybridised to the desired target sequences (*i.e.* long microsatellite repeats) remained bound to the membrane. Probe bound less stringently to short repeats and/or non-specific sequences was removed. The membranes were air dried and wrapped with "cling-film".

iv. Autoradiography

Hybridised membranes were exposed to Agfa CP-BU Medical X-ray film overnight (Figure 3.3, Step 4). The exact positions of the membranes relative to the X-ray film were marked so that the autoradiograph could later be lined up with the original microtitre plate. During hybridisation, the poly(dC-dA).(dG-dT) and poly(dG-dA).(dC-dT) probes bound to complementary microsatellite repeat sequences in the insert bat DNA, and showed up as black spots on the autoradiographs exposed to the hybridised membranes (Figure 3.4). Developed autoradiographs were aligned with the original microtitre plates, and the wells containing potentially positive clones identified.

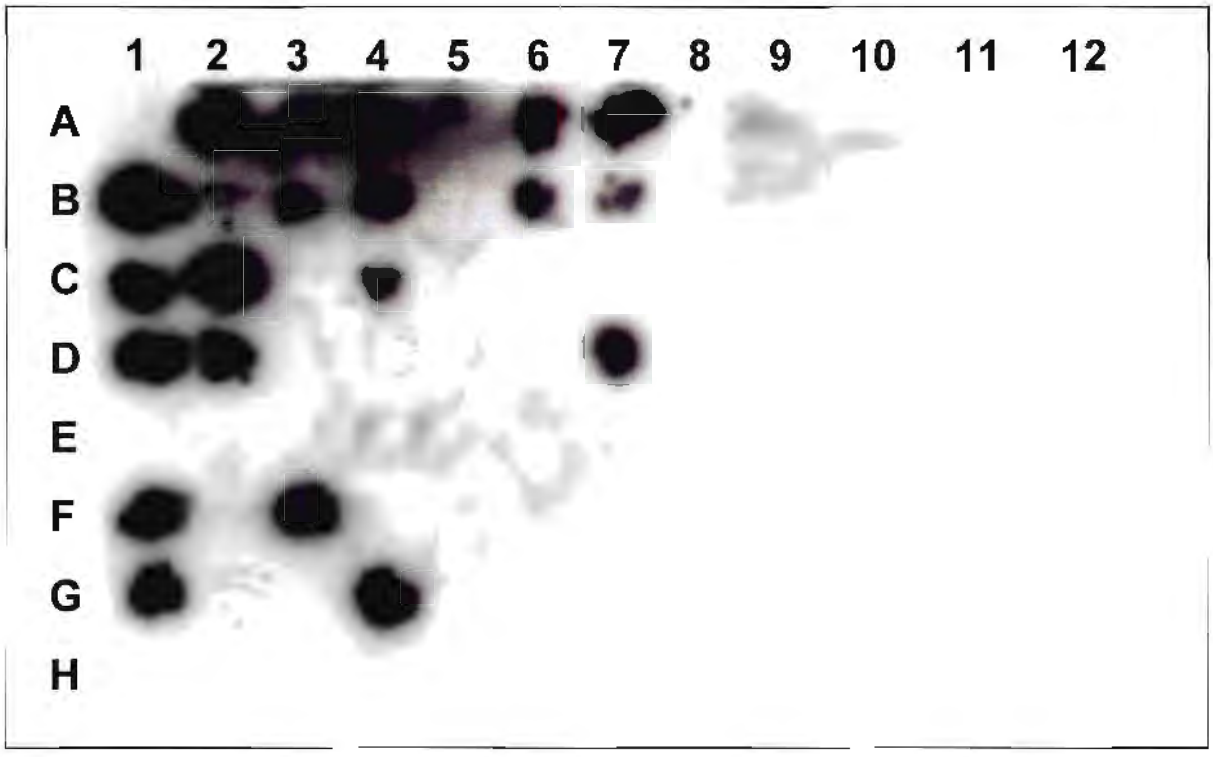

Figure 3.4. Example of an autoradiograph from a secondary screen, showing positive colonies. The letters and numbers identify the rows and columns, respectively, of the original microtitre dish.

v. Secondary screening

Potentially positive clones were screened a second time to confirm that they were indeed positive, and also to ensure that only one clone was present in each well of the microtitre dish (Figure 3.3, Step 5; Figure 3.4). If more than one clone was present, subsequent sequencing would fail. Fifty microlitres of each potentially positive clone were plated onto amp+ LB plates and grown overnight at 37°C. Between ten and 20 individual colonies were picked at random from these plates, and transferred to individual wells of a sterile microtitre dish containing amp+ Luria Broth. The "hedgehog", hybridisation and autoradiography procedures described above were repeated, and confirmed positive clones were identified.

vi. Mini-prepping

Aliquots of 5 ml amp+ Luria Broth were each inoculated with 50 µl of confirmed positive and single clones, and grown overnight at 37°C in an orbital shaker. One to 1.5 ml of each culture was mini-prepped to extract plasmid DNA, using a QIAprep Spin Miniprep Kit (QIAGEN, supplied by Southern Cross Biotechnology, Cape Town).

3.2.10. Sequencing and primer design

i. Sequencing

Mini-prepped plasmid DNA was sequenced to confirm the existence of a microsatellite repeat (Figure 3.2, Step 7). Sequencing was initially performed manually with a Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB; supplied by Amersham Pharmacia Biotech), according to the manufacturer's instructions (Figure 3.5). Thereafter, mini-prepped samples were sent to the Department of Molecular and Cellular Biology at the University of Cape Town (UCT), where they were sequenced with a Cy5TM Thermo Sequenase Dye Terminator Kit (Amersham Pharmacia Biotech), and then run on an ALFexpress DNA Automated Sequencer. In both cases, the –40 universal primer (sequence 5′-3′: GTT TTC CCA GTC ACG AC) was used in the sequencing reaction.

ii. Asymmetrical inserts

In total, thirteen different clones containing microsatellites were identified. However, seven of these clones contained asymmetrical inserts, *i.e.* 0 – 26 bp of insert DNA lay between one end of the microsatellite repeat and the vector sequence (*e.g.* Figure 3.5). In each case, the

microsatellite contained in the insert was long (13 to 30 repeats), and so likely to be polymorphic. Therefore, where more than 15 bp existed between the repeat region and the vector sequence, primers were designed to bind to the flanking regions, but clean, scorable microsatellites could not be amplified in any of the bat samples tested. This was probably because one of the primers lay too close to the microsatellite to allow for successful PCR amplification. Other workers in our laboratory have also found that primers designed to bind less than 20 bp away from a microsatellite, do not work efficiently in a PCR (C. O'Ryan and J. Cunningham, pers. comm.).

Multiple copies of each microsatellite repeat are likely to be present in the *M. schreibersii* library. Because only a partial restriction enzyme digest of the original stock genomic DNA was performed (Section 3.2.2), these microsatellites are likely to be contained within different sized inserts. Attempts were therefore made to amplify larger inserts that contained the same microsatellite repeats as those present in the asymmetrical inserts. To do this, two PCR primers were designed for each asymmetrical clone sequence: one, which would bind to the vector, and another, which would bind to the insert DNA on the far side of the repeat region (Figure 3.6). A miniprep of the *M. schreibersii* library was prepared (Section 3.2.9, step vi), and a PCR with these two primers was performed using the *M. schreibersii* genomic library as template material. It was hoped that the primer designed to bind to the insert region would be sufficiently stringent to allow amplification of only those inserts containing the identical sequence, and thus the same repeat region.

The PCR products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The products were run against a size ladder (λ*Hind*III or λ*Dra*I) as well as minipreps of the previously isolated asymmetrical clones. Individual amplified fragments larger than the original asymmetrical clone were electro-eluted from the gel (Section 3.2.3) and purified with a QIAquick PCR purification kit (QIAGEN). They were sent to the Department of Molecular and Cellular Biology, UCT where they were sequenced as described above. Unfortunately, only unreadable sequences were obtained. This was probably due to the presence of multiple inserts in the template material. In other words, the single PCR primer designed to bind to the insert region was not sufficiently stringent, and multiple PCR products of the same size must have been

extracted from the gel. Several attempts were made to reamplify the electro-eluted band, using a nested primer, but no readable sequences were obtained.

iii. Symmetrical inserts and primer design

Six positive clones contained $(CA)_n$ or $(GA)_n$ repeats near the centre of the insert, *i.e.* they contained symmetrical inserts (*e.g.* Figure 3.7). Primers were designed to bind to the regions flanking the microsatellite in each of these loci, with the assistance of the computer software program, *Oligo* (Rychlik 1989). Primers were designed to meet the following criteria:

- Primers were 18 – 22 bp in length.
- Guanine/cytosine content of the primers was 40 – 60%.
- Fewer than four consecutive bases within each primer and between the primers were complementary, to minimise hair-pin and primer-dimer formation respectively.
- The difference in melting temperature between the primers of each primer pair was ≤1°C.
- Product size was between 150 bp and 250 bp.

Primers were manufactured either by the DNA Synthesis Laboratory at the Department of Molecular and Cellular Biology, UCT or by Sigma-Genosys Ltd, a division of Sigma Aldrich. They were diluted to a working concentration of 100 pmol/µl and stored at -20°C. An aliquot of 50 µl was kept at 4°C for regular use.

Pairs of primers were initially optimised on ten *M. schreibersii* samples from different colonies (discussed in Chapter 4). Amplification of microsatellite loci with these pairs of primers was also attempted in fourteen *M. fraterculus* samples, as well as in a limited number of individuals belonging to the families Molossidae (*Chaerophon pumila* and *C. ansorgei*) and Rhinolophidae (*Rhinolphus capensis* and *R. clivosus*).

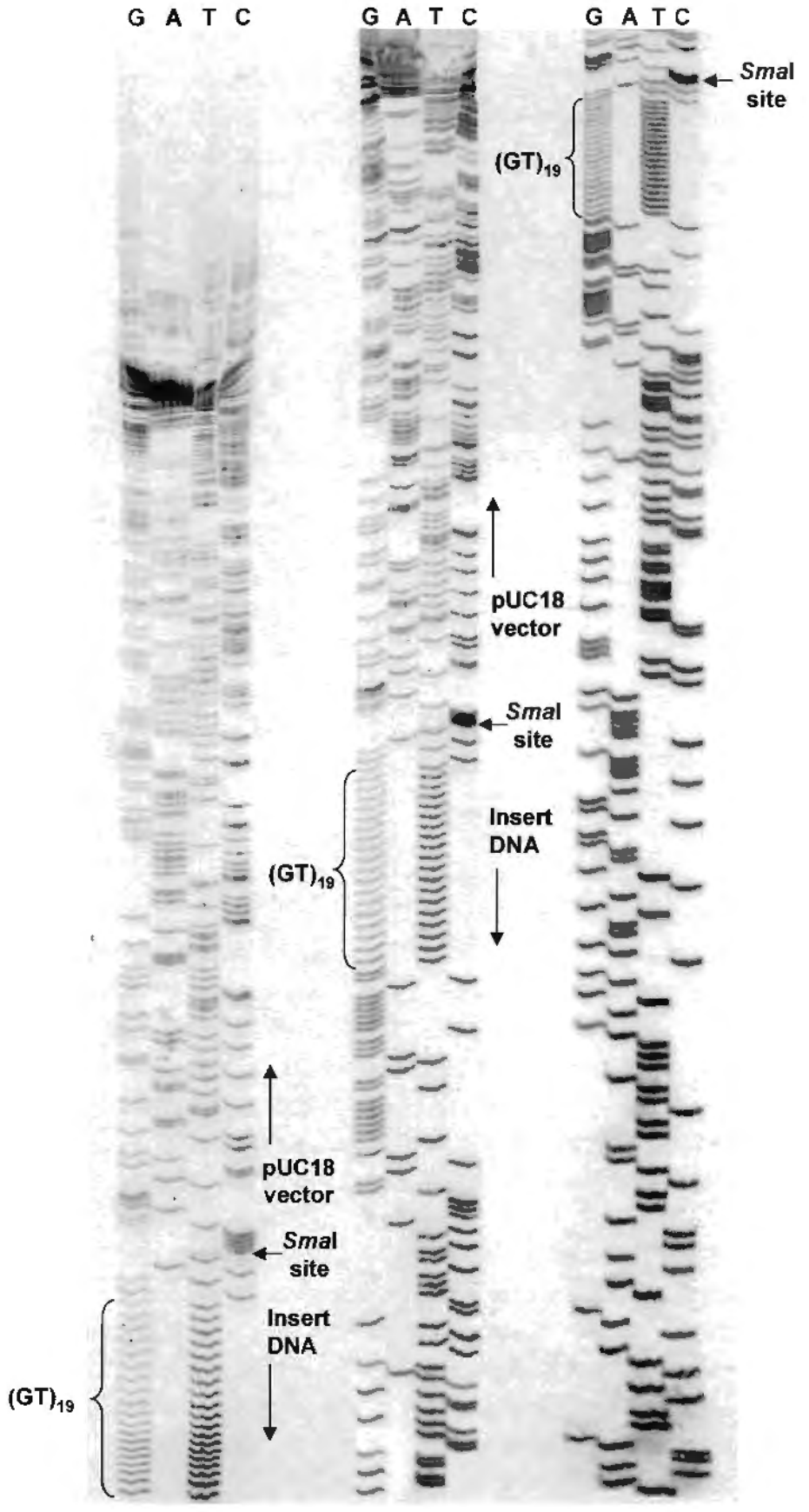

Figure 3.5. Example of a clone sequenced manually with the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit. The clone contains an asymmetrical insert with a $(CA)_{19}$ microsatellite.

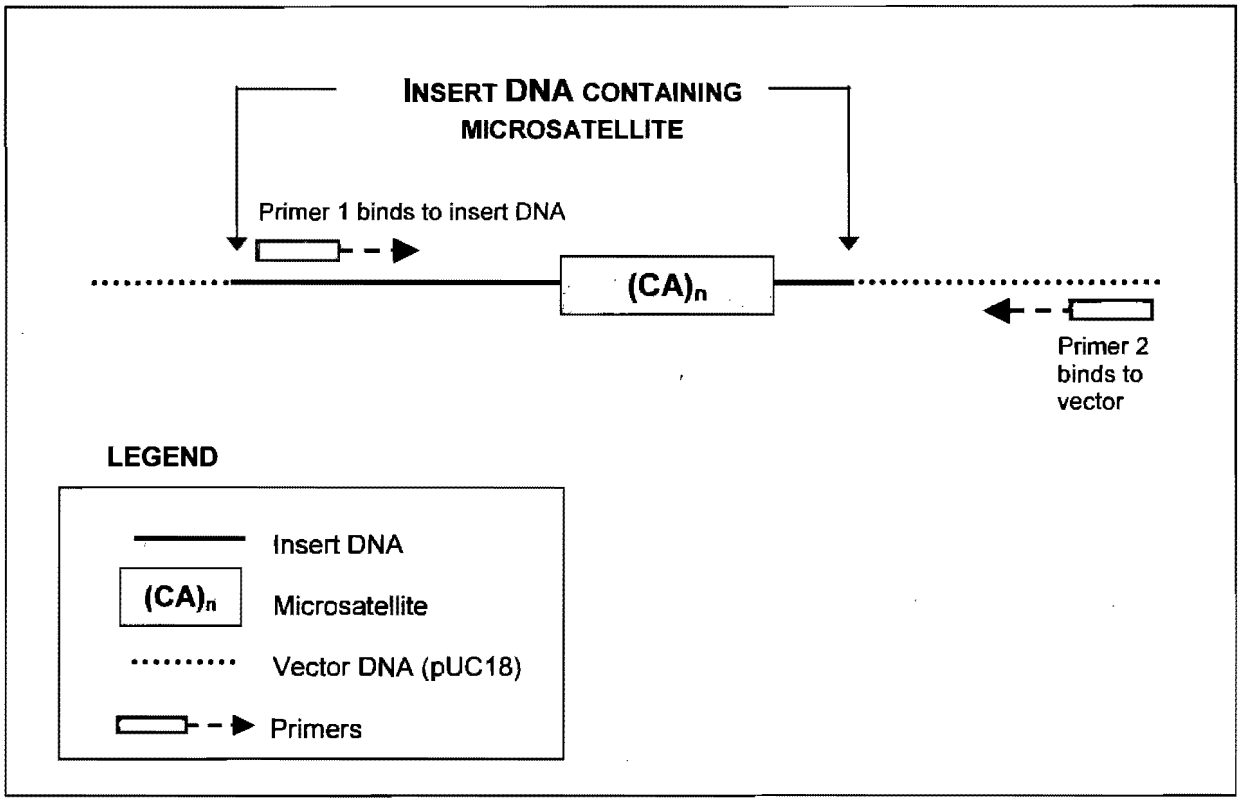

Figure 3.6. Schematic diagram showing a clone with an asymmetrical insert, and the binding sites of primers in the insert and the vector sequences.

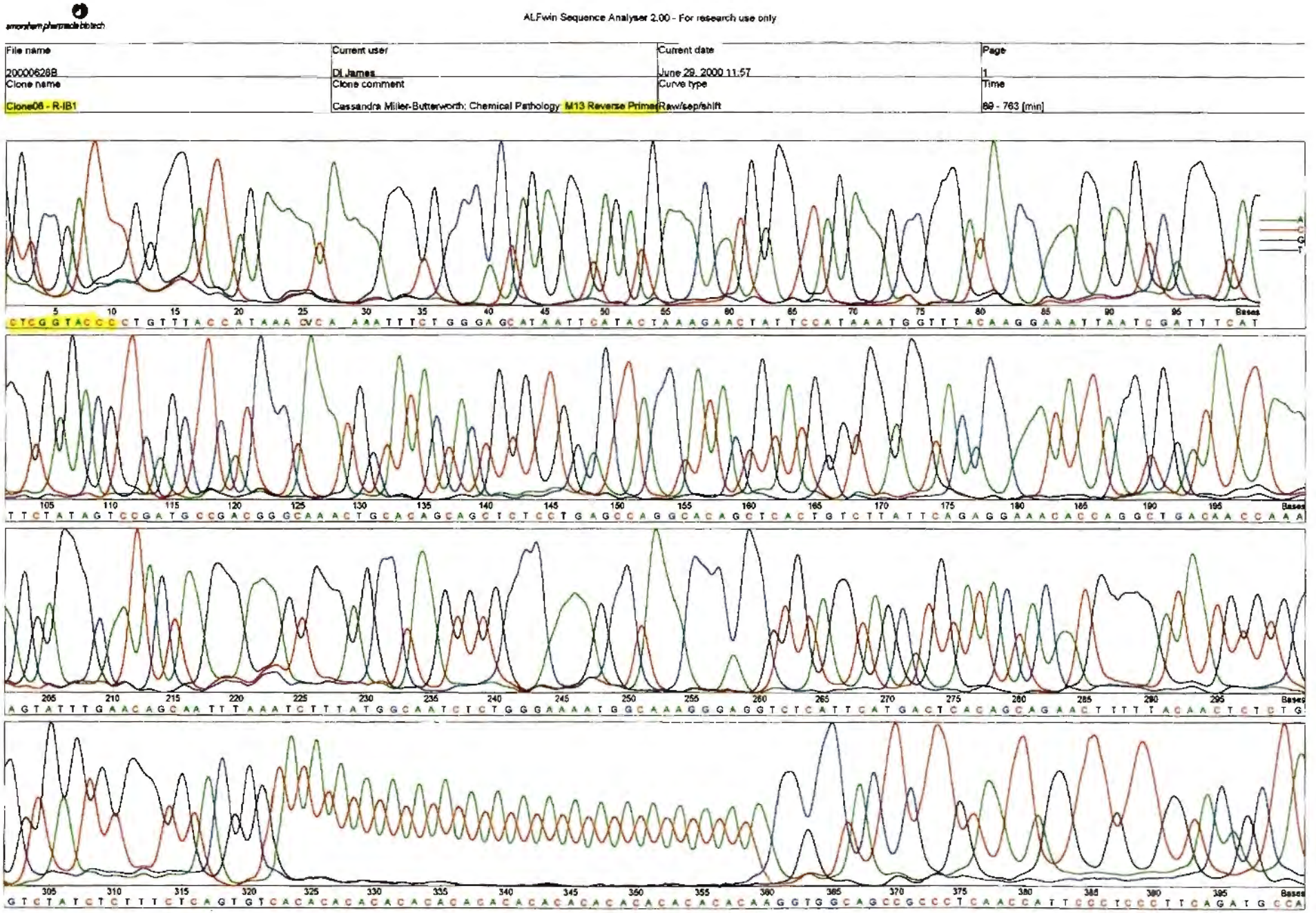

Figure 3.7. Example of a clone sequenced with a Cy5TM Thermo Sequenase Dye Terminator Kit and run on an ALFexpress DNA Automated Sequencer. The clone (locus Mschreib3) contains a symmetrical insert, with a $(CA)_{19}$ microsatellite.

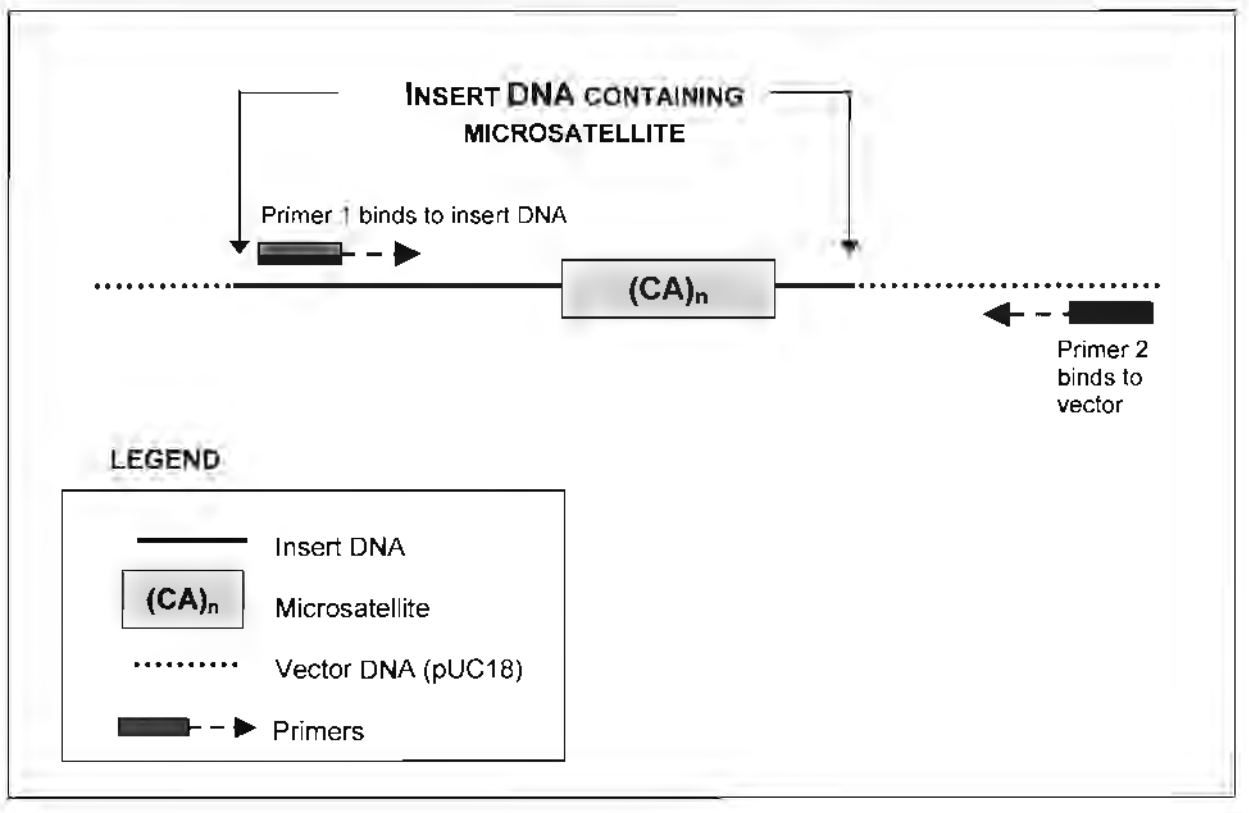

Figure 3.6. Schematic diagram showing a clone with an asymmetrical insert, and the binding sites of primers in the insert and the vector sequences.

3.3. RESULTS

In total, between 1500 and 2000 clones were screened, and 17 positive clones were identified and sequenced. Due to the stringent screening protocol followed, all 17 clones contained a microsatellite. However, five of these proved to be duplicate clones and therefore only 13 unique clones were identified. The proportion of recombinant clones found to contain microsatellites (0.65 – 0.86%) is within the lower range of the expected frequency of microsatellites in genomic libraries (0.5 – 2%; Bruford *et al.* 1996). Other workers (Baker *et al.* 1992; Bradley & Wichman 1994;Van den Bussche *et al.* 1995) have also reported a lower incidence of repetitive DNA in bat genomes, compared to other mammals (Chapter 4).

Seven of the 13 unique clones (54%) contained asymmetrical inserts, *i.e.* the repeat region lay between 0 bp and 26 bp from one end of the insert, and thus was too close to the vector for primers to be designed to these microsatellites (Table 3.3). This is considerably greater than the average expected frequency of asymmetrical inserts (5%) reported by Hillis *et al.* (1996) for cattle, horses, lizards and turtles. The remaining six clones contained microsatellites near the centre of the insert, and therefore had sufficiently long flanking regions for primers complementary to those sequences to be designed. Two of the symmetrical microsatellites were interrupted or compound, while the remaining four were perfect repeats (Table 3.4). One contained a $(GA)_{26}$ repeat, and the others contained a $(CA)_n$ element ranging in size from $(CA)_{10}$ to $(CA)_{28}$.

Once they had been optimised (Chapter 4), all pairs of primers amplified clean, scorable microsatellites in *M. schreibersii*. Loci Mschreib1 – Mschreib5 were polymorphic, and between 17 and 20 alleles were identified per locus (Table 3.4). Locus Mschreib6 was monomorphic in all South African *M. schreibersii* tested, as well as in the individuals from Australia, Madagascar and Israel.

Five polymorphic microsatellite loci (Mschreib1 – Mschreib5) were successfully amplified in *M. fraterculus* (Table 3.5), and two loci (Mschreib1 and Mschreib2) were found to be polymorphic in *Chaerophon sp.* However, microsatellite loci could not be amplified in *Rhinolophus sp.* using the Mschreib primers.

Table 3.3. Characterisation of asymmetrical microsatellite loci identified in *M. schreibersii*, including the name of each clone, description of each microsatellite repeat motif and the distance (in base pairs) between the repeat region and the vector sequence.

Clone name	Repeat motif	Distance between repeat and vector (base pairs)
A9	$(AC)_3TCAT(AC)_{13}$	0
B11	$(AC)_{19}$	9
G11	$(CA)_{15}$	11
D1	$(CA)_3CG(CA)_2TGCACG(CA)_5CG(CA)_3CT(CA)_6$	16
C9	$(AC)_{30}$	17
C5	$(AC)_{30}$	22
E4	$(CA)_{25}$	26

Table 3.4. Characterisation of microsatellite loci identified in *M. schreibersii*, including the name of each locus, forward (F) and reverse (R) primer sequences, description of each microsatellite repeat motif, allele size range in base pairs (sequenced clone product size is given in brackets), optimal PCR amplification temperature (T_a), the number of alleles identified at each locus (N_A) and the GenBank accession numbers.

Locus	Primer sequences (5'-3') F= forward primer; R = reverse primer	Repeat motif	Allele size range (clone size)	T_a (°C)	N_A	GenBank accession number
Mschreib1	F: GTTCTAGCAATCTAATGTAAAGC R: AAGATCACCACAATCAAGC	$(CA)_4(CG)_2(CA)_{13}$	169 - 203 (187)	55	17	AY056588
Mschreib2	F: ACTTCCTTTCATCACCTCAG R: CCAGCCACACCCTCAG	$(CA)_{18}$	174 - 222 (194)	58	20	AY056589
Mschreib3	F: AAATGGCAAAGGGAGGTC R: TCTGAAGGGAGGGAATGG	$(CA)_{19}$	142 - 172 (152)	58	17	AY056590
Mschreib4	F: GACTGGCAAGTTAGTCCATG R: TGATGAGTTGATGAGTTTTGAC	$(CA)_{17}$	171 - 205 (191)	55	18	AY056591
Mschreib5	F: TTCACTTGGCCACATTTC R: AGTCTTTGGCTCTCCCTG	$(GA)_{26}$	168 - 208 (201)	55	19	AY056592
Mschreib6	F: CAGCCCAGAATCTGGTCC R: GTTAAATTTGCAGCAATGAGC	$(CA)_{10}(CG)_5(TG)_3$	231 (231)	55	1	Not submitted

Table 3.5. Cross-species amplification of polymorphic microsatellite loci by Mschreib primers in *M. fraterculus* and *Chaerophon sp.* Abbreviations are as follows: N_A = number of alleles; n = number of individuals genotyped per locus.

Locus	*M. fraterculus*			*C. pumila*			*C. ansorgei*		
	N_A	Allele size range (bp)	n	N_A	Allele size range (bp)	n	N_A	Allele size range (bp)	N
Mschreib1	4	177 – 187	14	5	171 - 173	2	1	173	1
Mschreib2	3	182 – 186	14	7	202 – 216	5	3	200 – 214	2
Mschreib3	9	150 – 172	14	-	-	-	-	-	-
Mschreib4	8	187 – 207	13	-	-	-	-	-	-
Mschreib5	8	184 – 204	6	-	-	-	-	-	-

CHAPTER 4

EXAMINATION OF *M. SCHREIBERSII* POPULATION STRUCTURE WITH MICROSATELLITE MARKERS

AIMS: 1. TO DETERMINE WHETHER THE SOUTH AFRICAN POPULATION OF *MINIOPTERUS SCHREIBERSII* IS GENETICALLY SUBSTRUCTURED.

2. TO ESTIMATE THE EXTENT AND DIRECTION OF MIGRATION BETWEEN *M. SCHREIBERSII* COLONIES.

4.1. INTRODUCTION

4.1.1. Classification and distribution of microsatellites

Microsatellites or short tandem repeats are simple base sequences that consist of tandemly repeated units of one to five base pairs (bp) *e.g.* $(CA)_n$, $(AAT)_n$ or $(GATC)_n$ where n is the number of times the unit is repeated. Microsatellites are classified as mono-, di-, tri-, tetra- or pentanucleotide repeats depending on the number of base pairs within each repeat unit. They are

further categorised into three families: pure or perfect (*e.g.* CACACACACACA), interrupted (*e.g.* CACA**GGG**CACA**GG**CACA) and compound repeats (*e.g.* CACACA**GAGAGAGA**). Microsatellites are randomly and widely dispersed throughout eukaryotic genomes, occurring in organisms as evolutionarily diverse as yeast, *Drosophila* and vertebrates (Hamada *et al.* 1982; Tautz & Renz 1984). They have even been identified in some prokaryotes, such as *Neisseria gonorrhoeae* and *Hemophilus influenzae* (Moxon & Wills 1999). Eukaryotic taxa frequently differ in terms of which type of repeat motif is most common. In humans, as in most mammals, $(A)_n$ and $(CA)_n$ are the most common repeat motifs, followed by $(GA)_n$, while $(AT)_n$ is relatively rare (Primmer *et al.* 1997; Van den Bussche *et al.* 1995). On the other hand, $(AT)_n$ occurs more frequently than $(CA)_n$ in plants, and in insects, $(GA)_n$ is the most numerous repeat type (Primmer *et al.* 1997).

Mononucleotide, dinucleotide and tetranucleotide repeats are found only in non-coding regions of the genome. These types of microsatellites are generally highly polymorphic (Section 4.1.3 below), and mutations in them would disrupt the triplet reading frame of a coding region, thus affecting the resultant gene product. Trinucleotide repeats, however, are found in both non-coding and coding regions (exons) of the genome, because mutations within them do not disrupt the codon reading frame (Jarne & Lagoda 1996). However, abnormal expansion of trinucleotide repeats within coding regions is frequently the cause of neurodegenerative diseases, such as Huntington's Disease and spinal bulbar muscular atrophy (Yamada *et al.* 2000; Yu *et al.* 2000), as well as myotonic dystrophy and fragile X syndrome (Bruford & Wayne 1993; Hearne *et al.* 1992). In the case of the latter disorder, for example, the fragile X gene, which normally contains about 29 CGG repeats, expands to include as many as 200 repeats in infected individuals (Bruford & Wayne 1993). These so-called pathogenic microsatellites are stably inherited in normal individuals, from one generation to the next. Consequently, they are frequently studied in connection with human disease, and are used as genetic markers for tracing inheritance patterns in human pedigrees.

In general, microsatellites may comprise up to 5% of a typical eukaryotic genome (Tautz 1989), although the number and frequency of microsatellites differs among taxa. Humans, for example,

are estimated to have up to 500 000 microsatellites (Ashley & Dow 1994), of which about 50 000 are $(CA)_n$ repeats (Van den Bussche *et al.* 1995). Mice of the genus, *Mus* on the other hand, have about 100 000 $(CA)_n$ repeats (Hamada *et al.* 1982). Trinucleotide and tetranucleotide repeats are less common in the mammalian genome than dinucleotide repeats, but still number in the thousands.

Certain vertebrate groups, however, have less repetitive DNA. Bats have the smallest mammalian genome, ranging in size from 50% to 87% of that characteristic of other eutherians (Baker *et al.* 1992; Bradley & Wichman 1994). The total amount of repetitive DNA in the bat genome is also reduced relative to other mammals. Baker *et al.* (1992) examined the number of tandemly repeated ribosomal DNA (rDNA) sites in 50 species of bats and 40 species of rodents. They found that bats had on average 1.76 pairs of repeated rDNA sites per species, compared to an average of 4.19 pairs in rodents, a finding later confirmed by Van den Bussche *et al.* (1995). Bradley & Wichman (1994) compared the occurrence of rapidly evolving DNA sequences in the conservatively evolving genome of the bat species *Macrotus waterhousii,* to that in equids, which are considered to have a rapidly evolving genome. They found that *M. waterhousii* had both a lower number and fewer different kinds of repetitive DNA sequences than equids. Van den Bussche *et al.* (1995) estimated there were ~ 22 000 $(CA)_n$ repeats in the bat genome, compared to ~ 50 000 in humans and ~ 100 000 in *Mus*. They also found dinucleotide repeats occurred less frequently in bats than in other mammals. For example, $(CA)_n$ repeats occurred once in every 106 kilobases (kb) in bats, compared to 1/ 54 kb in humans, and 1/18 kb for *Mus*.

Primmer *et al.* (1997) reported that birds also have significantly fewer microsatellites than do most mammals. For example, dinucleotide repeats such as $(CA)_n$ and $(GA)_n$ occurred ten to fifteen times less frequently in birds than in humans. The average density of microsatellites (of any motif type) in the avian genome was determined to be one every 31 kb, while in humans it was estimated to be one in every 6 kb.

Primmer *et al.* (1997) found that, like bats, birds had one of the smallest genome sizes of all vertebrates, and also had relatively less intron DNA than mammals. They suggested that the reduced number of introns may be one explanation for why birds and bats had fewer microsatellites. Baker *et al.* (1992) and Van den Bussche *et al.* (1995) argued that in organisms with small genome size, such as birds and bats, the equilibrium between mechanisms that amplify and those that limit repetitive elements, is shifted towards reducing the amount of repetitive DNA. This is supported by Bradley & Wichman (1994) who further suggested that when such stabilising mechanisms fail, the accumulation or amplification of repetitive DNA regions results, as appears to occur in taxa that undergo karyotypic megaevolution. They cited as an example the bat species *Artibeus jamaicensis*, which had 16 chromosomal rearrangements compared to the *M. waterhoussii* karyotype, and so was considered to have a more rapidly evolving genome. *Artibeus* was also found to have a greater number of repetitive elements than *M. waterhousii*, although still considerably fewer than that found in equids or mice. The reason for the possible existence of a stabilising mechanism to control the occurrence of repetitive elements is unclear. However, it may be that animals with a particularly high metabolic rate (*e.g.* volant animals such as bats and birds) require a small genome size with fewer and shorter introns, and hence fewer microsatellites (Baker *et al.* 1992; Primmer *et al.* 1997; Van den Bussche *et al.* 1995).

4.1.2. Functions of microsatellites

As with most non-coding DNA, the functions of microsatellites are unclear. Several authors believe microsatellites may act as hot spots for genetic recombination (Stallings *et al.* 1991; Tautz & Renz 1984; Weber *et al.* 1993), and are thus a source of naturally occurring genetic variation and rearrangements, possibly playing a role in the evolution of new genes (Tautz & Renz 1984). This is supported by Bradley & Wichman's (1994) study. Their finding that conservatively evolving genomes have less repetitive DNA elements than more rapidly evolving genomes is consistent

with the hypothesis that rapidly evolving repetitive DNA sequences (such as tandem repeats) may be involved in chromosomal evolution.

It has also been proposed that microsatellites may be involved in chromatin-folding (Tautz & Renz 1984; Vogt 1990) or in the formation of telomeres (Tautz & Renz 1984). Alternatively, they may act as "tuning knobs", regulating the expression of genes (Moxon & Wills 1999; Stallings *et al.* 1991; Tautz & Renz 1984). Moxon & Wills (1999) point out that many human triplet-repeat diseases involve degeneration of neurological function (*e.g.* Huntington's Disease and spinal bulbar muscular atrophy), and none of these diseases has yet been reported in other primates. They suggest that long microsatellite sequences near certain genes might contribute to brain function, and that such diseases may represent a genetic cost incurred as a result of the rapid evolution of the human brain.

4.1.3. Advantages of microsatellites for conservation genetics studies

i. <u>High levels of polymorphism and heterozygosity</u>

The majority of microsatellites are found in non-coding regions (introns), and hence their alleles are selectively neutral. In other words, the alleles (as determined by the number of tandemly repeated units) are functionally equivalent, and have no affect on the fitness of the organism (Avise 1994). Consequently, they show great variation in the number of repeat units, *i.e.* they are highly polymorphic. Mutation rates for microsatellites have been estimated at between $5x10^{-6}$ and 10^{-4} per generation (Bruford & Wayne 1993), with pure and/or longer repeats (*i.e.* more than ten repeat units) generally being more polymorphic than interrupted and/or shorter microsatellites (Jarne & Lagoda 1996).

Variation in the length of the repeat region is believed to be caused by "slipped-strand mispairing" (Levinson & Gutman 1987; Schlotterer & Pemberton 1994). This process occurs during DNA replication when the newly formed and original template strands misalign, slipping either up or down a repeat unit. If this is not recognised by the DNA mismatch repair system, a repeat unit may be added to or deleted from the new strand. Because microsatellites are believed to mutate primarily through the addition or removal of individual repeat units, they are thought to follow a "stepwise" mutation model, rather than an infinite allele model, which is characteristic of allozymes (Bruford *et al.* 1996; Jarne & Lagoda 1996; Nielsen & Palsboll 1999). This is discussed in more detail in Section 4.2.6.

The data generated by microsatellite analysis are similar to those produced by allozymes, but being hypervariable and selectively neutral, microsatellites generally have more alleles and higher levels of heterozygosity (or genetic variation) than allozymes (Bruford *et al.* 1996). Allozyme loci are under strong selection pressure not to mutate, and thus frequently are invariant in many organisms, especially endangered species (Hedrick 1999). Microsatellites therefore have an important advantage over allozymes. They can be used to study populations with low levels of genetic variability, such as inbred populations or species that have been through a population bottleneck and have little detectable allozyme polymorphism. For example, Hughes and Queller (1993) found a mean observed heterozygosity of 0.62 at six polymorphic microsatellite loci in the social wasp, *Polistes annularis*. By comparison, the mean heterozygosity at 33 allozyme loci studied in the same populations was 0.035, and only three of the 33 allozyme loci were polymorphic. Similarly, little or no variation has been found at allozyme loci in cheetah, *Acinonyx jubatus*, yielding heterozygosity levels as low as 0.0004 in South African populations (O'Brien *et al.* 1983), while between two and eight alleles have been reported at some microsatellite loci (Harley *et al.* 2000; Menotti-Raymond & O'Brien 1995). Such high levels of variability make microsatellites invaluable as quantitative genetic markers.

ii. Ease of amplification

In 1985, Jeffreys *et al.* (1985) discovered hypervariable repetitive regions in the human genome, and named them minisatellites. Like microsatellites, minisatellites are tandem repeat regions, but their repeat units are much larger (up to 200 bp each), and their alleles may be up to 50 kb in length. The discovery of these hypervariable loci and their widespread application in "DNA fingerprinting" revolutionised both forensics and conservation genetics studies. A disadvantage of minisatellites, however, is the large size of their alleles. This limits the efficiency of the polymerase chain reaction or PCR (Saiki *et al.* 1988) when amplifying these loci, particularly when the alleles are over 10 kb long (Bruford *et al.* 1996). Consequently, Southern blotting and hybridisation techniques are used more commonly to reveal minisatellite variation at many loci simultaneously, but these protocols require relatively large quantities of source DNA, *e.g.* from muscle biopsies or large volumes of blood (Ashley & Dow 1994). In addition, using a hybridisation probe to the variable region itself results in a multitude of bands being visualised on gel electrophoresis. This type of multilocus fingerprint is less amenable to statistical analysis than visualisation of the alleles at only a single locus.

Microsatellite alleles, however, are usually less than 500 bp in length, and thus avoid many of the disadvantages associated with minisatellites, while retaining their advantages. Being smaller, microsatellites are easier to clone and to sequence (Hearne *et al.* 1992), and can be amplified efficiently by PCR. Use of primers (see below) specific for the flanking regions of a microsatellite means that only the variable region at that locus will be amplified, simplifying both visualisation and statistical analysis. The polymerase chain reaction is able to increase the quantity of target DNA in a sample substantially because each cycle of amplification theoretically doubles the amount of template DNA, resulting in exponential amplification. Microsatellite loci can be amplified reliably by PCR, therefore only minute quantities of starting material are required. This means that organisms such as highly endangered species, that are difficult if not impossible to study with minisatellites or allozymes (which require large quantities of source DNA) are now accessible to conservation

genetics studies. These organisms can be sampled non-destructively and non-invasively, because DNA can be extracted and amplified by PCR from hair follicles (Gagneux *et al.* 1997; Menotti-Raymond *et al.* 1997; Morin *et al.* 1994), sloughed skin (Valsecchi *et al.* 1998), saliva (Tautz 1989), feathers (Ellergren 1992) and even faecal material (Gerloff *et al.* 1999; Taberlet *et al.* 1997). In addition, ancient and extinct species can now also be examined. Museum specimens and even highly degraded material can be used for microsatellite studies because the typical microsatellite target sequence necessary for PCR amplification is sufficiently small (100 – 200 bp) that fragments of this size usually still persist in degraded material (Ashley & Dow 1994; Queller *et al.* 1993).

During PCR, short oligonucleotides of ~15 – 25 bp in length, called primers, are required to identify and amplify the region of interest. These primers bind to complementary regions of the DNA sequence flanking the area to be amplified, in this case, the microsatellite. Therefore, in order for the PCR to be successful, the unique DNA sequences on either side of the locus of interest must be known, so that primers specific for the locus can be designed. An advantage of microsatellites over minisatellites is that the locations of microsatellite sequences are frequently conserved between species (Moore *et al.* 1991). Thus PCR primers developed in one species can frequently be used in closely related taxa (Bruford *et al.* 1996). Moore *et al.* (1991) for example, examined 48 polymorphic sets of primer pairs developed in bovines, and found that 56% of these loci could be amplified successfully in sheep, and 42% showed polymorphisms.

However, if primers do not exist for a species, and those designed for related species do not work optimally, it is usually necessary to construct a genomic library to identify and characterise microsatellite loci unique to the species of interest. This was the case in the current study. It was found that primers developed for other bat species did not work optimally in *M. schreibersii*. It was therefore necessary to develop a genomic library for this species. A detailed description of the methodology followed in this procedure was given in Chapter 3.

iii. Ease of analysis

After a microsatellite has been amplified through PCR, its alleles are separated by electrophoresis on a vertical polyacrylamide gel. These gels have sufficient resolution to separate alleles that differ by as little as one base pair. For this reason, and because the alleles tend to differ from each other in a predictable way (*i.e.* by addition or deletion of multiples of the microsatellite repeat unit), microsatellite gels are easy to score. Most importantly, microsatellite alleles can be sized consistently and reliably, and are comparable across different gels, because a size marker is run on each gel alongside the samples, providing a reproducible standard.

As described above, multilocus DNA fingerprinting employs a hybridisation probe designed to bind to the variable minisatellite region itself, resulting in visualisation of several minisatellite loci simultaneously. Thus it is not possible to determine genotypes at specific, individual loci (Ashley & Dow 1994). On the other hand, individual microsatellite loci can be distinguished from one another. Individual genotypes can therefore be determined easily and reproducibly because the PCR primers used to amplify each microsatellite are specific to the unique flanking sequences for the repeat region of interest. Furthermore, microsatellites are codominantly inherited (Ashley & Dow 1994). This means that if a diploid organism inherits two different microsatellite alleles (*i.e.* if it is heterozygous), both alleles will be amplified during PCR and will be visible as separate bands on a gel. One will not dominate over or prevent the expression (or amplification) of the other, as in classic dominance-recessiveness relationships. If an individual inherits two identical alleles at a particular microsatellite locus (*i.e.* if it is homozygous) only one allele or band will be visible on the gel. In this way, the genotype of each individual (heterozygous or homozygous) can be determined (Figure 4.1). An advantage of codominant inheritance is that genotypic parameters such as levels of heterozygosity and inbreeding can be estimated more easily and reliably than under conditions of dominance, when heterozygotes cannot be distinguished from homozygous dominant individuals (Queller *et al.* 1993).

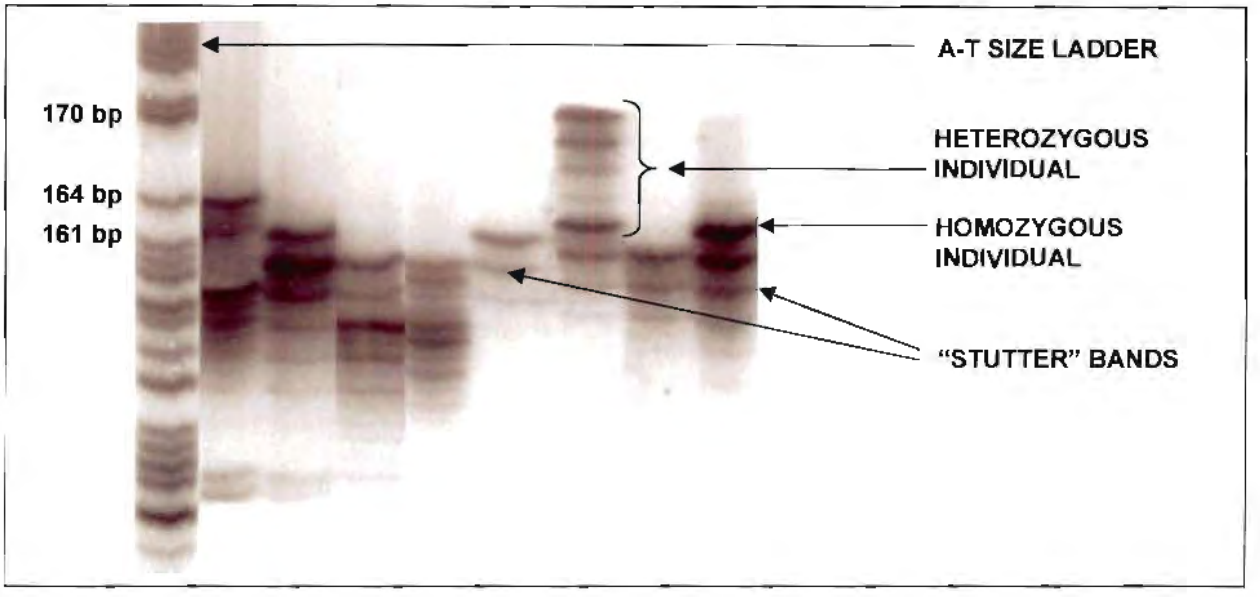

Figure 4.1 Example of a gel of a hypothetical microsatellite locus with six alleles, showing homozygous and heterozygous individuals and slippage products ("stutter bands").

4.1.4. Applications of microsatellites

As a consequence of the many advantages described above, in particular their ubiquity and high levels of polymorphism, microsatellites have become invaluable as neutral genetic markers for population genetics studies. Microsatellite data can be used for a variety of applications, including assessment of heterozygosity, as well as genetic relatedness or kinship (*e.g.* for the design of captive breeding programmes for endangered species), examination of social structure (*e.g.* Amos *et al.* 1993; Richard *et al.* 1996), hybridisation (*e.g.* Reich *et al.* 1999; Roy *et al.* 1994), current and historical population migration patterns (Hughes & Queller 1993), and for estimation of effective population size and levels of inbreeding (*e.g.* Reeve *et al.* 1991).

Microsatellites are particularly useful for determining reproductive success and for paternity analyses (*e.g.* Craighead *et al.* 1995; Gerloff *et al.* 1999; Harley *et al.* 2000; McCracken 1997; McCracken *et al.* 1999; Petri *et al.* 1997) because they show codominant, Mendelian inheritance (Ashley & Dow 1994). Furthermore, the ubiquity of microsatellite loci in eukaryotic genomes also makes them useful for linkage mapping (Bruford & Wayne 1993; Hearne *et al.* 1992; Menotti-Raymond *et al.* 1999). Most importantly for the purposes of this study, microsatellites are a precise tool for measuring levels of population substructuring, gene flow, dispersal and genetic variation in natural populations (Bruford & Wayne 1993; Schlotterer & Pemberton 1994).

4.1.5. Limitations of microsatellites

As mentioned in Section 4.1.3 (ii), one disadvantage of microsatellites is that it is frequently necessary to develop a genomic library in order to identify microsatellite loci in new species. Other disadvantages include non-amplification of alleles (due either to null alleles or "dropped" alleles) and "stutter bands", both of which can complicate analysis of microsatellite data. Null alleles result when a mutation in the regions flanking the microsatellite prevent optimal binding of the PCR primers in certain individuals, and thus one or more alleles in those individuals does not amplify. As a result, heterozygous individuals may be mistyped as homozygotes. If null alleles are common in the population, this may lead to an apparent excess of homozygous individuals in the population, leading to a deviation from Hardy-Weinberg equilibrium (Section 4.2.5; Callen *et al.* 1993; Pemberton *et al.* 1995; Schlotterer & Pemberton 1994). Similarly, random alleles may fail to amplify when very low quantities of template DNA are used in the PCR, such as when microsatellites are amplified from faeces (Taberlet *et al.* 1996). In this case, non-amplifying alleles are referred to as "dropped alleles", and if this occurs frequently, may also lead to an apparent excess of homozygotes in the population. The presence of both null and "dropped" alleles can cause significant deviation from Hardy-Weinberg equilibrium (Section 4.2.5).

A common complication associated with the amplification of dinucleotide repeats is the presence of one or more "stutter bands" visible on the polyacrylamide gel, usually underneath the main allele band (Figure 4.1). These amplification artefacts are most likely the result of slippage events during the PCR replication process, in which the template DNA and the newly synthesized strand misalign during elongation. This results in the amplification of additional truncated products (Hearne *et al.* 1992; Tautz 1989). Generally however, the stutter bands appear fainter on the gel than the main alleles, and thus can be disregarded fairly easily. In some cases, the presence of faint, regularly spaced stutter bands may even assist with sizing of the true alleles, as they can be used to indicate the exact spacing between alleles.

A final limitation of microsatellites is that they are generally typed by means of determining the length of a PCR product, as visualised on a gel, rather than through sequencing of each and every allele. Mutations are more likely to occur in the microsatellite itself than in the more conserved flanking regions, and therefore any observed differences in length of the PCR products are attributed to a change in the microsatellite repeat number, rather than to insertions or deletions in the flanking region. Nevertheless, if such mutations in the flanking regions do occur, and are mistakenly attributed to a mutation in the microsatellite, they may lead to an increase in variance, and thus inflate variance based genetic distances (Schlotterer & Pemberton 1994). However, insertions or deletions in the flanking regions occur relatively rarely. Furthermore, because the simultaneous occurrence of two mutation events is even less likely than a single mutation, when they do occur they generally alter the allele size by a single base pair, rather than by a multiple of the dinucleotide repeat unit. Abnormal variation in allele size can be detected on a polyacrylamide gel, drawing attention to a possible mutation in the flanking region. This can then be confirmed by direct sequencing of the PCR product. Generally, however, the advantages of microsatellites outweigh their limitations, and they are increasingly becoming the marker of choice for population genetics studies.

4.2. MATERIALS AND METHODS

4.2.1. Identification of species-specific microsatellite loci

New species-specific microsatellite loci were isolated in *M. schreibersii,* through the construction and screening of a genomic library (Chapter 3). Five novel, polymorphic microsatellite loci (named Mschreib1 – Mschreib5) were identified and characterised in *M. schreibersii.* Primers specific for these loci were designed and synthesised (Table 3.4, Chapter 3), and used to amplify the microsatellite loci as described below.

4.2.2. Polymerase Chain Reactions (PCR's)

i. End-labelling of primers

The forward primer for each locus was end-labelled with γ^{32}P-ATP, by incubation with T4 polynucleotide kinase (PNK, supplied by New England Biolabs, Cape Town). Stock γ^{32}P-ATP (150 mCi/ml) was supplied by Amersham Pharmacia Biotech (AP Biotech) and was diluted to a working concentration of 20 µCi/µl. End-labelling was allowed to continue for a minimum of 90 minutes at 37°C, and then stopped by incubation at 80°C for two minutes. A typical protocol for end-labelling of the forward primer is given below.

10x PNK Buffer (New England Biolabs)	2.0 µl
100 pmol/µl forward primer	4.5 µl
10 U/µl T4 polynucleotide kinase (PNK)	1.0 µl
Sterile deionised water	11.5 µl
20 µCi/µl γ^{32}P-ATP	1.0 µl
TOTAL VOLUME	20.0 µl

ii. PCR optimisation

Primers were optimised on between ten and twenty bat samples. An annealing temperature of 55°C was initially used for all loci. Depending on the results obtained in this first PCR, the annealing temperature was raised for some loci to increase specificity. Generally, 55°C proved to be optimal but some primer pairs functioned better at 58°C (Table 3.4, Chapter 3). A magnesium chloride ($MgCl_2$) titration was also conducted to optimise the concentration of $MgCl_2$ to be included in the PCR reaction. In general, 1.5 mM $MgCl_2$ was optimal. Generally, a final concentration of 0.2 mM deoxynucleotides (dNTP's) was used, but some primer pairs regularly produced multiple slippage products ("stutter bands"), and this was minimised by decreasing the concentration of dNTP's to 0.1 mM.

iii. PCR cocktails and cycles

Once a pair of primers had been optimised, bulk PCR cocktails were prepared to enable an entire colony of bat samples (~20 to 40 individuals) to be analysed in one PCR reaction. The above end-label cocktail was generally sufficient for up to 50 samples. Two different *Taq* DNA polymerases (BIOTAQ DNA Polymerase: Bioline USA; *Taq* DNA polymerase: Promega) and their associated buffers (10x magnesium-free reaction buffer and either 25 mM or 50 mM $MgCl_2$) were used, both supplied by Whitehead Scientific, Cape Town. Deoxynucleotides were supplied individually as 100 mM solutions by AP Biotech, and were combined in equal quantities to produce a working stock of 25 mM.

A typical PCR cocktail for 40 samples contained the following:

10x reaction buffer	40.0 µl	(final concentration 1x)
50 mM $MgCl_2$	12.0 µl	(final concentration 1.5 mM)
25 mM dNTP's	3.2 µl	(final concentration 0.2 mM)
100 pmol/µl reverse primer	4.0 µl	(final concentration 1 pmol/µl)
End-labelled forward primer	20.0 µl	(final concentration 1.1 pmol/µl)
5 U/µl *Taq* polymerase	2.6 µl	(final activity ~0.3 U)
Sterile deionised water	278.2 µl	
TOTAL	360.0 µl	

Each PCR was performed under mineral oil in a 10 µl reaction volume, containing 9 µl of the above cocktail and 1 µl template DNA. Template DNA concentration ranged between 10 – 50 ng/µl, depending on the sample. The PCR's were performed in a Stratagene RoboCycler96. A typical PCR cycle consisted of 2 minutes at 95°C, followed by 30 – 35 cycles of 50 seconds at 95°C, 50 seconds at the optimal annealing temperature (*e.g.* 55°C) and 1 minute at 72°C, followed by a single, final extension period of 10 minutes at 72°C. Each PCR was stopped by the addition of 4 µl formamide loading dye (Appendix 1), and stored at -20°C until electrophoresis.

4.2.3. Electrophoresis and scoring of alleles

Immediately prior to electrophoresis, the PCR products were denatured by heating to 94°C for two minutes. Denatured microsatellite alleles were separated by electrophoresis through a 6% denaturing polyacrylamide gel (Appendix 1) for three to four hours, at 60 – 65 W, and visualised by overnight exposure on Agfa CP-BU X-ray film. Examples of gels from two loci are shown in Appendix 3.

The genotype of each individual in each colony was determined as either homozygous or heterozygous, and the size of each microsatellite allele was estimated manually (scored) according to an M13mp18 AT sequencing ladder, containing fragments of known size. This ladder was prepared by dideoxy-sequencing (Chapter 5) of single-stranded M13mp18 DNA (Separations, Cape Town), using the –40 universal primer (sequence 5′-3′: GTT TTC CCA GTC ACG AC). Prior to sequencing, this primer was end-labelled by incubation with γ^{32}P-ATP and T4 PNK for 90 minutes at 37°C, according to the following protocol:

0.5 pmol/µl -40 universal primer		6.67 µl
10x PNK reaction buffer		1.0 µl
20 µCi/µl γ^{32}P-ATP		1.0 µl
10 U/µl T4 PNK		0.67 µl
Sterile distilled water		0.66 µl
	TOTAL	10.0 µl

Dideoxy-sequencing was performed with the Sequenase Version 2.0 Sequencing Kit (USB; supplied by Separations), according to the manufacturer's instructions. However, only two reaction tubes, containing dideoxyadenine (ddA) and dideoxythymine (ddT), were used instead of the usual four. These were combined after individual sequencing reactions to produce an AT ladder.

4.2.4. Published microsatellite locus

In addition to the five polymorphic loci identified in this study (Mschreib1 – Mschreib5), one published microsatellite locus (Moore *et al.* 1998) was also successfully amplified in the *M. schreibersii* samples. The NCAM locus is a polymorphic, dinucleotide microsatellite, located at the 3′ untranslated end of the neural cell adhesion molecule (NCAM) gene. It is conserved across

eutherian mammal species, and has previously been amplified successfully in several species, including humans, pinnipeds, bovids and cetaceans (Moore *et al.* 1998). It is likely that this is the first time this locus has been amplified in bats. Optimisation of this locus, subsequent PCR amplification (using an annealing temperature of 55°C) and electrophoresis were performed as described above for the Mschreib loci.

4.2.5. Statistical analysis of results

i. Locus characteristics

Any bat samples that did not amplify at four or more of the Mschreib and NCAM loci were excluded from the statistical analyses. In total seven samples were thus excluded (five from De Hoop, one from Jozini Dam and one from Madagascar). The total number of individuals from major South African colonies that were genotyped was therefore 301. The number of different alleles per locus, the frequency of each allele in each colony, and the observed (H_O) and expected (H_E) heterozygosity (average level of genetic variation) for each locus were determined with the assistance of the computer software package *AGARst* (Harley 2001).

Allele frequencies were tested for compliance with Hardy-Weinberg expectations by means of Fisher's exact test, which tests whether the distribution of allele frequencies is attributable to change sampling effects (Markov Chain parameters: 1000 dememorisations, 100 batches of 1000 iterations each; implemented in *GENEPOP*, Raymond & Rousset 1995a). The principle of Hardy-Weinberg equilibrium states that in the absence of selective pressure, migration and genetic drift, allele frequencies (at a two-allele locus) in a randomly mating population maintain a stable equilibrium at which the genotypic frequencies are $AA = p^2$, $Aa = 2pq$ and $aa = q^2$, where p is the frequency of allele A, and q is the frequency of the alternative allele a. Deviation of allele

frequencies from these expected levels may indicate (1) non-random sampling methods, (2) the individuals within the population are not randomly mating (*e.g.* due to assortative mating or high levels of inbreeding), (3) there is substructuring within the population, and genes from neighbouring populations may be migrating into this population, (4) selection may be acting on one or more of the alleles, or (5) there is non-random association of alleles during gamete formation (Murphy *et al.* 1996). An excess of homozygotes may also indicate the presence of significant numbers of non-amplifying alleles (see Section 4.1.5 and below). Compliance with or deviation from Hardy-Weinberg expectations was tested statistically by means of Fisher's exact probability test on contingency tables, implemented in the software package *GENEPOP* (Raymond & Rousset 1995a). This programme was also used to calculate F_{IS}, an estimate of the degree of inbreeding in a population (Section 4.2.6). Sequential Bonferroni corrections were used to compute critical significance levels for simultaneous tests of deviation from Hardy-Weinberg equilibrium (Rice 1989).

Due to the relatively low quantities of DNA extracted from the skin biopsies, "dropped" alleles (Section 4.1.5) were potentially a problem in this data set and could cause deviations from Hardy-Weinberg equilibrium. The incidence of these non-amplifying alleles was estimated in two ways. First, allele frequencies at each locus were tested with *NULLTEST* (Allen *et al.* 1995). This programme assumes that any excess of homozygotes detected in the data set is the result of non-amplifying alleles, and calculates the frequency of such alleles at each locus that would best account for this homozygote excess. A second estimate of the incidence of null alleles was obtained as follows. One hundred and thirty five individuals were selected at random from all colonies, and PCR's were repeated for these individuals at locus Mschreib2 (also chosen at random from the six loci). Individuals were scored as either heterozygous or homozygous, without reference to the previous analysis of this locus, and any discrepancies between the two sets of PCR's were recorded. Because "dropped" alleles are caused by low quantities of DNA, rather than a mutation in the primer binding site, their occurrence is likely to be random and not linked to any

particular locus. The results from locus Mschreib2 can therefore be generalised to the other loci, and it was not considered necessary to repeat the procedure for the other Mschreib loci.

Each locus was tested for linkage disequilibrium using *GENEPOP*, under the null hypothesis that loci are independent of one another. During this test, contingency tables for all pairs of loci in each colony were created, and tested for significant deviation from the null hypothesis by means of Fisher's exact test, using a Markov chain method (Raymond & Rousset 1995a) with 1000 dememorisations and 1000 batches of 1000 iterations each. The Markov chain involves a random examination of all possible contingency tables, under the assumption that the probability of a change from one state to another at any given site is independent of the history of that site (Swofford *et al.* 1996). In order to ensure that each batch of iterations of the Markov chain begins at a random position, a pre-defined number of initial dememorisation steps is explored before the newly generated contingency table is compared to the previous table. These dememorisation steps ensure the chain "forgets" its initial state, making it independent of its starting position (Schneider *et al.* 2000).

ii. Demographic history of *M. schreibersii* colonies

Garza & Williamson's (2001) *"M"* value was estimated using *AGARst* (in which this procedure is implemented) to examine the demographic history of each of the sampled *M. schreibersii* colonies in South Africa. *M* is the mean ratio between the number of microsatellite alleles (*k*) and the range of allele sizes (*r*), and can be used to detect historical reductions in population size. When a population experiences a bottleneck, the rate of genetic drift in that population increases and alleles are lost. Any loss of alleles will affect the value of *k*, but loss of only the smallest and/or largest alleles will affect the value of *r*. The rarest alleles (which are generally the first to be lost after a population bottleneck) are usually not the largest or the smallest in the range, because microsatellite allele frequency distributions in populations rarely conform to a normal distribution. The range of allele sizes therefore decreases more slowly than the number of alleles after a

reduction in population size. The ratio $M = {}^{k}/_{r}$ therefore decreases as the severity and duration of the bottleneck increases, and can be used to predict the demographic history of a population. For example, Garza and Williamson (2001) found that populations with a known history of a bottleneck tend to have $M < 0.65$.

iii. Colony differentiation

To assess the level of population subdivision, the genetic variation of one colony was compared to that of other colonies, or to that of the entire population by means of fixation indices. A brief description of the theory behind these fixation indices is given in Section 4.2.6. A global estimate of *Rho* (an estimate of R_{ST}, corrected for sample size differences, see Section 4.2.6) for *M. schreibersii* in South Africa was obtained with the assistance of the software packages *AGARst* and *R_{ST} CALC* (Goodman 1997). The former programme calculates the mean *Rho* value by averaging across all loci (Harley 2001), while *R_{ST} CALC* calculates the mean either by averaging across loci or across variance components (Goodman 1997). Pairwise comparisons between all South African colonies of *M. schreibersii* were performed in order to estimate the extent of genetic differentiation between individual colonies. These pairwise estimates of R_{ST} (and *Rho*) were calculated from data sets standardised for different sample sizes (Section 4.2.6), using three different software packages, namely *AGARst*, *R_{ST} CALC* and *Arlequin* (Schneider *et al.* 2000). In addition, both global and pairwise G_{ST} values were estimated using *AGARst*. Permutation tests were implemented in *R_{ST} CALC* and *Arlequin* to determine whether observed estimates of R_{ST} (*Rho*) differed significantly from zero. Critical significance values for these tests were computed using sequential Bonferroni corrections (Rice 1989).

The number of migrants exchanged between colonies per generation (N_m) and the genetic distance $(\delta\mu)^2$ (Goldstein *et al.* 1995) between colonies were estimated using *R_{ST} CALC*. N_m was

estimated from R_{ST} (*Rho*) as described in Slatkin (1995). The distance measure $(\delta\mu)^2$ was calculated from unstandardised allele size data (Section 4.2.6) according to the formula:

$$(\delta\mu)^2 = (\mu_A - \mu_B)^2$$

where μ_A and μ_B are the mean allele sizes in populations A and B respectively (Goldstein *et al.* 1995). They are determined by first calculating the mean allele size at each locus in each colony, and the squared difference in mean allele size is then averaged over loci. $(\delta\mu)^2$ was developed as a distance measure for microsatellite data, based on the stepwise mutation model (Section 4.2.6). It is independent of population size when populations are at equilibrium, has low variance and changes linearly over time. It thus provides a more reliable estimate of genetic distance for microsatellite data than other distance measures that are based on infinite allele models (Goldstein *et al.* 1995). A UPGMA (unweighted pair group method using arithmetic averages) phylogenetic tree was constructed from the $(\delta\mu)^2$ values with the assistance of the software packages *Populations* (Langella 2001) and *TreeView* (Page 1996).

Pairwise R_{ST} and $(\delta\mu)^2$ values between *M. schreibersii* colonies were calculated separately for males and females, and compared with Mann-Whitney rank sum tests (Zar 1984) to determine whether the pattern of colony differentiation differed significantly between the sexes. Mann-Whitney rank sum tests were performed with the assistance of *SigmaStat* (Jandel Corporation 1995). In addition, the extent of genetic differentiation between the sexes within individual colonies was estimated by calculating pairwise R_{ST} (*Rho*) values between males and females within each colony, using both *AGARst* and *R_{ST} CALC*.

Mantel tests were conducted to determine whether any correlation exists between geographic separation of colonies and genetic distances (*i.e.* $(\delta\mu)^2$) between them, or between geographic separation and the extent of genetic differentiation (*i.e.* pairwise R_{ST} values) among colonies. These tests were implemented in *Arlequin* and *GENEPOP* respectively.

To obtain a graphical representation of any colony differentiation that may exist, principle component analysis was performed on the microsatellite data with the assistance of *PCA-GEN* (Goudet 1999), incorporating 1000 randomisations. Principle component analysis is reviewed in Chapter 6 (Section 6.2.6).

iv. Assignment tests on individuals from major colonies

An additional means of assessing the degree of differentiation among colonies is through the implementation of an assignment test. This test, developed by Paetkau *et al.* (1995), provides an indication of how characteristic any particular individual's multi-locus genotype is of the colony from which it was sampled. Under the assumptions of random mating and no linkage disequilibrium, the assignment test calculates the expected frequency of each individual's genotype in each colony, and then assigns each individual to the colony where its expected genotypic frequency is highest. The greater the number of individuals "correctly" assigned to the colony from which they were actually sampled, the more characteristic the allele frequencies associated with that colony are likely to be. The test can also be used to assess where individuals of unknown colony affiliation are likely to have originated. Accordingly, an assignment test was performed on all individuals from all colonies to assess how characteristic their genotypes were of the colonies from which they were collected.

v. Assignment tests on smaller numbers of individuals from minor sample sites

In addition to the ten major sample sites in South Africa, a limited number of samples were also obtained from underneath the Pongola River Bridge (PRB, n = 6) in KwaZulu-Natal (Section 2.1.1, Chapter 2), and from Knysna (K, n = 2) and the Cedarberg Mountains (C, n = 1) in the Western Cape. These individuals were not sampled from large colonies, and so their affinity with major maternity and/or hibernating roosts is unknown. They may have originated from one of the sampled colonies or more probably, from other colonies that are closely related to the major

sample sites. Two assignment tests were performed to assess firstly, to which subpopulation (Section 4.3.2) the individuals belong, and secondly, to determine which of the ten major colonies is most closely related to these bats.

vi. Genetic differentiation between South African and international *M. schreibersii*

The extent of genetic differentiation between South African *M. schreibersii* and those individuals obtained from international locations was estimated by pooling all South African samples (n = 310), and calculating both R_{ST} (*Rho*) and $(\delta\mu)^2$ between all pairwise combinations of the four geographic regions (South Africa, Australia, Israel and Madagascar). R_{ST} and $(\delta\mu)^2$ were estimated as described above.

4.2.6. Fixation Indices

Genetic variation or diversity within a population is measured in terms of heterozygosity (H), which is the frequency of heterozygous loci in a population (Crow 1986). Wright (1951) developed the concept of fixation indices (also known as F-statistics), which partition heterozygosity into components that reflect the genetic structure of a population. These components are H_I (which represents the genetic variation within individuals), H_S (representing genetic diversity among individuals within a particular subpopulation) and H_T (the genetic variation in the total population).

These components can be used to measure the level of subdivision within a population by means of the fixation indices F_{IS}, F_{IT} and F_{ST}. F_{IS} is defined as the proportion of variation within individuals relative to the variation found in subpopulations, while F_{IT} is the proportion of variation within individuals relative to that of the total population. F_{ST} is the proportion of variation occurring in subpopulations relative to the total variation found in the population (Dobson 1998; Sugg *et al.*

1996), and provides an indication of the extent of population substructuring. A strongly positive F_{ST}, for example, suggests significant genetic substructuring (Pope 1998).

Fixation indices can therefore be used to measure the degree of inbreeding and differentiation between subpopulations. In a randomly mating population, both F_{IT} and F_{IS} should be close to zero. Positive results for these F-statistics suggest higher rates of inbreeding than would be expected under conditions of random mating (*i.e.* promotion of consanguineous matings), while negative results indicate an excess of heterozygotes, such as may result from rapid expansion after a population bottleneck (Dobson 1998; Dobson *et al.* 1998; Long *et al.* 1998).

Nei (1973) developed an equivalent measure to F_{ST} for use with molecular data, known as G_{ST} (the coefficient of gene differentiation), which also indicates the extent of genetic differentiation within and between subpopulations:

$$G_{ST} = \frac{\left(H_T - \overline{H}_S\right)}{H_T}$$

where H_T = heterozygosity of the total population, calculated as if it were a single randomly mating unit

$\overline{H}_S$ = average heterozygosity of several subpopulations

Values of G_{ST} between 0.1 and 1.0 suggest progressively greater amounts of population substructuring, while small values (less than 0.05) suggest little substructuring (Hartl & Clark 1997).

Fixation indices such as F_{ST} and G_{ST} have traditionally been used to analyse results obtained from allozyme studies. However, these measures have drawbacks when used to analyse microsatellite data because they assume no mutation, and do not take into account the process by which microsatellites are thought to evolve, namely stepwise mutation (Slatkin 1995). During this process, mutant alleles arise by addition or subtraction of one or more entire repeat units. Therefore, the size of the new allele depends on that of the "ancestral" allele (*i.e.* the process has a

"memory"). This, together with the high mutation rate of microsatellite loci, and the fact that the mutation process can be reversible over time, means that the assumptions made when using F_{ST} and G_{ST} (*i.e.* low mutation rate and an infinite allele model of mutation) may not always be valid (Slatkin 1995). Consequently, when used to analyse microsatellite data, these statistics tend to underestimate population differentiation.

Slatkin (1995) developed an analogue of F_{ST}, known as R_{ST}, which is the fraction of the total variance in allele size found between populations, and which takes which the above factors into account. Consequently, R_{ST} should provide a more reliable estimate of population differentiation in the case of microsatellites:

$$R_{ST} = \frac{\left(\overline{S} - S_W\right)}{\overline{S}}$$

where $\overline{S}$ = twice the variance in allele size in the collection of populations together

S_W = twice the average variance in allele size within populations

Like F_{ST} and G_{ST}, R_{ST} also measures gene flow (Valsecchi *et al.* 1997), but it does so by examining variation in microsatellite allele lengths (as determined by the number of repeat units within the microsatellite), rather than variation in gene frequencies (Slatkin 1995). However, R_{ST} measures have a higher variance than G_{ST} (*i.e.* greater deviation from the mean), since they include the variation due to mutation as well as that due to genetic drift. Therefore, results from more loci may be needed to maximise accuracy. R_{ST} values range from zero to one, where zero indicates a panmictic population, and one indicates total philopatry with no gene flow. As for G_{ST}, values of R_{ST} between 0.1 and 1.0 indicate moderate to high levels of population substructuring, while values less than 0.05 suggest very little differentiation (Hartl & Clark 1997).

Slatkin's (1995) derivation of R_{ST} is idealised, and corrections must be made for differences in sample sizes between populations, as well as for differences in the degree of variation of allele sizes between loci. This is because if sample sizes between populations differ significantly, those

populations with smaller sample sizes will contribute less to calculations of total variance than will those with larger sample sizes. Furthermore, if loci have very different variance values, then loci that have less variance (despite perhaps having many alleles) will contribute less to the final value of R_{ST} (Goodman 1997). Allele sizes should therefore be standardised across the entire data set in order to make different loci comparable. This is done by expressing allele sizes in terms of standard deviations from a global mean, rather than as the number of repeat units. This transformation of the data is performed with the assistance of the computer software package *R_{ST} CALC* (Goodman 1997). The standardised value of each allele (Y_S) at each locus is determined according to the following formula:

$$Y_S = \frac{(Y - GM)}{SD}$$

where Y = allele *n* at locus *i*

GM = mean allele size in repeat units for locus *i* over the whole data set (global mean)

SD = standard deviation in allele size in repeat units for locus *i* over the whole data set.

Once the data set has been standardised, software packages such as *R_{ST} CALC* and *AGARst* (Harley 2001) can be used to calculate G_{ST} and *Rho*, which is an unbiased estimate of R_{ST} that takes into account differing sample sizes by examining variance components. In *R_{ST} CALC*, *Rho* is calculated as follows:

$$Rho = \frac{S_b}{(S_b + S_W)}$$

where S_b = is the component of variance that is found between populations

S_W = twice the average variance in allele size within populations

The programme *AGARst* implements a similar procedure. *Rho* can be determined across all loci and all populations, or for each possible pairwise combination of populations, as discussed in Section 4.2.5.

4.3. RESULTS

4.3.1. Locus characteristics and demographic history of colonies

Between two and fifteen alleles were identified per Mschreib locus in each of the major *M. schreibersii* colonies (Table 4.1), and between one and five alleles were identified per colony for the NCAM locus. Private alleles (alleles that are unique to a colony) were found in six of the ten major colonies, as well as in individuals collected from the Pongola River Bridge, Madagascar and Israel (Table 4.2). Locus-specific allele frequency distributions for each colony (Figures 4.2 – 4.7) suggest that the South African *M. schreibersii* population may be genetically differentiated. Three relatively distinct groups of colonies can be discerned, based on the identity and frequency of different alleles found in each colony. These groups are (1) De Hoop and Die Hel; (2) Steenkampskraal and Koegelbeen and (3) Grahamstown, Maitland Mines, Shongweni Dam, Jozini Dam, Peppercorn and Sudwala. This potential population substructuring is quantified in Section 4.3.2.

No linkage disequilibrium was detected between any pairs of loci ($p \geq 0.20$). Observed and expected heterozygosity values were relatively high (> 0.6) for all colonies, although observed values were consistently lower than the expected values (Table 4.3), indicating an excess of homozygotes, and possible deviation from Hardy-Weinberg equilibrium. Indeed, after Bonferroni correction, six colonies were found to deviate significantly ($p \leq 0.05$) from Hardy-Weinberg expectations due to an excess of homozygotes, but only at one or two loci each (Table 4.4). No significant excess of heterozygotes was detected. F_{IS} values were also strongly positive at some loci (Table 4.4), which is indicative of an excess of homozygotes, possibly due to inbreeding.

Inbreeding is, however, unlikely in this case, for reasons discussed below. In three cases, the NCAM locus was responsible for the deviation from Hardy-Weinberg equilibrium. Polymorphism levels at this locus were much lower than at other loci. Although seven alleles were identified in total at this locus, most colonies contained only two or three alleles, and two (Grahamstown and Jozini Dam) were monomorphic at this locus.

Table 4.1. The number of alleles identified per locus in each *M. schreibersii* colony*, in other regions in South Africa and internationally. The number of individuals genotyped per locus is given in brackets.

Locus	Total alleles	Number of alleles identified per colony or region															
		DHP (39)	DHL (19)	SKK (20)	KB (40)	G (37)	MM (37)	SHD (29)	JD (28)	PC (19)	SW (33)	PRB (6)	K (2)	C (1)	A (6)	MD (3)	I (4)
Mschreib1	17	14	11	2	4	8	7	8	6	8	8	6	4	2	6	5	3
Mschreib2	20	9	8	7	10	10	11	12	15	15	13	8	2	2	3	3	3
Mschreib3	17	10	11	10	12	10	9	11	10	9	11	8	4	2	6	4	3
Mschreib4	18	11	10	8	12	9	9	12	12	14	13	8	2	2	6	4	6
Mschreib5	19	8	8	8	9	12	9	10	10	8	12	7	2	2	4	4	5
NCAM	7	5	3	3	3	1	2	2	1	2	2	1	1	1	1	1	2
TOTAL	98	57	51	38	50	50	47	55	54	56	59	38	15	11	26	21	22

Table 4.2. The number of private alleles identified per locus in each *M. schreibersii* colony*, in other regions in South Africa and internationally. The number of individuals genotyped per locus is given in brackets.

Locus	Total private alleles	Number of private alleles identified per colony or region															
		DHP (39)	DHL (19)	SKK (20)	KB (40)	G (37)	MM (37)	SHD (29)	JD (28)	PC (19)	SW (33)	PRB (6)	K (2)	C (1)	A (6)	MD (3)	I (4)
Mschreib1	6	3	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Mschreib2	3	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0
Mschreib3	2	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
Mschreib4	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mschreib5	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NCAM	4	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
TOTAL	17	5	2	2	1	0	0	0	1	3	0	1	0	0	0	1	1

* Names of colonies are abbreviated as follows:
DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown;
MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala,
PRB = Pongola River Bridge, K = Knysna, C = Cedarberg, A = Australia, MD = Madagascar, I = Israel

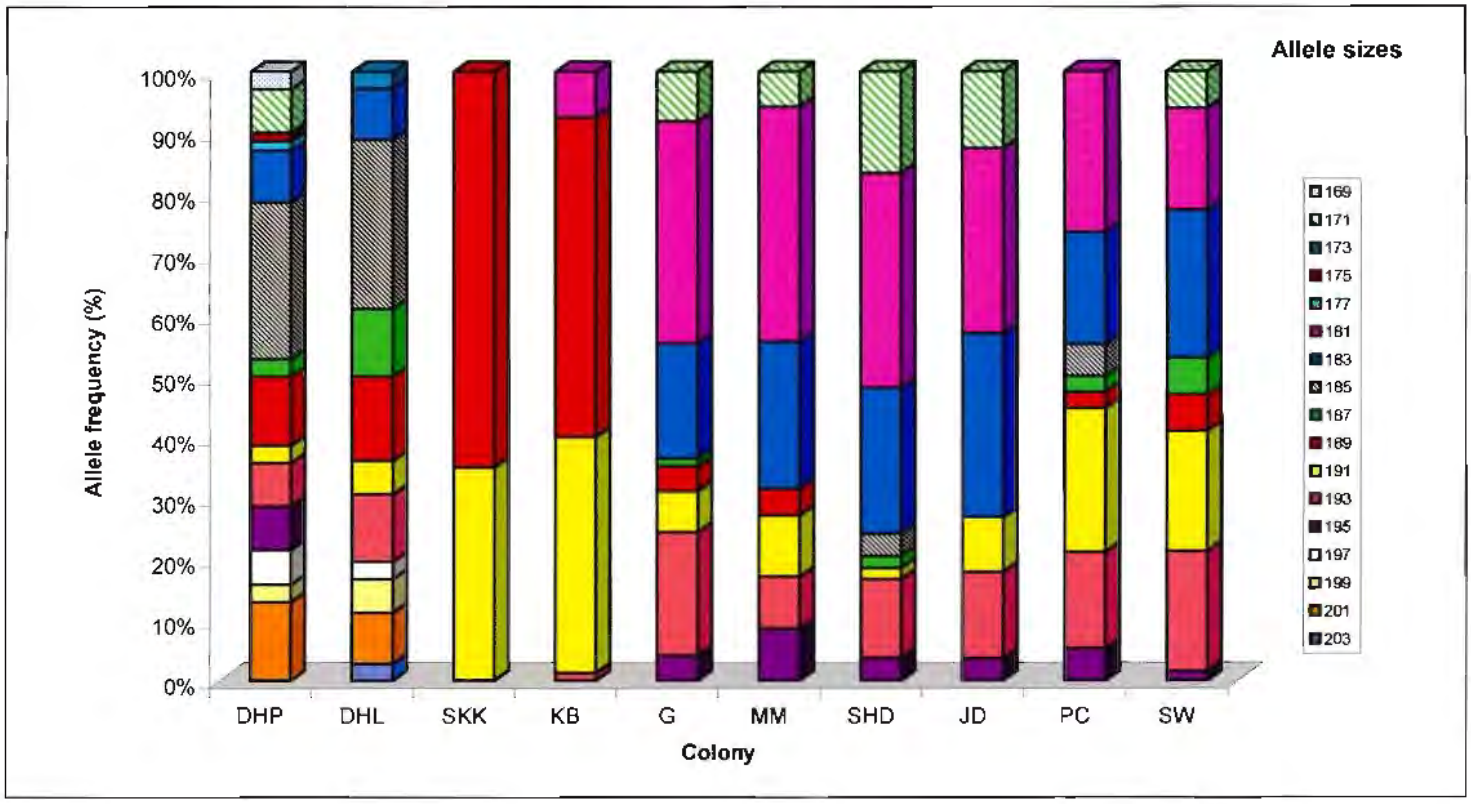

Figure 4.2. Allele frequency distribution for *M. schreibersii* colonies* at locus Mschreib1.

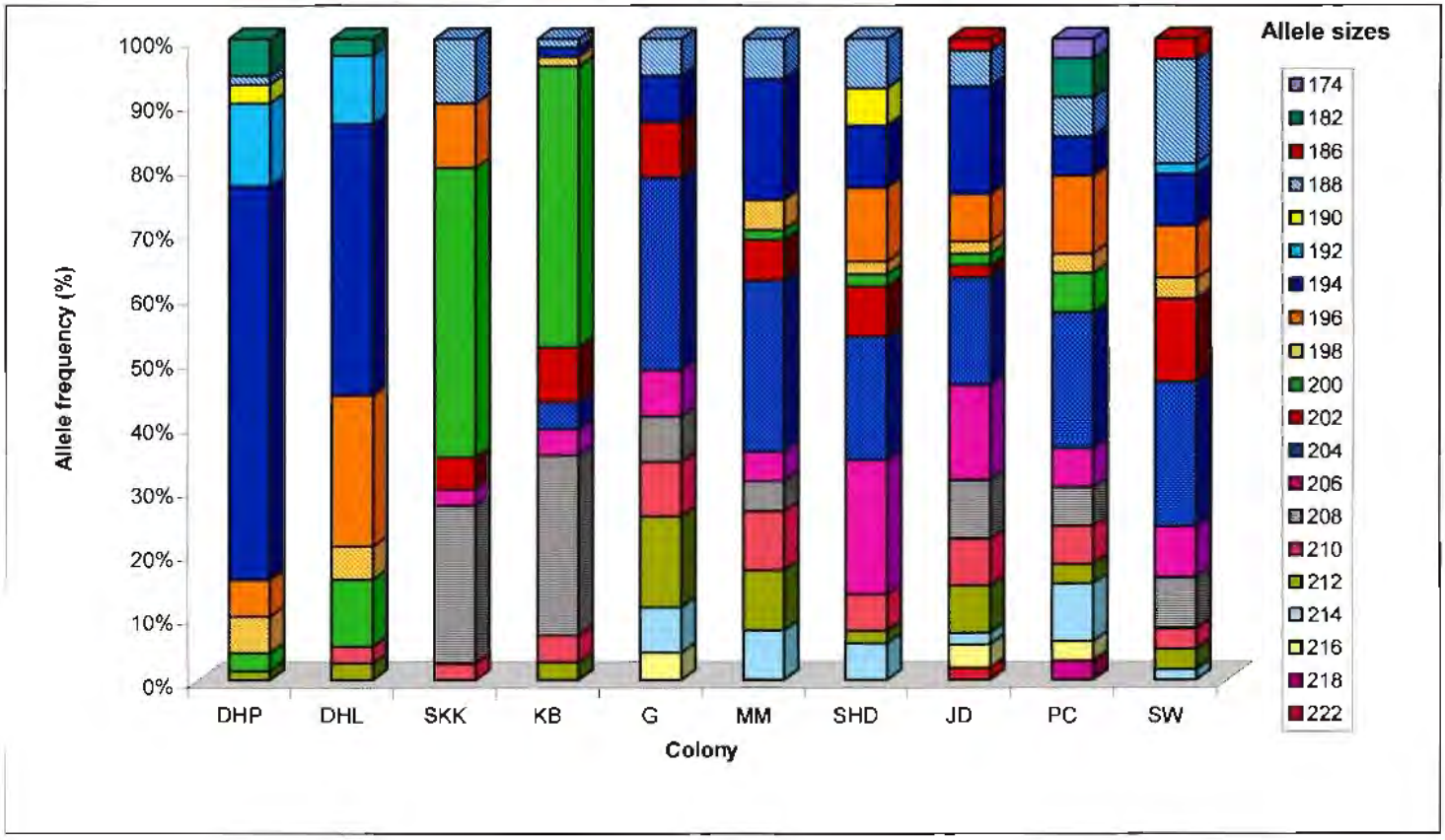

Figure 4.3. Allele frequency distribution for *M. schreibersii* colonies* at locus Mschreib2.

* Names of colonies are abbreviated as follows:
DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown;
MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala

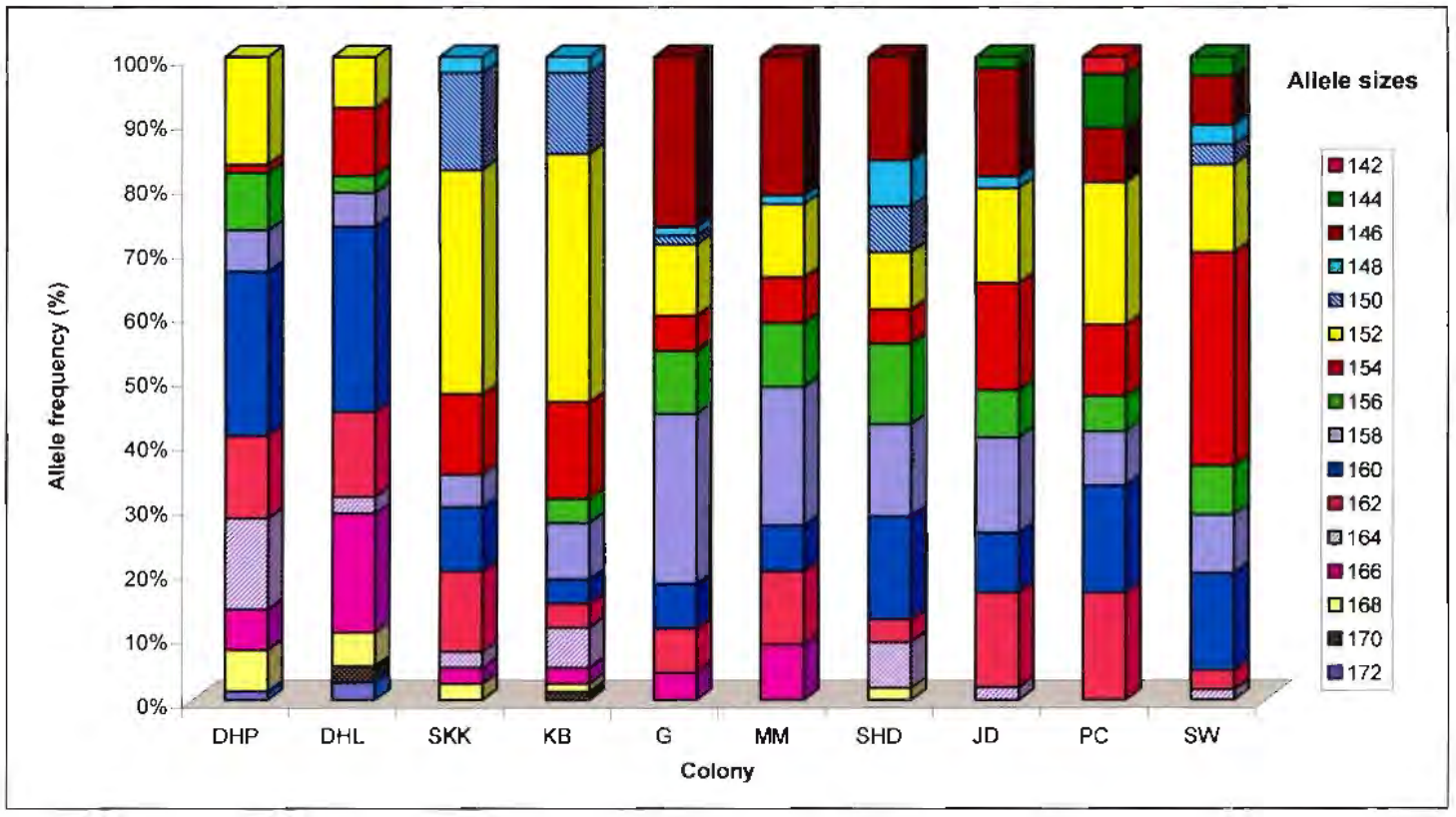

Figure 4.4. Allele frequency distribution for *M. schreibersii* colonies* at locus Mschreib3.

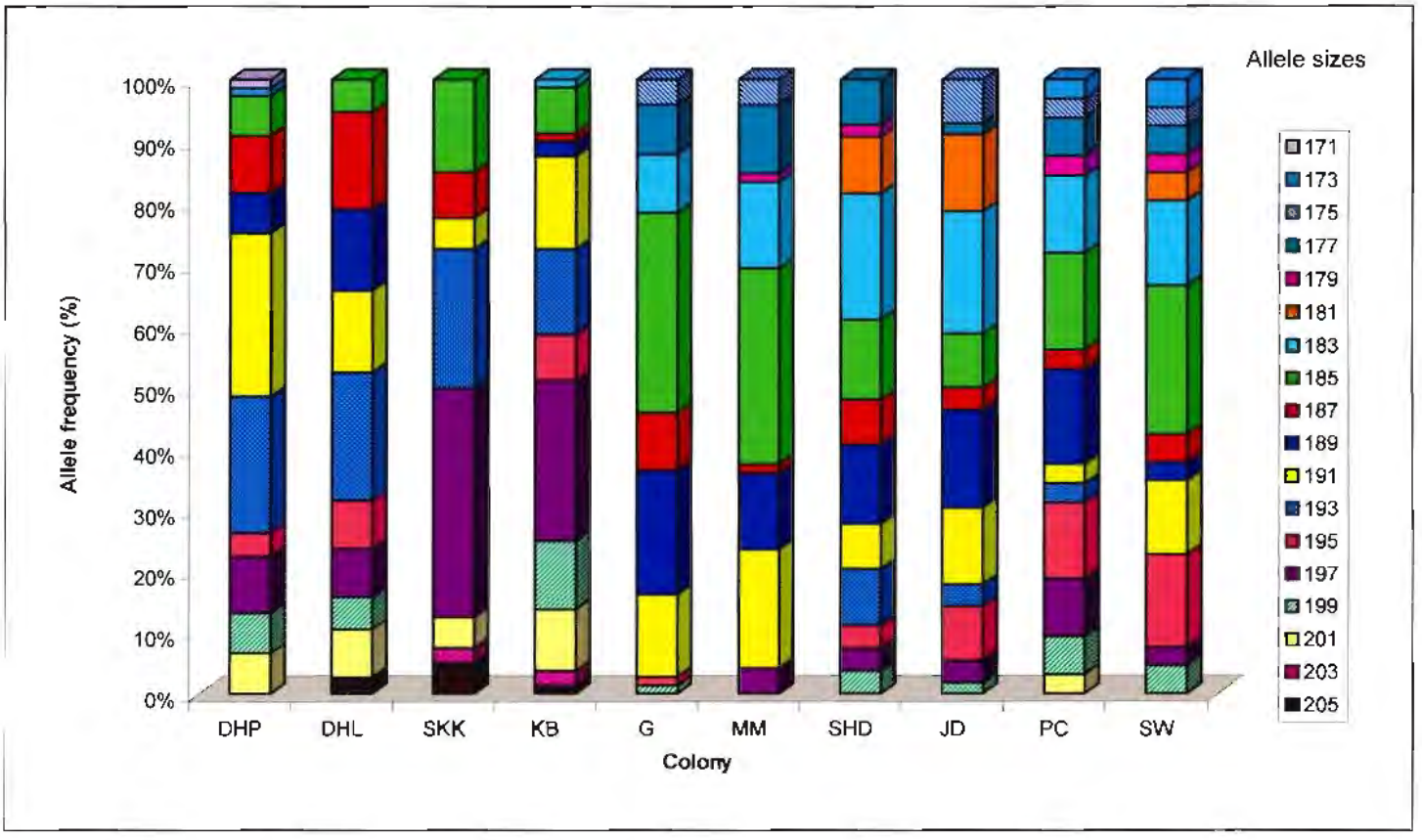

Figure 4.5. Allele frequency distribution for *M. schreibersii* colonies* at locus Mschreib4.

* Names of colonies are abbreviated as follows:
DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown;
MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala

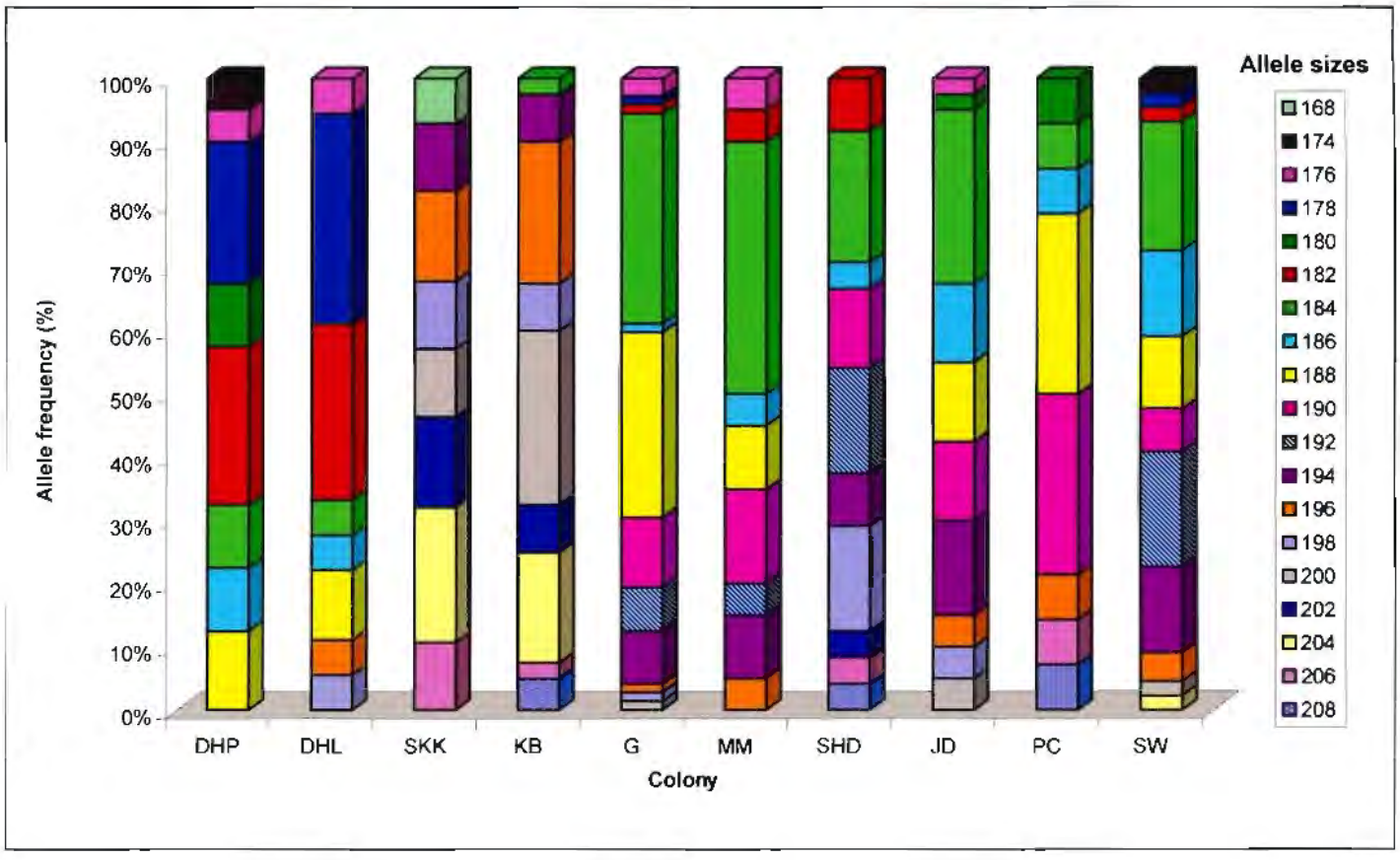

Figure 4.6. Allele frequency distribution for *M. schreibersii* colonies* at locus Mschreib5.

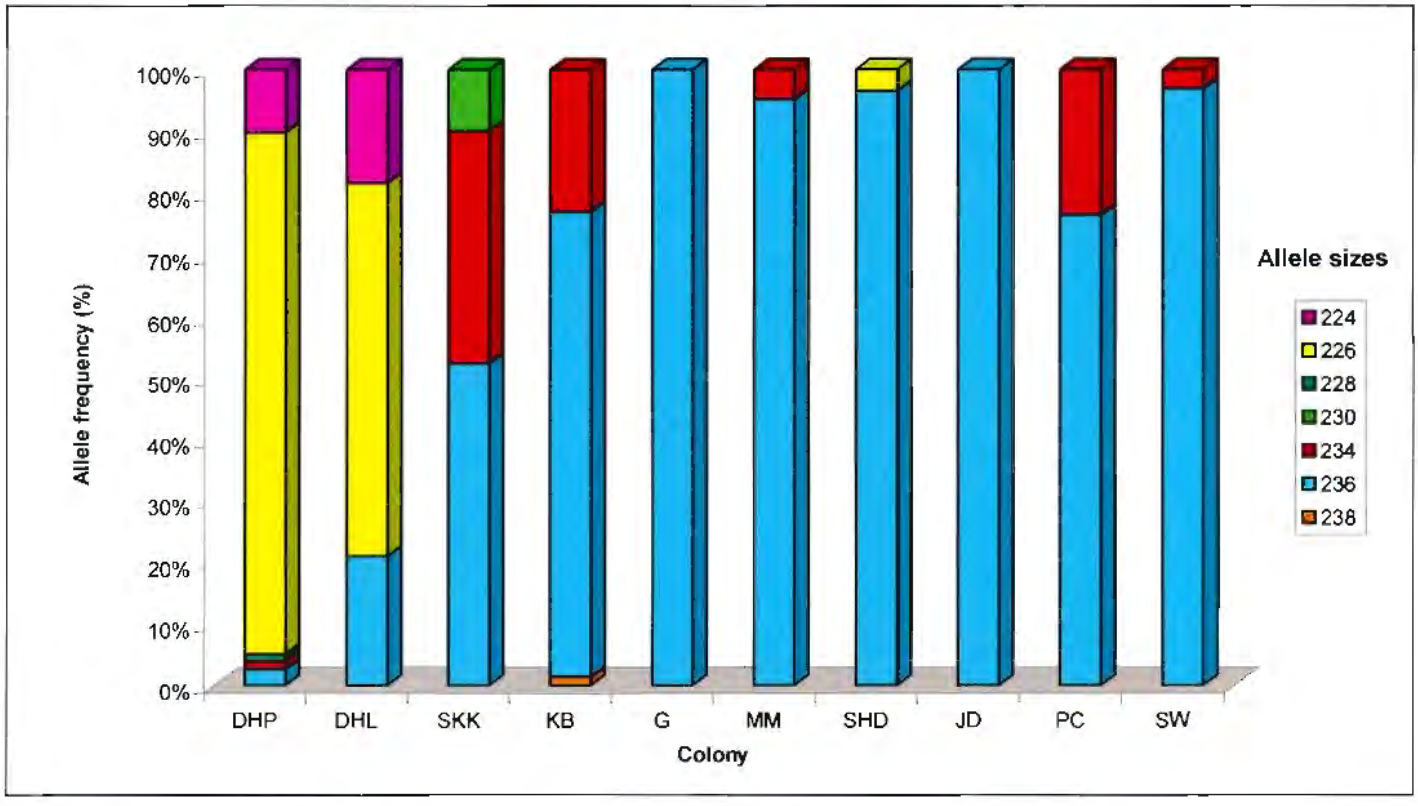

Figure 4.7. Allele frequency distribution for *M. schreibersii* colonies* at the NCAM locus.

* Names of colonies are abbreviated as follows:
DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown;
MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala

Table 4.3. Observed and expected heterozygosity values for each *M. schreibersii* colony.

Colony	n	Heterozygosity	
		Observed	Expected
De Hoop	39	0.626	0.712
Die Hel	19	0.621	0.774
Steenkampskraal	20	0.597	0.697
Koegelbeen	40	0.598	0.691
Grahamstown	37	0.650	0.673
Maitland Mines	37	0.654	0.693
Shongweni Dam	29	0.664	0.724
Jozini Dam	28	0.705	0.708
Peppercorn	19	0.649	0.772
Sudwala	33	0.652	0.720

Table 4.4. Probability of colonies deviating from Hardy-Weinberg equilibrium due to homozygote excess at each locus (*significance at $p \leq 0.05$), F_{IS} values for each colony† at each locus, and the frequency of non-amplifying alleles, as estimated by *NULLTEST*.

Locus	Probability (p) of homozygote excess									
	DHP	DHL	SKK	KB	G	MM	SHD	JD	PC	SW
Mschreib1	< 0.0001*	0.245	0.076	0.066	0.418	0.246	0.096	0.732	0.220	0.033*
Mschreib2	0.022*	0.088	0.992	0.895	0.436	0.217	0.300	0.213	0.252	0.179
Mschreib3	0.159	0.534	0.602	0.919	0.531	0.168	0.329	0.325	0.597	0.189
Mschreib4	0.362	0.184	1.746	0.015*	0.009*	0.216	0.281	0.958	0.721	0.305
Mschreib5	0.109	0.854	0.132	0.875	0.518	0.574	0.563	0.113	1.318	0.306
NCAM	0.056	< 0.0001*	0.071	0.004*	-	0.246	0.088	-	0.001*	0.080

Locus	F_{IS}									
	DHP	DHL	SKK	KB	G	MM	SHD	JD	PC	SW
Mschreib1	0.197	0.185	0.578	0.264	-0.032	0.003	0.057	-0.002	0.245	0.243
Mschreib2	0.053	0.172	-0.241	-0.091	0.005	0.106	0.094	0.063	0.118	0.059
Mschreib3	0.077	0.024	0.098	0.037	0.003	0.078	0.049	0.032	0.122	0.022
Mschreib4	0.016	0.121	-0.075	0.163	0.178	0.053	0.058	-0.079	-0.014	0.041
Mschreib5	0.069	0.067	0.004	0.114	0.085	0.034	-0.017	0.139	0.014	0.078
NCAM	0.539	0.910	0.666	0.576	-	0.659	1.000	-	1.000	1.000

Locus	Frequency of non-amplifying alleles
Mschreib1	0.136
Mschreib2	0.070
Mschreib3	0.045
Mschreib4	0.049
Mschreib5	0.075
NCAM	0.669

† Names of colonies are abbreviated as follows:
DHP = De Hoop, DHL = Die Hel,
SKK = Steenkampskraal, KB = Koegelbeen,
G = Grahamstown; MM = Maitland Mines,
SHD = Shongweni Dam, JD = Jozini Dam,
PC = Peppercorn, SW = Sudwala

Possible reasons why a colony may deviate from Hardy-Weinberg equilibrium include high levels of inbreeding and/or substructuring within that colony. These factors are unlikely to apply here because they would cause a significant deviation at all loci, not only at one or two. In addition, inbreeding is unlikely because the colonies concerned were polymorphic at all the Mschreib loci, and observed heterozygosity levels, although lower than the expected values, were still relatively high. Furthermore, these *M. schreibersii* colonies are large, many having in excess of 10 000 individuals, and inbreeding is unlikely to occur in such large populations. Finally, Garza and Williamson's (2001) *M* ratio was greater than 0.7 for all colonies (Table 4.5). These values of *M* are in accordance with those determined by Garza and Williamson (2001) for populations known to be historically large and outbred. This suggests that none of these *M. schreibersii* colonies has undergone a severe bottleneck in its recent history, and that they are all currently large and outbred.

A more likely explanation for the excess of homozygotes at a few of these loci is the presence of non-amplifying alleles. The frequency of these alleles was estimated by *NULLTEST* to be between 0.04 and 0.14 for the Mschreib loci (mean = 0.075), and 0.67 for the NCAM locus (Table 4.4). This programme assumes that any deviation from Hardy-Weinberg equilibrium is caused by an excess of homozygotes, which in turn is caused exclusively by the presence of non-amplifying alleles. The NCAM locus was found to deviate from Hardy-Weinberg equilibrium at three of the ten colonies, and was monomorphic in another two. It is possible that a mutation in the flanking region of one or more of its alleles has reduced the ability of one of the primers to bind, leading to a high incidence of null alleles at this locus.

However, null alleles are relatively rare, and are unlikely to be the primary source of homozygote excess in the majority of loci. In the case of the Mschreib loci (and possibly also the NCAM locus), "dropped" alleles may be a more likely source of non-amplifying alleles. The alternative method of estimating the incidence of "dropped" alleles at locus Mschreib2 (Section 4.2.5) produced an estimate of their frequency similar to that given by *NULLTEST* for locus Mschreib1. In 17 of the

135 (12.6%) PCR's that were repeated at locus Mschreib2, individuals formerly scored as homozygotes were secondarily scored as heterozygotes, or *vice versa*. These discrepancies are likely to be due primarily to the presence of "dropped" alleles. Therefore, while other factors causing deviation from Hardy-Weinberg equilibrium cannot be ruled out completely, due to the low quantities of DNA extracted from the tail biopsies, it is probable that "dropped" alleles are the primary reason for the excess of homozygotes at individual Mschreib loci.

Table 4.5. Mean value of Garza & Williamson's (2001) *M* for each colony. *M* is calculated as the mean ratio of number of alleles (*k*) to the range in allele sizes (*r*), across all loci.

Colony	*M*	Variance of *M*
De Hoop	0.783	0.025
Die Hel	0.699	0.050
Steenkampskraal	0.728	0.047
Koegelbeen	0.839	0.035
Maitland Mines	0.785	0.022
Grahamstown	0.761	0.021
Shongweni Dam	0.739	0.059
Jozini Dam	0.770	0.035
Peppercorn	0.823	0.038
Sudwala	0.860	0.023

4.3.2. Colony differentiation within South Africa

Mean *Rho* values indicate significant genetic substructuring exists in the *M. schreibersii* population in South Africa (Table 4.6). This is detectable both globally, as well as at each individual microsatellite locus. G_{ST} values are consistently much lower than corresponding *Rho* values, both when calculated for each locus and as a global mean across loci or across variance components.

This could be due to the fact that if significant mutational change has occurred since the ancestral population split into the different colonies, this may cause G_{ST} to underestimate the divergence. Furthermore, G_{ST} is based on an infinite allele model (Section 4.2.6), and so may underestimate the degree of population subdivision when applied to microsatellite data. However, it should be noted that not all microsatellite loci mutate in a stepwise manner, and the likelihood of single stepwise mutations occurring depends on the type, length and purity of the repeat region (P. Racey, pers. comm.). Therefore, in situations where a stepwise mutation model is not applicable, G_{ST} may in fact not underestimate the degree of population subdivision. Nevertheless, it is assumed that the dinucleotide repeats examined here mutate according to a stepwise mutation model, and R_{ST} (*Rho*) is therefore considered to be a more reliable measure of colony differentiation under these circumstances.

Table 4.6. Mean *Rho* and G_{ST} values for each locus, and global mean values across variance components (R_{ST} *CALC*) or across all loci (*AGARst*).

Locus	*Rho*		G_{ST}
	R_{ST} *Calc*	*AGARst*	(*AGARst*)
Mschreib1	0.150	0.145	0.137
Mschreib2	0.226	0.221	0.112
Mschreib3	0.143	0.143	0.056
Mschreib4	0.293	0.293	0.059
Mschreib5	0.469	0.454	0.095
NCAM	0.819	0.824	0.469
Global mean	0.360	0.347	0.155
Variance	< 0.00001	0.681	0.025
Std Error	0.001	0.107	0.064
Rho 95% confidence intervals: L = 0.338; U = 0.409 (1000 bootstrap pseudoreplications)			

The pattern of colony differentiation within South Africa can be ascertained from pairwise R_{ST} (*Rho*) and G_{ST} comparisons. Once again, G_{ST} values for each pairwise comparison are consistently much lower than all three estimates of R_{ST} or *Rho* (Table 4.7), and are considered less reliable than these latter measures. In the majority of comparisons, the R_{ST} or *Rho* values obtained from the three statistical packages correspond very closely. *R_{ST} CALC* and *Arlequin* assign probability values to each comparison, and after sequential Bonferroni correction, all but two of the 45 pairwise comparisons correspond at $p \leq 0.01$. However, in the comparison Peppercorn *vs.* Steenkampskraal, *Arlequin* calculated $R_{ST} = 0.103$ and assigned significance at $p \leq 0.05$, while *R_{ST} CALC* calculated *Rho* = 0.152 and assigned significance at $p \leq 0.01$. As *Rho* calculated by *AGARst* for this comparison (0.132) is more similar to that obtained by *R_{ST} CALC*, significant differentiation at $p \leq 0.01$ will be accepted. In the comparison Peppercorn *vs.* Grahamstown, *Arlequin* calculated $R_{ST} = 0.073$ and indicated these colonies are significantly different at $p \leq 0.05$, while *R_{ST} CALC* assigned a lower *Rho* value (0.042) to this comparison and did not find these colonies to be significantly different from one another. In this case, the *AGARst* value for *Rho* (0.072) is very similar to the R_{ST} determined by *Arlequin*, and therefore a significant difference between Peppercorn and Grahamstown will be accepted at $p \leq 0.05$.

Three major subpopulations of *M. schreibersii* can be discerned, based on the pairwise comparisons: 1. a southern subpopulation consisting of De Hoop and Die Hel;

2. a western subpopulation consisting of Steenkampskraal and Koegelbeen, and
3. a north-eastern subpopulation made up of the remainder of the colonies.

Within each of these subpopulations, R_{ST} (*Rho*) values are close to zero or negative, indicating that high levels of gene flow occur between the colonies and there is no genetic differentiation between them. Negative values of R_{ST} (*Rho*) result when the variance in allele size within populations (or colonies) is greater than that among them. This indicates that there is no differentiation between such colonies, which are therefore essentially panmictic. On the other hand, R_{ST} (*Rho*) values between colonies from different subpopulations are much higher (up to 0.686), indicating that gene flow among them is restricted.

Table 4.7. Pairwise comparisons of colony† differentiation. Pairwise G_{ST} values are given above the diagonal. Three R_{ST} or *Rho* values for each pairwise comparison are given below the diagonal: 1. *Rho,* averaged across variances, calculated by *R_{ST} CALC*; 2. *Rho*, averaged across loci, calculated by *AGARst*; 3. R_{ST}, calculated by *Arlequin*.

* = colonies differ significantly at $p \leq 0.01$ after sequential Bonferroni correction;

** = colonies differ significantly at $p \leq 0.05$ after sequential Bonferroni correction.

	DHP	DHL	SKK	KB	G	MM	SHD	JD	PC	SW
DHP	-	-0.004	0.145	0.161	0.187	0.169	0.161	0.171	0.110	0.169
DHL	0.013 0.021 0.019	-	0.111	0.089	0.107	0.109	0.107	0.118	0.093	0.105
SKK	0.590* 0.414 0.464*	0.498* 0.329 0.379*	-	0.014	0.096	0.106	0.106	0.113	0.073	0.089
KB	0.686* 0.508 0.572*	0.602* 0.438 0.525*	0.014 0.036 -0.033	-	0.099	0.091	0.086	0.096	0.030	0.074
G	0.594* 0.498 0.508*	0.520* 0.448 0.470*	0.382* 0.324 0.389*	0.445* 0.322 0.417*	-	-0.004	-0.007	0.010	-0.041	0.010
MM	0.551* 0.445 0.415*	0.475* 0.393 0.383*	0.374* 0.298 0.199*	0.449* 0.302 0.329*	0.005 0.005 -0.006	-	0.017	0.028	-0.005	0.021
SHD	0.546* 0.463 0.452*	0.477* 0.424 0.419*	0.269* 0.208 0.250*	0.336* 0.242 0.359*	0.068* 0.061 0.077*	0.045 0.042 -0.014	-	0.021	0.008	0.018
JD	0.555* 0.461 0.445*	0.481* 0.415 0.401*	0.320* 0.275 0.291*	0.386* 0.292 0.356*	0.007 0.005 0.013	-0.005 0.005 -0.043	0.005 0.008 -0.004	-	0.003	0.030
PC	0.482* 0.373 0.383*	0.400* 0.320 0.325*	0.152* 0.132 0.103**	0.215* 0.148 0.212*	0.042 0.072 0.073**	0.034 0.054 0.008	0.023 0.017 0.047	0.015 0.050 0.031	-	-0.009
SW	0.532* 0.419 0.401*	0.453* 0.358 0.357*	0.283* 0.223 0.244*	0.374* 0.261 0.335*	0.067* 0.042 0.081*	0.030 0.021 -0.015	0.040 0.030 0.039	0.024 0.015 0.029	-0.004 0.015 0.005	-

†Names of colonies are abbreviated as follows:
DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown;
MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala

To a lesser degree, some genetic substructuring is also evident within the north-eastern subpopulation. Gene flow between Grahamstown and three other colonies within this region is partially restricted, with significant differentiation being detected between Grahamstown and Shongweni Dam ($p \leq 0.01$), Peppercorn ($p \leq 0.05$) and Sudwala ($p \leq 0.01$). Nevertheless, R_{ST} (*Rho*) values for these pairwise comparisons were all relatively low (less than 0.10), indicating that gene flow among these colonies is still more extensive than between any of them and colonies within either the southern or western subpopulations.

No significant difference in pairwise R_{ST} (*Rho*) values calculated between colonies was found between males and females (Mann-Whitney T = 2018.00, p = 0.815). Furthermore, no significant genetic differentiation was found between males and females within any of the colonies ($p \geq 0.1$ for all comparisons). Pairwise R_{ST} (*Rho*) values calculated between males and females in each colony ranged between –0.040 and 0.065 (estimated using *R_{ST} CALC*) or between –0.005 and 0.054 (estimated using *AGARst*).

Based on the results of the initial pairwise comparisons (Table 4.7), colonies were pooled into the three proposed subpopulations (south, west and north-east) and pairwise R_{ST} (*Rho*) and genetic distances $(\delta\mu)^2$ recalculated. Genetic differentiation between the subpopulations was highly significant ($p \leq 0.0001$, Table 4.8; see also Table 4.10 below), and both R_{ST} (*Rho*) and genetic distances between the major subpopulations were large, particularly between the southern group and the other two regions.

The same pattern of colony differentiation into three major subpopulations is illustrated graphically by principle component analysis of the microsatellite data (Figure 4.8; Table 4.9). The first two principle components (PC1 and PC2) account for approximately 85% of the variance in the data (referred to as "inertia" by *PCA-GEN*), and significant differentiation among individual colonies is indicated by a global F_{ST} = 0.134 (p = 0.001). The western colonies (Steenkampskraal and Koegelbeen) load low on PC2, separating from the southern colonies (De Hoop and Die Hel), which load high on PC1, and from the north-eastern colonies, all of which load low on PC1.

Table 4.8. Pairwise comparisons between three proposed subpopulations of *M. schreibersii* in South Africa. Genetic distances $(\delta\mu)^2$ are given above the diagonal. Three R_{ST} or *Rho* values for each pairwise comparison are given below the diagonal, as for Table 4.7. * = subpopulations differ significantly at $p \leq 0.0001$.

	South	West	North-east
SOUTH (De Hoop & Die Hel)	-	4.550	2.563
WEST (Steenkampskraal & Koegelbeen)	0.623* 0.452 0.512*	-	2.037
NORTH-EAST (Grahamstown, Maitland Mines, Shongweni Dam, Jozini Dam, Peppercorn & Sudwala)	0.519* 0.434 0.406*	0.351* 0.255 0.252*	-

Table 4.9. Eigenvalues (or per axis inertia), percentage and cumulative variance (inertia) obtained from principle component analysis of microsatellite data by *PCA-GEN*.

	Per axis inertia (eigenvalue)	Percent inertia (variance)	Cumulative inertia (variance)
Principle Component 1	0.353	53.35	53.35
Principle Component 2	0.211	31.88	85.23

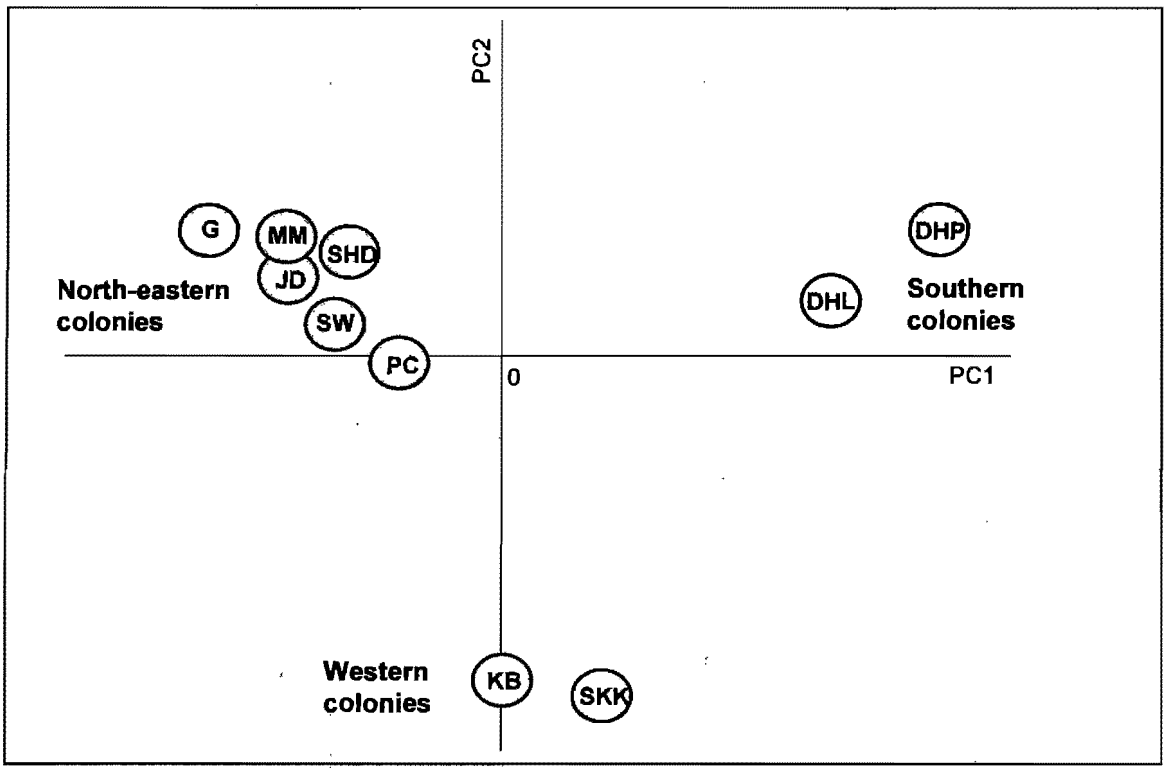

Figure 4.8. Principle component analysis plot of colony differentiation, generated from microsatellite data. Names of colonies are abbreviated as follows: DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown; MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala

The pattern of three major subpopulations is also confirmed by the genetic distances $(\delta\mu)^2$ between the *M. schreibersii* colonies (Table 4.10, Figure 4.9). Genetic distances among colonies within each subpopulation are low (0.098 – 0.446) while those among colonies from different subpopulations are considerably higher (1.033 – 5.286). This is clearly illustrated in the UPGMA tree generated from the genetic distance data (Figure 4.9). Bootstrap support for the division of the South African *M. schreibersii* population into three major clades (south, west and north-east) is high (96-100%; 1000 bootstrap pseudoreplications). However, support for the internal nodes of the north-eastern subpopulation is low, probably reflecting the close genetic relationships among these colonies. Males and females did not differ significantly in pairwise colony $(\delta\mu)^2$ values (Mann-Whitney T = 2075.00, p = 0.828).

The number of migrants exchanged between colonies per generation (N_m) may be used to estimate of the extent of gene flow among colonies (Table 4.10). It should be noted, however, that N_m is calculated under the assumption that populations are at equilibrium between the migration rate (leading to gain of alleles) and genetic drift (loss of alleles). This assumption may not be valid in many cases (Goodman 1997), particularly in recently established populations. For example, colonies such as Steenkampskraal and Maitland Mines are located in disused mine shafts, which were abandoned only towards the latter half of the twentieth century. These colonies thus cannot be more than 40 – 50 years old, and so are unlikely to have reached equilibrium. Nevertheless, although exact N_m values should be treated with caution, the general pattern and direction of gene flow among colonies can be ascertained from these data.

The N_m values indicate that gene flow among the major subpopulations is limited, with less than two migrants being exchanged per generation. On the other hand, gene flow among colonies within each of the three major subpopulations is far more extensive, with more than 50 migrants per generation being exchanged between some colonies. The colonies Peppercorn and Sudwala, and Maitland Mines and Jozini Dam are essentially panmictic. The number of migrants exchanged between these two pairs of colonies is too high to be quantified by the methods employed by *R_{ST} CALC* (Goodman 1997), and therefore is indicated by ∞ in Table 4.10.

Mantel tests indicate that neither pairwise R_{ST} (*Rho*) values nor genetic distance $(\delta\mu)^2$ is significantly correlated with geographic distance between colonies ($r = 0.167$ and $r = 0.133$ respectively; $p \geq 0.10$). This suggests that the limited gene flow among colonies from different subpopulations is not simply a reflection of their geographic separation. For example, Steenkampskraal and Die Hel are located relatively close to one another (~170 km; Table 5.10, Chapter 5) but are genetically very distinct and gene flow between them is limited (*e.g. R_{ST} CALC Rho* = 0.498; $(\delta\mu)^2 = 3.419$). In contrast, Steenkampskraal and Koegelbeen are geographically distant (~560 km), but are genetically very similar (e.g. *R_{ST} CALC Rho* = 0.014; $(\delta\mu)^2 = 0.098$). Factors other than physical distance must therefore influence the extent and direction of migration between colonies.

Table 4.10. Genetic distances $(\delta\mu)^2$ (above diagonal) and estimated number of migrants (N_m) per generation (below diagonal) between colonies* of *M. schreibersii* in South Africa.

	DHP	DHL	SKK	KB	G	MM	SHD	JD	PC	SW
DHP	-	0.105	4.242	5.286	3.326	2.635	3.203	2.899	2.666	2.265
DHL	18.437	-	3.419	4.313	2.842	2.259	2.794	2.487	2.157	1.931
SKK	0.174	0.252	-	0.098	2.408	2.445	1.524	1.959	1.033	1.646
KB	0.115	0.165	17.288	-	2.594	2.793	1.807	2.232	1.312	2.103
G	0.171	0.231	0.405	0.312	-	0.084	0.446	0.107	0.355	0.388
MM	0.204	0.277	0.419	0.306	55.646	-	0.344	0.062	0.319	0.206
SHD	0.208	0.275	0.678	0.495	3.414	5.335	-	0.138	0.234	0.257
JD	0.201	0.270	0.531	0.397	33.439	∞	50.943	-	0.194	0.176
PC	0.268	0.375	1.395	0.915	5.703	7.082	10.831	16.770	-	0.122
SW	0.220	0.302	0.634	0.418	3.464	8.110	6.041	10.120	∞	-

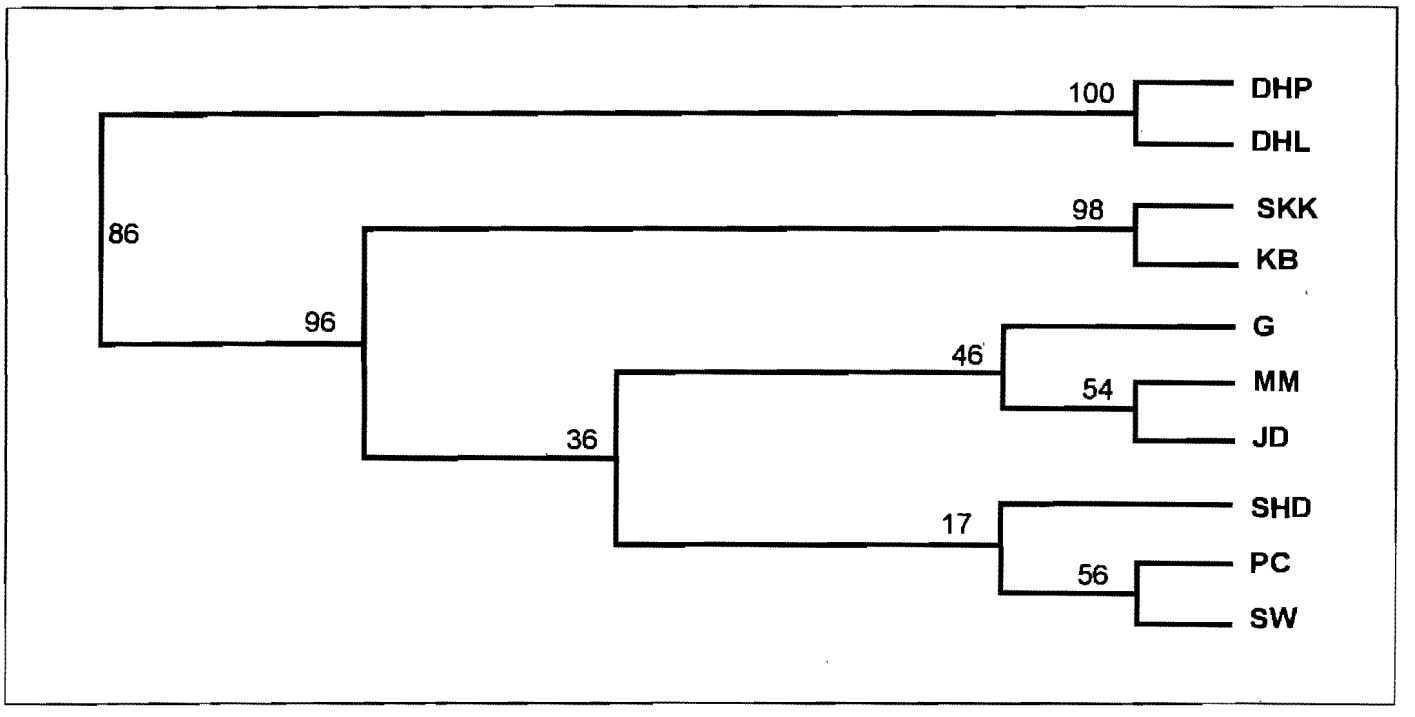

Figure 4.9. UPGMA tree constructed from genetic distances $(\delta\mu)^2$ between colonies* of *M. schreibersii*. Bootstrap support values (1000 pseudoreplications) are indicated on the internal nodes.

* Names of colonies are abbreviated as follows:
DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown;
MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala

i. Assignment tests on individuals from major colonies

In an assignment test, between 62% and 90% of individuals were correctly assigned to the colonies from which they were sampled (Table 4.11a), indicating that each of the ten colonies has characteristic genotypic frequencies. In the majority of cases, incorrect assignments placed individuals within alternative colonies that belonged to the same subpopulation as the one from which they were collected. For example, 82% of individuals collected at De Hoop were correctly assigned to this colony based on their genotype frequencies, and all remaining individuals from De Hoop (18%) were assigned to Die Hel, which is part of the same southern subpopulation. Colonies within each subpopulation were therefore pooled and the assignment test was repeated.

This second test confirmed that the three subpopulations have highly characteristic genotypic frequencies, with 95 – 100% of individuals being assigned correctly to one of the subpopulations (Table 4.11b). In addition to determining the colony to which an individual is most likely to belong, *AGARst* also calculates a likelihood ratio between that colony and the next most likely colony. Although the modal likelihood ratios for ten separate colonies were relatively low, ranging between 2.4 and 98.4 (Table 4.11a), they were several orders of magnitude greater when colonies were pooled into three subpopulations. For example, 95% of individuals were correctly assigned to the north-eastern subpopulation, and (based on the modal likelihood value) were 5.54×10^4 times more likely to have come from this subpopulation than from either of the other subpopulations (Table 4.11b).

Table 4.11a. Results of an assignment test on ten separate *M. schreibersii* colonies: the percentage of individuals correctly assigned to each colony, the modal and range of likelihood ratios between most likely and second most likely colonies, and the colonies to which individuals were incorrectly assigned are shown.
Names of colonies are abbreviated as follows: DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown; MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala

Colony	Percent correctly assigned	Modal likelihood ratio (most likely colony to 2nd most likely)	Range of likelihood ratios (most likely colony to 2nd most likely)	Incorrect assignments: colonies to which individuals were incorrectly assigned (% of individuals incorrectly assigned)
DHP	82	14.6	$1.2 - 1.3 \times 10^3$	DHL (18%)
DHL	74	18.4	$1.18 - 1.42 \times 10^3$	DHP & KB (10.5% each); SKK (5%)
SKK	90	98.4	$1.33 - 8.82 \times 10^2$	KB (10%)
KB	90	10.5	$1.11 - 2.43 \times 10^3$	SKK (10%)
G	70.3	5.1	1.13 - 111.0	MM (16.2%); JD (5.4%); SHD, PC & SW (2.7% each)
MM	62.5	2.41	1.08 - 27.7	G (24%); SHD (5.4%); KB, SKK & SW (2.7% each)
SHD	69.1	8.77	1.02 - 341.0	MM (13.8%); JD (6.9%); SW, PC & DHP (3.4% each)
JD	75.5	5.7	1.22 - 52.4	SHD, G & SW (7% each); MM (3.5%)
PC	79.5	3.07	$1.07 - 1.12 \times 10^3$	JD (10.5%); MM & G (5% each)
SW	73	8.16	$1.1 - 3.68 \times 10^3$	G (12%); JD (9%); PC (6%)

Table 4.11b. Results of an assignment test on three proposed *M. schreibersii* subpopulations. The percentage of individuals correctly assigned to each subpopulation, the modal and range of likelihood ratios between most likely and second most likely subpopulations, and the subpopulations to which individuals were incorrectly assigned are shown.

Sub-population	% correctly assigned	Modal likelihood ratio	Range of likelihood ratios	Incorrect assignments
South	95	1.06×10^8	$1.23 \times 10^3 - 1.94 \times 10^{12}$	West (5%)
West	100	7.36×10^5	$4.25 - 1.36 \times 10^{10}$	0%
North-east	95	5.54×10^4	$2.2 - 1.05 \times 10^9$	West (4.5%); South (0.5%)

ii. Assignment tests on individuals from minor sample sites

The assignment test on individuals from smaller groups suggest that the two individuals collected at Knysna, and the single individual collected in the Cedarberg Mountains most likely belong to the southern subpopulation. All three individuals were assigned to this subpopulation with high likelihood ratios (Table 4.12). Furthermore, all three were assigned to De Hoop, but the likelihood ratios of this colony over Die Hel are very low (2.6 – 7.6). With the exception of one individual, all six bats collected under the Pongola River Bridge were assigned with high likelihood ratios to the north-eastern subpopulation. One PRB individual was assigned to the western subpopulation in the first assignment test, but with a very low likelihood ratio (4.55) relative to the north-eastern region. The second assignment test, however, indicated that this individual is most closely related to bats at Sudwala (in the north-eastern subpopulation). The PRB bats were individually assigned to five of the six colonies within the north-eastern region, but in each case the likelihood ratio relative to other colonies in this area was low.

This illustrates once again that the subpopulations are genetically highly distinctive, so that individuals of unknown origin can be assigned with confidence to one of these major regions. However, it is more difficult to determine the individual colony within any of the subpopulations to which an unknown individual is most closely related, as colonies within the subpopulations are genetically more similar.

Table 4.12. Results of assignment test on individuals from Knysna (K), Cedarberg (C) and Pongola River Bridge (PRB).

Individual		Subpopulation of most likely origin (likelihood ratio)	Most closely related colony (1)	Second most closely related colony (2)	Likelihood ratio of 1:2
K	M1	South (9.20×10^8)	DHP	DHL	5.76
	M2	South (1.59×10^8)	DHP	DHL	2.57
C	M1	South (3.4×10^3)	DHP	DHL	7.64
PRB	F1	North-east (10)	SHD	PC	2.81
	F2	North-east (4.99×10^3)	JD	SHD	3.84
	F3	North-east (8.5×10^4)	JD	SHD	1.82
	F4	North-east (6.7×10^7)	MM	G	7.43
	F5	North-east (386.2)	SHD	JD	5.04
	M1	West (4.55)	SW	JD	3.14

4.3.3. Differentiation between South African and international *M. schreibersii*

Pairwise R_{ST} (*Rho*) and $(\delta\mu)^2$ comparisons between samples from South Africa and those obtained from international locations (Australia, Madagascar and Israel) were uninformative (Table 4.13). Large discrepancies in pairwise R_{ST} (*Rho*) values were found between the three statistical packages used to calculate these values, namely *R_{ST}CALC*, *AGARst* and *Arlequin*. Only *Arlequin* calculated realistically large R_{ST} values between the South African samples and those from Israel and Madagascar (but not from Australia), and found significant genetic differentiation between them ($p \leq 0.01$). In addition, despite complete geographic separation between individuals from these four regions and the resulting improbability of any gene flow between them, less genetic differentiation was detected than that found among colonies within South Africa, and in some cases negative values of *Rho* were obtained. A possible reason for these results is the low sample size from the international locations compared to the number of individuals in the South African

sample (South Africa: n = 310; Australia: n = 6; Madagascar: n = 3; Israel: n = 4). Although R_{ST} *CALC* and *AGARst* standardise for differences in sample size, perhaps the differences involved in this comparison were too large, even after standardisation, to allow for meaningful comparisons.

Table 4.13. Pairwise comparisons between South African *M. schreibersii* and international samples. Genetic distances $(\delta\mu)^2$ are given above the diagonal. Three R_{ST} or *Rho* values for each pairwise comparison are given below the diagonal: 1. *Rho,* averaged across variances, calculated by R_{ST} *CALC*; 2. *Rho*, averaged across loci, calculated by *AGARst*; 3. R_{ST}, calculated by *Arlequin*.
* = populations differ significantly at $p \leq 0.01$.

	South Africa	Australia	Madagascar	Israel
South Africa	-	0.419	0.218	1.222
Australia	0.113 0.098 0.026	-	0.639	0.869
Madagascar	-0.060 -0.025 0.789*	-0.031 -0.004 0.109	-	0.723
Israel	0.266 0.253 0.700*	0.268 0.217 0.055	0.033 0.063 -0.013	-

4.4. DISCUSSION

The South African population of *Miniopterus schreibersii* is genetically substructured, with many of the major colonies being genetically differentiated from one another. This suggests that these bats exhibit strong levels of philopatry to their breeding sites, as suggested by previous behavioural studies (e.g. van der Merwe 1973b, 1975). This is also supported by the fact that male and female bats within each colony are closely related (as indicated by low R_{ST} (*Rho*) values between the sexes), which suggests they breed either with one another, or with bats from closely related colonies. High levels of philopatry have led to the formation of three major subpopulations within South Africa, namely a southern subpopulation consisting of the colonies De Hoop and Die Hel, a western subpopulation comprised of Steenkampskraal and Koegelbeen, and a north-eastern subpopulation made up of the remainder of the colonies. Colonies within each subpopulation are genetically similar, but significant differentiation exists between the three regions. Some degree of substructuring exists within the north-eastern subpopulation, as Grahamstown is genetically differentiated from other colonies within this region. Nevertheless, gene flow among colonies within the north-east region is far more extensive than that between the north-eastern subpopulation and either the southern or western regions.

No obvious barriers to dispersal are immediately apparent, and this pattern of colony differentiation is not simply a reflection of the geographic separation of colonies: only a very weak correlation was found between the extent of genetic differentiation and the geographic distance between colonies. Furthermore, *M. schreibersii* are known to migrate seasonally over hundreds of kilometres, both in South Africa (Herselman & Norton 1985) and in other parts of their range (Dwyer 1963, 1969; Wimsatt 1970). Migration between colonies such as Steenkampskraal and Die Hel (a distance of ~170 km) is therefore well within their migratory abilities, but these colonies are genetically very distinct, suggesting movement between them is limited. Clearly other factors must play a major

role in determining how *M. schreibersii* move between colonies. The extent and likely direction of this movement are discussed in further detail in Chapter 8.

Male and female *M. schreibersii* did not differ significantly in pairwise R_{ST} or $(\delta\mu)^2$ values between colonies, which suggests that the sexes exhibit similar levels of colony differentiation, as detected by microsatellite markers. They may therefore show similar patterns of philopatry and/or dispersal. However, this cannot be determined conclusively by microsatellites alone. As nuclear markers, microsatellites are subject to genetic recombination and so represent an average of any genetic differentiation present in the sexes. One must examine a sex-linked genetic marker, such as the Y-chromosome or maternally-inherited mitochondrial DNA to determine whether males and females differ in their patterns of dispersal and philopatry. Mitochondrial DNA was selected in the present case, and this aspect of the study is discussed in Chapter 5.

CHAPTER 5

ANALYSIS OF MITOCHONDRIAL DNA CONTROL REGION

AIM: To determine whether male and female *Miniopterus schreibersii* exhibit similar levels of philopatry and/or dispersal, by comparing estimates of population subdivision based on maternally-inherited mitochondrial DNA with those obtained from nuclear microsatellite markers (Chapter 4).

5.1. INTRODUCTION

5.1.1. The mitochondrial genome

Sequencing homologous fragments of DNA from different organisms is one of the most direct and powerful ways of examining the evolution of genes, reconstructing interspecific phylogenies and, in the present case, examining geographic variation and gene flow among intraspecific populations (Harrison 1989; Hillis *et al.* 1996). Isolation of homologous nuclear sequences may be difficult, often requiring the development of a genomic library, as in the case of microsatellites

(Chapters 3 and 4). Mitochondrial DNA, however, is relatively easy to isolate, as there are multiple copies per cell (some tissues contain over 2000 mitochondria; Quinn 1997), and conserved sequences often allow the use of so-called "universal" primers, which recognise mitochondrial sequences in a wide range of species. Most importantly, the mitochondrial genome represents a homologous DNA sequence, which can be compared between intraspecific populations or across animal species (Harrison 1989). Gene phylogenies that indicate the relationships between different mitochondrial alleles (or haplotypes) present in populations, coupled with geographic information on those populations, can be used to evaluate the genetic structure of groups of organisms (Hillis *et al.* 1996).

Mitochondria and chloroplasts are the principle organelles outside the nucleus that contain DNA (Stryer 1988). They are believed to have arisen from free-living, aerobic bacteria that formed a symbiotic relationship with anaerobic eukaryotic cells (Quinn 1997; Rand 1993; Voet & Voet 1990). Although mitochondria still resemble bacteria in size and shape (Voet & Voet 1990), their genome size has been reduced considerably. A typical vertebrate mitochondrial genome is approximately 1% the size of an extant bacterial genome (Quinn 1997). Mitochondrial genome size was reduced early in evolutionary history to the minimum size possible (Brown *et al.* 1979). Redundant genes (*i.e.* those also present in the nuclear genome) were eliminated, and other functional genes were translocated to the nucleus (Quinn 1997). Only essential genes associated with respiration remain in the mitochondrial genome.

5.1.2. Structure of mitochondrial DNA

Animal mitochondrial DNA (mtDNA) consists of a circular, double-stranded DNA molecule, of between 16 300 and 19 200 bp. It is the smallest, simplest eukaryotic genome known (Brown 1985) and is extraordinarily economical in gene content. It contains only single copies of its

genes, several of which have overlapping reading frames (Brown 1985; Quinn 1997; Stryer 1988). It also has no non-coding sequences (introns) within the structural genes, and sequence rearrangements such as inversions and transpositions are rare (Brown 1985; Quinn 1997). This contrasts with the nuclear genome, a large proportion of which contains both introns and sequence rearrangements, and which frequently has up to several hundred thousand copies of certain DNA sequences (Brown 1985).

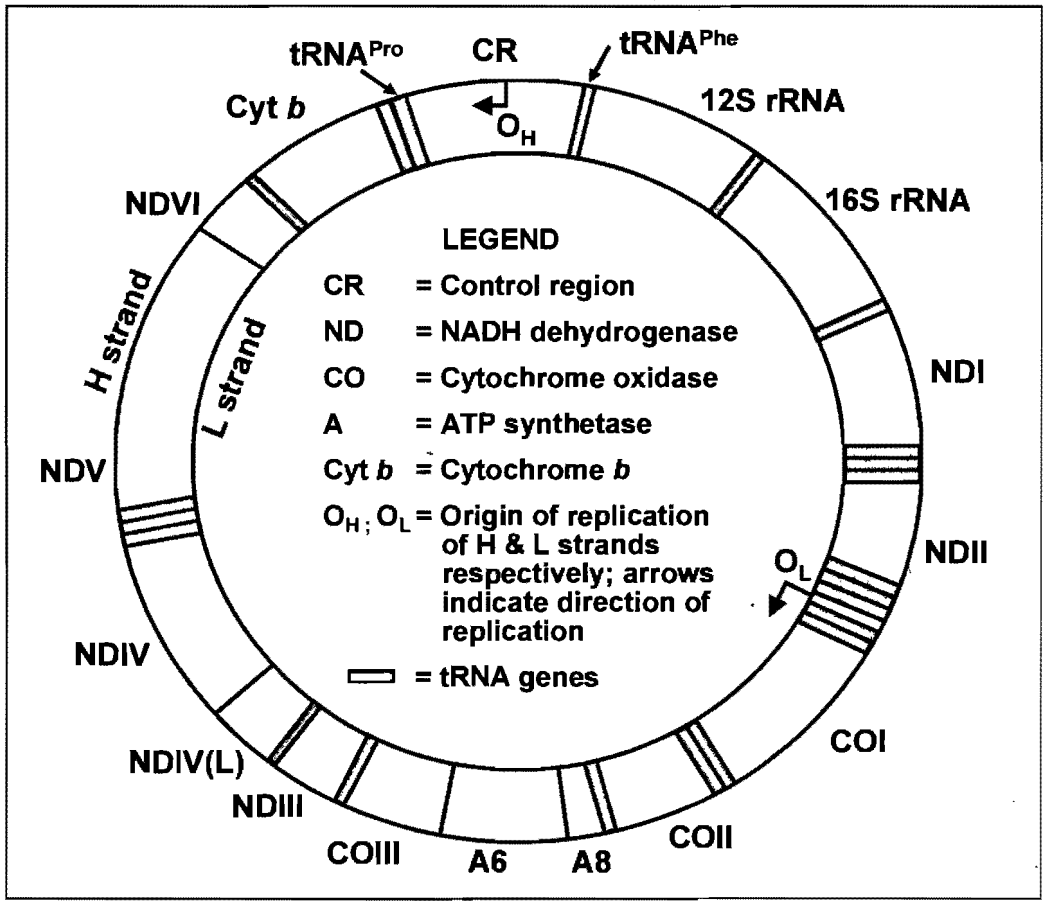

Figure 5.1. Schematic map of a generalised vertebrate mitochondrial genome, showing content and arrangement of genes. Modified from Brown (1985) and Harrison (1989).

Unlike the mtDNA of plants and protists, the vertebrate mitochondrial genome (Figure 5.1) is remarkably conserved across taxa, in terms of both gene content and order (Brown 1985; Harrison 1989). It encodes 22 transfer RNA's (tRNA), two ribosomal RNA's (rRNA) and 13 proteins involved in the electron transport chain (Harrison 1989). The majority of these gene

products are encoded by the heavy (H) strand, which is the denser of the two DNA strands, due to differences in base composition. Only one protein (NADH dehydrogenase IVL) and 14 tRNA's are encoded by the light (L) strand (Stryer 1988).

5.1.3. The control region

Essential regulatory sequences of mtDNA are concentrated within the control region (Figure 5.2), which generally lies between the proline tRNA ($tRNA^{Pro}$) and phenylalanine tRNA ($tRNA^{Phe}$) genes. The control region is approximately 1000 bp long and contains sites for the initiation of H-strand replication (O_H) and RNA transcription, as well as other sequences involved in the control of gene expression and mtDNA replication, such as the H- and L-strand promoters (Brown 1985; Taberlet 1996; Wilkinson & Chapman 1991).

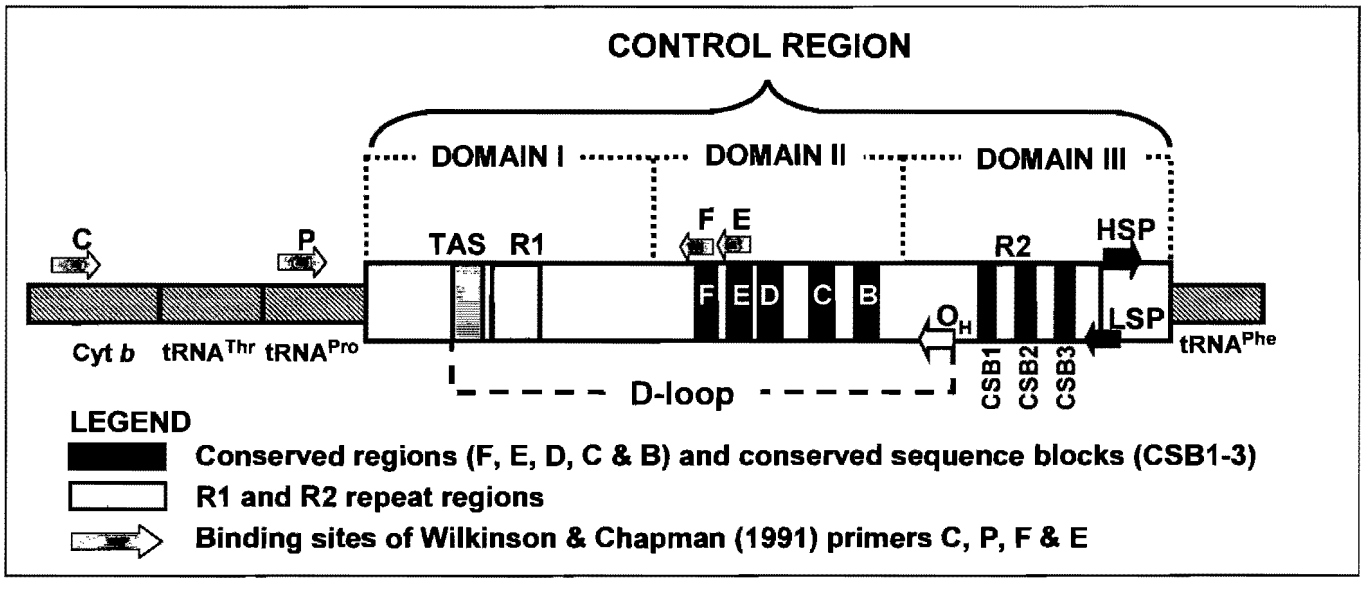

Figure 5.2. Schematic diagram of the organisation of the control region of bat mtDNA. Modified from Taberlet (1996) and Wilkinson *et al.* (1997). Binding sites and amplification direction of primers (Wilkinson & Chapman 1991; Section 5.2) are also shown.

Abbreviations are as follows:
Cyt *b* = Cytochrome *b*; $tRNA^{Thr}$, $tRNA^{Pro}$ & $tRNA^{Phe}$ = threonine, proline & phenylalanine tRNA's, respectively;
TAS = termination-associated sequence;
O_H= Origin of H-strand replication; HSP & LSP = H- & L-strand promoters, respectively.

i. Structure of the control region

The control region is conventionally divided into three regions or domains, based on differing base composition and degree of variability (Figure 5.2). The central, highly conserved region of ~200 bp, is referred to as domain II. It is G-C rich and is involved in regulation of H-strand replication and the formation of the displacement loop (see below; Baker & Marshall 1997; Fumagalli *et al.* 1996; Hoelzel *et al.* 1994). The two regions flanking domain II, known as domains I and III, are A-T rich, and vary extensively in size and sequence. Domain I, which lies closest to the $tRNA^{Pro}$, has the highest A-T content of the control region, and contains short termination associated sequences (TAS), which terminate replication of the H-strand in the displacement loop (see below). Domain III, which lies closest to the $tRNA^{Phe}$, contains the origin of H-strand replication (O_H) and both the H- and L-strand promoters (HSP and LSP), which initiate transcription of mtDNA (Baker & Marshall 1997). It also has three conserved sequence blocks (CSB's), which lie between the O_H and the LSP (Hoelzel *et al.* 1994).

ii. The displacement loop

In vertebrates, the control region is characterised by the presence of a displacement loop or D-loop (Figure 5.2), which is formed as a result of H-strand replication. Replication of the H-strand begins at the O_H within the control region, and precedes that of the L-strand, which acts as a template (Voet & Voet 1990). In 95% of replication cycles, DNA synthesis is terminated by termination associated sequences (TAS) within the control region, and the newly formed strand of DNA remains attached to the template L-strand, displacing the original H-strand (Wilkinson *et al.* 1997). The result is a 500 to 600 nucleotide triple-stranded structure known as the displacement loop, which may function in the binding of hydrophobic regulatory proteins (Wilkinson & Chapman 1991). The D-loop undergoes frequent cycles of degradation and re-synthesis (Voet & Voet 1990), until replication of the entire mtDNA molecule occurs. At this time, the D-loop is extended until it reaches and exposes the origin of replication of the L-strand

(O_L), at which point synthesis of this other strand begins (Voet & Voet 1990; Wilkinson *et al.* 1997).

iii. Repetitive sequences in the control region

Unlike other regions of the mitochondrial genome, a large proportion of the control region is non-coding (Brown 1985; Fumagalli *et al.* 1996). Variable domains I and III frequently contain repetitive sequences, which may originate through DNA slippage mechanisms, similar to those responsible for the formation of microsatellites (Fumagalli *et al.* 1996; Wilkinson & Chapman 1991; Wilkinson *et al.* 1997). These repetitive elements have been found in a wide range of vertebrates, including carnivores, shrews, pinnipeds, pigs, rabbits and bats (Fumagalli *et al.* 1996; Hoelzel *et al.* 1994; Wilkinson & Chapman 1991), and differences in the length of mtDNA between species is frequently due to variation in the number of tandem repeat sequences within the control region (Fumagalli *et al.* 1996; Wilkinson & Chapman 1991).

There are two major types of repetitive elements within the control region. R1 repeats contain repeat units of approximately 40, 80 or 160 bp, and are found in domain I (Figure 5.2), associated with the termination sites (TAS) for the D-loop. Many vespertilionid bat species contain up to nine tandem copies of an ~81 bp R1 repeat, but only a single R1 sequence has been found in *Miniopterus schreibersii* (Wilkinson *et al.* 1997). Smaller tandem repeat units of between six and 30 bp (R2 repeats) are located in domain III, upstream from the O_H, and have also been reported in a wide variety of species, including bats (Wilkinson *et al.* 1997). The function of the repeat regions is unclear, although Wilkinson *et al.* (1997) speculate that because domain I has binding sites for regulatory proteins (*e.g.* TAS), multiple R1 repeats may provide a buffer in case a mutation in one repeat affects the binding ability of regulatory proteins. Furthermore, they suggest that repeat duplication and deletion could be a means of eliminating damaged repeat sequences, with the effect that multiple repeats may increase longevity.

Knowledge of the structure of the control region is essential if sequences of this area are to be used in population genetic and phylogenetic studies (Baker & Marshall 1997). The central, conserved domain may be the most appropriate for cross-species studies because sequence alignment and homology are the least ambiguous in this region. However, intraspecific comparisons (such as the present study) may require sequencing of regions with higher variability, such as domains I and III. In this case, knowledge of the existence or absence of repeat regions is required. Uncertain homology of such hypervariable regions may complicate measures of sequence diversity and phylogeny reconstruction (Baker & Marshall 1997).

This is particularly true if individuals have more than one form (or haplotype) of mtDNA, a condition known as heteroplasmy. New mtDNA variants arise from mutations within a single molecule of a single cell. If this mutant mtDNA molecule is replicated and a new variant cell lineage results, the organism will have two different cell lineages containing both the original and new type of mtDNA. These may differ in size or (less frequently) in nucleotide sequence (Harrison 1989). Both length and sequence heteroplasmy can be transmitted from mother to offspring, because variant cell lineages can arise in the germ line (Petri *et al.* 1996). Heteroplasmy that arises as a result of length variation is relatively common due to variation in the number of R1 repeats, and is well recorded in bat mitochondrial genomes (Petri *et al.* 1996; Rand 1993; Wilkinson & Chapman 1991; Wilkinson *et al.* 1997).

5.1.4. Advantages and applications of mtDNA in conservation genetics studies

Many of the advantages of using mtDNA as a molecular marker in conservation genetics studies are similar to those associated with microsatellites. For example, a major advantage of mitochondrial DNA is that it is highly polymorphic (see below), and specific regions of interest are easily amplified from even minute amounts of unpurified tissue by means of PCR. Amplified

regions can either be sequenced or analysed with restriction enzymes, or both (Section 5.2). The existence of both hypervariable and more conserved regions within the mitochondrial genome allows one to obtain genetic information about many different groups, ranging from intraspecific populations to highly divergent taxa (Harrison 1989). Furthermore, by obtaining sequences of DNA rather than amino acids (proteins), the genotype rather than the phenotype can be examined (Dowling *et al.* 1996). Both coding and non-coding regions can be examined, providing greater information about the process of gene evolution (Li *et al.* 1985).

Another major advantage of mtDNA for population genetics studies is that it is exclusively maternally inherited (see below), and therefore is haploid and not subject to recombination. This reduces its effective population size (see below), which enhances its resolution (Harrison 1989; Moritz 1994a).

i. High mutation rate of mtDNA

Because mitochondrial function is fundamental to animal life, one might expect the mitochondrial genome to be highly conserved and its evolution to be tightly constrained. Contrary to expectation, however, comparative studies indicate that species which show limited differences in their single-copy nuclear genes frequently exhibit extensive differences between their mtDNA sequences (Brown *et al.* 1979). These studies indicate that the mutation rate of mtDNA is at least five to ten times higher than that of coding regions in the nucleus. Variable blocks within the non-coding control region evolve four or five times faster still (Brown 1985; Brown *et al.* 1979; Taberlet 1996). The mutation rate of the bat control region, for example, has been estimated at ~10^{-2} per generation (Wilkinson & Chapman 1991). This is at least two orders of magnitude faster than that of microsatellites (10^{-6} to 10^{-4} per generation; Bruford & Wayne 1993). Mutations occur in the form of base substitutions, insertions and deletions (indels) and, more rarely, sequence rearrangements. The frequency of all of these mutations is highest in the control region. More conserved regions (*e.g.* the tRNA and rRNA) genes have slower mutation

rates. For example, the base substitution rate for tRNA and rRNA genes in primates is half that of mtDNA protein genes, but still ~100 times higher than the substitution rate of equivalent genes in the nuclear genome (Brown 1985).

The reason for this extraordinarily high mutation rate is that mtDNA lacks any form of DNA repair mechanism, and the editing functions of its replication enzymes are also inefficient or lacking (Barton & Jones 1983; Brown *et al.* 1979). Furthermore, the turnover rate of mtDNA in tissues is higher than that of nuclear DNA, which results in more cycles of replication in which errors can be generated. Much of the control region of mtDNA is non-coding and selectively neutral. Mutations that accumulate in these neutral sections have no fitness effect on their bearers and are not eliminated (Avise 1994). This leads to particularly high variation in both length and nucleotide sequence (Brown 1985; Harrison 1989; Wilkinson & Chapman 1991).

In terms of population genetics studies, the rapid mutation rate of mtDNA, and in particular of the control region, means high resolution sequences can be obtained. Such sequences allow the detection of variation between closely related species, and even between conspecific populations (Harrison 1989; Taberlet 1996). In addition, the presence of regions that are relatively conserved across taxa (*e.g.* Cytochrome *b* and the conserved sequence blocks within the control region) provides binding sites for "universal" primers that frequently work for many vertebrate species (Kocher *et al.* 1989; Taberlet 1996). This obviates the need for designing a genomic library.

Highly variable mtDNA sequences are particularly useful for demonstrating patterns of population substructuring within a species, and for quantifying the extent of genetic differentiation between closely related, or even cryptic, species (Barton & Jones 1983; Moritz 1994a; Taberlet 1996). Each mitochondrial haplotype has a unique combination of linked mutations, and evolves independently of other haplotypes. It can therefore be used to trace phylogenetic histories (Barton & Jones 1983). Mitochondrial DNA can also provide information on historical

demographic events. Like other hypervariable regions, such as microsatellites, lack of variation in the control region can frequently be indicative of the occurrence of population bottlenecks and/or founder events in the recent evolutionary history of a population (Harrison 1989).

ii. Maternal inheritance of mtDNA

Sperm generally do not contribute cytoplasm to the fertilised egg, therefore mtDNA is exclusively maternally inherited and is effectively haploid (Barton & Jones 1983; Taberlet 1996). Alternate allelic forms of mtDNA (which differ either in sequence or length) are thus known as haplotypes (Avise 1994). The highly polymorphic and haploid nature of mtDNA makes it useful for tracing maternal phylogenies (Harrison 1989; Taberlet 1996). Furthermore, the asexual nature of mtDNA inheritance means it does not undergo recombination; therefore its genes are completely linked (*i.e.* they are always inherited as a single unit). Evolutionary histories determined through mtDNA are thus more straightforward than those assessed by nuclear genes, which may be complicated by recombination (Harrison 1989; Quinn 1997).

The effective population size for mtDNA is four times smaller than that of nuclear (diploid) genes (Randi *et al.* 1994) because only a fraction of the population (*i.e.* only females) passes on its mtDNA to the next generation (Barton & Jones 1983; Harrison 1989). Mitochondrial haplotype frequencies will therefore be more susceptible to stochastic changes in haplotype frequencies, because chance processes such as genetic drift occur more rapidly in smaller populations (Harrison 1989). The advantage of this is that the resolution of the marker is increased. Patterns of distribution of mitochondrial genes frequently appear more subdivided than those of nuclear genes (Barton & Jones 1983; Moritz 1994a).

A disadvantage, however, is that analyses of population differentiation based exclusively on mtDNA sequence divergences may over-estimate the degree of population subdivision. This is particularly true when species exhibit sex-biased dispersal, with males being the primary

dispersing sex (which is common in mammals; Greenwood 1980). Mitochondrial estimates of population differentiation are affected only by female movement patterns, and do not reflect gene flow due to male dispersal. Situations of female philopatry, therefore, will increase estimates of population subdivision relative to those obtained by nuclear genes, where the overall dispersal rate is the average of both sexes (Barton & Jones 1983; Moritz 1994a; Taberlet 1996). Ideally, therefore, analysis of mtDNA sequences should be accompanied by comparative studies of nuclear loci (as in this study), in order to provide a more balanced view of population structure.

5.1.5. Comparative studies between mtDNA and nuclear genes

Comparison of patterns of variation seen in maternally inherited mtDNA with those obtained from biparentally inherited nuclear DNA markers can indicate differences in male and female population structure and thus in ecology and behaviour (Harrison 1989). For example, if females are philopatric and males disperse, mtDNA differences between populations should be large, but nuclear genetic differences small, because females mate with males that have dispersed from unrelated populations. On the other hand, if neither sex (or only males) is philopatric, both mtDNA and nuclear differences are likely to show little population substructure.

These sorts of comparative studies are thus a useful means of assessing differential dispersal behaviour of males and females. The aim of this component of the present study, therefore, was to compare estimates of population differentiation based on microsatellite markers (Chapter 4) with those obtained from mtDNA control region sequences and restriction enzyme analysis, to determine whether male and female *M. schreibersii* exhibit similar patterns of philopatry and/or dispersal.

5.2. MATERIALS AND METHODS

A. OVERVIEW

Geographical variation in *M. schreibersii* mtDNA control region was examined by means of sequencing and analysis of restriction fragment profiles. One bat sample from each colony, as well as from each international colony, was initially chosen at random for sequencing, to determine whether colonies contained unique haplotypes. Unique mtDNA haplotypes were recognized by aligning the sequences from the colonies. This allowed the identification of restriction enzyme recognition sites that would characterise each haplotype. Five additional bats from each colony were examined using restriction fragment length polymorphisms (RFLP's), and the frequency at which individual haplotypes occurred in each colony was estimated. Genetic relationships among colonies were assessed, and the patterns of population differentiation indicated by mtDNA were compared to those obtained by microsatellite analysis to determine whether male and female *M. schreibersii* exhibit similar dispersal behaviours.

B. SEQUENCING OF MTDNA CONTROL REGION

5.2.1. Amplification and purification of mtDNA control region

Wilkinson & Chapman (1991) primers C and E (Table 5.1) were used to amplify approximately 550 bp of mtDNA control region in a limited number of *M. schreibersii* from each location (Table 5.2). The amplified region extended from within the Cytochrome *b* gene, through the variable

domain I of the control region to conserved block E within domain II, incorporating part of the D-loop (Figure 5.2). Primers were synthesised by the DNA Synthesis Laboratory at the Department of Molecular and Cellular Biology, University of Cape Town (UCT). They were diluted to a working concentration of 100 pmol/µl and stored at -20°C. An aliquot of 50 µl was kept at 4°C for regular use. *Miniopterus fraterculus* (five specimens, see Chapter 7) was used as an outgroup. Initially the control region of only one *M. schreibersii* specimen was sequenced from each colony. However, after restriction fragment haplotypes had been identified (Section 5.2.5), the control region of eight additional *M. schreibersii* was sequenced so that the nucleotide sequence of at least one example of each restriction fragment haplotype was determined. The final number of samples sequenced per colony thus depended on the number of RFLP haplotypes identified in each colony (Table 5.2).

Table 5.1. Sequences and binding sites of primers (Wilkinson & Chapman 1991) used to amplify (C and E) and sequence (E and P) the mtDNA control region of *M. schreibersii*.

Primer name	Binding region	Strand to which primer binds	Direction of amplification	Sequence (5'-3')
C	Cytochrome *b*	L	Into control region	TGA ATT GGA GGA CAA CCA GT
E	Conserved sequence block E	H	Towards $tRNA^{Pro}$	CCT GAA GTA GGA ACC AGA TG
P	$tRNA^{Pro}$	L	Into control region	TCC TAC CAT CAG CAC CCA AAG C

Table 5.2. Number of *M. schreibersii* and *M. fraterculus* individuals sequenced per colony or region.

Species	Colony or region	Number of individuals sequenced	Haplotype names
M. schreibersii		**TOTAL = 22**	
M. s. natalensis	**SOUTH AFRICA:**	**TOTAL = 18**	
	De Hoop	2	DHP1, DHP2
	Die Hel	2	DHL1, DHL2
	Steenkampskraal	1	SKK
	Koegelbeen	1	KB
	Grahamstown	2	G1, G2
	Maitland Mines	2	MM1, MM2
	Shongweni Dam	1	SHD
	Jozini Dam	2	JD1, JD2
	Peppercorn	1	PC
	Sudwala	3	SW1, SW2, SW3
	Pongola River Bridge (near Jozini Dam)	1	PRB
M. s. manavi	**MADAGASCAR**	**TOTAL = 1**	MD
M. s. pallidus	**ISRAEL**	**TOTAL = 1**	I
	AUSTRALIA:	**TOTAL = 2**	
M. s. bassanii	Naracoorte, S. Australia	1	AB
M. s. orianae	Darwin, N. Australia	1	AO
M. fraterculus (see Chapter 7)		**TOTAL = 5**	
	Shongweni Dam	1	MF1
	Peppercorn	1	MF2
	Sudwala	3	MF3, MF4, MF5

The PCR's were performed under mineral oil in 50 µl reactions, containing 1.5 mM magnesium chloride ($MgCl_2$), 0.2 mM deoxynucleotide mix, 1x reaction buffer, 0.5 pmol each primers C and E, 1% bovine serum albumin (BSA; Promega, supplied by Whitehead Scientific, Cape Town), 0.3 U *Taq* polymerase and 2 – 5 µl template DNA (10 – 50 ng/µl, depending on the sample). Two different *Taq* DNA polymerases (BIOTAQ DNA Polymerase: Bioline USA; *Taq* DNA polymerase: Promega) and their associated buffers (10x magnesium-free reaction buffer and either 25 mM or 50 mM $MgCl_2$) were used, both supplied by Whitehead Scientific. Deoxynucleotides (dNTP's) were supplied individually as 100 mM solutions by Amersham Pharmacia Biotech (AP Biotech), Cape Town. They were combined in equal quantities to produce a working stock of 25 mM, and stored at -20°C. The PCR's were performed in either a Stratagene RoboCycler96 or an Eppendorf Mastercycler Gradient. A typical PCR cycle consisted of 2 minutes at 95°C, followed by 30-35 cycles of 50 seconds at 95°C, 50 seconds at 50°C and 1 minute at 72°C, followed by a single, final extension period of 10 minutes at 72°C. Although Wilkinson & Chapman (1991) used an annealing temperature of 55°C to amplify the control region of *Nycticeius humeralis*, primers C and E functioned better at 50°C when used on *M. schreibersii*.

To confirm the specificity of the PCR, a 5 – 10 µl aliquot of each amplified product was electrophoresed at 100 V through a 1% agarose gel containing 1% ethidium bromide, against a λ*Dra*I marker (Chapter 3). The product was visualised under ultraviolet (UV) light, and if only one band of the correct size (~550 bp) was visible on the gel, the remainder of the PCR product (40 – 45 µl) was purified with a QIAquick PCR purification kit (QIAGEN, supplied by Southern Cross Biotechnology, Cape Town). The concentration of the purified PCR product was estimated by electrophoresing 5 µl of the purified sample through a 1% agarose gel, containing 1% ethidium bromide. The fluorescence of the sample was compared to that of a known standard, such as a 100 bp DNA ladder (Promega), as described in Chapter 2, Section 2.3.2.

5.2.2. Sequencing of the control region

Two different methods were employed for sequencing of the control region: (i) dideoxy sequencing and (ii) cycle sequencing.

i. Description of dideoxy sequencing

The dideoxynucleotide chain-termination method of sequencing was developed by Sanger *et al.* (1977), and involves the controlled interruption of enzymatic DNA replication (Hillis *et al.* 1996). A DNA template is denatured to produce single-stranded molecules, and a short oligonucleotide primer, which is complementary to one of the strands, is allowed to anneal to a target sequence. The sample is divided into four subsamples, to which is added DNA polymerase as well as the four deoxynucleotides or dNTP's (dATP, dTTP, dCTP and dGTP), one of which is radioactively labelled. In addition, one dideoxynucleotide (ddATP, ddTTP, ddCTP or ddGTP) is added to each subsample.

During the sequencing reaction, DNA polymerase synthesises a complementary copy of the single-stranded DNA template by incorporating dNTP's into the growing strand. Each dNTP is attached to the chain by means of a phosphodiester bond between the 3' hydroxyl group of the growing chain and the 5' phosphate group of the new dNTP. Occasionally a dideoxynucleotide (ddNTP) is incorporated in place of a dNTP, which terminates elongation of the growing DNA strand. This is because ddNTP's lack a hydroxyl group on the 3' carbon, thus preventing further elongation of the strand. The position at which a ddNTP is incorporated into each new strand is random, and each subsample therefore contains a collection of extended primer chains of differing lengths. The fragments share a common point of origin, determined by the annealed primer, but differ in length depending on where the ddNTP was incorporated. The radioactive

fragments in each subsample are loaded into separate, adjacent wells of a denaturing polyacrylamide gel, separated by electrophoresis and visualised by autoradiography (Ausubel *et al.* 1994; Hillis *et al.* 1996).

ii. Description of cycle sequencing

Cycle sequencing is based on dideoxy sequencing, but involves amplification of labelled DNA (which is complementary to the original sequence) by means of a linear polymerase reaction (Hillis *et al.* 1996). As for dideoxy sequencing, a primer anneals to single-stranded target DNA and is elongated by incorporation of dNTP's. This continues until a ddNTP is added to the chain, which terminates the elongation process. The major difference between the methods is that cycle sequencing involves linear amplification of the product through successive cycles of denaturation, annealing and synthesis, catalysed by a thermostable DNA polymerase (*e.g. Taq* DNA polymerase). The principle is similar to PCR, but the amplification process is linear rather than exponential because, unlike PCR, only one DNA strand is replicated during each cycle, rather than both. In cycle sequencing, either the primer or one of the dNTP's is labelled radioactively (*e.g.* with α-^{32}P or α-^{35}S) or, in the case of automated sequencing, with fluorescent dyes that are detected by a laser during electrophoresis (Hillis *et al.* 1996). One advantage of cycle sequencing over traditional dideoxy sequencing is that it can utilise double-stranded as well as single-stranded templates (template molecules are denatured during the thermal cycling procedure and therefore do not need to be denatured prior to sequencing). Furthermore, because it involves linear amplification of the sequenced product, it reduces the amount of template material necessary for the sequencing reaction (Hillis *et al.* 1996).

iii. Sequencing of mtDNA PCR products

Purified PCR products (Section 5.2.1) were sequenced using either primer P or E (Table 5.1); primer E generally produced cleaner, longer sequences. Initially, automated sequencing was

performed by the Department of Molecular and Cellular Biology, UCT. Samples were sequenced according to the dideoxy method, using a Cy5TM Thermo Sequenase Dye Terminator Kit (AP Biotech), and then run on an ALFexpress DNA Automated Sequencer. Thereafter, samples were cycle-sequenced in-house with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Cape Town), and then sent to the Central DNA Sequencing Facility at the University of Stellenbosch, where they were run on an ABI Prism 3100 Genetic DNA Analyzer (Applied Biosystems).

5.2.3. Statistical analysis of mtDNA sequences

To confirm that the sequences were from the mitochondrial control region, an online BLAST (Basic Local Alignment Search Tool) search was performed with the assistance of *BLASTN* 2.2.1 (Altschul *et al.* 1997). This set of similarity search programmes compares nucleic acid or protein sequences to a selection of online databases managed by the National Centre for Biotechnology Information (NCBI). It identifies database records that contain regions of similarity with the submitted sequence, to aid identification of the sequence of interest. The search results (Appendix 4) indicated close alignment of these sequences (Sections 5.2.1 and 5.2.2) with Cytochrome *b* and control region sequences of *M. schreibersii* and other bat species.

Control region sequences of *M. schreibersii* were aligned manually with the aid of the computer software package *DAPSA* (Harley 2000), and unique mtDNA haplotypes were identified in each South African colony and for the international samples. A genetic distance matrix was constructed for the sequences, and sequence divergence estimates were corrected for multiple hits by the Kimura two-parameter model (Kimura 1980), which accounts for differences in the rate at which transitions and transversions occur. Gene diversity was determined with the assistance of the software package, *Arlequin* (Schneider *et al.* 2000). Gene diversity is

equivalent to the expected heterozygosity for diploid data, and can be defined as the probability that two haplotypes chosen at random from a sample are different (Nei 1987).

To examine intra- and inter-colony genetic diversity, analysis of molecular variance (AMOVA) was conducted, with the assistance of *Arlequin*. During this statistical procedure, a matrix of the squared Euclidean distances (see below) among all pairs of haplotypes is generated, and information on haplotypic divergence is derived directly from this matrix through an hierarchical analysis of variance. In addition to estimates of variance components, fixation indices analogous to the traditional F-statistics (Chapter 4, Section 4.2.6) are calculated, known as Φ-statistics. The format of the analysis is very similar to that employed for F-statistics, but allows for haploid transmission of mitochondrial genomes. The significance of each Φ-statistic is estimated by comparing it to a null distribution of estimates obtained by multiple (~1000) randomised permutations of the columns and rows within the squared distance matrix. Three hierarchical components of diversity can be examined by AMOVA: (1) within populations (or colonies), (2) among populations (or colonies) within regions, and (3) among regions. The corresponding Φ-statistics for these hierarchies are Φ_{ST}, Φ_{SC} and Φ_{CT} respectively. For example, Φ_{ST} examines the correlation between random haplotypes drawn from within a colony and random pairs of haplotypes drawn from the species as a whole, while Φ_{CT} examines haplotypes drawn from groups of colonies (*i.e.* regions), and correlates them with haplotypes drawn from the whole species (Excoffier *et al.* 1992). Please note that while *Arlequin* refers to Φ_{ST}, Φ_{SC} and Φ_{CT} as F_{ST}, F_{SC} and F_{CT} respectively, to avoid confusion with traditional F-statistics (Chapter 4), these indices will be referred to as Φ-statistics here, according to their original definition in association with AMOVA (Excoffier *et al.* 1992).

Within- and among-colony variance components were examined for *M. schreibersii*, and Φ_{ST} was determined to provide an indication of the extent of population subdivision within South Africa. Pairwise colony comparisons were not conducted because only single sequences were obtained for many colonies, and the sample size for these colonies was thus too low to provide a meaningful estimate of pairwise colony differences. Analysis of molecular variance was also performed comparing South African *M. schreibersii* to the international samples (*i.e.* from Australia, Madagascar and Israel) and to *M. fraterculus*. In this case, three fixation indices (Φ_{ST}, Φ_{SC} and Φ_{CT}) were determined because all hierarchies of variance components could be examined.

A minimum spanning tree was constructed, based on squared Euclidean distances between the haplotypes. This tree indicates the relationships between the sequenced haplotypes. Squared Euclidean distances were determined by AMOVA, with the assistance of *Arlequin,* and are an estimate of the minimum number of mutational steps between haplotypes. They can be used to construct a tree, which indicates the extent of evolutionary divergence between haplotypes (Excoffier *et al.* 1992). Although connection lengths on the minimum spanning tree are based on squared Euclidean distances and are measures of evolutionary divergence, they may not always equal the phenetic distance between haplotypes. This may be due either to homoplasy (convergent or back mutations) or because the translation process between the actual number of mutational events and that inferred on the tree may not be linear (Excoffier *et al.* 1992).

Phylogenetic reconstruction of the relationships between the South African *M. schreibersii* control region haplotypes and those of the international samples was performed, implementing neighbour-joining (distance) and maximum likelihood methods. *Miniopterus fraterculus* was used as an outgroup for both methods. Neighbour-joining trees were constructed using *MEGA* (Kumar *et al.* 1993), and the stability of branches in the neighbour-joining tree was evaluated by means of 1000 bootstrap pseudoreplications. Maximum likelihood trees were constructed with the assistance of *Tree-Puzzle* 5.0 (Strimmer & von Haeseler 1996), using 10 000 quartet puzzling steps.

C. RESTRICTION FRAGMENT ANALYSIS OF THE CONTROL REGION

5.2.4. Restriction fragment analysis

Although sequencing of mtDNA haplotypes is the most direct and reliable means of examining haplotype variation, it is expensive and impractical for large numbers of samples. An alternative procedure is the analysis of restriction fragment profiles. This method examines haplotype diversity indirectly by identifying variation in the number and size of DNA restriction fragments separated by electrophoresis. This method offers less resolution than sequencing, but is a cost effective alternative when large numbers of individuals are being screened (Dowling *et al.* 1996). Restriction fragment analysis was therefore conducted on a large subsample of the bats (n = 58) to estimate the number and relative frequencies of haplotypes in each colony, and to determine whether any population substructuring of these haplotypes was evident.

Sequence differences resulting from base substitutions and from insertions and deletions (indels) can be detected by restriction enzymes, which cut DNA at specific nucleotide sequences. Each restriction enzyme has a unique recognition or cut site where it cleaves the DNA, and the specificity of these enzymes means that reproducible arrays of fragments will be produced after complete digestion of specific DNA sequences (Dowling *et al.* 1996). Alteration of the nucleotide sequences may either create or eliminate restriction enzyme recognition sites, which affects the number and size of fragments produced by digestion with any particular enzyme. This alters the restriction fragment profile (or pattern) visible on an electrophoretic gel, and variations in fragment profiles resulting from these mutations are known as restriction fragment length polymorphisms (RFLP's).

Individuals may differ in the number and/or size of restriction fragments due to base substitutions, insertions or deletions that alter the recognition site of the restriction enzyme, or due to changes in the amount of DNA (*e.g.* the number of repeat regions) within the area of interest. Knowledge of the structure of the region to be examined is therefore important for correct interpretation of restriction fragment patterns. For example, although R1 tandem repeats are found in the control region of many vespertilionid bat species (Section 5.1.3), no such repeats have been found in *M. schreibersii* (Wilkinson *et al.* 1997). Therefore, any variation in size between restriction fragments produced from the control regions of different individuals of this species is likely to be due to substitutions or indels that alter enzyme recognition sites.

5.2.5. Screening the control region sequences for restriction enzyme cut sites

Control region sequences were screened, with the assistance of *DAPSA* (Harley 2000), for restriction enzyme cut sites that characterised each haplotype. Six restriction enzymes were identified (Table 5.3), which together characterised eight haplotypes.

Table 5.3. Recognition sites of the restriction enzymes used to characterise mtDNA control region haplotypes of *M. schreibersii.* Sizes (in base pairs) of resulting restriction fragments (RF; based on an original PCR product size of 550 bp), optimum assay temperature (T), activity of the enzyme, manufacturer (and supplier) are also given.

Restriction enzyme	Recognition site	RF (bp) if cut site is present	RF (bp) if cut site is absent	Activity of enzyme	T (°C)	Supplier
*Acc*I	GTMKAC	128, 128, 294	128, 422	5 U/µl	37	Roche Diagnostics, Cape Town
*Bcl*I	TGATCA	57, 204, 289	204, 346	10 U/µl	50	Roche Diagnostics
*Bgl*I	GCCNNNNNGGC	136, 414	550	10 U/µl	37	Roche Diagnostics
*Hae*III	GGCC	37, 513	29, 37, 484	10 U/µl	37	Amersham Life Science (AP Biotech, Cape Town)
*Mae*II	AGCT	254, 296	550	2 U/µl	50	Roche Diagnostics
*Vsp*I	ATTAAT	32, 518	550	12 U/µl	37	Promega (Whitehead Scientific, Cape Town)

5.2.6. Restriction enzyme digests

Five bats (in addition to those initially sequenced) were chosen at random from each colony for restriction fragment analysis. A sequenced sample, known to contain the relevant recognition site was used as a positive control in each restriction digest. Negative controls were also included for those digests involving enzymes that had multiple recognition sites, only one of which was diagnostic for the haplotypes (*Acc*I, *Bcl*I and *Hae*III). Samples that did not originate from major colonies, or were obtained from locations outside of South Africa were not included in the RFLP analyses. The restriction fragment profiles of the *M. fraterculus* samples collected from Shongweni Dam (MF1) and Peppercorn (MF2) were included as outgroups.

Each sample was amplified by PCR using primers C and E, as described in Section 5.2.1. Once the specificity of each PCR had been confirmed by the presence of a single product of ~550 bp on a 1% agarose gel (Section 5.2.1), amplified products were individually digested with each restriction enzyme. An example of a typical protocol for *Hae*III is given below. Similar protocols were followed for the other restriction enzymes, and adjusted for varying enzyme activities when necessary (Table 5.3).

10x restriction enzyme buffer	2.0 µl	(final concentration: 1x)
10 U/µl *Hae*III	0.5 µl	(final activity: 0.25 U)
10 µg/µl acetylated BSA	0.5 µl	(final concentration: 0.25 µg/µl)
≥ 20 ng/µl PCR product	10.0 µl	(final concentration ≥ 10 ng/µl)
Sterile distilled water	7.0 µl	
TOTAL VOLUME	20.0 µl	

Restriction digests were carried out overnight in a water bath at either 37°C or 50°C, depending on the enzyme (Table 5.2), and then stopped by incubation at 65°C for 15 minutes. Restriction fragments were electrophoresed at 100 V through 2% agarose gels containing 1% ethidium bromide, and visualised under UV light. Restriction fragments from *Vsp*I digests were examined on 4% rather than 2% agarose gels, because of the small size difference between digested (518 bp) and undigested (550 bp) fragments. After digestion with all six enzymes, the composite banding pattern of each sample was determined and designated as a haplotype (Section 5.2.7).

i. Purification of PCR products prior to digestion

The activity of certain restriction enzymes is affected by inhibitors in the PCR cocktail. Therefore, the following experiment was conducted to determine whether it was necessary to purify PCR products prior to digestion with each restriction enzyme. One microlitre of Lambda (λ) DNA (425 mg/ml; Amersham Life Science, Cape Town) was added to a "blank" PCR cocktail (*i.e.* a PCR cocktail containing all components except the template DNA). A restriction digest was conducted with each restriction enzyme, according to the protocol described above, but 10 µl of the "λ-blank PCR" was included in place of the usual PCR product. The cocktail was incubated overnight at either 37°C or 50°C, depending on the enzyme, followed by 15 minutes at 65°C. It was then electrophoresed and visualised as described above.

If digested fragments of λ DNA were visible, it was deemed that no purification of the PCR products was necessary prior to restriction enzyme digestion. This was true for all but one of the restriction enzymes: only *Mae*II was unable to digest λ DNA in the "blank" PCR cocktail. Prior to digestion with *Mae*II, therefore, each PCR product was purified as follows. Approximately 15 µl of PCR product was pipetted onto a 0.025 µm nitrocellulose filter (Millipore) of diameter 25 mm, and floated on sterile distilled water (Figure 5.3). Inhibitors in the PCR cocktail were dialysed out through the nitrocellulose filter. After ten minutes, 10 µl of the filtered PCR product

was removed for use in the restriction digest. This purification protocol was tested using the "λ-blank PCR", and digestion with *Mae*II following purification was successful.

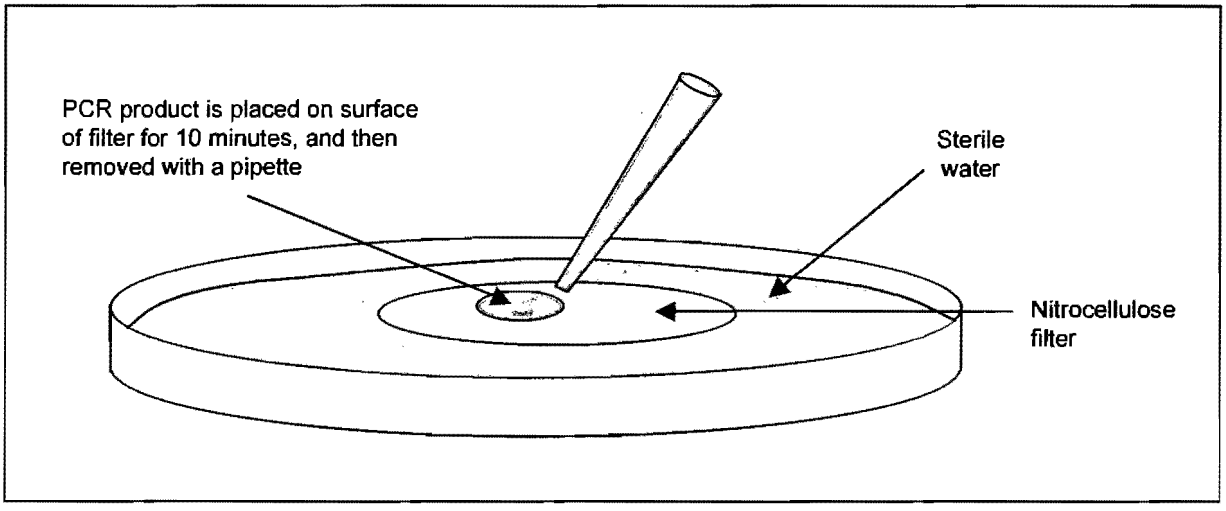

Figure 5.3. Schematic illustration of the process of purifying PCR products using a nitrocellulose filter (Millipore), floated on water.

5.2.7. Statistical analysis of RFLP data

The composite restriction fragment profile of each sample was converted to a binary code, in which 1 and 0 represented the presence and absence of a restriction site respectively. The restriction profile of each of the sequenced individuals was also determined by searching for the characteristic base sequences, with the assistance of *DAPSA*. Gene diversity (Section 5.2.3) was determined, and both global and pairwise exact tests of colony differentiation based on haplotype frequencies were performed, using *Arlequin*. The exact test is analogous to Fisher's

exact test, and examines whether or not there is a random distribution of haplotypes among the colonies (Raymond & Rousset 1995b). During this procedure, haplotype frequencies are arranged into a contingency table, and possible states of this contingency table are examined by a 6000 step Markov chain model (which assumes that the probability of a change at any site from one state to another is independent of the mutational history of that site; Swofford *et al.* 1996). The p-value associated with the exact test indicates the probability of obtaining a contingency table less or equally likely than the observed configuration of haplotypes in the sample, under a null hypothesis of panmixia (Raymond & Rousset 1995b).

Varying frequencies of restriction haplotypes were analysed by means of fixation indices, with the assistance of *Arlequin*. An analysis of molecular variance (AMOVA; Section 5.2.3) based on pairwise distances between the restriction fragment haplotypes was performed to determine whether the distribution of the haplotypes in South Africa was homogenous. Pairwise Φ_{ST} and their associated probability values were also determined from the haplotype frequency distributions. A Mantel test was conducted using *Arlequin*, to determine whether a correlation existed between the pairwise Φ_{ST} values and the geographical distances between the *M. schreibersii* colonies. Finally, a minimum spanning network of the evolutionary relationships between the restriction fragment haplotypes was constructed, based on squared Euclidean distances obtained from the AMOVA.

5.3. RESULTS

5.3.1. Sequenced haplotypes

i. Indices of molecular diversity and genetic distances

Seventeen unique haplotypes were identified among the 18 South African *M. schreibersii* mtDNA control region sequences (Table 5.4). No evidence of heteroplasmy was detected. Gene diversity was very high (0.994 ± 0.02), which is indicative of the fact that almost all haplotypes were different. However, this index should be treated with caution, because the sample size is relatively small. Of 48 polymorphic sites in the aligned South African sequences, 37 were phylogenetically informative. When all *M. schreibersii* and *M. fraterculus* sequences were aligned, 147 sites were found to be polymorphic, 101 of which were phylogenetically informative (Figure 5.4, Appendix 5). This latter alignment also included 11 informative indels.

Table 5.4. Molecular diversity indices for sequenced mtDNA control region haplotypes for *M. schreibersii* and *M. fraterculus.*

	South African *M. schreibersii* only	**All *M. schreibersii* and *M. fraterculus***
Nº of samples sequenced	18	27
Nº of different haplotypes identified	17	25
Nº of polymorphic sites	48	147
Phylogenetically informative sites	37	101
Nº of indels (Nº informative)	1 (0)	40 (11)
Transition : transversion ratio	6.8 : 1	3.4 : 1
Nº of pairwise differences	14.40 ± 6.77	37.20 ± 16.70
Gene diversity ± SD	0.994 ± 0.02	1.00 ± 0.01

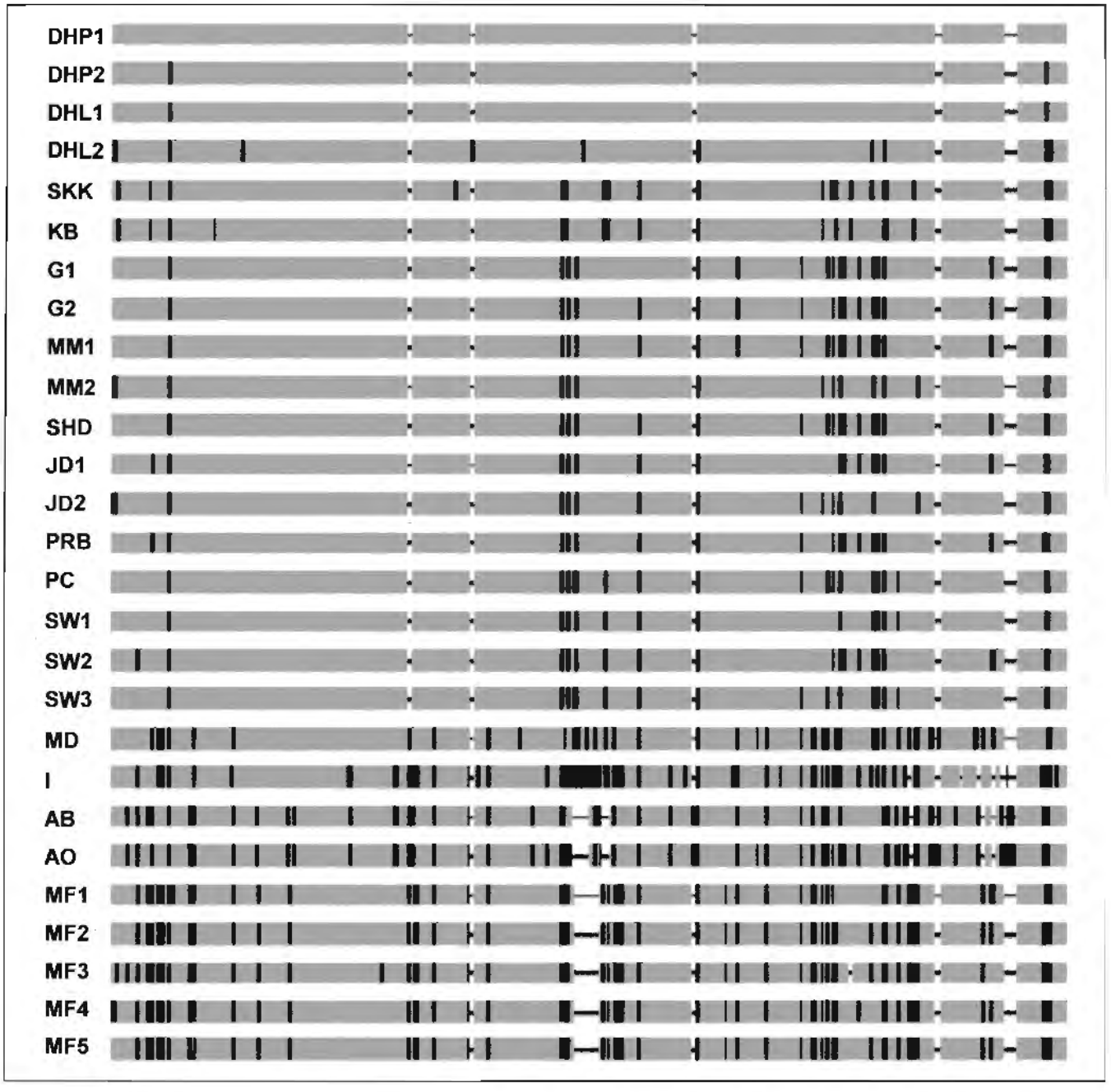

Figure 5.4. Graphical representation of the alignment of *M. schreibersii* and *M. fraterculus* control region sequences from South Africa and from international locations, generated by *DAPSA*. Haplotype DHP1 was arbitrarily chosen as a standard, and the alignment of other sequences is shown relative to this haplotype. Grey areas indicate regions of complete alignment, vertical black bars indicate polymorphic sites, white areas with dashes (-) are gaps inserted for alignment purposes. Details of haplotype names and origins are given in Table 5.2.

Mean pairwise Kimura two-parameter distances were determined from Table 5.5. The mean distance between pairs of South African colonies of *M. schreibersii* was 0.026 ± 0.014. This was significantly lower (Mann-Whitney Rank Sum test: T = 17775.00, $p < 0.001$) than the mean distance between *M. schreibersii* and *M. fraterculus* sequences (0.092 ± 0.005). It was also significantly less than the mean pairwise distance (0.107 ± 0.02) between South African and international *M. schreibersii* sequences, but was significantly greater than the mean distance (0.012 ± 0.006) between pairs of *M. fraterculus* sequences (T = 13572.00, $p < 0.001$ and T = 352.5, p = 0.006 respectively). The mean pairwise distance between *M. fraterculus* sequences was significantly lower than the mean distance (0.105 ± 0.01) between *M. fraterculus* and international *M. schreibersii* sequences (T = 45.0, $p < 0.001$). The differences between *M. schreibersii* and *M. fraterculus* are discussed in Chapter 7.

ii. Analysis of Molecular Variance

Analysis of molecular variance (AMOVA) indicated significant sequence heterogeneity exists within and among South African colonies of *M. schreibersii* (Table 5.6), suggesting strong genetic substructuring. More than two thirds of haplotype diversity (69.15%) was distributed among different colonies of *M. schreibersii*, compared to 30.85% within colonies. In this initial AMOVA, all South African *M. schreibersii* were treated as belonging to a single region, and colonies within this region were compared to each other. In a second AMOVA, the South African *M. schreibersii* were compared to individuals from three additional regions: Australia, Madagascar and Israel. The results of this AMOVA indicated that, as would be expected, 96.28% of haplotype variation was distributed among the geographical regions (South Africa, Australia, Madagascar and Israel; Table 5.6). However, although the variance components of other hierarchical levels were low, there was nevertheless significant heterogeneity of haplotypes both within colonies and among colonies within the geographical regions (Table 5.6).

The extent of divergence of restriction fragment haplotypes can be represented by a minimum spanning network (Figure 5.10). *Miniopterus schreibersii* haplotypes differ from each other by a minimum connection length of 1.0. The two *M. fraterculus* haplotypes also differ by a minimum connection distance of 1.0, but are separated from *M. schreibersii* haplotypes by a minimum distance of 2.0. Haplotypes found in the same *M. schreibersii* colony tend to cluster together in the minimum spanning network, and are more closely related to one another than haplotypes found in geographically separate colonies. Different haplotypes within any colony are separated by between one and three branches, each of connection length 1.0, while differing haplotypes from different colonies may be separated by up to five branches.

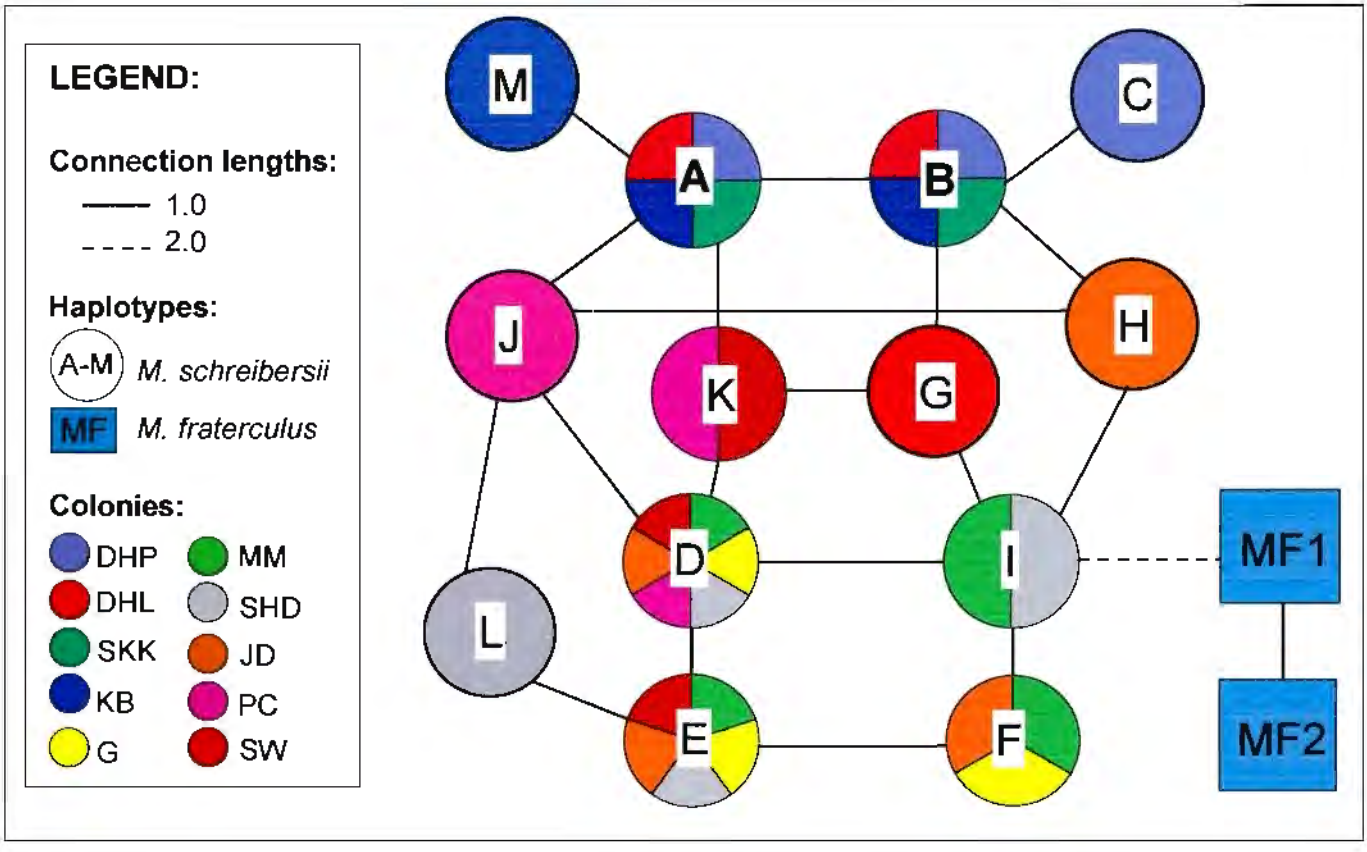

Figure 5.10. Minimum spanning network of mitochondrial restriction fragment haplotypes A-M. The colours indicate colonies in which the haplotypes were identified.
Names of colonies are abbreviated as follows: DHP = De Hoop, DHL = Die Hel; SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown, MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala.

The global exact test indicated that the distribution of restriction fragment haplotypes among colonies deviated significantly from panmixia ($p < 0.00001$). This was confirmed by the AMOVA, which indicated significant heterogeneity of restriction fragment haplotypes within and among *M. schreibersii* colonies (Table 5.10, A). Although the variance components were equally distributed within and among colonies, the overall Φ_{ST} of 0.503 ($p < 0.000001$) suggests strong genetic substructuring. Probability values obtained from pairwise exact tests of differentiation as well as pairwise Φ_{ST} comparisons between colonies (Table 5.11) indicate that this substructuring is primarily between a south-western subpopulation consisting of Die Hel, De Hoop, Steenkampskraal and Koegelbeen, and a north-eastern group consisting of Grahamstown, Maitland Mines, Shongweni Dam, Peppercorn and Sudwala. The sequenced haplotypes suggested there are three subpopulations (south, west and north-east), but as sequencing offers greater resolution than restriction fragment analysis, it is not surprising that the latter method has identified only two subpopulations.

The pattern of substructuring suggested by the pairwise Φ_{ST} values was quantified by a second AMOVA, in which the colonies were divided into a south-western (SW) region and a north-eastern (NE) region (Table 5.10, B). Approximately 58% of the haplotype variance was distributed between the two regions, compared to only ~5% among colonies within the regions. Although considerable haplotype diversity (~36%) is still found within each colony, the fixation indices and their associated p-values once again indicate significant heterogeneity among the restriction fragment haplotypes at all levels of the AMOVA hierarchy. A Mantel test comparing the pairwise colony Φ_{ST} values to the geographical distances between the colonies (Table 5.11) indicated there is a weak but significant correlation between geographical separation and colony differentiation (1000 permutations; correlation coefficient = 0.48; $p = 0.018$).

Table 5.10. Results of analysis of molecular variance of restriction fragment haplotypes. Comparisons were conducted (A) with all colonies considered as a single group, and (B) with colonies divided into a south-western (SW) region and a north-eastern region (NE); DF = degrees of freedom.

Source of variation	A. Single group of colonies				B. SW *vs.* NE colonies			
	DF	Variance components	Percent of variation	Fixation index	DF	Variance components	Percent of variation	Fixation indices
Among regions	-	-	-	-	1	0.777	58.05	Φ_{CT} = 0.580 p < 0.0001
Among colonies within regions	9	0.493	50.29	-	8	0.074	5.52	Φ_{SC} = 0.074 p < 0.0001
Within colonies	48	0.488	49.71	Φ_{ST} = 0.503 p < 0.0001	48	0.488	36.43	Φ_{ST} = 0.636 p < 0.0001
Total	57	0.981	100	-	57	1.339	100	-

Table 5.11. Pairwise comparisons of haplotype frequencies between *M. schreibersii* colonies[†], and their correlation with geographical distance. Pairwise Φ_{ST} values are above the diagonal; geographical distances (km) between the colonies are below the diagonal. Colonies that are significantly differentiated ($p \leq 0.05$) according to pairwise exact tests and Φ_{ST} comparisons are indicated by an asterisk (*) and bold text respectively. Negative values indicate the colonies are effectively panmictic.

	DHP	DHL	SKK	KB	G	MM	SHD	JD	PC	SW
DHP	-	-0.120	0.050	0.040	**0.730***	**0.690***	**0.576***	**0.480***	**0.462***	**0.700***
DHL	189.11	-	0.050	0.040	**0.700***	**0.656***	**0.540***	**0.443***	**0.381***	**0.657***
SKK	347.23	168.04	-	-0.125	**0.796***	**0.768***	**0.632***	**0.591***	**0.522***	**0.769***
KB	718.46	653.50	561.44	-	**0.754***	**0.730***	**0.591***	**0.553***	**0.447***	**0.714***
G	583.70	692.62	752.98	614.61	-	-0.059	-0.050	0.240	**0.372**	0.188
MM	456.64	583.77	666.18	639.62	138.32	-	0.025	0.100	**0.401**	0.296
SHD	1099.58	1160.08	1160.34	733.69	550.54	687.80	-	0.028	0.157	0.086
JD	1362.85	1397.96	1370.88	866.92	844.05	978.42	304.56	-	0.275	0.323
PC	1429.67	1401.82	1320.77	759.39	1051.41	1160.33	655.58	463.37	-	0.027
SW	1416.75	1416.35	1358.05	808.92	969.86	1092.26	500.85	264.16	204.52	-

[†] Names of colonies are abbreviated as follows:
DHP = De Hoop, DHL = Die Hel; SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown, MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala

Although the AMOVA in which the colonies were divided into SW and NE regions indicates only ~5% of the genetic variation is located among colonies within these regions (Table 5.10, B), several of the pairwise comparisons between colonies within the north-eastern region have high Φ_{ST} values. However, these values are not indicated as being significantly different from zero at $p \leq 0.05$ by either the AMOVA or the exact tests of differentiation (Table 5.11). For example, Jozini Dam and Sudwala (Φ_{ST} = 0.323) apparently do not differ significantly from each other, although $\Phi_{ST} \geq 0.25$ is normally considered indicative of restricted gene flow and extensive genetic differentiation (Hartl & Clark 1997). Other pairwise colony comparisons have Φ_{ST} values of between 0.100 and 0.296, that would generally be considered high, but surprisingly, these Φ_{ST} values are also apparently not significantly different from zero at $p \leq 0.05$. Gene flow within the north-eastern group is certainly more extensive than that between this region and either the southern or western colonies. However, Φ_{ST} values of ≥ 0.10, which generally indicate moderate to high levels of genetic differentiation (Hartl & Clark 1997), suggest that gene flow may partially be restricted even within this subpopulation. This is supported by the microsatellite data (Chapter 4), which also indicated that gene flow between certain north-eastern colonies is partially restricted.

5.4. DISCUSSION

Analysis of maternally-inherited mitochondrial DNA and nuclear microsatellite markers (Chapter 4) indicates that the *M. schreibersii* population within South Africa is strongly substructured. Both mtDNA sequences and restriction fragment patterns indicate significant heterogeneity exists among *M. schreibersii* haplotypes. Genetic diversity is high, as reflected both by high gene diversity values ($\geq$ 0.6 for most colonies, based on the restriction fragment analysis) and the high within-colony variance component resulting from the analysis of molecular variance. This is in agreement with the high observed heterozygosity values estimated from the microsatellite data ($\geq$ 0.6; Chapter 4). However, the genetic diversity is compartmentalised. The *M. schreibersii* population is genetically subdivided, as indicated by highly significant Φ_{ST} values obtained from the AMOVA of both sequence and restriction fragment data.

The general pattern of population subdivision suggested by the mtDNA data agrees with that indicated by the microsatellites. Patterns of sequence and restriction fragment haplotype divergence (Figures 5.5 and 5.10), phylogenetic analysis of the sequenced haplotypes (Figures 5.6 and 5.7) and the restriction fragment AMOVA strongly support the division of the *M. schreibersii* population into three major subpopulations: (1) a southern subpopulation, consisting of De Hoop and Die Hel, (2) a western subpopulation made up of Steenkampskraal and Koegelbeen, and (3) a north-eastern subpopulation, consisting of Grahamstown, Maitland Mines, Shongweni Dam, Jozini Dam, Peppercorn and Sudwala. Gene flow between these subpopulations is relatively limited. This may be due in part to geographic separation, as a Mantel test indicated a weak but significant correlation (r = 0.48) between geographic distance and genetic differentiation of mtDNA haplotypes. However, virtually no correlation between geographic and genetic separation was found based on the microsatellite data (Chapter 4), and thus geographic separation alone cannot entirely explain this pattern of population differentiation. This is illustrated, for example, by the fact that Maitland Mines and De Hoop,

which are ~450 km apart, differ extensively genetically (Φ_{ST} = 0.69), while Steenkampskraal and Koegelbeen, which are ~560 km apart, are effectively panmictic, as indicated by the negative Φ_{ST} = -0.025.

Colonies within the north-eastern subpopulation are relatively closely related to each other, indicating significant gene flow must occur between these colonies on a regular basis. However, even within this group, gene flow between certain colonies may partially be restricted, suggesting that female *M. schreibersii* are strongly philopatric to their maternity colonies and hibernacula within this region, although, once again, this may be due in part to geographic separation of colonies. Less genetic differentiation within the north-eastern subpopulation was detected by the nuclear microsatellites than is suggested by mtDNA. The microsatellite results (Chapter 4) suggest that while gene flow between Grahamstown and the other north-eastern colonies is partially restricted, the remainder of the colonies within this region are effectively panmictic, with very low R_{ST} values between them. However, AMOVA based on the mtDNA haplotypes produced high Φ_{ST} values between several colonies within the north-eastern group, suggesting gene flow between them may be restricted (although this did not always imply significant differentiation between them). This may indicate that while both males and females are strongly philopatric to their breeding colonies, or at least to the geographic regions of the subpopulations, as indicated by the nuclear markers, females may be even more so. This leads to greater differentiation within the north-eastern region, as detected by the maternally-inherited marker. Alternatively, the difference in the extent of differentiation detected by the two markers may simply be due to the fact that mtDNA is haploid, and therefore its effective population size is smaller. This makes it more susceptible to the effects of genetic drift than are diploid, nuclear markers (Barton & Jones 1983; Harrison 1989). Mitochondrial DNA therefore provides greater resolution and enhanced detection of genetic differentiation between populations (Section 5.1.4).

Restriction fragment analysis of mtDNA grouped the southern and western populations into a single south-western group, probably because this method offers less resolution than either

sequencing or microsatellite analysis. However, even AMOVA based on restriction fragment patterns indicates that gene flow within the south-western subpopulation is restricted. Pairwise Φ_{ST} values obtained from restriction fragment patterns indicate moderate gene flow between the southern and western colonies (De Hoop/Die Hel *vs.* Steenkampskraal: $\Phi_{ST} = 0.05$; De Hoop/Die Hel *vs.* Koegelbeen: $\Phi_{ST} = 0.04$). However, the extent of evolutionary divergence of the sequenced haplotypes (Figure 5.5), as well as the phylogenetic clustering indicated by both neighbour-joining and maximum likelihood trees (Figures 5.6 and 5.7) suggest the south-western group can be subdivided further into southern (De Hoop and Die Hel) and western subpopulations (Steenkampskraal and Koegelbeen), a pattern also indicated by the nuclear microsatellite markers.

In summary, the close correlation between results obtained from mitochondrial and nuclear markers indicates that both male and female *M. schreibersii* are strongly philopatric in South Africa, females perhaps more so than males. Both sexes appear to return to the same breeding sites each year, and females return annually to their natal maternity colonies to give birth and raise their young. Annual migrations between these wintering and summer sites may be extensive in terms of distance travelled, but appear to be restricted to certain geographical regions. Gene flow between these regions is limited, leading to strong population substructuring. This is discussed in further detail in Chapter 8.

With the exception of Madagascar, mtDNA sequences from international specimens appear to differ more from the South African *M. schreibersii* sequences, than do the *M. fraterculus* haplotypes. Considerable differences are expected to exist between individuals from South Africa, Israel and Australia due to the improbability of any gene flow between such geographically separate populations. However, it is surprising that individuals that are considered to be members of the same species are more divergent than individuals of separate species. This suggests that members of *M. schreibersii* in different parts of the world are on an evolutionary trajectory towards speciation, and the global taxonomy of this species should perhaps be re-examined.

CHAPTER 6

WING MORPHOLOGY AND ECOLOGY

AIM: To determine whether genetic differences between colonies of *Miniopterus schreibersii* are reflected in their wing morphology.

6.1. INTRODUCTION

6.1.1. Correlation between morphology and feeding ecology

The relationship between form and function is a well established phenomenon in evolutionary biology, and a correlation can frequently be made between an organism's morphology and its ecological role (Findley & Wilson 1982). Such a correlation has been demonstrated in insectivorous bats by Norberg & Rayner (1987). They concluded that behavioural and ecological differences between species correlate with specialisation in flight morphology, and that flight performance has a dominant influence on adaptation in bats. However, Norberg & Rayner (1987) examined the entire range of species without regard for detailed analysis of bat communities in particular habitats. It is therefore possible that detailed analyses at the community level may identify other factors (*e.g.* skull morphology or echolocation) as the dominant factors in the evolution of bats. For example, Freeman (1979, 1981) has shown that

jaw morphology in animal-eating bats is closely correlated with diet. Bowie *et al.* (1999) and Jacobs (2000), on the other hand, have shown that differences in the form and use of echolocation is a better predictor of diet than morphology. Nevertheless, there is evidence that wing morphology does play an important role in the ecology of bats. Aldridge & Rautenbach (1987), for example, examined 26 sympatric insectivorous bat species in the Kruger National Park, South Africa, and found a significant correlation between wing morphology and habitat use. Differences in wing morphology between the various species have apparently led to spatial partitioning of available habitat. Measurements of wing morphology allowed the authors to make predictions about each species' flight performance and habitat use, and suggested a link between wing morphology and habitat. Similarly, Saunders & Barclay (1992), working on morphologically similar species, concluded that large morphological differences may influence habitat use, while small morphological differences may influence prey availability within the same habitat.

Analysis of microsatellite markers and mitochondrial DNA (Chapters 4 and 5) indicate that the *M. schreibersii* population in South Africa is comprised of three sub-populations. Furthermore, the sub-populations appear to be restricted to certain vegetation zones to which they may have become locally adapted (discussed in Chapter 8). If this is the case, then given the relationship between wing morphology and ecology in insectivorous bats, one might expect the genetic differences among *M. schreibersii* colonies to be reflected in morphological variation among the colonies.

6.1.2. Wing parameters

Body mass, wing area and wingspan are the primary morphological parameters that provide an indication of wing design. Wing area is the combined area of both wings, the body area between the wings (excluding the head) and the entire tail membrane. Wingspan is the distance between the wingtips, with both wings extended. These measures are used to derive two additional parameters that describe the aerodynamic characteristics of the wing, namely wing loading and aspect ratio (Norberg & Rayner 1987).

Wing loading is a measure of the size of the wing. It is related to the mean pressure on the wings, and is given by the following formula (Norberg & Rayner 1987):

$$Wing\ loading\ \left(N.m^{-2}\right) = \frac{Body\ mass\ (kg) \times g}{Wing\ area\ \left(m^2\right)}$$

where g is acceleration due to gravity (9.81 ms^{-1})

The higher the wing loading, the smaller the area of lifting surface available per unit weight (Vaughan 1970). Therefore bats that have a high wing loading need to be fast fliers so that they generate sufficient lift to remain airborne (Findley *et al.* 1972). Bats that have lower wing loading are able to fly at lower speeds without "stalling" (Norberg & Rayner 1987). Bats with high wing loading also tend to be less manoeuvrable and less agile, *i.e.* less able to turn tightly and quickly (Norberg & Rayner 1987).

Aspect ratio is an index of the shape of the wing and is given by the formula (Norberg & Rayner 1987):

$$Aspect\ ratio\ = \frac{\left[Wingspan\ \ (m)\right]^2}{Wing\ area\ \left(m^2\right)}$$

Wings that are long and narrow have a high aspect ratio (Findley *et al.* 1972; Norberg & Rayner 1987), which generally corresponds to greater aerodynamic efficiency (Norberg & Rayner 1987). As aspect ratio increases, so drag on the wing decreases, permitting greater speed, but at the same time, reducing lift on the wing. Therefore the bat needs to fly faster to generate lift and to remain airborne (Findley *et al.* 1972). Short, broad wings have low aspect ratio and generate a large amount of drag at high speed (making flight more energetically expensive at high speed), but they provide maximal lift at low speed (Findley *et al.* 1972).

Aspect ratio and wing loading tend to be positively correlated with each other (Findley *et al.* 1972; Norberg & Rayner 1987). Thus bats with high wing loading tend to have high aspect ratios (*e.g.* the molossids). These bats are able to fly fast, but have low manoeuvrability. They therefore tend to forage in open areas, catching airborne prey on the wing, and are generally unable to forage effectively in cluttered environments, *i.e.* habitats with many obstacles such as vegetation (Fenton 1990; Jacobs 1999). Because high wing loading and aspect ratio promote energetically inexpensive flight (Fenton 1990; Jacobs 1999), these features are also characteristic of migratory species (Findley *et al.* 1972; Norberg & Rayner 1987).

Bats that have low wing loading and low aspect ratio (*e.g.* rhinolophids) fly slower, and their flight is generally more energetically expensive (Norberg & Rayner 1987). However, they are more manoeuvrable, and so are able to forage in cluttered habitats, such as forests (Findley *et al.* 1972; Jacobs 1999). Some of these bats are also able to hover or to glean insects from branches. These species are not restricted to cluttered environments however, and also have access to open spaces (Aldridge & Rautenbach 1987; Fenton 1990). In fact, some studies (*e.g.* Saunders & Barclay 1992) have indicated that prey distribution plays an important role, and in some cases this may influence habitat use more than wing morphology.

6.1.3. Wing morphology of *Miniopterus schreibersii*

As its common name suggests, Schreibers' long-fingered bat has long, narrow wings, and therefore average to high aspect ratio. Accordingly, it generally makes use of rapid flight (~16 ms^{-1}, McDonald *et al.* 1990b) with low manoeuvrability, in open areas (Jacobs 1999). However, the average to high aspect ratio is coupled with relatively low wing loading, which suggests it may also be able to forage in cluttered habitats (Jacobs 1999; Norberg & Rayner 1987). Jacobs (1999), however, found that this was not due to each individual having the morphology that permitted flight in both habitat types, but due to intraspecific variation which allowed some individuals with shorter wingspans (and thus lower aspect ratio) to forage in both open and cluttered environments, while others with longer wingspans were restricted to open spaces.

Jacobs' (1999) research was conducted on a single colony of *M. schreibersii* at the De Hoop Nature Reserve in the Western Cape region of South Africa (Figure 2.1, Chapter 2). Because *M. schreibersii* appear to show intraspecific variation in wing morphology, allowing members of a single colony to utilise different foraging habitats, it is possible that these bats also exhibit differences between colonies, located in different environments or biomes. This component of the study, therefore, investigated whether or not genetic differences identified among South African colonies of *M. schreibersii* (Chapters 4 and 5) are reflected in differences in wing morphology. If *M. schreibersii* colonies do differ significantly in their wing morphology, this may suggest morphological adaptation to local habitats.

6.2. MATERIALS AND METHODS

6.2.1. Sampling procedure

Morphological measurements were taken at the same time and from the same bats as the genetic samples (Chapter 2, Section 2.2.2). The times of the year at which samples were collected are given in Table 2.1, Chapter 2. Mass (to the nearest 0.5 g) and forearm length (to the nearest 0.1 mm) of each bat were measured, and age was recorded as either juvenile or adult, based on the presence or absence, respectively, of cartilaginous epiphyseal plates between the metacarpal and phalangeal joints (Anthony 1988). The reproductive status of females was assessed by means of gentle palpation of the abdomen to determine if they were pregnant (Racey 1988).

Wing tracings or digital photographs were taken from the extended right wing of each bat, according to the method described in Saunders & Barclay (1992). Wing-tracings were made from bats collected at Steenkampskraal, Die Hel and De Hoop. Digital photographs were taken of all other bats. In the case of the latter method, the bat was photographed against a white background with an Olympus digital camera, model C-800L, in low-resolution mode (512 x 384 pixels) with built-in flash illumination. A ruler was included in each photograph as a scale. Both wing tracings and digital photographs were taken for ten bats (chosen at random) from Grahamstown to compare the two methods.

6.2.2. Analysis of wing tracings and digital photographs

Wing tracings and digital photographs were scanned and downloaded, respectively into a personal computer, and saved as JPEG files. Prior to scanning, a 10 cm line was drawn onto each wing tracing, as a scale. Wingspan and wing area were calculated from scanned or downloaded images by means of a custom-written software package, *BatWing* Version 1.0 (Harley & Miller-Butterworth 2000). Prior to analysis with *BatWing*, the digital photographs and wing tracings were processed in *Paint Shop Pro* Version 5.01 (Jasc Software, Inc.), as shown in Figures 6.1 and 6.2 respectively. Hand-wing, arm-wing and shoulder-midline (including tail membrane) areas were selected and coloured black, red and grey respectively, which are the colours recognised by *BatWing* for these area calculations. Coloured marker files named A1, A2, B1, B2, C1 and C2 were pasted onto the wing images in *Paint Shop Pro*, as shown in Figures 6.1 and 6.2. Processed files were converted to portable pixelmaps (.ppm extension), which is the file type recognised by *BatWing*. Thereafter, files were loaded into *BatWing*, and the programme executed to calculate hand-wing, arm-wing and shoulder-midline areas (in cm^2) and lengths (in cm).

BatWing calculates areas by finding and counting all pixels within an image that have a particular colour (determined by the programme). It converts this value to a proportion of the total pixel count, and then to an area by reference to a known length measurement (the ruler or scale line). Similarly, it calculates distances (*e.g.* length of the hand-wing, HWL; Figures 6.1 and 6.2) by reference to the ruler or scale line. *BatWing* uses the distance between the A1 and A2 marker files as a 10 cm reference, and they are therefore placed accordingly on the ruler or scale bar. Hand-wing (HWL), arm-wing (MWL) and shoulder-midline (SML) lengths are calculated by determining the number of pixels between markers B1 and B2, B2 and C1, and C1 and C2 respectively, and comparing these to the reference distance A1 – A2. For further details of how to process images in *Paint Shop Pro* and *BatWing*, please refer to Harley & Miller-Butterworth (2000).

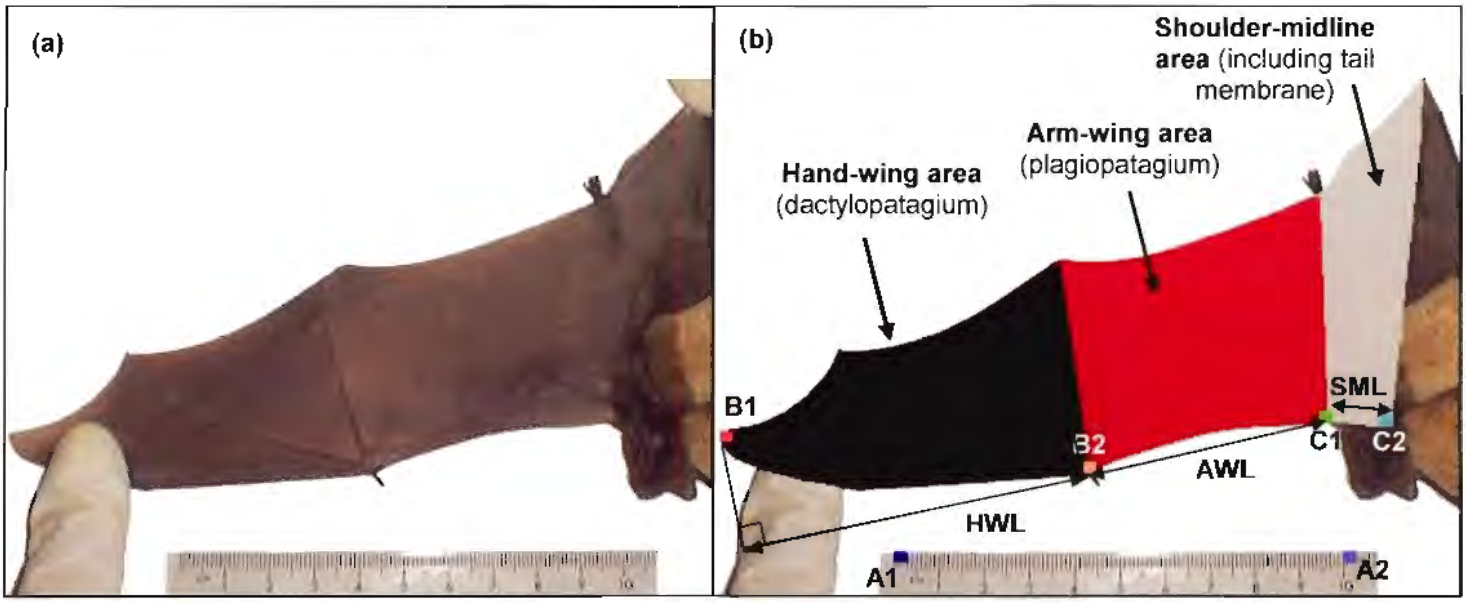

Figure 6.1. (a) Photograph of the extended right wing of an *M. schreibersii*, taken with an Olympus digital camera (model C-800L); (b) The same photograph after processing in *Paint Shop Pro*, showing areas coloured for calculation of hand-wing, arm-wing and shoulder-midline areas. Positions of markers A1, A2, B1, B2, C1 and C2 for calculation of linear measurements are also shown; HWL = hand-wing length, AWL = arm-wing length, SML = shoulder to midline length.

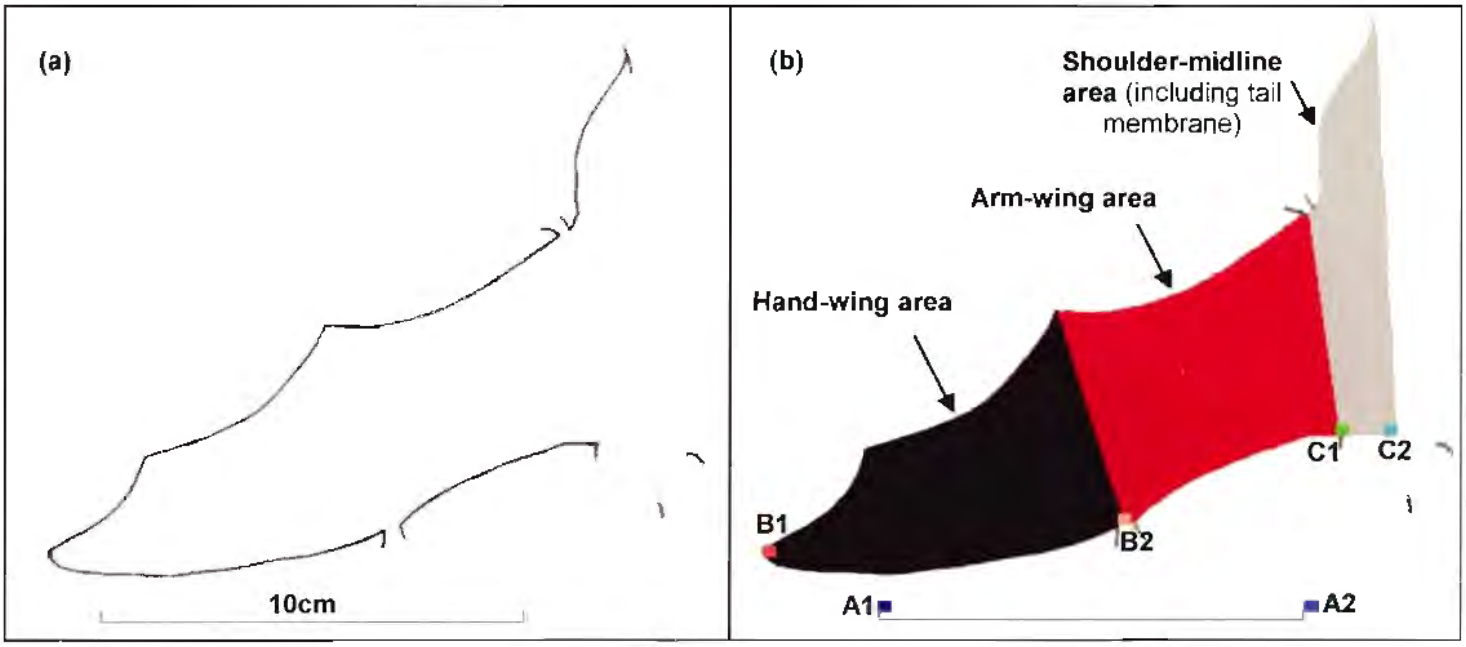

Figure 6.2. (a) Hand-drawn wing tracing of the extended right wing of an *M. schreibersii.*
(b) The same wing tracing after processing in *Paint Shop Pro* for calculation of area and linear measurements in *BatWing*.

6.2.3. Testing the accuracy and precision of *BatWing*

One photograph was separately processed ten times in *Paint Shop Pro*, as described above, to determine the precision of analysing digital images of wings with *BatWing*. Linear and area measurements were determined for each replicate using *BatWing*. The coefficient of variation was 0.008 for areas, and 0.005 for linear measurements.

Area measurements obtained with *BatWing* were compared to alternative, more conventional methods as follows. Ten photocopies were made of a printed version of the photograph described above. Areas were determined by cutting out the individual wing areas, weighing them, and comparing the weights to that of a 10 cm x 10 cm square cut from similar paper. Mean total wing areas for the weighing method and for *BatWing* were 146.52 ± 1.11 cm^2 and 146.19 ± 1.14 cm^2 respectively. These were not significantly different ($t = -0.642$, degrees of freedom (DF) = 18, $p = 0.53$), as indicated by a two-tailed t-test (Zar 1984).

The accuracy of linear (wingspan) measurements obtained by *BatWing* was estimated as follows. Wingspans were measured with a ruler on eleven hand-drawn wing tracings. These measurements were compared to those obtained from digital photographs of the same eleven bats, analysed with *BatWing*. The mean for both methods was 30.5 cm, with variances of 3.3 and 3.1 for the *BatWing* and ruler methods respectively, which did not differ significantly ($t = 0.785$, DF = 14, $p = 0.44$).

correlation between each variable and each principal component is given by a factor loading. These factor loadings give the coordinates at which the variables should be plotted on the principal component axes (Kleinbaum *et al.* 1988). The magnitude of the differences between the variables is therefore reflected by their placement in principal components morphological space (Findley & Wilson 1982), and the variance explained by each principal component is given as an eigenvalue (Kleinbaum *et al.* 1988). Similarly, component scores are obtained for each individual bat included in the analysis. These scores give the coordinates at which each of these individuals should be plotted on the axes.

Due to the large number of bats (between ten and 40) from each colony, principal components plots showing component scores of individual bats from all colonies were unclear. To clarify these plots, the mean component score and its standard deviation were determined from the individual bats from each colony, and these mean points and standard error bars were plotted instead. This gave a clearer picture of how the colonies were positioned in principal components morphological space. Juvenile *M. schreibersii* were not included in the principal components analyses or subsequent analysis of variance (see below).

iii. Analysis of Variance

Principal components analysis merely reflects trends of variation between populations (Norberg & Rayner 1987), and the differences between variables that load on opposite extremes of one or both of the principal component axes must be quantified. Accordingly, those variables that were identified by principal components analysis as likely to differ among colonies were tested by one-way analysis of variance (ANOVA). Variables that were normally distributed and had equal variances across the colonies were tested with a standard one-way ANOVA, while those that deviated from normality and/or had unequal variances were tested with a Kruskal-Wallis test, a non-parametric one-way ANOVA (Zar 1984). These ANOVA calculations were performed with the assistance of the computer software package *Minitab* (Minitab Inc. 2000).

If the ANOVA tests indicated a significant difference among colonies for any particular variable, a Dunn's test (Zar 1984) was performed, to assess which variables were responsible for the difference. Dunn's test is a non-parametric multiple comparison test, which allows comparisons between populations with different sample sizes and unequal variances (Zar 1984). It was used to perform pairwise comparisons between the colonies to determine exactly which colonies differed significantly from each other.

iv. Multifactor Analysis of Variance

Multifactor ANOVA (MANOVA) was performed using *Minitab* (Minitab Inc. 2000) to determine if any significant difference in morphology existed between males and females within the different colonies. These tests simultaneously examined the effects of both sex and colony on the mean of each morphological variable (Zar 1984).

6.3. RESULTS

6.3.1. Descriptive statistics

The Kolmogorov-Smirnov tests indicated that wing area, wingspan and aspect ratio of *M. schreibersii* were normally distributed (Table 6.1). However, the distributions of mass, forearm and wing loading differed significantly from normal. With the exception of wing area and forearm length, none of the variables had equal variances across the colonies. Mean, standard deviation and range for each morphological variable for all *M. schreibersii* for which wing tracings were

taken are given in Table 6.1. The same statistics are given for each colony in Table 6.2, and for each sex within each colony in Table 6.3. Descriptive statistics for mass for all *M. schreibersii*, and for a subset which excludes females known to be pregnant (n = 32), are given in Table 6.1.

Table 6.1. Descriptive statistics for morphological variables of 286 adult *Miniopterus schreibersii* in South Africa. The Kolmogorov-Smirnov D statistics for the normality test and their associated probability (p) values are given for those variables whose distributions deviated from normality. Bartlett's or Levene's test statistics (TS) and associated p values are given for variables that had unequal variances.

	Wing area (cm^2)	**Wingspan (cm)**	**Mass (g) [excluding 32 pregnant females]**	**Forearm length (cm)**	**Wing loading (Nm^{-2})**	**Aspect ratio**
Mean ± SD	137.72 ± 12.77	29.95 ± 1.27	11.94 ± 1.70 [11.54 ± 1.31]	4.59 ± 0.14	8.57 ± 1.42	6.55 ± 0.57
Range	91.14 - 175.5	26.26 - 33.38	9.0 - 17.5 [9.0 - 15.0]	4.2 - 5.8	5.81- 12.87	5.11 - 8.03
Variance	162.07	1.61	2.89 [1.72]	$2.1x10^{-2}$	2.02	0.32
Normal distribution?	Yes	Yes	No (D = 0.056; p < 0.04)	No (D= 0.141; p < 0.01)	No (D = 0.061; p < 0.01)	Yes
Equal variances across colonies?	Yes	No (Bartlett's TS = 26.72; p = 0.002)	No (Levene's TS = 5.35; p < 0.0001)	Yes	No (Levene's TS = 2.47; p = 0.01)	No (Levene's TS = 2.01; p = 0.038)

Table 6.3. Mean (± standard deviation; SD) and range of morphological variables for male (♂) and female (♀) *M. schreibersii* in each colony. No wing traces were made for females from Die Hel. Please refer to Table 6.2 for descriptive statistics for this colony.

Colony	Wing area (cm^2)		Wingspan (cm)		Mass (g)		Forearm (cm)		Wing loading (Nm^{-2})		Aspect ratio	
De Hoop	♂ (n=23)	♀ (n=15)	♂ (n=23)	♀ (n=15)	♂ (n=23)	♀ (n=15)	♂ (n=23)	♀ (n=15)	♂ (n=23)	♀ (n=15)	♂ (n=23)	♀ (n=15)
Mean	145.40	137.86	30.29	29.59	12.40	12.44	4.66	4.59	8.39	8.86	6.33	6.36
± SD	± 11.48	± 7.49	± 1.51	± 1.54	± 1.19	± 1.46	± 0.27	± 0.16	± 0.79	± 1.07	± 0.47	± 0.48
Range	122.50-160.84	123.62-153.22	27.24-33.12	28.04-32.64	10.0 -15.0	10.0 -16.0	4.46 – 5.80	4.20 - 4.83	6.90 - 10.01	7.32 -12.01	5.38-7.19	5.41-7.13
Grahams-town	♂ (n=18)	♀ (n=16)	♂ (n=18)	♀ (n=16)	♂ (n=18)	♀ (n=16)	♂ (n=18)	♀ (n=16)	♂ (n=18)	♀ (n=16)	♂ (n=18)	♀ (n=16)
Mean	145.95	137.38	30.07	29.04	10.55	10.12	4.57	4.60	7.13	7.32	6.21	6.16
± SD	± 13.86	± 10.76	± 1.84	± 1.12	± 0.76	± 0.72	± 0.08	± 0.05	± 0.92	± 0.84	± 0.46	± 0.40
Range	126.74-175.5	114.57-152.52	26.40-33.16	26.26-30.72	9.5 - 12.0	9.0 - 11.5	4.45 - 4.72	4.49 - 4.68	5.87 - 8.73	6.21- 9.42	5.39 - 7.15	5.67 - 6.86
Jozini Dam	♂ (n=9)	♀ (n=20)	♂ (n=9)	♀ (n=20)	♂ (n=9)	♀ (n=20)	♂ (n=9)	♀ (n=20)	♂ (n=9)	♀ (n=20)	♂ (n=9)	♀ (n=20)
Mean	118.70	125.72	29.29	29.82	10.78	11.00	4.64	4.60	9.04	8.60	7.29	7.09
± SD	± 14.44	± 6.91	± 1.35	± 0.78	± 0.67	± 0.97	± 0.15	± 0.08	± 1.38	± 0.78	± 0.57	± 0.33
Range	91.14-138.2	108.66-136.46	27.06-31.54	28.66-31.44	10.0 - 12.0	10.0 - 13.0	4.49 - 4.99	4.42 - 4.73	7.79 - 11.84	7.19 - 9.96	6.27 - 8.03	6.43 - 7.66
Koegelbeen	♂ (n=20)	♀ (n=20)	♂ (n=20)	♀ (n=20)	♂ (n=20)	♀ (n=20)	♂ (n=20)	♀ (n=20)	♂ (n=20)	♀ (n=20)	♂ (n=20)	♀ (n=20)
Mean	136.97	139.52	30.19	30.48	12.88	15.28	4.60	4.61	9.27	10.76	6.67	6.68
± SD	± 9.52	± 10.68	± 1.00	± 1.10	± 0.87	± 1.38	± 0.05	± 0.08	± 0.92	± 0.87	± 0.42	± 0.33
Range	116.88-155.52	108.1-155.54	27.96-31.74	28.26-33.12	11.0 - 14.0	12.0 -17.5	4.46 - 4.69	4.46-4.74	7.70 - 10.78	8.80-12.25	5.72 - 7.32	6.15-7.39
Maitland Mines	♂ (n=16)	♀ (n=20)	♂ (n=16)	♀ (n=20)	♂ (n=16)	♀ (n=20)	♂ (n=16)	♀ (n=20)	♂ (n=16)	♀ (n=20)	♂ (n=16)	♀ (n=20)
Mean	141.87	151.12	28.81	30.44	11.38	10.88	4.55	4.59	7.88	7.07	6.27	6.15
± SD	± 9.44	± 10.33	± 1.07	± 1.14	± 0.76	± 1.06	± 0.10	± 0.07	± 0.57	± 0.67	± 0.26	± 0.45
Range	128.90-165.46	132.54-172.50	28.24-31.86	27.82-32.42	10.0 - 12.5	9.0 - 13.5	4.34 - 4.73	4.47 - 4.74	6.65 - 8.56	6.18 - 8.67	5.90 - 6.86	5.26 - 6.89

Table 6.3 cont. Mean (± standard deviation; SD) and range of morphological variables for male (♂) and female (♀) *M. schreibersii* in each colony.

Colony	Wing area (cm^2)		Wingspan (cm)		Mass (g)		Forearm (cm)		Wing loading (Nm^{-2})		Aspect ratio	
Peppercorn	♂ (n=9)	♀ (n=10)	♂ (n=9)	♀ (n=10)	♂ (n=9)	♀ (n=10)	♂ (n=9)	♀ (n=10)	♂ (n=9)	♀ (n=10)	♂ (n=9)	♀ (n=10)
Mean	128.72	126.10	30.00	30.16	12.22	13.65	4.56	4.51	9.34	10.67	7.02	7.24
± SD	± 8.40	± 10.36	± 0.77	± 0.72	± 1.00	± 1.75	± 0.11	± 0.06	± 0.72	± 1.57	± 0.47	± 0.47
Range	119.74-147.72	114.04-147.68	29.02 - 31.16	29.40 - 31.40	10.5 - 13.5	10.0 - 15.0	4.39 - 4.74	4.45 - 4.63	8.13 - 10.32	7.98 - 12.87	6.04 - 7.76	6.59 - 8.02
Shongweni Dam	♂ (n=15)	♀ (n=14)	♂ (n=15)	♀ (n=14)	♂ (n=15)	♀ (n=14)	♂ (n=15)	♀ (n=14)	♂ (n=15)	♀ (n=14)	♂ (n=15)	♀ (n=14)
Mean	130.63	132.25	29.99	30.61	11.73	12.46	4.56	4.60	8.86	9.31	6.90	7.11
± SD	± 9.34	± 11.66	± 0.92	± 1.19	± 0.98	± 1.15	± 0.08	± 0.10	± 1.05	± 1.16	± 0.34	± 0.32
Range	106.34-141.44	117.36-150.24	28.52 - 31.36	28.92 - 32.82	10.0 - 13.5	11.0 - 15.0	4.44 - 4.73	4.46 - 4.77	7.63 - 11.99	7.84 - 12.13	6.33 - 7.68	6.54 - 7.66
Steenkamps-kraal	♂ (n=12)	♀ (n=8)	♂ (n=12)	♀ (n=8)	♂ (n=12)	♀ (n=8)	♂ (n=12)	♀ (n=8)	♂ (n=12)	♀ (n=8)	♂ (n=12)	♀ (n=8)
Mean	139.74	141.09	29.79	29.51	10.29	9.63	4.62	4.65	7.25	6.73	6.37	6.19
± SD	± 13.53	± 10.47	± 1.42	± 0.82	± 0.81	± 0.52	± 0.07	± 0.05	± 0.48	± 0.70	± 0.19	± 0.28
Range	119.92-169.52	118.98-151.84	27.58 - 32.66	28.50 - 30.50	9.0 - 12.0	9.0 - 10.0	4.52 - 4.75	4.59 - 4.76	6.47 - 8.01	5.81 - 8.25	6.12 - 6.65	5.97 - 6.83
Sudwala	♂ (n=17)	♀ (n=14)	♂ (n=17)	♀ (n=14)	♂ (n=17)	♀ (n=14)	♂ (n=17)	♀ (n=14)	♂ (n=17)	♀ (n=14)	♂ (n=17)	♀ (n=14)
Mean	136.70	138.88	30.35	29.79	12.06	13.39	4.51	4.66	8.68	9.56	6.75	6.43
± SD	± 8.32	± 14.20	± 1.38	± 1.18	± 1.12	± 0.96	± 0.09	± 0.38	± 0.97	± 1.26	± 0.45	± 0.50
Range	122.56-158.02	115.58-156.86	27.9 - 33.38	27.96 - 31.56	10.5 - 14.0	11.0 - 14.5	4.35 - 4.63	4.41 - 5.55	7.14 - 10.41	7.42 - 11.88	5.75 - 7.57	5.77 - 7.74

6.3.2. Principal components analysis incorporating all variables

Principal components analysis was initially performed on all morphological variables (wing area, wingspan, mass, forearm length, wing loading and aspect ratio). The first two principal components (PC1 and PC2) accounted for 64.2% of the variance in the data (Table 6.4). Wing loading and aspect ratio, which are positively correlated with each other (Findley *et al.* 1972), loaded low on both PC1 and PC2 (Figure 6.3). In contrast, wing area loaded high on both principal components. Wingspan and forearm both fell towards the centre of PC1, but loaded high and low on PC2, respectively. Mass loaded low on PC1 but towards the centre of PC2. Varimax rotation did not alter the distribution of the variables appreciably.

Table 6.4. Eigenvalues for principal components analysis on all morphological variables.

Principal Component	Eigenvalue	% Total variance	Cumulative percentage
1	2.272	37.858	37.858
2	1.578	26.294	64.153

The principal components plot of mean component scores for each colony (Figure 6.4) shows considerable overlap between the colonies, as expected when dealing with a single species. However, there is a tendency for the north-eastern colonies (Shongweni Dam, Jozini Dam, Sudwala and Peppercorn) to group together in the negative half of PC1, and for the southern and western colonies (De Hoop, Die Hel, Maitland Mines, Grahamstown and Steenkampskraal) to group together within the positive half of PC1. This is in general agreement with the patterns

obtained from the genetic analyses (Chapters 4 and 5), in particular the analysis of restriction fragment haplotypes, which also identified two subpopulations (south-west and north-east) of *M. schreibersii* (Chapter 5). However, the genetics results suggest that Grahamstown and Maitland Mines should group more closely with the north-eastern than the southern or western colonies. Furthermore, genetic analysis suggests that Steenkampskraal and Koegelbeen should be positioned close together in principal components space, as they are genetically closely related. This is not the case. Grahamstown and Maitland Mines fall well within the south-western group, while Koegelbeen falls outside of the south-western grouping, and loads low on PC1, in the quadrant diagonally opposite to Steenkampskraal. The plot of variable factor loadings (Figure 6.3) suggests that mass and therefore wing loading might be responsible for the separation of Koegelbeen from Steenkampskraal in principal components space. It may also be responsible for the separation of Grahamstown and Maitland Mines from the north-eastern colonies.

Differences in the time of year that sampling took place are likely to cause differences in mass between colonies, and therefore also influence wing loading (the ratio of mass to wing area). The majority of colonies were sampled during summer (November to January), when insect populations were likely to be abundant and the bats well fed. However, Steenkampskraal was sampled in winter (July), and both Grahamstown and Maitland Mines were sampled in early spring (September; Table 2.1; Chapter 2). Bats typically lose 20 – 30% of their body weight during hibernation (Bernard 1980; Norton & van der Merwe 1978), and bats from Steenkampskraal had the lowest mean mass of all the colonies (10.33 g ± 0.77; Table 6.2), followed by Grahamstown (10.35 g ± 0.76), Jozini Dam (10.93 g ± 0.88) and Maitland Mines (11.10 g ± 0.96). This is probably because Steenkampskraal bats were sampled during their winter hibernation, and Grahamstown and Maitland Mines shortly thereafter, before they had time to replenish their fat reserves. The reason for the low mean mass of Jozini Dam bats is uncertain, as these bats were sampled in November. However, they were captured during a period of two weeks of continuous, heavy rain, which may have affected their foraging success, as insect abundance is likely to be reduced during heavy rain.

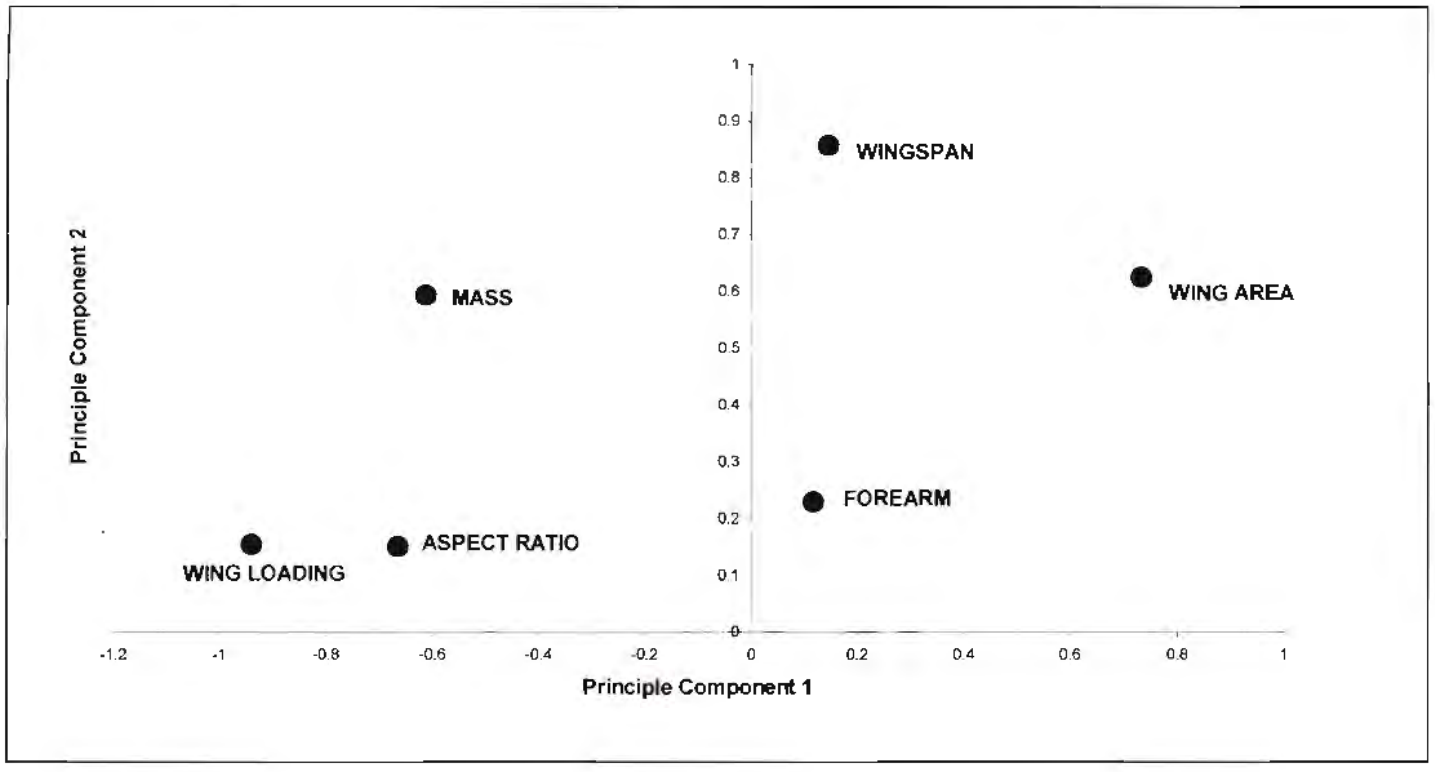

Figure 6.3. Principal components plot of all morphological variable factor loadings.

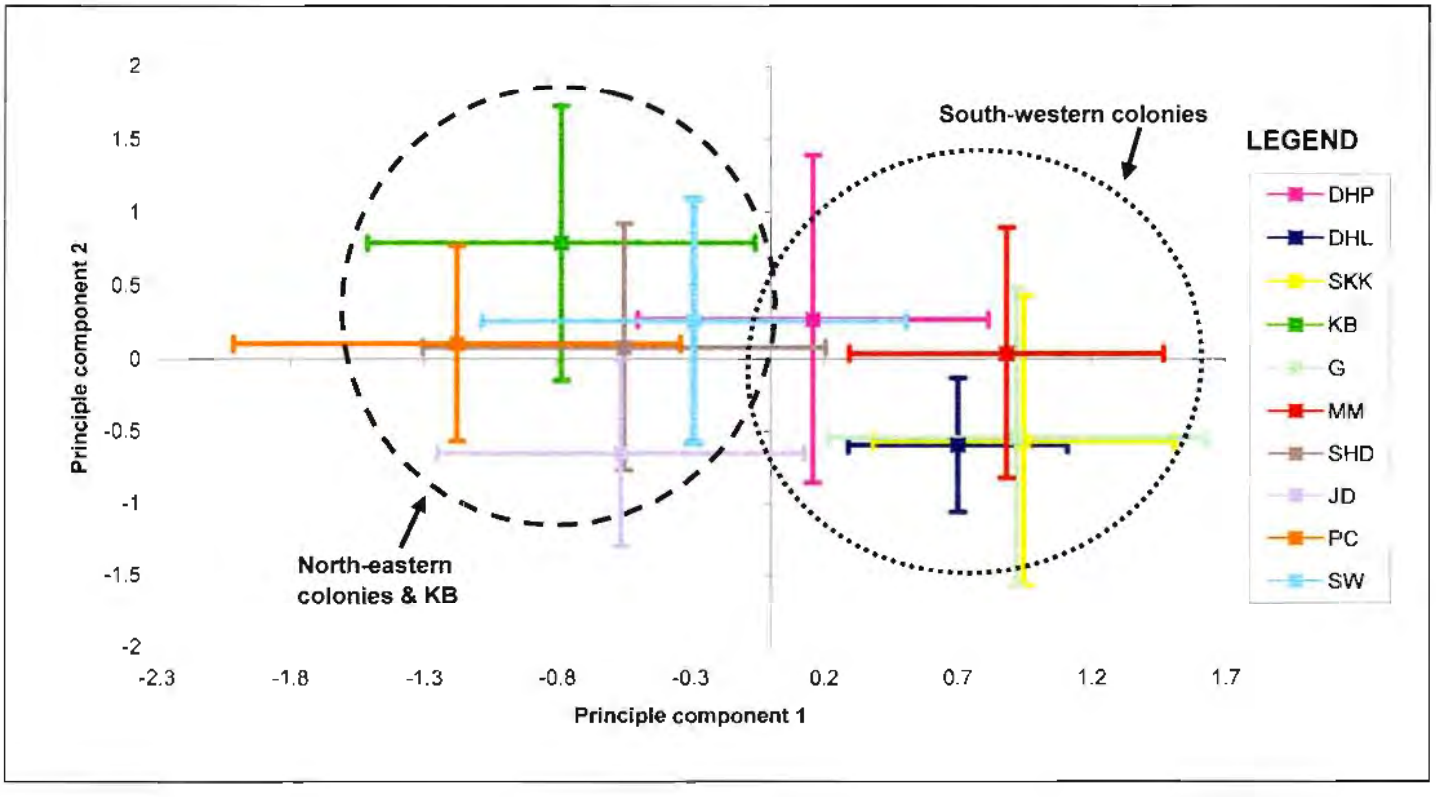

Figure 6.4. Principal components plot of mean component scores and standard errors for *M. schreibersii* colonies, incorporating all morphological variables.

Names of colonies are abbreviated as follows: DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown, MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala.

The lower mass and wing loading for these colonies (Steenkampskraal, Grahamstown, Maitland Mines and Jozini Dam) are probably the result of these bats being sampled when they were, for one reason or another, unable to forage. This may have distorted the principal components analysis. Both mass and wing loading were therefore eliminated from the data set, and the principal components analysis repeated excluding these variables. Variables were eliminated rather than colonies because the purpose was to determine whether the morphology grouped the colonies in the same way that genetic analyses did.

6.3.3. Principal components analysis excluding mass and wing loading

When mass and wing loading were excluded from the principal components analysis, the first two principal components accounted for 75.85% of the variance in the data (Table 6.5). Aspect ratio loaded high on PC1 and high on PC2 (Figure 6.5), while the other variables all loaded low on PC1. Once again, Varimax rotation did not alter the distributions appreciably.

Table 6.5. Eigenvalues for principal components analysis on morphological variables, excluding mass and wing loading.

Principal component	Eigenvalue	% Total variance	Cumulative percentage
1	1.658	41.456	41.456
2	1.376	34.398	75.854

The plot of mean component scores and standard errors for each colony (Figure 6.6) indicates a distribution pattern for the colonies generally in accordance with that suggested by the genetics results (Chapters 4 and 5), except that Grahamstown and Maitland Mines still group more closely with the south-western group than with the north-eastern colonies. Two major geographical groupings of the colonies can be identified, separated by their relative positions along PC1. A north-eastern group, consisting of Shongweni Dam, Jozini Dam, Peppercorn and Sudwala, loads on the positive half of PC1, while a south-western group, consisting of De Hoop, Die Hel, Koegelbeen, Steenkampskraal, Grahamstown and Maitland Mines, loads on the negative half of PC1. Like Steenkampskraal, Koegelbeen now also loads in the negative half of PC1. The standard error bars for each colony are large, resulting in considerable overlap between the colonies, but this is to be expected as the bats are of the same species, and therefore complete separation of colonies would be unlikely.

Relating the plot of variable factor loadings (Figure 6.5) to that of mean component scores for the colonies (Figure 6.6) indicates that aspect ratio is the primary morphological variable causing the separation of the two major geographical groups along PC1. Indeed, the colonies did differ significantly in aspect ratio (Kruskal-Wallis test: $H = 141.16$, $DF = 9$, $p < 0.0001$). However, because wing loading was eliminated from the analysis, one cannot exclude the possibility that wing loading might also contribute substantially to morphological variation within this species.

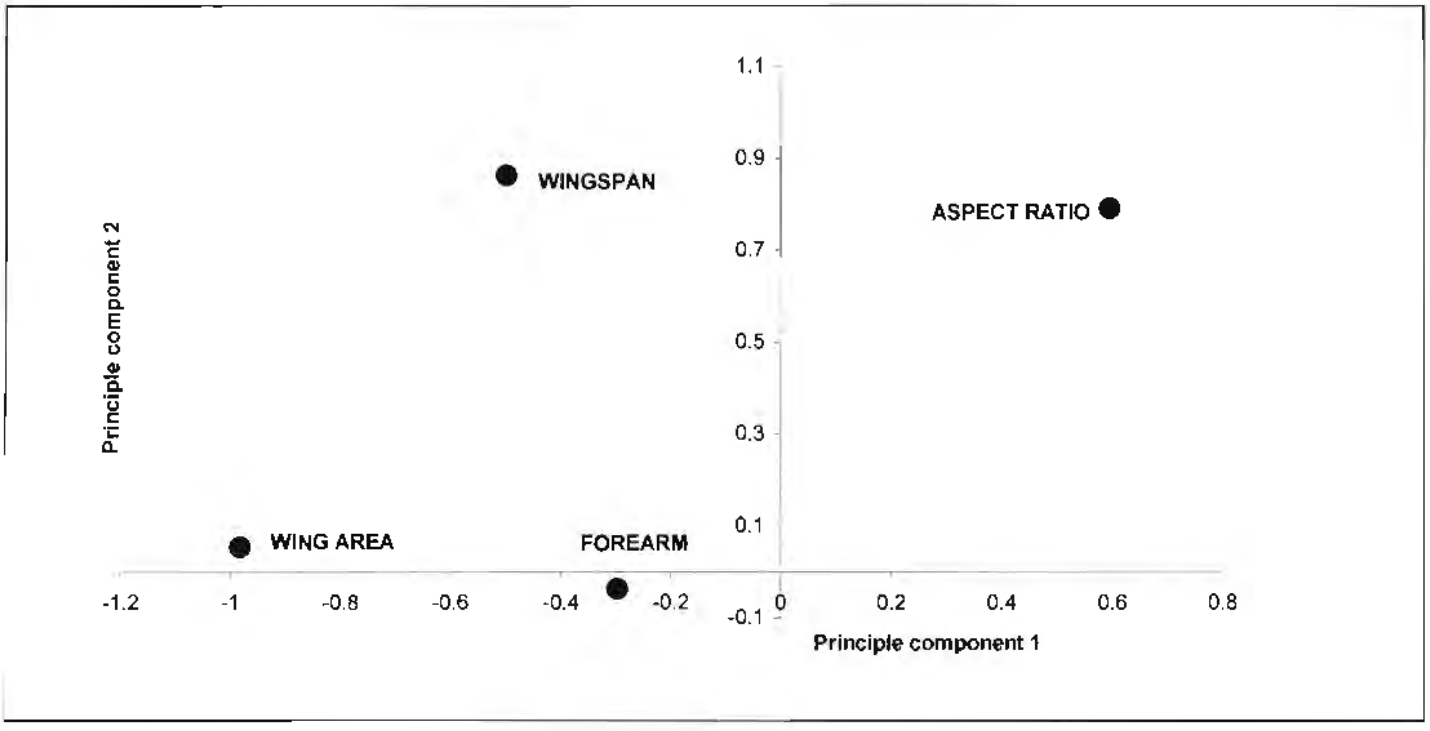

Figure 6.5. Principal components plot of morphological variable factor loadings, excluding mass and wing loading.

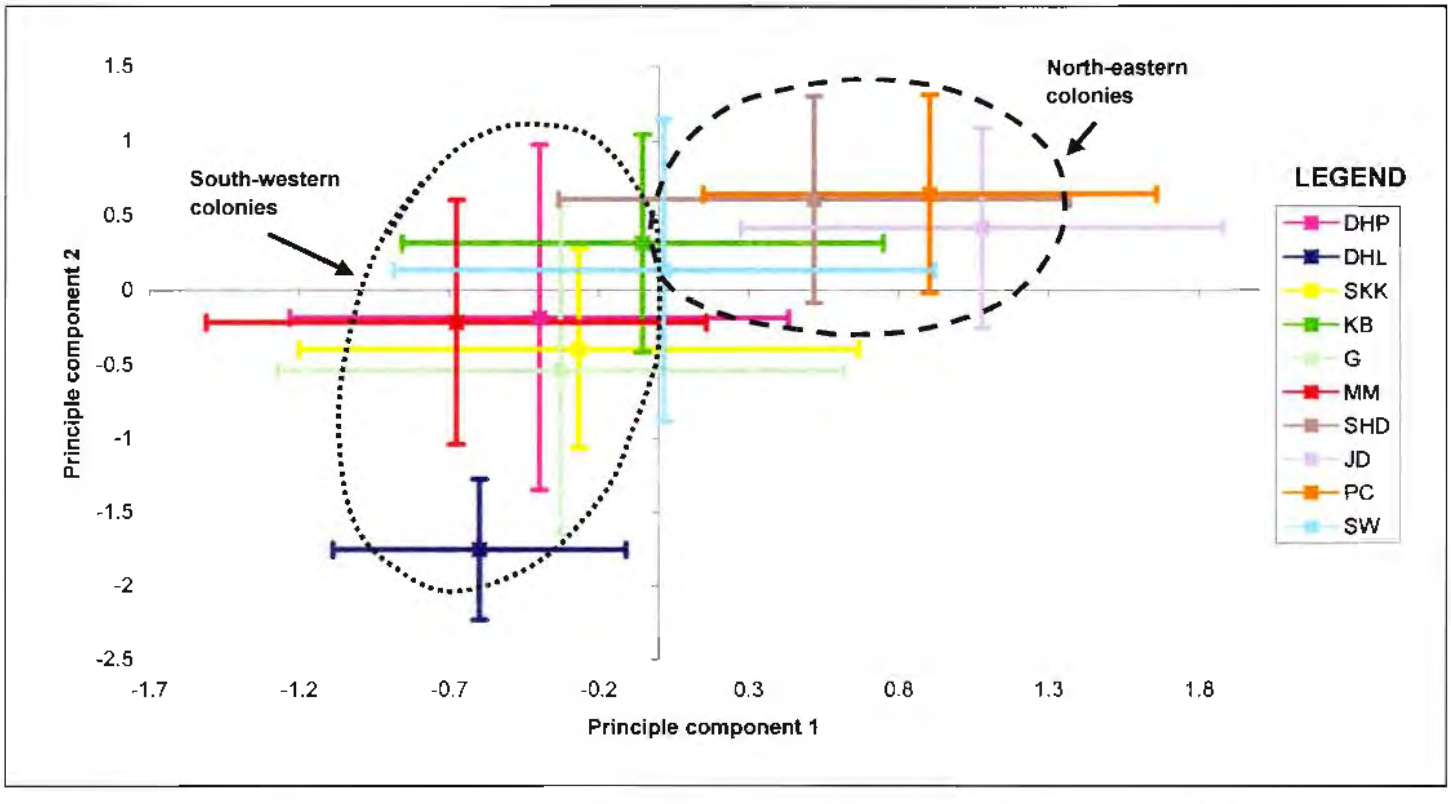

Figure 6.6. Principal components plot of mean component scores and standard errors for each *M. schreibersii* colony, excluding mass and wing loading.

Names of colonies are abbreviated as follows: DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown, MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala.

The aspect ratio data were reorganised into a north-eastern group, consisting of Shongweni Dam, Jozini Dam, Peppercorn and Sudwala (n = 108), and a south-western group, including De Hoop, Die Hel, Steenkampskraal, Koegelbeen, Grahamstown and Maitland Mines (n = 178). A two-tailed t-test confirmed that the north-eastern and south-western geographical groups differ significantly in aspect ratio ($t = -11.03$, $DF=217$, $p < 0.0001$), but the pattern of colony differentiation resulting from multiple pairwise comparisons (Dunn's method; Zar 1984) was not as clear cut (Table 6.6). There is a general trend for the colonies to fall into the two geographical groups, but Koegelbeen and Sudwala do not conform to this pattern. De Hoop, Die Hel, Steenkampskraal, Grahamstown and Maitland Mines do not differ significantly from each other in terms of aspect ratio, and can be grouped together to form the south-western group. In general, aspect ratio of colonies in this group differs significantly from that of Shongweni Dam, Jozini Dam and Peppercorn, which form the north-eastern group. None of the colonies in the north-eastern group can be distinguished from each other on the basis of aspect ratio.

Although the principal components plot of colony means (Figure 6.6) suggests that Koegelbeen can be grouped with the south-western colonies, in accordance with the genetics results, the analysis of variance indicates that the mean aspect ratio of bats in this colony differs significantly from those at Die Hel, Grahamstown and Maitland Mines (Table 6.6, Figure 6.7). The aspect ratio of Koegelbeen bats, however, does not differ from that of colonies in the north-eastern group. Bats from Sudwala do not differ in aspect ratio from any colonies in the north-eastern group, as would be expected from the genetics results, but neither do these bats differ significantly in aspect ratio from any of the south-western colonies, other than Die Hel. Hence the intermediate positioning of Sudwala between the bulk of the colonies making up the south-western group and those making up the north-eastern group in the principal componentsanalysis (Figure 6.6).

No significant difference in any of the morphological variables was found between males and females within the colonies. The sexes differ significantly in mass only when pregnant females are included in the data set, as would be expected. This indicates that there is no sexual dimorphism within this species, and therefore any morphological differences between the colonies cannot be ascribed to varying sex ratios in the samples.

Table 6.6. Pairwise comparisons of aspect ratio between *M. schreibersii* colonies (Dunn's method); critical $Q_{0.01;10}$ = 3.692; NS, not significant at p = 0.01.
Names of colonies are abbreviated as follows:
DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown, MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala.

	DHL	SKK	KB	G	MM	SHD	JD	PC	SW
DHP	NS	NS	NS	NS	NS	Q=5.005	Q=5.689	Q=4.917	NS
DHL	_	NS	Q=5.154	NS	NS	Q=6.596	Q=7.056	Q=6.569	Q=4.499
SKK		_	NS	NS	NS	Q=4.865	Q=5.445	Q=4.874	NS
KB			_	Q=3.953	Q=4.047	NS	NS	NS	NS
G				_	NS	Q=6.009	Q=6.695	Q=5.817	NS
MM					_	Q=6.118	Q=6.793	Q=5.903	NS
SHD						_	NS	NS	NS
JD							_	NS	NS
PC								_	NS

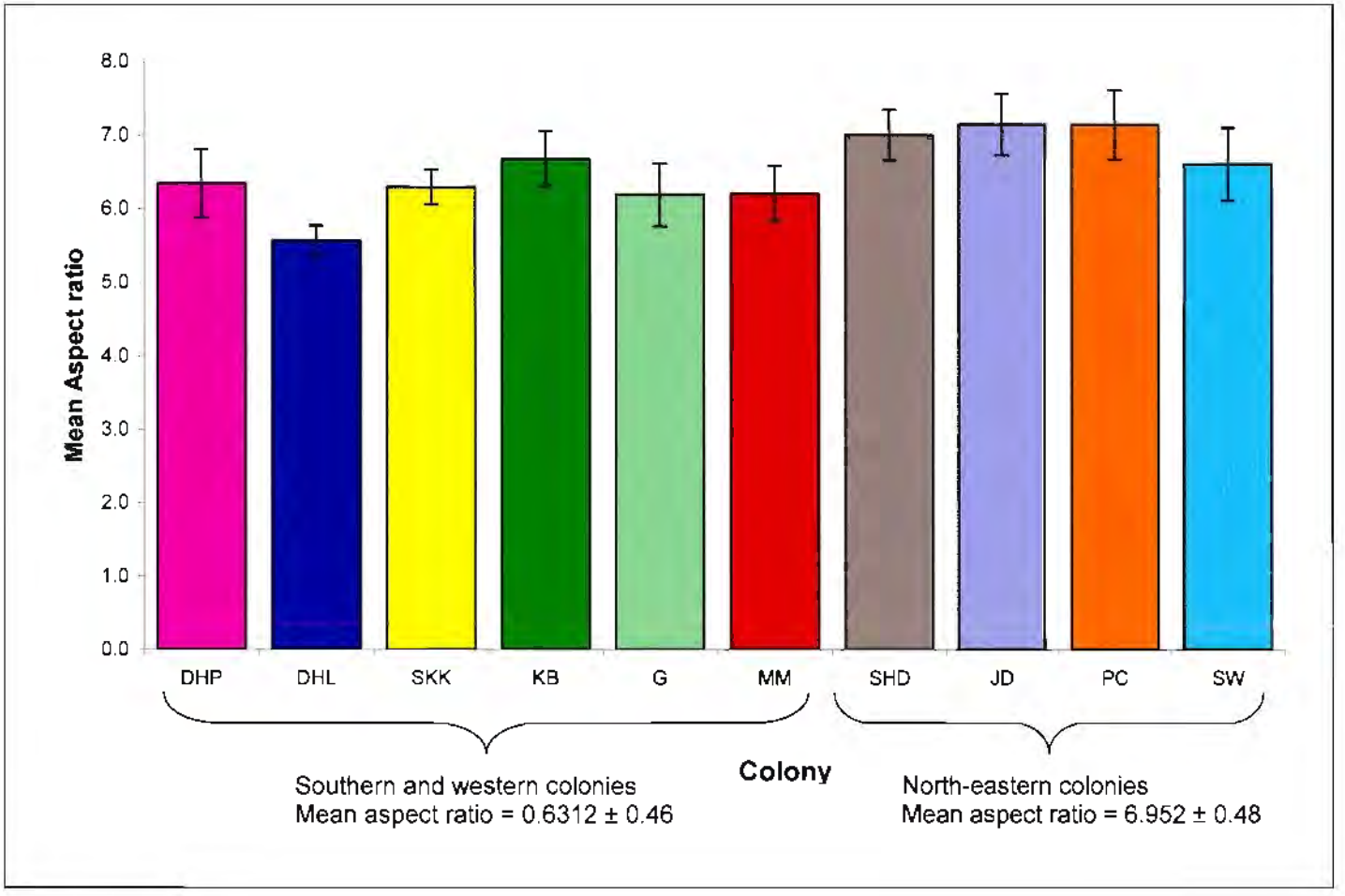

Figure 6.7. Mean aspect ratio of *M. schreibersii* from different colonies. Colonies are colour-coded as for Figures 6.4 and 6.6. Names of colonies are abbreviated as follows:

DHP = De Hoop, DHL = Die Hel, SKK = Steenkampskraal, KB = Koegelbeen, G = Grahamstown, MM = Maitland Mines, SHD = Shongweni Dam, JD = Jozini Dam, PC = Peppercorn, SW = Sudwala.

6.4. DISCUSSION

6.4.1. Intraspecific morphological variation in *M. schreibersii*

Two major geographical groupings of colonies (south-west and north-east) are discernable based on wing morphology. This is in broad agreement with the three subpopulations (southern, western and north-eastern) identified by the microsatellite and mtDNA sequence data (Chapters 4 and 5 respectively). The morphological data collapses the southern and western subpopulations identified by the genetic data into one group, a south-western group. This is similar to that identified by the relatively low resolution mtDNA restriction fragment analysis (Chapter 5), except that Grahamstown and Maitland Mine are now included in this group rather than the north-eastern subpopulation. Complete agreement between morphological and genetic data is unlikely because morphological characters are influenced by natural selection, and are likely to evolve more slowly than neutral genetic markers. Morphological data are therefore unlikely to provide as fine a resolution of population differentiation as genetic data. In general, however, the variation in wing morphology among the different colonies supports the pattern of population differentiation indicated by the genetic markers.

Both the principal components analysis and ANOVA results indicate that there is considerable morphological variability within this species. Very little is currently known about the environmental conditions within which these bats operate and to which they have adapted. It is therefore not possible at this stage to explain fully the morphological differences between colonies. However a possible explanation may relate to the migratory characteristics of *M. schreibersii*.

6.4.2. Morphological adaptation for long-distance flight

During flight, the curvature or camber of the wing causes greater air pressure to build up below the wing than above it, generating lift. Some of the air under the wing spills upward around the wing tip, creating turbulence, which increases the drag on the wing and reduces the lift. Long, narrow wings that have high aspect ratio lose less lift as a result of this turbulence than do short, broad wings (Vaughan 1970). Therefore, bats that are adapted for long distance flight such as migration, typically have high aspect ratios, which results in faster flight that is energetically efficient, although less manoeuvrable (Findley *et al.* 1972). These species are also frequently large and have high wing loading, which is not essential for endurance, but facilitates rapid flight, thus reducing travel time (Norberg & Rayner 1987). This is because high wing loading is associated with low wing area, which in turn is associated with reduced drag (Vaughan 1970). Free-tailed bats of the genus *Tadarida*, for example, migrate thousands of kilometres between summer and winter roosts, and have the highest aspect ratios recorded for any bat species (up to 14.3), in conjunction with wing loadings of up to 20.2 Nm^{-2} (Norberg & Rayner 1987). Other long-distance fliers (*e.g. Lasiurus sp.*, *Miniopterus australis* and *M. inflatus*) typically have slightly lower aspect ratios (6.7 - 8.7) and more average wing loadings (6.2 - 14.4 Nm^{-2}; Norberg & Rayner 1987). South African *M. schreibersii* wing parameters fall within this range for migratory bats, particularly in the case of individuals from the north-eastern subpopulation (Tables 6.1 and 6.2).

6.4.3. Wing morphology of the north-eastern colonies

The bats within the north-eastern subpopulation are known to migrate seasonally hundreds of kilometres between maternity caves and hibernacula (Section 1.3, Chapter 1; van der Merwe 1973b, 1973c, 1975). During the migration to the maternity roosts, the majority of adult females are likely to carry the additional weight burden of pregnancy, as implantation occurs before they leave the hibernacula (Strahan 1998). Therefore it is essential that flight be as energetically efficient as possible and any intrinsic flight costs be minimised as much as possible. High aspect ratio and wing loading would thus be an advantage for these bats, particularly for females, although no significant difference in aspect ratio or wing loading was found between males and females. In general however, this group of bats does have higher mean aspect ratio and wing loading than other colonies (Table 6.2, Figure 6.7).

6.4.4. Wing morphology of the Western Cape and Eastern Cape bats

The migration patterns of *M. schreibersii* in the Western and Eastern Cape provinces of South Africa (Figure 1.4; Chapter 1) are less well known. However, the genetics results (Chapters 4 and 5) suggest that these bats may not migrate as long distances as the north-eastern subpopulation, especially in the case of individuals from De Hoop and Die Hel. Both the nuclear and mitochondrial DNA (mtDNA) markers suggest that these bats may remain within the Western Cape fynbos biome, to which they may have become locally adapted (discussed in Chapter 8; Figure 8.1). When they migrate between summer and winter roosts, they may do so over relatively shorter distances than the north-eastern bats. High aspect ratio and wing loading may therefore not be as great a benefit to them as it is for the north-eastern subpopulation.

Rather, it may be more of an advantage to have lower aspect ratio, permitting foraging within a variety of habitats (Jacobs 1999). Although bats that are adapted to foraging in open habitats are restricted to these areas by their morphology, those that are able to fly at lower speeds and are more manoeuvrable have access to both open and cluttered environments (Fenton 1990; Jacobs 1999).

Jacobs (1999) observed *M. schreibersii* foraging in both open and cluttered habitats in the vicinity of De Hoop Guano Cave and found that those bats foraging in dense vegetation tended to have lower aspect ratios. This intraspecific variation in *M. schreibersii* morphology is supported by the findings of this study, where aspect ratio of De Hoop bats ranged from 5.38 to 7.19. Selective pressures will tend to minimise energy expenditure and maximise energy intake, altering wing design accordingly until a particular style of flight is achieved that maximises overall fitness for the species concerned (Aldridge & Rautenbach 1987; Norberg & Rayner 1987). Norberg & Rayner (1987) suggest that bat evolution might represent a compromise between different selective pressures, without optimising any one factor in particular, thus avoiding excessive specialisation. This may be the case for the bats at De Hoop and Die Hel, where wing morphology may reflect a compromise between the varying adaptive pressures of foraging habitat and medium- to long-distance migration.

The genetics results (Chapters 4 and 5) suggest that bats in the Eastern Cape (Grahamstown and Maitland Mines) exchange more gene flow with colonies in the north-eastern parts of the country than with those in the southern and western regions. However, Grahamstown and Maitland Mines group closely with the south-western colonies in the principal components analysis of morphology, and their mean aspect ratio is significantly less than that of bats in the north-eastern subpopulation (Table 6.6). This paradox may be due to circumstances similar to those suggested to be operating in the Western Cape. The microsatellite results (Chapter 4) also suggested that while Grahamstown and Maitland Mines bats are very closely related, gene flow between bats from Grahamstown and several other colonies in the north-eastern

subpopulation is partially restricted. This is supported by the morphological findings. The majority of Grahamstown and Maitland Mines bats may not migrate extremely long distances between summer and winter roosts, moving instead mainly between these two sites, which are located ~130 km apart (Table 5.11, Chapter 5). Like the bats at De Hoop and Die Hel, Eastern Cape bats may also have a lower mean aspect ratio because it allows them to utilise a range of foraging habitats. Like the De Hoop bats, these colonies show a wide range in aspect ratio (5.39 - 6.86 and 5.26 - 6.89 for Grahamstown and Maitland Mines respectively; Table 6.2). This intraspecific variation in wing morphology may mean that while many of the Grahamstown and Maitland Mines bats have shorter wingspans and may remain within the Eastern Cape year-round, others are likely to have sufficiently high aspect ratios to permit energetically efficient medium- to long-distance migration, which would allow gene flow to occur between these bats and others in the north-eastern subpopulation.

6.4.5. Morphology of the Northern Cape populations

The wing morphology of the bats in the Northern Cape province (Figures 1.4 and 2.1, Chapters 1 and 2) appears anomalous, particularly in the case of the Koegelbeen population. Although genetically the Koegelbeen bats are most similar to the bats in the other southern and western colonies, particularly Steenkampskraal, the analysis of variance on aspect ratio suggests their wing morphology is more similar to that of the north-eastern subpopulation. It is possible that this is because the Koegelbeen bats, like the north-eastern colonies, migrate large distances. The genetics results indicate that bats in the Northern Cape migrate over 500 km between Koegelbeen and Steenkampskraal. Furthermore, the vegetation in this region is sparse and shrub-like, with very few areas with dense vegetation. The bats therefore are likely to forage primarily in open, uncluttered habitats. Their wing morphology may thus reflect both local

adaptation to an uncluttered foraging environment, which permits rapid, less manoeuvrable flight, as well as selective pressure for energetically efficient long-distance migration.

Another possible explanation for the high aspect ratio of Koegelbeen bats relates to prey availability. Koegelbeen is located within the semi-arid succulent Karoo biome (Figure 8.1, Chapter 8), in which insect populations are likely to be relatively low, even during summer. Furthermore, suitable roost sites are probably scarce because the region is flat and has few hills or mountains to provide caves. Both Koegelbeen and Steenkampskraal may thus be the only suitable roosts for hundreds of kilometres. Therefore, bats from these colonies may not only have to migrate seasonally between widely dispersed roosts, but may need to commute long distances each night to and from the cave to find suitable foraging areas. High aspect ratio and wing loading are generally associated with species that fly long distances nightly to locate food sources (Jones *et al.* 1995) because these features minimise both cost of transport and time taken to reach foraging areas (Norberg & Rayner 1987). The ability of bats to operate efficiently in different habitats is determined both by the costs of flight and the potential rate of prey capture (Fenton 1990). Therefore, faster, low-cost flight (due to high aspect ratio and wing loading) may allow these bats to maintain the same rate of energy intake in a habitat with low insect density as bats that forage in better feeding areas and have slower, more energetically costly flight (Aldridge & Rautenbach 1987).

6.4.6. Conclusions

Wing morphology is influenced by a wide range of environmental conditions, rather than by any single factor (Norberg & Rayner 1987). It is therefore possible that the bats within each colony have adapted to local environmental conditions, as well as to the varying requirements of their migratory lifestyle. Their wing morphology may thus reflect a compromise between these

selective pressures. Differences in time of sampling (*e.g.* seasons) are unlikely to cause the patterns of differentiation indicated by the morphological variables because mass and wing loading were eliminated from the principal components analysis and ANOVA tests. Neither are differences in the sex ratio of different samples, because there is no sexual dimorphism in this species. It is more likely therefore that these differences in wing morphology, and in particular aspect ratio, are the result of adaptation to varying environmental factors. This is confirmed by genetic analysis, which also suggests that the South African population of *M. schreibersii* is substructured, and gene flow between the major subpopulations is restricted. Isolation of colonies not only enhances genetic differentiation due to genetic drift, but also is likely to promote morphological differentiation. If bats within any particular geographic region or biome exchange minimal gene flow with individuals from other regions, they will be able to adapt to local environmental conditions and develop morphological characteristics suited to their unique habitat and/or way of life. These adaptations will not be diluted by limited gene flow from different ecological regions, thus promoting further specialisation and adaptation. This is discussed in further detail in Chapter 8.

CHAPTER 7

MINIOPTERUS FRATERCULUS

AIMS: 1. TO IDENTIFY AND EXCLUDE ANY *MINIOPTERUS FRATERCULUS* THAT MAY BE PRESENT IN THE SAMPLE SET.

2. TO COMPARE THE GENETIC AND MORPHOLOGICAL CHARACTERISTICS OF *MINIOPTERUS FRATERCULUS* AND *MINIOPTERUS SCHREIBERSII*.

7.1. RATIONALE

Miniopterus schreibersii and *M. fraterculus* resemble one another closely in overall appearance and pelage colouration, and there is much morphological overlap between them. *Miniopterus fraterculus* has a forearm range of 4.1 – 4.4 cm and mass ranging from 6.0 – 11 g. *Miniopterus schreibersii* has a forearm which ranges from 4.2 – 4.8 cm, and a mass of 9.0 – 17.0 g; Herselman & Norton 1985; Mills & Hess 1997; Taylor 2000). A plot of mass *vs.* forearm length is therefore frequently used to distinguish between the two species (Taylor 2000). Their southern African distribution ranges overlap (Figure 1.3, Chapter 1), and they frequently occupy the same roosts, although *M. fraterculus* generally occurs in lower numbers than *M. schreibersii* (Mills & Hess 1997;

Taylor 2000). Consequently, in the field, *M. fraterculus* individuals may be mistaken for small or juvenile *M. schreibersii*.

The possibility therefore existed that such incorrectly identified individuals were present within the *M. schreibersii* samples collected in this study. This would influence the genetics results (Chapters 4 and 5), and could cast doubt on the validity of any conclusions drawn about population differentiation. For example, if large numbers of *M. fraterculus* were present in the data set, it could be argued that a significant proportion of the population differentiation detected by the genetic markers was due to the presence of another species within certain colonies, rather than due to genuine differences within the *M. schreibersii* population itself. For this reason, an effort was made to identify any potential *M. fraterculus* within the sample set, so that they could be excluded prior to statistical analysis of the genetics (Chapters 4 and 5) and morphological (Chapter 6) data.

One *M. fraterculus* specimen was collected at Shongweni Dam in 1997, and its identity was confirmed by Dr Peter Taylor of the Durban Museum, South Africa, through a series of cranial and post-cranial measurements. The genetic characteristics of this specimen were used as a reference for all subsequent identifications. Additional *M. fraterculus* individuals were identified through a combination of mitochondrial DNA sequencing (Section 7.2) and comparative analysis of microsatellite allele frequencies (Section 7.3).

7.2. MITOCHONDRIAL DNA ANALYSIS

7.2.1. Methods

i. Sequence divergence between *M. fraterculus* and *M. schreibersii*

Approximately 550 bp of mitochondrial DNA (mtDNA) control region of the Shongweni Dam *M. fraterculus* specimen (MF1) were amplified and sequenced, as described in Chapter 5, Sections 5.2.1 and 5.2.2. The sequence was aligned manually with homologous sequences from *M. schreibersii* specimens (Chapter 5), with the assistance of the software package *DAPSA* (Harley 2000). The extent of sequence divergence between species was estimated and corrected for multiple hits by the Kimura two-parameter model (Kimura 1980), using *Arlequin* (Schneider *et al.* 2000), as described in Chapter 5 (Section 5.2.3).

For comparative purposes, homologous regions of mtDNA were also amplified and sequenced from tail membrane biopsies (Chapter 2) taken from a single specimen each of *Rhinolophus capensis* and *R. clivosus* (collected by Dr David Jacobs from De Hoop Guano Cave). These sequences were aligned and corrected for multiple hits as described above. Although *R. capensis* and *R. clivosus* are also similar in size, they exhibit clear differences in their dentition, echolocation call frequencies and distribution ranges (Taylor 2000). The extent of sequence divergence between these well-recognised species could therefore be used as a guideline against which to compare the sequence divergence between *M. schreibersii* and *M.fraterculus.*

ii. Identification of additional *M. fraterculus* specimens by mtDNA sequencing

Four bats were initially suspected of being *M. fraterculus* rather than *M. schreibersii,* based on their low mass and forearm length (Table 7.5, Section 7.4). These included one individual collected from Peppercorn (MF2) and three individuals collected from Sudwala (MF3, MF4 and MF5). Accordingly, mtDNA control region from these individuals was amplified and sequenced, as described above. Sequences were aligned with the known *M. fraterculus* sequence MF1, as well as with homologous *M. schreibersii* sequences (Chapter 5). The extent of sequence divergence between these sequences and those of MF1 and the *M. schreibersii* samples was estimated and corrected for multiple hits by the Kimura two-parameter model (Kimura 1980) using *Arlequin,* as described in Chapter 5 (Section 5.2.3).

7.2.2. Results

i. Sequence divergence between *M. fraterculus* and *M. schreibersii*

The corrected sequence divergence between the mtDNA control region of MF1 and *M. schreibersii* ranged between 0.084 and 0.098 (Table 5.5, Chapter 5). This is significantly higher (Mann-Whitney Rank Sum test: $T = 2925.00$, $p \leq 0.001$) than divergence estimates between sequences of *M. schreibersii* obtained from different South African colonies (0.002 – 0.056; Table 5.5, Chapter 5). The most distinctive difference between mtDNA sequences of the two *Miniopterus* species is the presence of a 13 bp deletion in the *M. fraterculus* sequence, at position 266 of the alignment (Figure 7.1; Figure 5.4, Chapter 5; Appendix 5). The extent of mtDNA control region sequence divergence between *M. fraterculus* and *M. schreibersii* is similar to that found between homologous sequences of *R. capensis* and *R. clivosus* (corrected distance = 0.1343). This supports the current classification of *M. fraterculus* and *M. schreibersii* as separate species, despite their morphological similarities.

ii. Identification of additional *M. fraterculus* specimens by mtDNA sequencing

The mtDNA sequences from individuals MF2 – MF5 aligned closely with that of the known *M. fraterculus* individual, MF1, but differed substantially from all *M. schreibersii* sequences. All had the characteristic 13 bp deletion identified in MF1 (Figure 7.1; Figure 5.5, Chapter 5). The bats MF2 – MF5 were therefore classified as *M. fraterculus*, and were excluded from the *M. schreibersii* data set. Corrected sequence divergence estimates between MF1 and the MF2 – MF5 samples ranged from 0.011 – 0.023, while corrected distances between individuals MF2 – MF5 and *M. schreibersii* varied between 0.083 and 0.102 (Table 5.5, Chapter 5). As discussed above, these *M. fraterculus* – *M. schreibersii* divergence estimates are similar to that found between the two *Rhinolophus* species (0.134). The mean corrected distance between pairs of all five *M. fraterculus* sequences (MF1 – MF5; 0.012 ± 0.006) was significantly lower (Mann-Whitney Rank Sum test: $T = 55.00$, $p < 0.001$) than that between *M. schreibersii* and *M. fraterculus* sequences (0.092 ± 0.005). Similarly, the mean distance between *M. schreibersii* sequences from different colonies (0.026 ± 0.014) was significantly less than that between *M. schreibersii* and *M. fraterculus* ($T = 17775.00$, $p < 0.001$). The extent of sequence divergence between the five *M. fraterculus* sequences was also significantly less than that between individual *M. schreibersii* from different colonies ($T = 352.5$, $p = 0.006$).

A minimum spanning network showing the evolutionary relationships between the sequenced haplotypes (Figure 5.5, Chapter 5) illustrates the extensive divergence between *M. fraterculus* and *M. schreibersii* mtDNA sequences. This network indicates that the *M. fraterculus* sequences are separated from the closest *M. schreibersii* sequence (JD1), by a minimum connection length of 338, while one *M. fraterculus* sequence is separated from another by connection lengths of between five and eight. The neighbour-joining and maximum likelihood trees (Chapter 5, Figures 5.6 and 5.7, respectively) also clearly illustrate the well supported, tight clustering of haplotypes MF2 – MF5 with MF1, rather than with any of the *M. schreibersii* haplotypes. These figures provide further support for the identification of samples MF2 – MF5 as *M. fraterculus*, and for the current classification of *M. schreibersii* and *M. fraterculus* as distinct species.

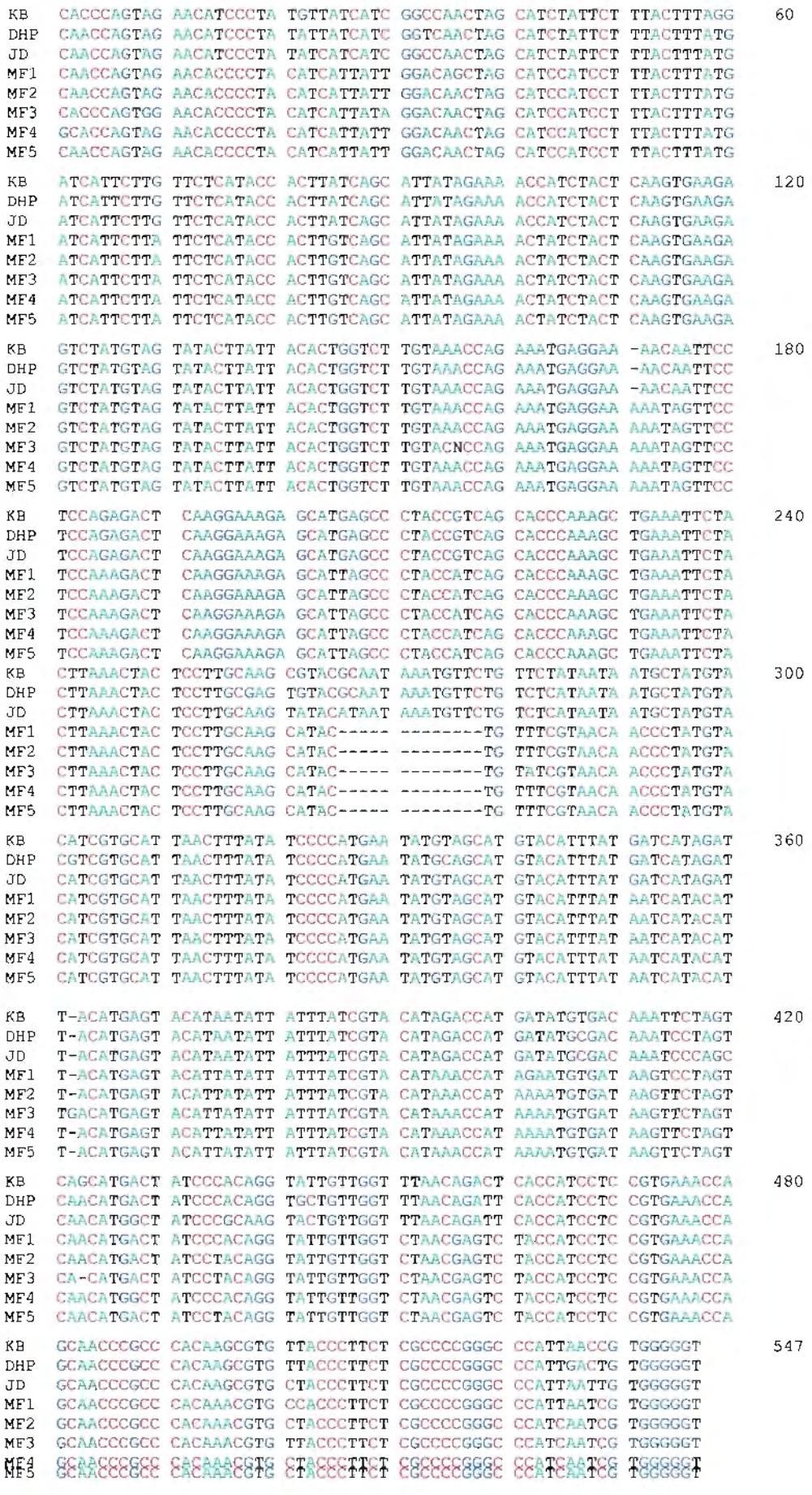

Figure 7.1. Alignment between *M. fraterculus* sequences MF1 – MF5 and examples of *M. schreibersii* sequences from each of the major subpopulations (West: Koegelbeen, KB; South: De Hoop, DHP; North-east: Jozini Dam, JD). See also Figure 5.4, Chapter 5 and Appendix 5.

7.2.3. Attempted design of species-specific mtDNA primers

Although significant differences were identified between the mtDNA sequences of *M. schreibersii* and *M. fraterculus*, it was not practical to sequence all remaining samples to confirm their identification as *M. schreibersii*. An attempt was therefore made to design PCR primers that would bind to the characteristic region spanning the 13 bp deletion that was identified in *M. fraterculus*. It was hoped these primers would preferentially amplify mtDNA from either *M. schreibersii* (no deletion) or *M. fraterculus* (deletion present). The differential amplification of specimens could then be used to distinguish between the species.

Accordingly, two PCR primers were designed (Table 7.1), one to bind to the regions flanking the deletion in the *M. fraterculus* mtDNA sequence, and the other to bind to the corresponding "non-deleted" sequence in *M. schreibersii*. These oligonucleotides were designed to function as forward primers in conjunction with Wilkinson & Chapman's (1991) primer C. The region corresponding to the *M. fraterculus* deletion is highly polymorphic in *M. schreibersii*, which necessitated the design of a degenerate primer for use in the latter species. Primers were synthesised and diluted as described in Chapter 5, Section 5.2.1. Polymerase chain reactions were conducted as described in Chapter 5, Section 5.2.1, using an annealing temperature of 50°C. Unfortunately, neither of the new primers functioned reliably or reproducibly, as judged by lack of amplification of specimens used as positive controls and/or the presence of amplification products in negative controls.

Table 7.1. Sequences of PCR forward primers designed to amplify either *M. schreibersii* or *M. fraterculus* mtDNA control region in conjunction with reverse primer C (Wilkinson & Chapman 1991).

	Primer sequence (5′ – 3′)
***M. schreibersii* primer**	GCA TTA TTA T (AG) (AG) (AG) AC AGA ACA TTT ATT
***M. fraterculus* primer**	GGT TGT TAC GAA ACA GTA TGC T
Primer C	TGA ATT GGA GGA CAA CCA GT

7.3. MICROSATELLITE ANALYSIS

Because species-specific mtDNA primers (Section 7.2.3) were unreliable, an alternative means of identifying potential *M. fraterculus* specimens was required. Although the NCAM locus (Chapter 4) was monomorphic, the five Mschreib microsatellite loci were polymorphic in *M. fraterculus* (Table 7.2). Because *M. fraterculus* and *M. schreibersii* differ significantly in their mtDNA, it is likely that their respective multilocus microsatellite allele frequencies would also differ, as would be expected for different species. If so, examination of their distinguishing allele frequencies could be used to identify other potential *M. fraterculus* specimens within the microsatellite data set.

Table 7.2. Amplification of microsatellite loci in *M. fraterculus* by primers for the Mschreib and NCAM loci; N_A = number of alleles; *n* = number of individuals genotyped per locus.

Locus	N_A	Allele size range (bp)	*n*
Mschreib1	4	177 – 187	14
Mschreib2	3	182 – 186	14
Mschreib3	9	150 – 172	14
Mschreib4	8	187 – 207	13
Mschreib5	8	184 – 204	6
NCAM	1	236	14

An assignment test (Chapter 4, Section 4.2.5) was conducted with the assistance of *AGARst* (Harley 2001) to establish whether the multilocus microsatellite genotypes of the known *M. fraterculus* individuals were indeed identifiably different from those of *M. schreibersii*. The genotypes of all assumed *M. schreibersii* samples (n = 319) were pooled into one "population" and the five known *M. fraterculus* specimens were placed in another. An assignment test was then run comparing every individual in each of the two "populations" to all others to assess how likely each individual was to belong to either of the two groups.

All five *M. fraterculus* individuals were correctly assigned to their "population", with very high likelihood ratio values ranging between 5.3×10^6 and 2.1×10^{10} (Table 7.3). In addition, 97% of the assumed *M. schreibersii* were correctly assigned to their "population", with likelihood ratios of being *M. schreibersii* rather than *M. fraterculus* ranging between 1.49 and 8.31×10^{10}. However, nine of these assumed *M. schreibersii* were assigned to the *M. fraterculus* "population" with high likelihood ratios ranging between 446.4 and 1.9×10^7. One of these individuals (DHLM8) was collected from Die Hel, three (MMM12, MMM15 and MMM17) were from Maitland Mines, and the remaining five (SWF11, SWF14, SWF15, SWF17 and SWF19) were from Sudwala.

Because the forearm lengths of all these individuals were ≤ 4.46 cm (Table 7.5), there was a reasonable possibility that they were indeed *M. fraterculus* rather than *M. schreibersii*. The mitochondrial DNA control region of each of these bats was therefore sequenced, as described above (Sections 7.2.1 and 7.2.2), and all sequences were found to align closely with the known *M. fraterculus* sequences (Figure 7.2). Corrected (Kimura two-parameter) sequence divergences (Table 7.4) between these potential *M. fraterculus* sequences and those of known *M. fraterculus* individuals ranged between 0.01 and 0.05 (compared to 0.08 – 0.1 for *M. fraterculus vs. M. schreibersii*; Section 7.2.2). These individuals were therefore classified as *M. fraterculus*, and were eliminated from the *M. schreibersii* sample set.

An additional seven assumed *M. schreibersii* samples had low likelihood ratios (1.49 – 8.8) of being *M. schreibersii* rather than *M. fraterculus*. The mtDNA control region of these individuals was amplified and sequenced as described above. All seven sequences aligned closely with *M. schreibersii*. Corrected (Kimura 2-parameter) divergences between these seven sequences and a representative *M. schreibersii* sequence from Koegelbeen ranged between 0.026 and 0.072. On the other hand, corrected distances between these sequences and the *M. fraterculus* specimen, MF1 ranged between 0.103 and 0.126. These seven individuals were therefore not excluded from the *M. schreibersii* sample set.

Observed (H_O) and expected (H_E) heterozygosity for the fourteen *M. fraterculus* samples were estimated (with the assistance of *AGARst*) to be 0.4267 and 0.5982 respectively. These are considerably lower than H_O (0.6445) and H_E (0.8199) estimates for all South African *M. schreibersii* (n = 310), but this may simply be due to the smaller *M. fraterculus* sample size.

Table 7.3. Results of an assignment test comparing *M. schreibersii* and *M. fraterculus*.

Species	Likelihood ratios
M. schreibersii (n = 319)	***M. schreibersii vs. M. fraterculus*** Modal: 8.7×10^5 Range: 1.49 - 8.31×10^{10}
Known *M. fraterculus:*	***M. fraterculus vs. M. schreibersii***
MF1	2.1×10^{10}
MF2	1.1×10^8
MF3	5.7×10^7
MF4	8.3×10^7
MF5	5.3×10^6
Possible *M. fraterculus:*	***M. fraterculus vs. M. schreibersii***
DHL M8	1.0×10^4
MM M12	4.1×10^5
MM M15	5.0×10^4
MM M17	1.3×10^3
SW F11	446.4
SW F14	2.4×10^5
SW F15	2.0×10^7
SW F17	9.0×10^3
SW F19	452.3

Table 7.4. Corrected sequence divergence estimates between known *M. fraterculus* samples (MF1 – MF5) and potential *M. fraterculus* identified through an assignment test.

	MF1	MF2	MF3	MF4	MF5	DHLM8	MMM12	MMM15	MMM17	SWF11	SWF14	SWF15	SWF17
MF1	-												
MF2	0.010	-											
MF3	0.022	0.012	-										
MF4	0.012	0.006	0.014	-									
MF5	0.010	0.000	0.012	0.006	-								
DHLM8	0.024	0.022	0.030	0.020	0.022	-							
MMM12	0.026	0.024	0.032	0.026	0.024	0.014	-						
MMM15	0.012	0.012	0.024	0.018	0.012	0.026	0.026	-					
MMM17	0.022	0.022	0.034	0.028	0.022	0.037	0.039	0.016	-				
SWF11	0.041	0.037	0.047	0.035	0.037	0.053	0.060	0.045	0.037	-			
SWF14	0.022	0.016	0.026	0.014	0.016	0.014	0.020	0.028	0.039	0.049	-		
SWF15	0.039	0.028	0.034	0.030	0.028	0.026	0.028	0.036	0.047	0.055	0.016	-	
SWF17	0.020	0.016	0.026	0.022	0.016	0.037	0.039	0.024	0.030	0.043	0.032	0.045	-
SWF19	0.022	0.012	0.024	0.018	0.012	0.034	0.034	0.020	0.034	0.045	0.028	0.036	0.016

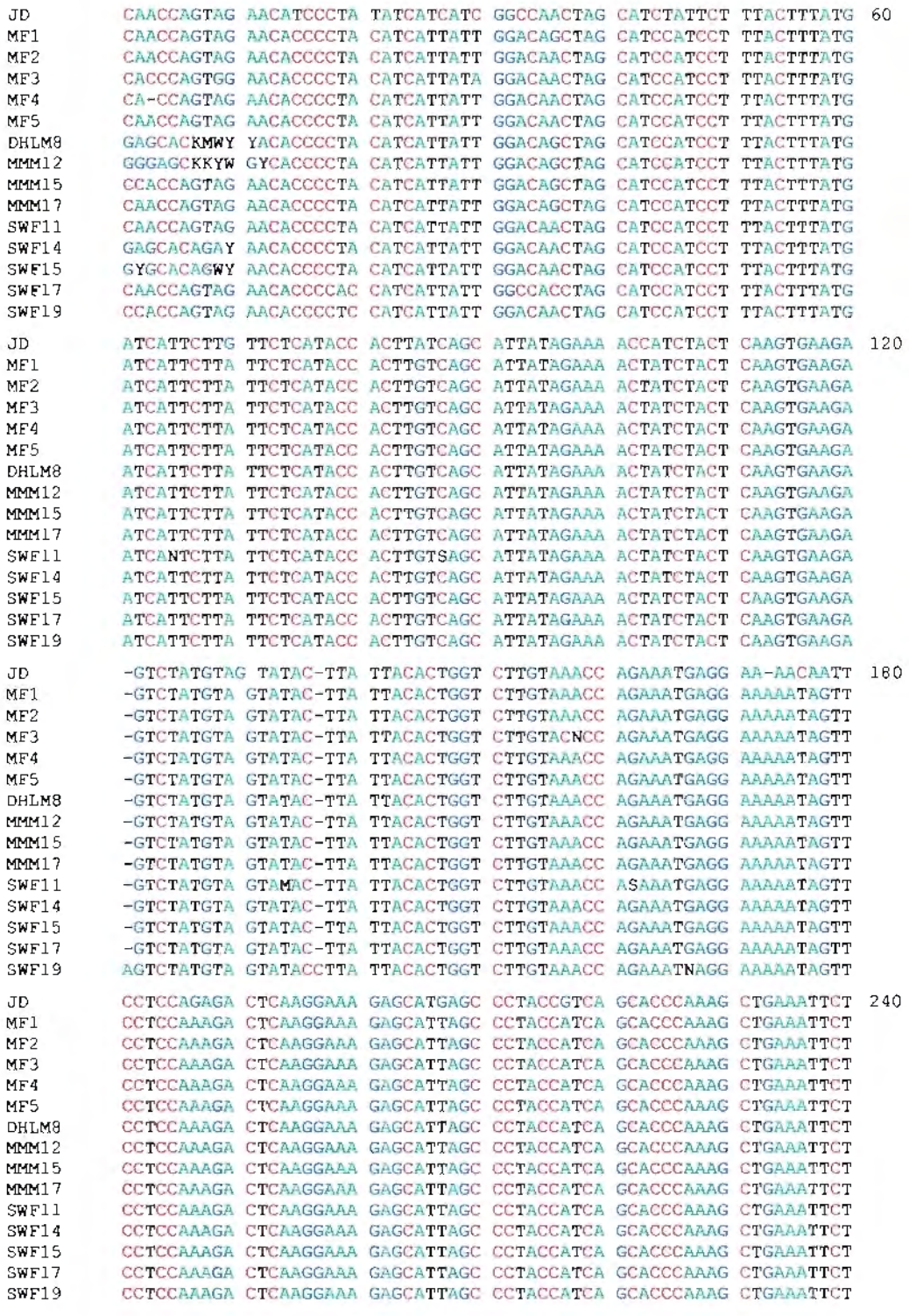

Figure 7.2. Alignment of potential *M. fraterculus* sequences with a Jozini Dam *M. schreibersii* (JD) and with *M. fraterculus* specimens MF1 – MF5.

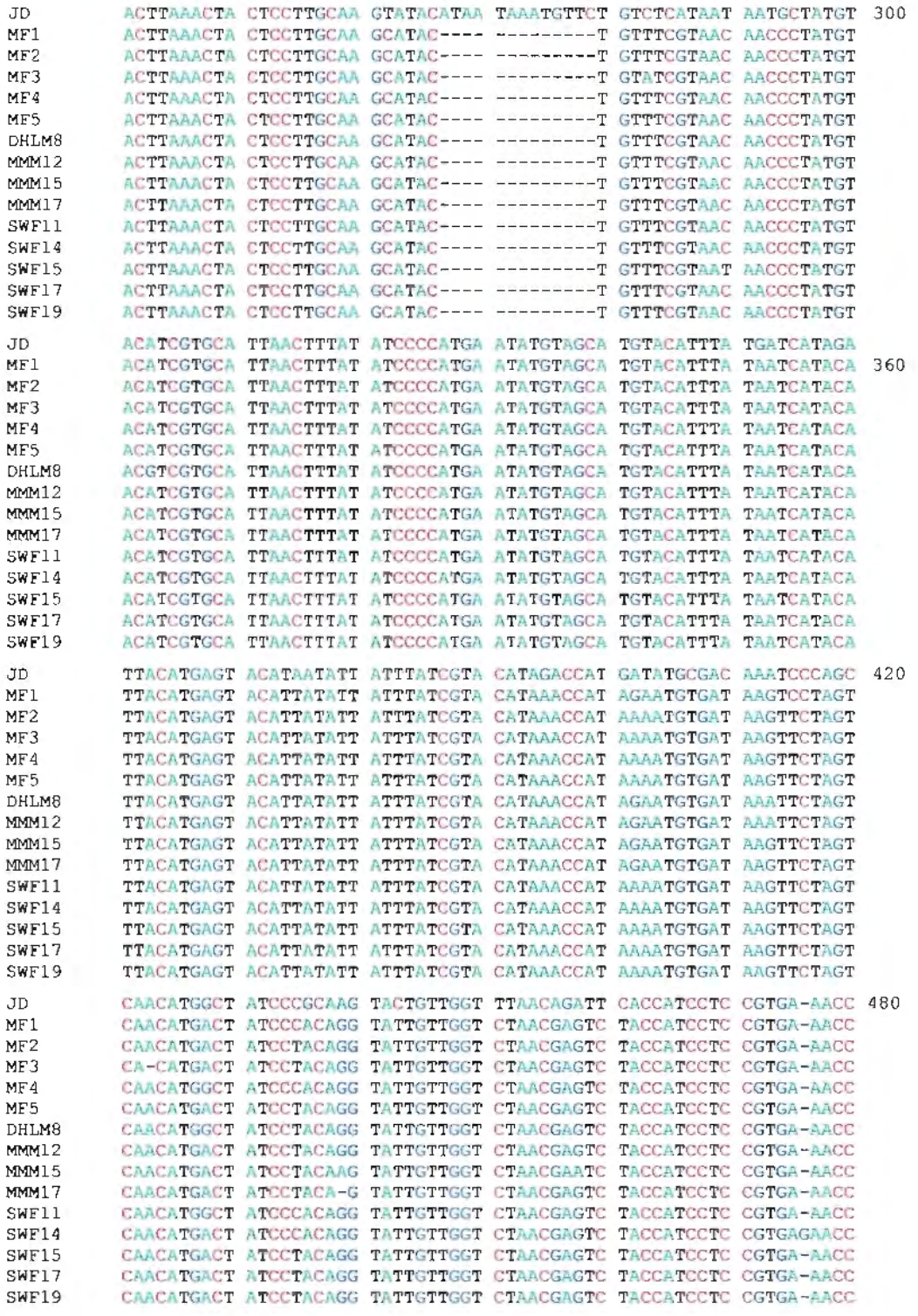

Figure 7.2. cont. Alignment of potential *M. fraterculus* sequences with a Jozini Dam *M. schreibersii* (JD) and with *M. fraterculus* specimens MF1 – MF5.

7.4. MORPHOLOGICAL EXAMINATION

Mass and forearm length of the fourteen *M. fraterculus* individuals (Table 7.5) were compared to those of *M. schreibersii* by means of Mann-Whitney rank sum tests (Zar 1984). Although mean mass and forearm length of *M. fraterculus* were significantly lower than *M. schreibersii* (mass: *M. fraterculus vs.* male and non-pregnant *M. schreibersii*, $T = 412.5$, $p < 0.001$; forearm length: $T=191.0$, $p < 0.001$), there is considerable overlap in their morphology. This is illustrated in the plot of mass *vs.* forearm length (Figure 7.3). Although the fourteen *M. fraterculus* fall at the lower end of the distribution, the high end of their mass and forearm range overlaps that of smaller *M. schreibersii*.

The identities of all *M. schreibersii* individuals with forearm lengths ≤ 4.5 cm and mass ≤ 11.0 g were confirmed by means of an assignment test (Section 7.3 and Chapter 4, Section 4.2.5). The likelihood ratios classifying these individuals as *M. schreibersii* rather than *M. fraterculus* ranged between 2.7×10^3 and 1.4×10^8, and they were therefore considered *M. schreibersii*.

Table 7.5. Mass and forearm length of *M. fraterculus* and *M. schreibersii* (see also Table 6.1, Chapter 6).

		Mass (g)	Forearm (cm)
M. fraterculus	**MF1**	11.0	4.30
	MF2	9.0	4.32
	MF3	9.0	4.32
	MF4	8.5	4.34
	MF5	8.5	4.30
	DHLM8	9.0	4.34
	MMM12	9.5	4.40
	MMM15	9.0	4.42
	MMM17	9.5	4.42
	SWF11	9.0	4.27
	SWF14	10.0	4.31
	SWF15	9.5	4.46
	SWF17	11.0	4.34
	SWF19	10.0	4.46
MEAN		9.5 ± 0.8	4.36 ± 0.06
M. schreibersii **MEAN** (n = 286)		11.54 ± 1.31 (excl. pregnant females)	4.59 ± 0.14

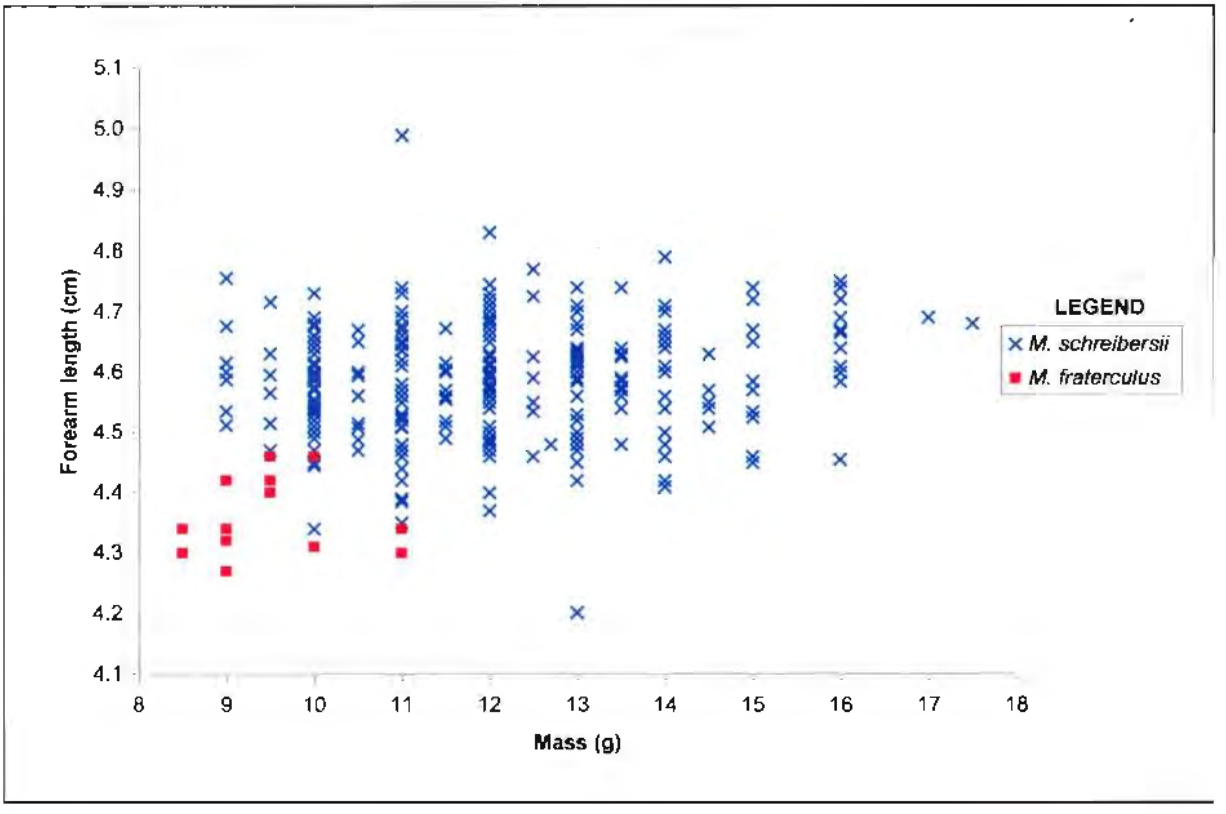

Figure 7.3. Plot of mass *vs.* forearm length for *M. schreibersii* and *M. fraterculus*

7.5. CONCLUSIONS

7.5.1. Identification and exclusion of *M. fraterculus* from the *M. schreibersii* sample

Thirteen *Miniopterus fraterculus* specimens were identified within the *M. schreibersii* sample set by means of a combination of mitochondrial DNA sequencing and a microsatellite assignment test. These genetic techniques allowed previously unrecognised *M. fraterculus* specimens to be identified and excluded from the *M. schreibersii* sample set, prior to statistical analysis of the data.

While it is possible that other *M. fraterculus* specimens remain undetected within the *M. schreibersii* data set, it is likely that the effect of their presence is negligible. The differences in *M. fraterculus* and *M. schreibersii* multilocus allele frequencies suggests that if large numbers of *M. fraterculus* remained in the data set, their presence within one or more of the *M. schreibersii* colonies would cause those colonies to deviate significantly at all loci from Hardy-Weinberg equilibrium, due to the effect of substructuring within that colony. However, no significant deviation from Hardy-Weinberg equilibrium was detected in any of the *M schreibersii* colonies, when examined over all six microsatellite loci (Chapter 4, Section 4.3.1). It can therefore be assumed that the majority, if not all of the *M. fraterculus* individuals have been identified and excluded from the data set, and the presence of any remaining *M. fraterculus* in the sample should be too few to affect the interpretation of the extent of population substructuring in *M. schreibersii.*

7.5.2. Recommendations for future studies of *M. schreibersii* and *M. fraterculus*

Although *M. schreibersii* and *M. fraterculus* differ significantly in mass and forearm length, there is a wide range of morphological variation in both species, identified here (Section 7.4 and Chapter 6) and in other studies, (*e.g.* Jacobs 1999). This leads to considerable overlap in the morphology of the two species. Therefore, where possible, sequencing of the mitochondrial DNA control region and/or use of an assignment test to examine microsatellite allele frequencies may be a more reliable means of distinguishing the two species, or should at least supplement the more traditional morphological techniques for individuals that fall within the overlap region. The morphology of the two species should also be re-examined more closely to identify characters that could be used to distinguish between them in the field.

7.6. NEW DISTRIBUTION RECORD FOR *M. FRATERCULUS*

One of the individuals (DHLM8) identified here as being *Miniopterus fraterculus* was collected from Die Hel Cave (33°05'S, 19°05'E) in the Western Cape Province. Although Roberts (1951, cited in Herselman & Norton 1985) recorded *M. fraterculus* from Knysna (Figure 1.4, Chapter 1), most current distribution records for this species (*e.g.* Mills & Hess 1997; Skinner & Smithers 1990; Taylor 2000) mark the western limit of its distribution as being near Port Elizabeth in the Eastern Cape. No records of *M. fraterculus* occurring in the Western Cape province have been reported since 1951, and no individuals of this species have ever been reported west of Knysna. The identification of *M. fraterculus* at Die Hel Cave therefore marks a new distribution record for this species in South Africa.

CHAPTER 8

DISCUSSION

8.1. PHYLOGEOGRAPHY OF *M. SCHREIBERSII*

8.1.1. Summary of results

Both microsatellites and mitochondrial DNA (mtDNA) indicate that *Miniopterus schreibersii* in South Africa exhibits significant genetic heterogeneity. The species is genetically subdivided into three major subpopulations: (1) a southern subpopulation including the colonies De Hoop and Die Hel, (2) a western subpopulation consisting of Steenkampskraal and Koegelbeen, and (3) a north-eastern subpopulation including Grahamstown, Maitland Mines, Shongweni Dam, Jozini Dam, Peppercorn and Sudwala. The locations of these subpopulations correspond broadly to four vegetation zones or biomes (discussed in Section 8.4). Although there is little genetic differentiation between colonies within each of the major regions, colonies from different subpopulations differ significantly from each other, as indicated by high R_{ST} (*Rho*) and genetic distance values. Microsatellite allele frequencies are strongly characteristic of each region, and individual bats can be assigned with 95 – 100% confidence to one of the three subpopulations. Smaller numbers of bats sampled from other sites (Knysna, Cedarberg and Pongola River Bridge)

within these major geographical regions also show strong genetic affinity with major colonies associated with these regions.

In addition, mitochondrial DNA (mtDNA) haplotypes show significant phylogeographic substructuring (see below). Closely related sequenced haplotypes cluster together within each of the three subpopulations, and are separated from haplotypes belonging to other subpopulations by a connection length of at least 22. No sequenced haplotypes are shared between subpopulations. Restriction fragment haplotypes also show phylogeographic substructuring, although with lower resolution. Two subpopulations (south-west and north-east) are discernable, which do not share any haplotypes. The population differentiation indicated by the genetic markers is broadly supported by differences in wing morphology, in particular aspect ratio.

8.1.2. Phylogeographic structure

Phylogeography is the study of geographic patterns of genetic variation among species or populations (Avise 1994). Examination of such patterns of inter-population genetic diversity can provide valuable information about both historical and current evolutionary processes affecting a species, which can be used for conservation and management purposes. Avise *et al.* (1987) have defined five categories of phylogeographic structure, based on a survey of intraspecific geographic variation in mtDNA haplotypes. The primary distinction between the categories is determined by whether or not the mtDNA phylogenies show deep phylogeographic discontinuities. Phylogenies that show distinct genetic differences, with large numbers of mutational steps separating their respective haplotypes, are generally indicative of long-term separation of populations due to extrinsic barriers to gene flow (*e.g.* mountains or rivers). Such divergent phylogenies may occur in different geographical regions (Category I) or may be found sympatrically (Category II). The latter condition of phylogeographic discontinuity without spatial separation is relatively rare, but may

occur if previously isolated populations secondarily become sympatric, or if there is an intrinsic barrier to gene flow, *e.g.* if sibling species have differing mating strategies. Alternatively, mtDNA phylogenies may be separated by small numbers of mutational steps. These genetically continuous haplotypes may be geographically localised (Category III), which indicates restricted gene flow between populations that have not been separated for long periods of time. Alternatively, similar phylogenies may not be spatially separated (Categories IV and V), which indicates intermediate to extensive gene flow, with no population subdivision.

Terrestrial mammals that have limited dispersal abilities generally fall into Category I (Avise *et al.* 1987). For example, pocket gophers, *Geomys pinetis* (Avise *et al.* 1979) and deer mice, *Peromyscus maniculatus* (Lansman *et al.* 1983) show extensive population subdivision, with geographically isolated assemblages of mtDNA haplotypes separated by 3 – 5% sequence divergence. On the other hand, very mobile animals such as birds generally fall into Categories IV or V (Avise *et al.* 1987). For example, red-winged blackbirds (*Agelaius phoeniceus*) show very minor population subdivision across the continental United States of America (USA), with a maximum of 0.8% sequence divergence between mtDNA haplotypes (Ball *et al.* 1988). High levels of dispersal have resulted in high levels of gene flow, and consequently little or no phylogeographic clustering has been detected in this species (Ball *et al.* 1988).

8.1.3. Phylogeography of very mobile bat species

Like birds, many species of bats are very mobile, and thus have the potential for high levels of dispersal. This is particularly true of migratory species, such as the New World Mexican free-tailed bat, *Tadarida brasiliensis mexicana,* and one might therefore expect similarly low levels of phylogeographic substructuring in this species. This is indeed the case: *T. b. mexicana* is highly gregarious (some colonies number in the millions), and several populations of this species

undertake long-distance, seasonal migrations between summer maternity roosts located in central and south-western USA, and wintering roosts in Mexico (McCracken *et al.* 1994; Svoboda *et al.* 1985). In addition, other non-migratory populations of this species are found in the western USA (McCracken & Gassel 1997). However, despite the existence of both migratory and non-migratory populations, the return of the migratory bats to the same roost sites each year, and the use of at least two distinct Mexico-USA flyways, only very weak genetic differentiation has been found among the *T. b. mexicana* populations (McCracken & Gassel 1997; McCracken *et al.* 1994; Svoboda *et al.* 1985). High levels of genetic diversity in the *T. b. mexicana* populations further indicate that these bats have large breeding populations, and that there is considerable gene flow between them. Gene flow may be facilitated by substantial interchange of individuals between the flyways (McCracken *et al.* 1994). Although one cannot assign *T. b. mexicana* to one of Avise *et al.*'s (1987) phylogeographic categories because all genetic studies on this species to date have been based on allozymes rather than mtDNA, one can nevertheless conclude that *T. b. mexicana* shows high rates of dispersal, possibly in both sexes. This contrasts strongly with the results of the present study on *M. schreibersii*.

Miniopterus schreibersii may be considered an Old World analogue of *T. b. mexicana*. Like the latter species, *M. schreibersii* is migratory and gregarious. However, contrary to expectations based on other volant and mobile species, such as the Mexican free-tailed bat and red-winged blackbirds, the phylogeographic pattern shown by *M. schreibersii* in South Africa does not fall into Avise *et al.'s* (1987) Categories IV or V (genetic continuity with high levels of gene flow). Connection lengths of at least 22 and 34 separate the mtDNA sequence haplotypes of the southern subpopulation of *M. schreibersii* from those of the north-eastern and western subpopulations respectively. The mean sequence divergence estimate between colonies from different subpopulations is 3.9%. The levels of control region divergence shown by *M. schreibersii* are within the upper range of those reported in other very mobile, vertebrate species (Norman *et al.* 1994; Wilmer *et al.* 1994). Greater than 3% sequence divergence is generally considered unusual

among very mobile animals, which usually show less than 1% divergence, unless they are geographically isolated (Avise 1989; Wilkinson & Fleming 1996).

The strong phylogeographic structuring of *M. schreibersii* mtDNA sequences into geographically distinct assemblages appears similar to that shown by Category I terrestrial mammals, such as pocket gophers (Avise *et al.* 1979) and deer mice (Lansman *et al.* 1983). These animals have very low rates of dispersal and gene flow, due to long-term separation. However, sequence divergence estimates in the current study are based on the control region, which mutates four or five times faster than other parts of the mtDNA molecule (Brown *et al.* 1979). These estimates cannot therefore be compared directly with those obtained for pocket gophers and deer mice. The latter studies examined restriction fragment patterns obtained from restriction enzyme digests of the entire mtDNA molecule (Avise *et al.* 1979; Lansman *et al.* 1983), the majority of which is more conserved than the control region (Brown 1985).

Therefore, despite the apparent large divergence of sequenced haplotypes, a more conservative interpretation of *M. schreibersii* phylogeography would be a Category III classification, rather than Category I. Category III is seen in species that have historically limited gene flow, but have not been separated by long-term extrinsic or intrinsic barriers, and thus show less extreme subdivision (Avise *et al.* 1987). This categorisation is supported by mtDNA RFLP analysis, which does not show as extreme geographic separation as the sequenced haplotypes. The *M. schreibersii* RFLP haplotypes are geographically separate, with different haplotypes being found in the north-eastern subpopulation compared to the western and southern subpopulations, which share several haplotypes. However, in many cases only one restriction site change separates a north-eastern haplotype from either a western or a southern one. This less extreme population subdivision is possibly due to the lower resolution obtained from RFLP's compared to sequencing.

There are no immediately obvious zoogeographic barriers to *M. schreibersii* dispersal in South Africa. Extrinsic barriers to dispersal may therefore not be responsible for the pattern of population subdivision indicated by both nuclear and mitochondrial DNA markers. If so, the limited dispersal of both sexes, suggested by the population substructure, may be due to high levels of philopatry in both sexes.

8.2. PHILOPATRY IN *M. SCHREIBERSII*

Dispersal and philopatry may substantially influence the genetic structure of populations. Dispersal is usually the major mechanism of gene flow among populations, and if extensive may lead to panmixia and prevent the development of local adaptations (see below and Section 8.4). On the other hand, if gene flow is limited (*e.g.* by philopatry) genetic differentiation and local adaptation of neighbouring groups may be promoted (Greenwood 1980).

The agreement between the results obtained from both nuclear and mitochondrial markers in this study suggests that both male and female *M. schreibersii* are philopatric, or at least are faithful to the geographic regions encompassing the three major subpopulations (Section 8.4). If *M. schreibersii* exhibited sex-biased (*e.g.* male) dispersal, one would expect to see significant differentiation in the maternally-inherited mtDNA marker, while limited substructuring would be indicated by microsatellite markers. Further support for philopatry in both sexes is provided by the absence of a significant difference between the pairwise colony R_{ST} (*Rho*) and genetic distance values of males and females. Pairwise *Rho* comparisons between the sexes within individual colonies indicate that males and females of each colony are closely related. This suggests that the bats mate either with individuals from within their own colony, or with individuals from closely related colonies that are located within the same subpopulation. Females may be somewhat more

philopatric than males, because greater genetic differentiation among colonies was detected by mtDNA than by microsatellites. However, as discussed previously (Chapter 5), this may be the result of the smaller effective population size of the mtDNA maker, which increases its resolution when detecting population differentiation.

8.2.1. Other studies on *M. schreibersii*

The findings of the present study support those of previous behavioural and banding studies in South Africa (Laycock 1976; Van der Merwe 1973b, 1975), Australia (Dwyer 1966, 1969) and Europe (Palmeirim & Rodrigues 1995; Serra-Cobo *et al.* 1998), all of which suggest that both male and female *M. schreibersii* show high levels of roost fidelity. However, van der Merwe (1975) and Serra-Coba *et al.*'s (1998) studies involved only adult individuals. Therefore, the relatively high recapture rates of individuals at the same hibernacula or maternity roosts at which they were banded (~53% in both studies) imply only that adult bats show roost fidelity. No conclusions can be drawn about philopatry or dispersal rates, and therefore the findings of these studies do not necessarily imply philopatry to natal colonies.

Dwyer (1966, 1969), on the other hand, examined site attachment in different ages classes (juveniles, yearlings and adults). He identified two biologically and geographically separate subpopulations of *M. schreibersii* in south-eastern Australia, possibly separated by physiographical features such as mountains or large bodies of water. Each subpopulation was associated with a major maternity roost, used annually by the majority of females from each region, and individuals were found to move within these subpopulation ranges more frequently than they moved between them (Dwyer 1969). Dwyer's (1966) findings were similar to those of van der Merwe (1973b, 1975) in that colonies were relatively permanent in terms of the individuals comprising them. Dwyer (1966) found no differences in site attachment between females of different ages, but noted that

roost fidelity in males tended to increase with age (home recoveries: 48% in juveniles, 64% in yearlings and 84% in adults), and immature bats appeared temporarily at a wide range of colonies before they reached maturity. However, many juveniles were unable to establish themselves at foreign roosts and therefore returned to their natal colonies after the first year. He concluded that *M. schreibersii* social organisation involved relatively stable groups, based on fixed home sites. Such stable groups would explain the genetic substructuring found in the present study.

However, Dwyer (1966) found that recovery rates of females (of all age classes) at their home roosts were lower than those of males (48.5% and 77.6% respectively). On the other hand, Palmeirim & Rodrigues (1995) did not observe any cases of natal dispersal in juvenile or adult females in Portugal, and found that during the breeding season, males were also more likely to be present in their natal colony than any other. Furthermore, although individuals of both sexes visited foreign roosts outside of the breeding season, they returned to their natal colonies to give birth or to mate. The authors argued that the high levels of philopatry shown by *M. schreibersii* were due to strong imprinting during nursing, rather than due to lack of knowledge of alternative roosts. Dwyer (1966) also noted that knowledge of many roosts within a region may be important, but maintained that attachment to a specific roost was not developed at birth, but was learned with maturity.

The extent of philopatry and/or dispersal displayed by *M. schreibersii* clearly varies across this species' very wide distribution range, and probably depends on the unique selective pressures faced by each colony (Section 8.4). Such variation in dispersal tendencies has also been reported in the brown long-eared bat, *Plecotus auritus*. In the United Kingdom, males of this species display strong philopatry to their natal colonies (Section 8.3.3), while in Germany, they associate with different colonies each year (Entwistle *et al.* 2000). In the case of *M. schreibersii*, however, several studies indicate both sexes are philopatric, although the degree of natal site fidelity may vary, as described above.

The results of this study suggest that in South Africa, both sexes of *M. schreibersii* are faithful to the geographic regions of the subpopulations. Colonies within each of the subpopulations are relatively closely related, while those from different subpopulations are genetically relatively distinct. This pattern of population differentiation could be the result of high levels of natal philopatry in both sexes. On the other hand, genetic differentiation among subpopulations may also occur in the presence of dispersal, provided the majority of dispersing individuals move to new roosts located in the same geographic region as their natal colonies, and thereafter remain faithful to the new roost. The small number of bats that may disperse to roosts outside their natal geographic region would allow for the limited gene flow between subpopulations. However, an assignment test (Chapter 4) indicated that between 62% and 90% of bats could be assigned correctly to the colony from which they were sampled, suggesting that individual colonies within subpopulations are genetically distinctive. Furthermore, males and females within each colony are closely related, as indicated by low R_{ST} (*Rho*) and genetic distance values. These genetic results, coupled with van der Merwe (1973b, 1975) and Palmeirim & Rodrigues' (1995) respective findings of strong site attachment and philopatry, suggest that dispersal, even within a subpopulation, may be limited. The pattern of population differentiation exhibited by this species in South Africa may therefore be the result of natal philopatry in both sexes, rather than merely adult roost fidelity.

Bats from several different maternity colonies within a subpopulation may, however, utilise a common hibernaculum, as has been observed in Portugal (Palmeirim & Rodrigues 1995). This would provide opportunities for bats to mate with individuals from different summer colonies, while still remaining philopatric to both their winter and summer roosts, because mating is believed to occur at the wintering sites (Dwyer 1966; van der Merwe 1973b). This sharing of communal wintering (mating) roosts by several colonies belonging to a single subpopulation would allow gene flow to occur between related colonies, even without dispersal, but would limit gene flow to other subpopulations. Although Dwyer (1966, 1969) reported that in Australia maternity colonies tended to be focal points for a number of hibernacula, rather than the reverse, he also proposed the existence of discrete "breeding populations", within which there would be high levels of gene flow,

but between which gene flow would be restricted. This could lead to population divergence and the development of local behavioural and physiological adaptations (Section 8.4).

8.3 PHILOPATRY & DISPERSAL IN OTHER BAT SPECIES

8.3.1. Female philopatry and male-biased dispersal

Whether members of a population remain at home or disperse depends on the relative fitness benefits and costs associated with each behaviour pattern (Gompper *et al.* 1998). Individuals that are philopatric benefit from remaining within a familiar area, but may face increased risks of inbreeding and competition for mates (Burland *et al.* 2001). Dispersers may suffer high energetic costs in searching for a new place to live, and may face increased risks of predation, reduced familiarity with new foraging areas, disrupted social bonds, the risk of not finding an alternative suitable home and/or not being able to establish themselves in a new territory (Lewis 1995; Palmeirim & Rodrigues 1995). In many species, these risks are outweighed by the benefits of greater exposure to reproductive opportunities with unrelated individuals (inbreeding avoidance).

In the majority of mammalian species studied, males disperse and females are philopatric (Dobson 1982; Greenwood 1980). Females invest heavily in their offspring, while males usually invest relatively little, and must compete for access to females, the limiting sex. In such cases, males benefit more than females from gaining access to a large number of mates. Therefore, the

increased risks associated with (male) dispersal are offset by the benefits of increased reproductive opportunities associated with greater mobility (Greenwood 1980).

This very common mammalian pattern of female philopatry and male dispersal (Greenwood 1980) has been reported in a number of bat species, several of which also show weak to moderate levels of population differentiation. Examples include *Desmodus rotundus* (Wilkinson 1985b), *Myotis myotis* (Castella *et al.* 2000; Petri *et al.* 1997), *Nycticeus humeralis* (Wilkinson & Chapman 1991), *Pipistrellus pipistrellus* (Thompson 1992) and *Nyctalus noctula* (Petit & Mayer 1999, 2000; Petit *et al.* 1999). The noctule bat, *N. noctula* is of particular interest because, like *M. schreibersii*, this species is migratory, moving up to 1000 km between summer and winter roosts each year (Petit *et al.* 1999). Female *N. noctula* are philopatric to their natal maternity colonies, resulting in significant differentiation of mtDNA haplotypes among colonies. However, unlike *M. schreibersii*, microsatellite markers detected little population differentiation in this species, suggesting high rates of male dispersal (Petit & Mayer 1999).

Very low levels of female dispersal have also been found in the non-migratory Bechstein's bat, *Myotis bechsteinii* (Kerth *et al.* 2000). Female Bechstein's bats form maternity colonies of 20 – 40 individuals, and although the colony frequently changes roosts within its home range, inter-colony dispersal rarely occurs. As a result, minimal overlap of mtDNA haplotypes was found between colonies, as indicated by a very high F_{ST} of 0.68 (Kerth *et al.* 2000). However, many of the solitary males roosting in the vicinity of the female maternity roosts had mtDNA haplotypes that did not match those of local females, indicating that they had immigrated into the area from other colonies (Kerth *et al.* 2000). The authors suggest that high cohesiveness of *M. bechsteinii* maternity colonies is related to communal rearing of their young, which favours colony stability and long-term associations with specific individuals. Female philopatry and cooperation has also been shown to play an important role in long-term, non-random associations of female vampire bats, *D. rotundus* (Wilkinson 1985a).

Possibly the most extreme population substructuring in bats reported to date is that found in the non-migratory ghost bat, *Macroderma gigas* (Worthington Wilmer *et al.* 1994). Complete segregation of mtDNA control region haplotypes was found between four Australian populations of this species, and a mean sequence divergence of 4.5% was estimated between these populations, although some differed by as much as 7.3%. Within populations, however, sequence divergence estimates averaged only 0.68% (Worthington Wilmer *et al.* 1994). Although segregation of most mtDNA haplotypes in *M. schreibersii* is not as extreme as that found in *Macroderma*, estimates of inter-subpopulation differences are similar to those found between populations of *M. gigas*. Mean inter-subpopulation sequence divergence estimates for *M. schreibersii* range between 3.2% (southern vs. north-eastern) and 4.8% (western vs. north-eastern), while divergences of up to 5.6% occur between Steenkampskraal and Grahamstown haplotypes. Within the major *M. schreibersii* subpopulations, however, mean sequence divergences are much lower, and are similar to those found within *M. gigas* populations, ranging between 0.7% (Steenkampskraal *vs.* Koegelbeen) and 1.4% (colonies within north-eastern subpopulation). The extreme levels of population substructuring detected in *M. gigas* were attributed to long-term female philopatry, resulting in negligible female-mediated gene flow among ghost bat populations (Worthington Wilmer *et al.* 1994). However, because the *M. gigas* study was conducted using only mtDNA, no conclusions can be made about male dispersal and/or philopatry in this species.

8.3.2. Male philopatry and female-biased dispersal

Although uncommon among mammals, male philopatry and female-biased dispersal (Greenwood 1980) has been reported in the white-lined bat, *Saccopteryx bilineata* (McCracken 1984). Males of this species exhibit high roost fidelity and are recruited into their natal groups, while females disperse. As a result, although very weak genetic structure may exist in the adult male population of *S. bilineata*, no genetic structuring has been detected in females (McCracken 1987).

8.3.3. Philopatry in both sexes

In his review of mammalian mating systems and dispersal, Greenwood (1980) reported no cases of philopatry occurring in both sexes of the same species. However, the results of this and other studies on *M. schreibersii* (Dwyer 1966, 1969; Palmeirim & Rodrigues 1995; Serra-Cobo *et al.* 1998; Van der Merwe 1973b, 1975) suggest that both males and females may be philopatric. In a highly gregarious species such as *M. schreibersii*, the risks associated with inbreeding and intrasexual competition are likely to be low (Palmeirim & Rodrigues 1995), and therefore these factors, which normally would promote dispersal, may be outweighed by the benefits of philopatry, leading to low rates of dispersal in both sexes.

In cases where individuals live in small groups, compromises may be reached in which these individuals may benefit from staying at home, while still managing to avoid the risk of inbreeding. For example, the brown long-eared bat, *Plecotus auritus* displays natal philopatry in both sexes in certain parts of its range (Burland *et al.* 2001). Long-term ringing studies conducted on *P. auritus* in the United Kingdom found that individuals of both sexes and all age groups showed long-term association with their colonies. Emigration of either adults or juveniles was extremely rare, accounting for less than 1% of all recaptures in both sexes and all age classes (Burland *et al.* 2001; Entwistle *et al.* 2000). However, genetic structuring between the *P. auritus* summer colonies was found to be weak, and within-colony relatedness low, suggesting that extra-colony copulation occurs, and is the primary mechanism of gene flow (Burland *et al.* 1999, 2001). This contrasts with the findings of the present study on *M. schreibersii* in which there are large inter-colony differences between subpopulations, and within-colony differences between males and females are very low. *Plecotus auritus* colonies are far smaller than those of *M. schreibersii*, numbering up to ~50 individuals (Entwistle *et al.* 2000). Under these circumstances, extra-colony copulations may represent a compromise between the advantages of long-term association with conspecifics and use of familiar roost sites, and the risks of inbreeding and competition for mates (Burland *et al.* 2001).

8.3.4. Why stay at home?

In addition to inbreeding-avoidance, the balance of the cost-benefit relationship determining whether individuals disperse or stay at home depends on each species' unique social structure, as well as a number of factors associated with its immediate environment. For example, utilisation of long-term, stable roost sites (such as caves, in the case of *M. schreibersii*) is likely to promote philopatry (in both sexes) because there is a high probability that the same roost will be available throughout the lifetime of any individual and of its offspring. Other species that utilise more ephemeral roosts, such as foliage, are less able to rely on the long-term stability of their roost, and so may be more likely to disperse (Palmeirim & Rodrigues 1995).

The availability of other suitable (stable) roost sites, within efficient foraging distances, will also influence the likelihood of dispersal *vs.* philopatry. *Miniopterus schreibersii* appears to have very specific temperature and humidity requirements (Chapter 1) for both maternity roosts and hibernacula (Churchill *et al.* 1997; Dwyer 1968; Dwyer & Harris 1972; McDonald *et al.* 1990a; Van der Merwe 1973a, 1973c). Roosts must therefore not only be able to house large numbers of bats, but must also provide the necessary environmental conditions for the particular season, and must be within energetically efficient access to reliable foraging areas. A number of potential alternative roost sites may be available within an area, but if they do not meet all these requirements, they may be unsuitable, and thus roosts may in effect be limited (Fenton & Rautenbach 1986).

Situations of limited suitable alternative roosts will tend to reduce dispersal and enhance philopatry (Palmeirim & Rodrigues 1995). This may apply to several of the South African colonies of *M. schreibersii*. Suitable sites for maternity colonies, such as caves, are rare in the fynbos biome (Section 8.4) in which De Hoop and Die Hel are located (McDonald *et al.* 1990b). Maternity roosts are also likely to be limited in the very flat terrain of the succulent- and Nama-Karoo biomes

(Section 8.4), in which Steenkampskraal and Koegelbeen are situated. Although caves are relatively numerous in the dolomite outcrops of the interior plateau of South Africa (where bats from Peppercorn hibernate; Van der Merwe 1975), those that have suitably low temperatures for hibernation may be scarce (Norton & van der Merwe 1978). In thermal preference experiments conducted during winter, clusters of *M. schreibersii* were found to select chambers that had lower temperatures than the majority of South African hibernacula examined (Brown & Bernard 1994). This suggests there may be a shortage of suitably cool hibernacula in South Africa, requiring that the bats utilise warmer than optimal roosts (Brown & Bernard 1994; Norton & van der Merwe 1978). If optimally cool winter roosts are scarce, this is likely to enhance philopatry should a suitable one be identified.

Even when suitable alternative roost sites are available, they may be located too close to other roosts that already support large colonies, and thus may increase competition for foraging resources to unsustainable levels. The presence of large aggregations of bats may inhibit the establishment of additional roosts nearby by imposing a spatial limit on foraging areas (Dwyer 1966). As a result, dispersing individuals may be forced to travel extensively in order to find a new home, thus compounding any risks already associated with dispersion. Furthermore, if suitable new roosts are located very far apart (either due to lack of suitable alternatives close by or a spatial foraging limit), they may be situated in regions with different ecological conditions (Palmeirim & Rodrigues 1995). If individuals of different populations have become locally adapted to their unique immediate environments, they may be less likely to disperse. They may be at an adaptive disadvantage in a new environment, and run the risk of being out-competed by better adapted locals (Hewitt & Butlin 1997; Palmeirim & Rodrigues 1995). In this case, selection will act in favour of philopatry (in both sexes) because it enhances adaptation to the local environment, which may otherwise be lost through gene flow. This is discussed in further detail in Section 8.4.

8.4. LOCAL ADAPTATION AND MIGRATION PATTERNS

Both male and female *M. schreibersii* in South Africa show high levels of roost fidelity, and possibly also philopatry. Furthermore, when they migrate between summer and winter roosts, the bats appear to remain within localised geographic regions, resulting in the formation of three genetically differentiated subpopulations (south, west and north-east). Thus, factors other than simply a tendency towards philopatry must influence their migration patterns within South Africa. The locations of the three subpopulations correspond to four of the major South African biomes (Figure 8.1). A biome is the largest ecological unit, recognised at a continental or subcontinental level. It is characterised primarily by its plant communities, and secondarily by its zoological components, climatic and soil conditions (Hickman & Roberts 1994; Rutherford 1997). Rutherford (1997) recognises seven major biomes in southern Africa (Figure 8.1), namely savanna, grassland, Nama-Karoo, succulent-Karoo, forest, desert and fynbos. The locations of the *M. schreibersii* subpopulations correspond broadly to the savanna, fynbos, Nama- and succulent-Karoo biomes. The distinguishing characteristics of each of these four biomes will be discussed briefly below.

8.4.1. The savanna biome

The majority of the *M. schreibersii* colonies that make up the north-eastern subpopulation (Shongweni Dam, Jozini Dam, Peppercorn and Sudwala) are found in the savanna biome (Figure 8.1). Grahamstown and Maitland Mines lie in a transition zone between the savanna, Nama-Karoo, fynbos and grassland biomes. The vegetation in this region is highly variable but the major type is referred to by Acocks (1988) as "false thornveld". It consists predominantly of shrubs and

clumps of trees as well as some grasses, and may be invaded by both Karoo and fynbos vegetation types (see below). Rainfall occurs throughout the year (30 – 130 mm per year) but is highest in the summer months, October to March (Bernard & Tsita 1995).

The remainder of the north-eastern colonies are found in the true savanna, which is the dominant vegetation type in Africa, and occupies 54% of southern Africa (Scholes 1997). Commonly referred to as the "bushveld" in South Africa, the savanna extends from about 34°S in the Eastern Cape province (Figures 1.4 and 8.1), along the eastern side of the country below 1000 m altitude, into the northern parts of the country (the "Lowveld"). From there, it spreads west along the northern edge of the "Highveld" plateau near Pretoria, into the Kalahari (Botswana) and Namibia (Acocks 1988; Scholes 1997). Unless there are abrupt changes in altitude (which cause sharp temperature differentials between regions, affecting vegetation types), the savanna grades continuously at its boundaries into neighbouring biomes, the grasslands and the Nama-Karoo.

The savanna biome contains both grasses and trees, and canopy cover ranges from 5 – 90% (Figure 8.2). It forms part of a continuum of vegetation types that differ in the extent and height of canopy cover, ranging from shrublands in drier areas (similar to that near Grahamstown and Maitland Mines) to woodlands in more moist regions. Rainfall is highly seasonal, and the climate in this region is therefore characterised by a hot wet season (mean temperature > 30°C, rainfall 500 – 1000 mm per year) between October and April (summer), and a warm dry season in the remainder of the year (Scholes 1997; Schulze 1997). In contrast to the neighbouring grassland biome, winters in the savanna are generally mild (> 8°C), with few severe frosts (Scholes 1997; Schulze 1997).

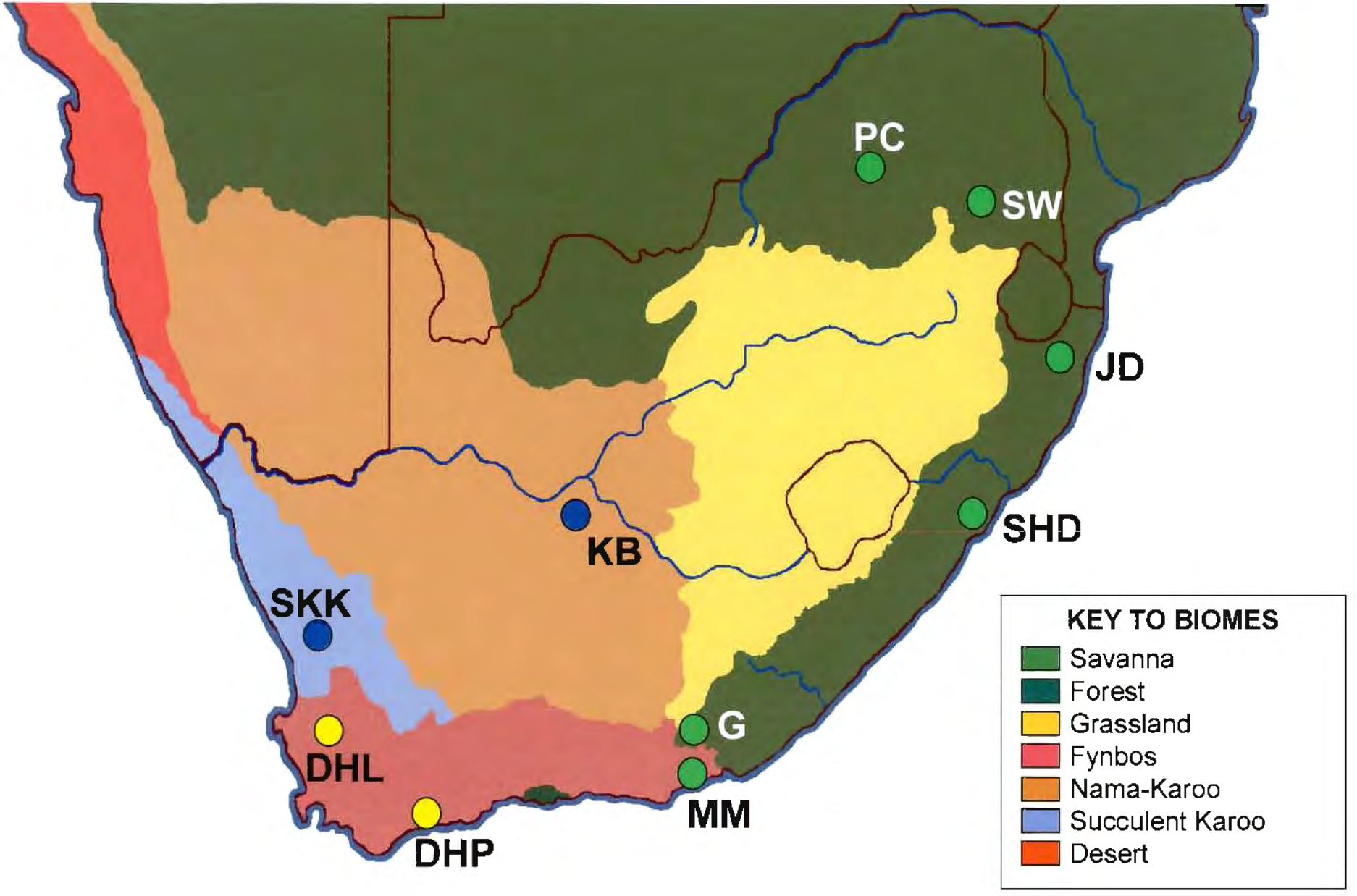

Figure 8.1. Location of *M. schreibersii* colonies in relation to the major biomes (after Rutherford 1997) of southern Africa.

Figure 8.2. Vegetation surrounding the Peppercorn site, an example of the savanna biome.

Figure 8.3. Vegetation surrounding the De Hoop site, an example of the fynbos biome.

8.4.2. The fynbos biome

The *M. schreibersii* colonies De Hoop and Die Hel are located in the fynbos biome (Figures 8.1 and 8.3) in the extreme southern and south-western parts of South Africa. This is primarily a winter rainfall area (April – September), and the range in annual rainfall is the largest of any southern African biome. Coastal areas receive 200 – 400 mm per year, but up to 3000 mm may fall each year in the mountainous areas (Cowling *et al.* 1997). Temperatures are generally mild near the coast (annual mean: 14° – 20°C) but may reach freezing with snowfalls in the mountains. The name "fynbos" is a vernacular term that literally means "fine-leaved bush", and it describes the predominant vegetation type in this region: an evergreen, sclerophyllous shrubland (Figure 8.3), which is adapted to nutrient-poor soils and warm, dry summers. The most characteristic plant types of fynbos are proteas, ericoid shrubs and restioid "grasses" (Acocks 1988; Cowling *et al.* 1997). In addition to true fynbos vegetation, this biome also includes shrub-like renosterveld (meaning "rhinoceros bush"), which occurs in the inland valleys, and a subtropical thicket or "strandveld", which is found on the coastal dunes (Cowling *et al.* 1997). The fynbos biome coincides with the Cape Floral Kingdom, which is recognised as one of the world's six floristic kingdoms, and has more endemic species per unit area than any other floristic kingdom. There are about 7300 plant species within this biome, about 80% of which are endemic to the region (Cowling *et al.* 1997).

8.4.3. The succulent- and Nama-Karoo biomes

Steenkampskraal and Koegelbeen are found in the succulent- and Nama-Karoo biomes respectively (Figures 8.1 and 8.4). Both of these biomes are semi-arid regions, characterised by dwarf shrublands. They differ primarily in their relative proportions of succulent and grassy vegetation types. The low altitude (< 550 m) succulent Karoo is characterised by low levels of mainly winter rainfall (July – August; 20 – 300 mm per year), mild winters (> 4°C) and warm, relatively arid summers (< 30°C). As its name suggests, the vegetation here is more succulent and less grassy than that of the Nama-Karoo. Rainfall is relatively reliable as much of the precipitation is brought by coastal fog and predictable cyclonic systems (Cowling *et al.* 1997).

The Nama-Karoo, which is located on the inland plateau of South Africa (550 – 1500 m), has low levels of highly seasonal summer rainfall (December – March; 60 – 400 mm per year). Summers are hot (> 30°C), and winters are frosty and cold (< 0°C; Cowling *et al.* 1997; Palmer & Hoffman 1997). Precipitation is less predictable than the succulent-Karoo, as it depends on irregular convectional rainfall, such as summer thunderstorms. The vegetation in the Nama-Karoo is variable (Figure 8.4), but it generally contains more shrubs and grasses and fewer succulents than the succulent-Karoo (Cowling *et al.* 1997). However, pastoral land use and annual variation in rainfall seasonality affect the proportion of succulent and grassy vegetation types in both biomes, and Cowling *et al.* (1997) therefore note that the boundaries between the Nama- and succulent-Karoo biomes are "fluid and blurred".

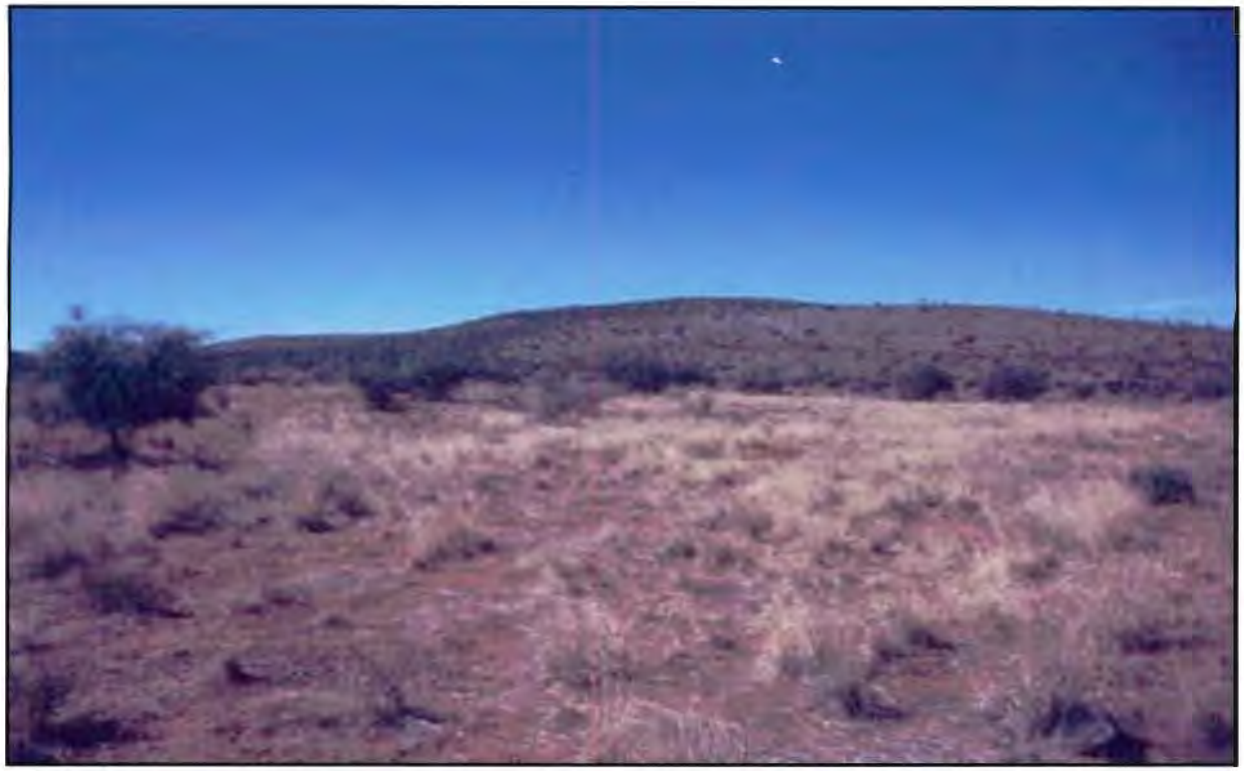

Figure 8.4. Vegetation surrounding the Koegelbeen site, an example of the Nama-Karoo biome.

8.4.4. Adaptation of *M. schreibersii* to local environmental conditions

The close association between the locations of *M. schreibersii* subpopulations and major South African biomes suggests that the bats may be adapted to local environmental conditions surrounding their roosts. This may involve adaptation to local vegetation types (which may influence their foraging habits), to local climatic conditions (*e.g.* seasonality and amount of rainfall), which may affect the timing of their reproductive cycle, and/or to regional differences in prey abundance (which, in turn, depends on vegetation and climate).

Bats from different subpopulations were found to differ significantly in their wing morphology (Chapter 6), particularly in terms of aspect ratio. However, differences in aspect ratio among colonies did not correspond closely to differences in vegetation types. High aspect ratio is

associated with fast flight and low manoeuvrability (Fenton 1990), which suggests that bats in more open areas (*i.e.* fewer trees, such as in the Karoo biomes) would have higher aspect ratios than bats foraging in more cluttered areas (*i.e.* more trees, such as in the "bushveld"). However, the habitat use of *M. schreibersii* in these vegetation zones is not known and their foraging habitat cannot be classified as cluttered or open. For example, *M. schreibersii* might forage above the tree canopy in the "bushveld", in which case its foraging habitat would still be classified as open despite there being more trees in the "bushveld". Indeed, bats in the north-eastern parts of the country were found to have high aspect ratios, despite living in a habitat with more trees. It is possible that the differences in wing morphology are adaptations for differing patterns of migration, rather than for differing foraging habitats (Chapter 6). If this is the case, then *M. schreibersii* is probably not adapting directly to differences in vegetation types between the different biomes. Instead, climate (*i.e.* amount and seasonality of rainfall) and its effect on insect abundance may play a more important role in promoting adaptation to the biomes.

The timing of the *M. schreibersii* reproductive cycle depends on environmental conditions (Bernard 1980; Happold & Happold 1990; McWilliam 1988; Medway 1971). *Miniopterus schreibersii* is able to alter the timing of copulation, length of the gestation period and the timing of parturition according to seasonal variation in temperature, rainfall and food supply (Bernard 1994; Richardson 1977). This adaptive response to climatic change may play a role in enhancing local adaptation to geographical regions, and may therefore influence migration patterns of these bats.

The biomes differ in seasonality and amount of rainfall, as well as in annual temperature range. Insect abundance is closely correlated with rainfall (Rautenbach *et al.* 1988) and therefore differences in timing and amount of rain will affect seasonal availability of insect prey. For example, insect biomass in the savanna of the Kruger National Park peaks in January, during the time of highest rainfall (mid-summer), and drops almost to zero during the dry winter months, June to August (Rautenbach *et al.* 1988). However, at De Hoop in the fynbos biome, insect abundance peaks in October and November (spring and early summer), largely due to an increase in the

abundance of dipterans at this time (McDonald *et al.* 1990b). Although insect availability does drop in winter, the abundance of lepidopterans is relatively constant throughout the year, even during the rainy winter months (McDonald *et al.* 1990b). The bats within the three subpopulations may respond to these regional differences in prey availability by specialising on locally available prey, and may adapt their foraging strategies and/or their reproductive cycles to ensure that parturition coincides with the time of greatest insect availability.

Furthermore, the genetic differentiation found among *M. schreibersii* subpopulations suggests that when these bats undertake their seasonal migrations between mating (winter) and maternity colonies, they may do so within the particular ecological zone to which they may have become locally adapted. It is possible that the reproductive cycles of bats living within each of the subpopulations (*i.e.* within the same biome) are synchronised (*e.g.* to track changes in prey abundance), but differ from those of bats adapted to living in other biomes. If bats from different biomes have sufficiently different reproductive cycles (in terms of, for example, timing of mating, implantation and parturition; Bernard 1994), this may restrict interbreeding between different subpopulations or biomes.

It is likely that a combination of several factors, affecting both the bats themselves and their prey populations, influences adaptation to local environmental conditions. Such local adaptation to conditions surrounding a roost may enhance a tendency towards philopatry because dispersing individuals may be at a selective disadvantage if they move to other ecologically different locations (Hewitt & Butlin 1997; Palmeirim & Rodrigues 1995). Philopatry (and habitat preference) in turn limits gene flow, which further enhances genetic differentiation and the development of unique local adaptations that may otherwise be broken up by genetic exchange with individuals from different areas (Hewitt & Butlin 1997). A feedback loop thus results. Alleles that promote habitat preference and restricted movement among different environments rapidly become differentiated among the differing habitat types. This leads to increased mating by individuals from the same habitat, further enhancing the development of local adaptations (Hewitt & Butlin 1997; Winker 2000).

8.4.5. Migration patterns of *M. schr*eibersii in South Africa

Based on the pattern of genetic subdivision and possible local adaptation indicated by the genetic markers, it is possible to propose a migration pattern for *M. schreibersii* in South Africa (Figure 8.5). Although *M. schreibersii* characteristically use two separate roosting sites in summer and winter, several of the roosts sampled here are occupied year round, namely De Hoop (McDonald *et al.* 1990b), Koegelbeen (Dr C. Anderson, McGregor Museum, pers. comm.), Maitland Mines (Bernard & Bester 1988) and Grahamstown (Brown & Bernard 1994). However, numbers of bats at these sites fluctuate during the year, and are generally higher in summer. For example, approximately 20 000 *M. schreibersii* utilise De Hoop Guano cave in winter, but up to 260 000 are present in summer (McDonald *et al.* 1990a, 1990b). These fluctuations may be related to the thermal requirements of the bats at different seasons.

Miniopterus schreibersii appear to select roosts depending on their differing thermal and humidity requirements in summer and winter (Churchill *et al.* 1997; Dwyer 1968; Dwyer & Harris 1972; McDonald *et al.* 1990a; Van der Merwe 1973a). Studies by Baudinette *et al.* (1994) and Dwyer (1968) further suggest that by being highly gregarious, *M. schreibersii* may modify the microclimate of a roost in order to improve conditions necessary for raising young. This suggests that large numbers of bats at maternity roosts may be an advantage because it raises temperatures in the roost, facilitating both pre- and postnatal development, as well as digestion (Bernard & Bester 1988). In winter, however, large aggregations of bats may be a disadvantage because they may prevent lowering of the roost temperature to levels suitable for hibernation. In cases where a roost is utilised year round, this may require a proportion of the bats to leave the summer roost to find an alternative, cooler wintering site. Once these bats have left, the roost temperature may drop to levels suitable for the remaining bats to hibernate for the duration of the winter.

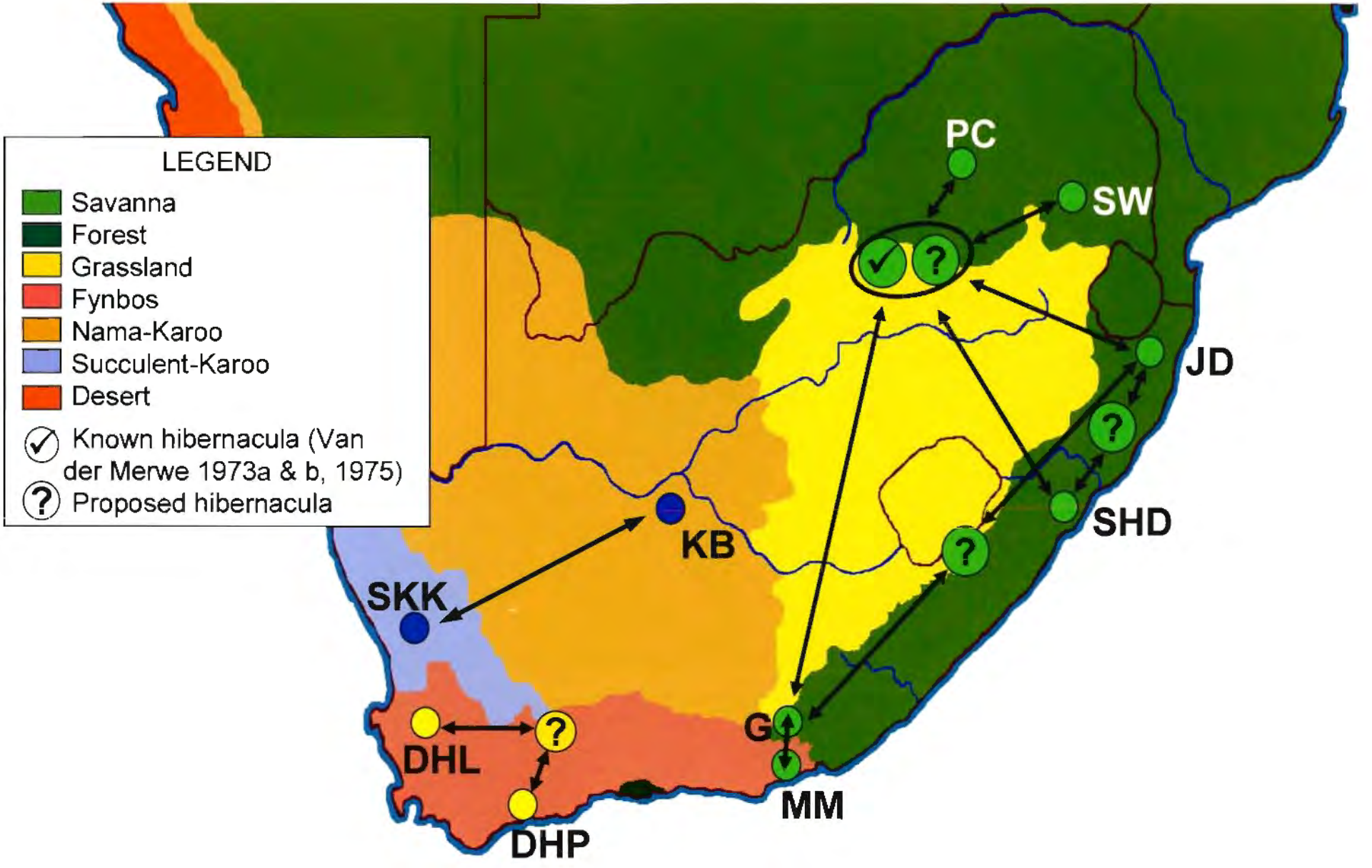

Figure 8.5. Proposed major migration routes for *M. schreibersii* in South Africa.

i. Western subpopulation

The results indicate that bats from Koegelbeen may migrate to Steenkampskraal to hibernate (Figure 8.5). Firstly, bats from Koegelbeen (occupied year-round) are genetically closely related to bats from Steenkampskraal. Secondly, Steenkampskraal is occupied only in winter, suggesting that the bats utilising this abandoned mine may be a subgroup of the bats that use Koegelbeen as a maternity roost in summer. If the number of bats using Koegelbeen in summer is too great to allow energetically efficient hibernation, a group of these bats may be required to leave this cave in autumn and migrate to an alternative, cooler wintering site, namely Steenkampskraal. Mating may occur prior to migration, or more probably at the hibernaculum itself, as is generally believed to occur (Bernard *et al.* 1996; van der Merwe 1973a, 1973b). If the latter proposal is true, *i.e.* that the Steenkampskraal bats mate at the hibernaculum, while the remaining bats mate at Koegelbeen, one might expect Steenkampskraal bats to be genetically distinct from those at Koegelbeen, unless a different group of individuals migrates to Steenkampskraal each year. This is unlikely if strong philopatry is exhibited by both sexes of this species. However, changes in allele frequencies (which lead to population differentiation if the populations are isolated) develop due to random genetic drift, and accumulate slowly in large populations (Hartl & Clark 1997; McCracken 1987; Slatkin 1987). The mine at Steenkampskraal was abandoned only in the 1960's, therefore the colony of *M. schreibersii* utilising this site cannot be more than 40 years old. Because both Steenkampskraal and Koegelbeen are relatively large populations, numbering in the thousands to tens of thousands, it is possible that insufficient time has passed for significant genetic differentiation to develop between these colonies.

The distance between Koegelbeen and Steenkampskraal (~560 km) is considerably greater than that previously recorded for *M. schreibersii* migrations in South Africa. Van der Merwe (1975) reported migrations of up to 250 km between maternity roosts and hibernacula in the northern parts of South Africa. Relatively short migrations of up to 190 km between winter and summer roosts have been reported for *M. schreibersii* in Austria, Hungary and Czechoslovakia, although, movements of up to 550 km have been recorded in France (Wimsatt 1970). Dwyer (1969) found that in the majority of cases, *M. schreibersii* in Australia moved less than 160 km (100 miles) between roosts. However, he did record some movements of between 320 km and 1500 km (200 – 650 miles), and the longest recorded migration was 1300 km (810 miles). A migration of 560 km between Steenkampskraal and Koegelbeen is therefore well within the capabilities recorded for this species. As discussed in Chapter 6, the wing morphology of the bats at Steenkampskraal and Koegelbeen may also be adapted for long-distance migration. Their wings have high aspect ratio, which may be an adaptation for long-distance, energetically efficient flight.

ii. Southern subpopulation

Like Koegelbeen, De Hoop is occupied year-round, but is used primarily as a maternity roost. Over 200 000 bats leave this cave each winter and migrate to a number of hibernacula within the Western Cape, some of which have been identified near the towns of Porterville, Ceres, Albertinia and Montagu (McDonald *et al.* 1990a). However, it is likely that there are several more hibernacula in this region that have yet to be identified. The genetics results indicate that the bats at De Hoop are closely related to those at Die Hel, which is occupied only in summer, as a maternity roost. It is possible, therefore, that at least some of the bats from De Hoop and Die Hel share one or more hibernacula in the Western Cape (Figure 8.5), and as mating is believed to occur at the wintering sites (Bernard *et al.* 1996; van der Merwe 1973a, 1973b), this would allow gene flow to occur between these colonies. Small numbers of bats obtained from other colonies within the Western Cape, namely Knysna and Cedarberg, were also found to be closely related to

the bats from De Hoop and Die Hel, which suggests that these bats may also utilise hibernacula within this subpopulation.

If bats that spend the summer in the fynbos migrate to hibernacula also located within this biome, they probably do not cover large distances during migration. This is supported by the wing morphology data (Chapter 6), which indicate that the fynbos bats have lower aspect ratio than those of the other subpopulations. Although De Hoop and Die Hel are ~190 km apart, if the bats from these colonies share one or more common hibernacula, the winter roosts are likely to be located somewhere between the two summer caves, reducing the required migration distance.

The reduced insect biomass around De Hoop probably cannot support the enormous *M. schreibersii* population during winter, necessitating migration away from this site by the majority of bats (McDonald *et al.* 1990a). Furthermore, as has been proposed for Koegelbeen, it is possible that these individuals also leave De Hoop in winter because the presence of such large numbers of bats makes the cave too warm for hibernation. In summer, temperatures inside De Hoop cave reach 31°C, and even during winter, with a reduced population size, temperatures may rise above 19°C (McDonald *et al.* 1990a). This is considerably warmer than other hibernacula in South Africa (*c.f.* Gauteng hibernacula: 10.5° – 12.6°C, van der Merwe 1973c); Eastern Cape hibernacula: 9.9° – 17.8°C, Brown & Bernard 1994). It has also been noted that the clustering behaviour of *M. schreibersii* at De Hoop varies depending on the season. In summer, the bats cluster closely together in very large groups, while in winter they roost separately (D. Jacobs, pers. comm.), perhaps to facilitate lowering of their body temperature to allow hibernation. The formation of smaller, looser clusters in winter has also been reported at Grahamstown (Brown & Bernard 1994) and in Namibia (Churchill *et al.* 1997). It is possible that the bats are able to survive the winter in a cave as warm as De Hoop because the winter rainfall pattern characteristic of the fynbos region may result in greater insect biomass during this season than is found in summer rainfall areas (McDonald *et al.* 1990a).

iii. North-eastern subpopulation

The genetics results indicate that considerable gene flow occurs between all colonies within the north-eastern subpopulation. This suggests that these bats share one or more mating (wintering) colonies. Van der Merwe (1973a, 1973b, 1975) identified three *M. schreibersii* hibernacula in the "Highveld" region of South Africa, near Pretoria (Figures 1.4 and 8.5). He also reported that some of the bats from these hibernacula utilise Peppercorn as a maternity roost, while others use different maternity sites, also located in the Northern Province (*e.g.* Sandspruit and Rookpoort Guano Caves; Van der Merwe 1975). It is therefore possible that at least some of the bats that use Sudwala as a maternity roost also migrate to these Highveld caves in winter, where they mate with the bats from Peppercorn. The genetics results further indicate that bats from Jozini Dam, Shongweni Dam and Maitland Mines are also closely related to those from Peppercorn and Sudwala, which suggests these bats may also migrate to the Highveld hibernacula. It is probable that there are many other roosts (both winter and summer) utilised by *M. schreibersii* from the north-eastern subpopulation, in addition to those already identified in the Highveld. Van der Merwe (1975) noted that only a small percentage of the bats from the hibernacula he identified migrated to Peppercorn in summer, therefore it is likely that these bats utilise other as yet unidentified hibernacula in this region, which they may share with bats from the other north-eastern colonies.

Although the bats at Grahamstown are genetically more closely related to individuals in the north-eastern subpopulation than to bats in either the southern or western subpopulations, they appear to show a small degree of genetic differentiation from some colonies within the north-eastern subpopulation, namely Shongweni Dam, Peppercorn and Sudwala. They are, however, very closely related to the bats at Maitland Mines and at Jozini Dam. Maitland Mines and Grahamstown are located close to one another (~138 km), and it is known that considerable movement occurs between these two sites during the year (R. Bernard, pers. comm.). It is therefore not surprising that there is significant gene flow between these colonies. The morphological results (Chapter 6) indicate that bats at Grahamstown have a wide range in aspect ratio. This intraspecific variation

may allow some of these bats (with low aspect ratio) either to remain at Grahamstown or to migrate to and breed at Maitland Mines in winter, while other Grahamstown bats with higher aspect ratios may be better adapted for long distance migration. These latter bats may migrate north to a winter (mating) roost that they share with some of the bats from Jozini Dam, allowing gene flow to occur between these colonies (Figure 8.5). Less extensive gene flow also occurs between the Grahamstown bats and those from the other north-eastern colonies. This may occur through the common utilisation of the same hibernacula, or as a result of interbreeding between the Grahamstown bats and those at Maitland Mines and Jozini Dam. Bats from Maitland Mines and Jozini Dam in turn interbreed with individuals from the remainder of the north-eastern subpopulation.

According to the biome boundaries defined by Rutherford (1997), the hibernacula identified by van der Merwe (1975) near Pretoria are located within the grassland biome rather than the savanna. However, O'Connor & Bredenkamp (1997) note that the vegetation around Pretoria is transitional between grassland and savanna, and is frequently invaded by "islands" of temperate bushveld, including tree and bush species characteristic of the savanna (*e.g. Acacia caffra* and *Rhus leptiodictya*). Acocks (1988) also noted the transitional nature of the vegetation in this region, and named it "false grassland". The major distinguishing feature between true savanna and true grassland is that grassland winters are colder: the amount and seasonality of rainfall is the same in the two biomes (O'Connor & Bredenkamp 1997). Therefore, even if the bats have adapted to local savanna-type conditions surrounding their maternity roosts, the "false grassland" conditions of their hibernacula are likely to be very similar in terms of rainfall and vegetation.

The lower winter temperatures of the hibernacula in the grassland biome are likely to be an advantage for hibernating bats, because lower ambient temperatures permit greater energy savings during hibernation (Brown 1999; Nagel & Nagel 1991). Climatic conditions in the warmer "bushveld" may be unsuitable for hibernation, requiring the bats to migrate to alternative, cooler wintering sites. These may be located either within the same biome (which have not yet been

identified), or at the boundary with another biome, which is ecologically sufficiently similar not to pose any adaptive disadvantage to the bats if they are adapted to savanna conditions. The same may apply to Grahamstown and Maitland Mines. The "false thornveld" (Acocks 1988) of this region is not true savanna, but the vegetation and/or rainfall patterns may be sufficiently similar to those in the true "bushveld" not to pose an adaptive disadvantage to bats moving into and out of this region.

8.5. ARE THE *M. SCHREIBERSII* SUBPOPULATIONS SEPARATE SUBSPECIES?

Cardinal & Christidis (2000) examined morphological and molecular (mtDNA) characteristics of *M. schreibersii* in Australia, and identified three geographically separate forms of this species, which they treated as separate subspecies. Prior to their study, two subspecies of *M. schreibersii* were recognised in Australia, namely *M. s. orianae* and *M. s. oceanensis*. However, based on significant differences in skull morphology, and large sequence divergences (3.05 – 4.66%) in cytochrome *b* and nicotinamine dehydrogenase subunit 2 (ND), the authors identified a third form, which they named *M. s. bassanii*. Because the sequence divergences measured in Cardinal & Christidis' (2000) study are based on cytochrome *b* and ND genes, they cannot be compared directly to the results of the present study, which examined the control region. However, because cytochrome *b* and ND are coding regions of mtDNA, they are likely to mutate slower than the non-coding control region (Brown *et al.* 1979). A sequence divergence estimate of 3.0 – 4.7% based on these genes probably represents greater differentiation than the equivalent degree of divergence in control region sequences (as detected between *M. schreibersii* subpopulations in South Africa). It is therefore premature to assign subspecific status to each of the South African *M.*

schreibersii subpopulations, because they do not appear to have diverged to the same extent as recognised subspecies of *M. schreibersii* in Australia. However, if the current patterns of philopatry and local adaptation continue in the long-term, it is likely that the three subpopulations will continue to diverge, which may ultimately result in speciation.

It is possible that the three subpopulations of *M. schreibersii* in South Africa are adapting to differing ecological conditions surrounding their roosts, and may be developing into "habitat races", as described by Kawecki (1997). Although allopatric speciation is the most commonly accepted method by which speciation events occur (White 1968), sympatric speciation is increasingly receiving recognition, particularly in the case of ecological speciation. This occurs when populations or subpopulations existing in contrasting environments (differing, for example, in climate, resources, competition or predation pressure) are subject to divergent selection (which selects for extreme, better adapted phenotypes rather than intermediate forms). This leads either directly or indirectly to the evolution of assortative mating and thus reproductive isolation (Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999; Schluter 2001; Tregenza & Butlin 1999). As a result, different "ecological races" of a species may gradually diverge genetically while coexisting geographically, until they constitute distinct species (Morell 1999; White 1968).

An extension of this theory of ecological speciation is that of habitat specialisation driven by deleterious mutations (Kawecki 1997). Under this model, organisms possess mutations that are deleterious in one habitat but neutral or advantageous in others. Alleles leading to specialisation and better adaptation to a specific habitat are selected for in preference to those that code for more generalised individuals, which may be able to survive in a range of environments, but do so less successfully. Selection that favours habitat fidelity leads to subdivision of the population into "habitat races". These are genetically isolated populations using differing habitats, but which are located within dispersal distance of one another. "Habitat races" are not considered full species, but once gene flow becomes restricted, assortative mating and reproductive isolation may result, and speciation may ultimately occur.

8.6. RECOMMENDATIONS FOR CONSERVATION AND FUTURE RESEARCH

Conservation-orientated legislation traditionally affords protection to species and subspecies, while divergent populations within a species' range may escape recognition and thus much-needed protection. However, the preservation of the long-term evolutionary potential of a species frequently depends on conserving such genetically differentiated populations. Fortunately, managing the genetic diversity of divergent populations is increasingly becoming a conservation priority, and such populations are thus gaining recognition as appropriate units for conservation action, regardless of their taxonomic status (Moritz 1994a). Accordingly, the concept of "evolutionarily significant units" or ESU's was developed to provide a basis for prioritising taxa for conservation priority. The concept provides a means of ensuring that the genetic diversity and thus evolutionary heritage of populations is recognised and protected (Moritz 1994b). To qualify as ESU's, populations must be historically isolated and exhibit significant divergence in allele frequencies. For example, Moritz (1994a, 1994b) recommends that ESU's should be monophyletic for different mtDNA haplotypes, and exhibit significant divergence of allele frequencies at nuclear loci. To achieve monophyly of mtDNA haplotypes, populations generally need to be separated for about $4N$ generations, where N is the population size.

In many cases, populations exhibit significant differentiation of both nuclear and mitochondrial alleles, but are not necessarily monophyletic for mtDNA haplotypes. Such populations cannot be classified as separate ESU's, but are nevertheless important for conservation attention because they represent populations that exchange such low levels of gene flow that they are functionally and demographically independent. Accordingly, they are recognised as "management units" or MU's (Moritz 1994b). While ESU's are important for preserving historical population structure and

planning of long-term conservation goals, MU's address current population structure and short-term management issues.

The three subpopulations of *M. schreibersii* in South Africa fulfil Moritz's (1994a, 1994b) genetic requirements of MU status, and it is therefore recommended that in future they be considered as such. The degree of mtDNA control region differentiation between the different subpopulations is frequently seen only in isolated populations of mammals (Avise 1989; Wilkinson & Fleming 1996), and this argues in favour of their recognition as separate MU's. It may even be argued that they are approaching ESU status, because the mtDNA haplotypes of the north-eastern subpopulation are monophyletic with respect to the southern and western subpopulations. However, the southern and western subpopulations share certain RFLP haplotypes, and are thus not monophyletic. Therefore the more conservative MU status rather than ESU status will be recommended. However, the possibility that the bats are becoming locally adapted to differing ecological conditions surrounding their roosts indicates that the subpopulations are functionally independent units, and could possibly even be considered distinct "habitat races" (Kawecki 1997). As separate MU's, the subpopulations should be managed independently, and the unique environmental conditions facing each subpopulation considered in the formulation of a management strategy.

Protection of wintering sites from disturbance is of particular importance. Arousal is energetically very costly and can account for over 75% of winter fat depletion (Thomas 1995). If bats are forced to arouse unnecessarily during their hibernation, and are unable to replenish their fat reserves due to low insect availability in winter, this may result in high levels of over-winter mortality (Thomas 1995). Studies have shown that handling bats during the hibernation period can have a significant effect on use of winter fat stores, and therefore on winter survival rates (Thomas 1995). Even non-tactile stimuli have been found to disturb hibernating bats, causing them to arouse and fly around the roost (Thomas 1995). Juveniles are particularly at risk because during their first summer and autumn they use most of the energy they gain from food for growing rather than laying down fat reserves (Norton & van der Merwe 1978). They are thus more susceptible to depleting their winter

fat supplies prematurely, leading to disproportionately high mortality rates (Barclay 1991; Thomas 1995). Human disturbance of wintering roosts should therefore be prevented or at least kept to a minimum to prevent increased mortality due to forced arousal and consequent premature depletion of fat reserves (Thomas 1995).

It is recommended that future research should focus on identifying potential aspects of each biome to which the bats may be becoming adapted, in order to form additional priorities and criteria for conservation attention. This should include a detailed dietary study, examining, for example, whether the biomes differ significantly in terms of seasonal prey abundance, primary targeted prey species, and seasonal changes in the bats' foraging strategies. This should be accompanied by an in depth examination of the vegetation types and climatic conditions surrounding each roost. In addition, future research should attempt to determine exactly where the bats are mating (at hibernacula, at maternity roosts, or at transitory mating roosts), and should examine whether bats belonging to different subpopulations have significantly different reproductive cycles. For example, do bats living in different biomes differ substantially in terms of timing of mating, implantation and parturition? Furthermore, virtually nothing is known about the mating behaviour of *M. schreibersii* in South Africa. Future studies could examine whether *M. schreibersii* does in fact follow a traditional, polygynous mating system, as has been assumed, whether males form harems or defend territories, and whether females mate with multiple males. Because microsatellite primers have now been developed for this species, a behavioural study on *M. schreibersii* mating behaviour should be combined with a microsatellite study examining, for example, kinship of females within a maternity colony. If males are also present in a maternity roost, this could be accompanied by paternity studies.

Although seasonal changes in the numbers of bats and the temperature of the roost have been monitored at some colonies that are occupied year-round, *e.g.* De Hoop (McDonald *et al.* 1990a), Grahamstown and Maitland Mines (Brown & Bernard 1994), this information is lacking for other roosts, such as Koegelbeen. It has been proposed here that the presence of large numbers of

bats in winter roosts may prevent hibernation because the roost would remain too warm, which necessitates that some bats migrate in winter to find alternative, cooler hibernacula. Future studies should address this issue by quantifying changes in temperature and number of individuals during the seasonal influx and efflux of bats.

Conservation efforts should also focus on changing peoples' negative attitudes towards bats. The many beneficial roles played by bats in the environment should be stressed during education campaigns, not least of which is that of controlling insect pest populations. The colony of *M. schreibersii* at De Hoop, for example, is estimated to consume 100 tons of insects per year, including many crop pest species (McDonald *et al.* 1990b).

Identification, genetic analysis and subsequent protection of as many *M. schreibersii* roosts as possible should be a conservation priority. Although numerous, the tendency of *M. schreibersii* to roost in few, isolated sites, in large numbers makes them vulnerable. Furthermore, the potentially strongly philopatric nature of this species suggests that they may have low capacity to re-colonise any sites where the species has become locally extinct. We do not fully understand the habitat requirements of most bat species, including *M. schreibersii*, and this substantially reduces our ability to predict the impact of habitat disturbance or destruction on bats (Fenton 1997). In many cases we do not know what should be the primary conservation priority: the bats themselves, their roosts or their foraging areas. Ideally, a conservation programme with a strong research base should incorporate all of these factors, particularly in the present case where it is apparent that *M. schreibersii* may have become adapted to local environmental conditions. It is therefore not sufficient only to protect the roosts themselves, but their surrounding ecosystems, particularly in terms of plant and insect diversity, also require urgent conservation attention.

CHAPTER 9

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APPENDIX 1

BUFFERS AND MEDIA

1. STORAGE BUFFERS

Extraction buffer

- 50 mM Tris-HCl pH 7.5
- 400 mM sodium chloride (NaCl)
- 100 mM ethylenedinitrilo tetra-acetic acid (EDTA)
- 0.5% sodium dodecyl sulphate (SDS)

1x Tris-EDTA (TE)

- 10 mM Tris-HCl (pH 7.4)
- 1 mM EDTA (pH 8)

2. CULTURE MEDIA

SOC medium (1000 ml)

- 20 g tryptone
- 5 g yeast extract
- 0.5 g NaCl
- 10 ml 250 mM KCl

Adjust to pH7 with 5 N NaOH.

Make up to volume with deionised water and autoclave.

Cool to 60°C, then add 20 ml 1M glucose and 5 ml 2 M $MgCl_2$

Luria Broth Plates (1000 ml)

- 10 g tryptone
- 5 g yeast extract
- 5 g NaCl
- 15 g agar

Make up to volume. Autoclave. Cool to 55°C and add ampicillin to 60 µg/ml before pouring plates.

Luria Broth (1000 ml)

- 10 g NaCl
- 10 g tryptone
- 5 g yeast extract

Adjust to volume. Autoclave. Cool to 55°C and add ampicillin to 60 µg/ml.

Glycerol stocks of frozen cells

- 65% glycerol
- 0.1 M $MgSO_4$
- 0.025 M Tris-Cl pH 8

Add 1ml freshly saturated culture to 1 ml glycerol solution and store at -70°C.

3. HYBRIDISATION BUFFERS

Church & Gilbert Solution (Prehybridisation / Hybridisation buffer)

- 1 mM EDTA
- 7% SDS
- 0.5 M phosphate buffer (see below)
- 0.5% non-fat milk powder

Medium stringency wash buffer

- 1% SDS
- 40 mM phosphate buffer (see below)
- 1 mM EDTA

1M Phosphate buffer (1000 ml) pH 7.2

- 134 g $Na_2HPO_4.7H_20$

pH to 7.2 with 85% H_3PO_4

20x SSC (sodium chloride - sodium citrate)

- 3 M NaCl (175 g/l)
- 0.3 M $Na_3Citrate2H_20$ (88 g/l)

Adjust to pH7 with 1 M HCl

3. MICROSATELLITE ANALYSIS

Polyacrylamide working solution for denaturing polyacrylamide gel

- 6% polyacrylamide stock solution (40% w/v acrylamide : bisacrylamide solution, 19:1; manufactured by Gibco BRL, supplied by Laboratory Specialist Scientific, Cape Town)
- 8 M urea
- 1x TBE buffer (see below)
- Distilled water to volume

To polymerise (100 ml) add:

- 350 µl 20% ammonium persulphate
- 80 µl TEMED (*N,N,N',N'*-Tetramethylethylenediamine)

10x stock TBE (Tris-Borate-EDTA) buffer

- 890 mM Tris-HCl
- 890 mM boric acid
- 20 mM EDTA (pH8)

Formamide stop solution and loading dye

- 98 % deionised formamide
- 10 mM EDTA (pH8)
- 0.025% xylene cyanol FF
- 0.025% bromophenol blue

APPENDIX 2

Trouble-shooting ligations

Table A2.1. Possible explanations for why initial ligations performed with the Fast-Link Ligation Kit (Epicentre Technologies) ceased working, summary of the methods used to test those explanations, and details of whether or not those explanations were valid.

Possible explanation	How validity of explanation was tested	Results of test	Likely explanation (yes or no)?
The ligations were working but electroporations were not.	Control transformations with undigested pUC18 were performed.	The control transformations worked well, therefore it was the ligations that were not working.	No
The plasmid was degraded or improperly cut (*i.e. Sma*I was not working properly).	Both uncut pUC18 and *Sma*I-pUC18 were electrophoresed through a 1% agarose gel with λ*Hind*III marker.	Both cut (linear) and uncut (circular) pUC18 produced bands in the expected positions (Harley and White, 1973) relative to the size marker.	No
Residual *Sma*I was inhibiting the ligations.	*Sma*I-pUC18 was placed in a 70°C water bath for 15 minutes before being used in the ligations, to inactivate any residual *Sma*I.	No ligation product was obtained.	No (but this would improve ligations when insert DNA was present).
The concentration of vector DNA in the ligation mix was not optimal.	The concentration of plasmid at which there should be equal amounts of linearised and recircularised plasmid was determined to be ~40 ng/µl for pUC18*. Higher concentrations of plasmid promote linearisation; lower concentrations promote re-circularisation. Ligations containing plasmid at concentrations of 20, 40 and 80 ng/µl were performed. * [plasmid] (mg/ml) = $\frac{51.1}{(\text{Nº base pairs} \times 660)^{\frac{1}{2}}}$	No ligation product was obtained at any of the plasmid concentrations tested.	No (although varying DNA content should influence ligation efficiency once ligations are working).

Table A2.1 cont. Possible explanations for why initial ligations performed with the Fast-Link Ligation Kit (Epicentre Technologies) ceased working, summary of the methods used to test those explanations, and details of whether or not those explanations were valid.

Possible explanation	How validity of explanation was tested	Results of test	Likely explanation (yes or no)?
Ligation reactions were not conducted for long enough duration.	The instructions in the Fast-Link Ligation and Screening Kit (Epicentre Technologies) suggested 5 minutes; ligation times of 15 minutes and overnight were attempted.	Longer ligation times did not produce any ligation product.	No
The ligation temperature was not optimal, or it varied during the ligation reaction.	The instructions in the kit suggested room temperature. Ligation reactions were placed in a 20°C water bath to maintain a constant temperature.	No ligation product was obtained.	No
The ligation buffer was contaminated.	Three different Fast-Link Ligation Kits were tested (see below).	No ligation product obtained with any of the kits.	Possible (see below)
The ligase and/or ATP supplied with the kit were no longer active.	• Three different Fast-Link Ligation Kits available in the lab were tested on *Sma*I-pUC18 and λ *Hind*III. • New T4 DNA ligase with buffer (Promega) was purchased. Ligations were performed with this ligase and new ATP on both *Sma*I-pUC18 and λ*Hind*III fragments.	• All three kits failed to ligate either *Sma*I-pUC18 or λ*Hind*III (ligation products of different sizes, including larger than the original *Sma*I-pUC18 or λ*Hind*III fragments should be visible on the gel; they were not). • The new ligase ligated both *Sma*I-pUC18 and λ*Hind*III; fragments larger than the original (unligated) fragments were visible on a 1% agarose gel.	**Yes** All kits were more than six months old; it is possible that the ligase and/or ATP had become inactive, or that the kits were contaminated. The content of the buffers provided in the kits is unknown, thus one cannot replace individual constituents to assess their efficiency.

APPENDIX 3

EXAMPLES OF MICROSATELLITE GELS

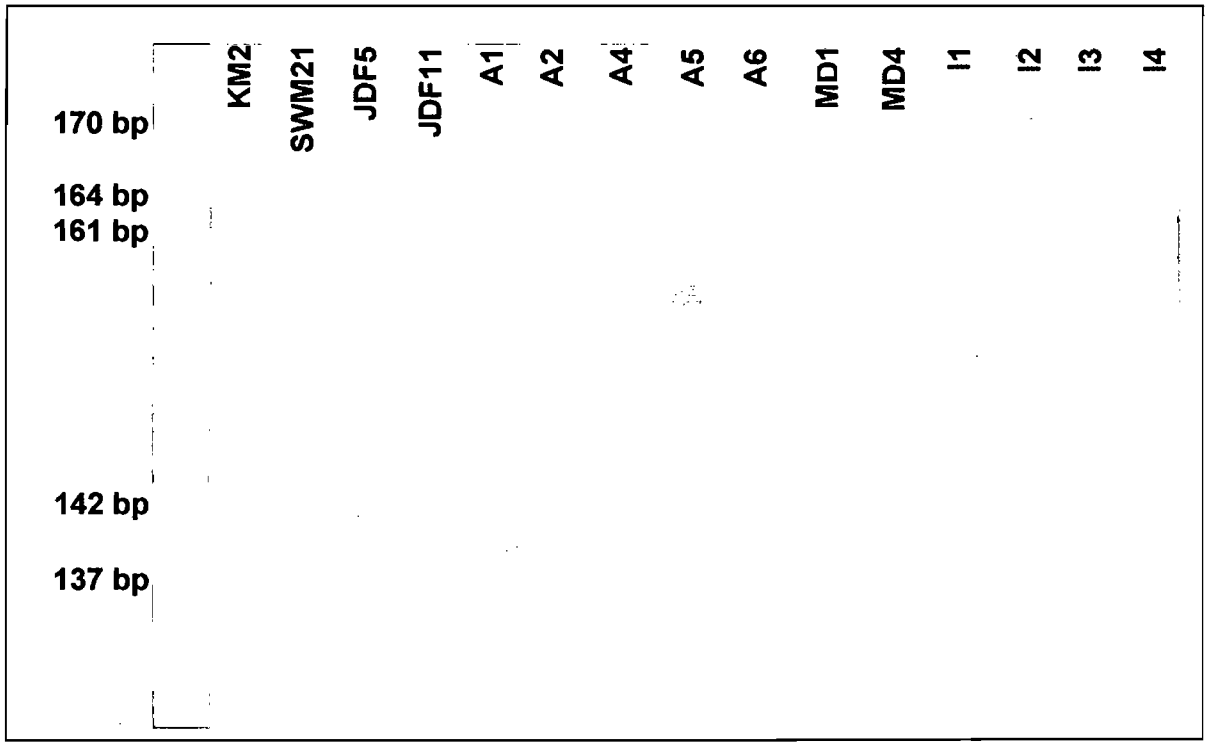

Figure A3.1. Example of a gel from locus Mschreib3. Individuals from Knysna (KM2), Sudwala (SWM21), Jozini Dam (JDF11), Australia (A1 – A6), Madagascar (MD1 and MD4) and Israel (I1 – I4) are shown.

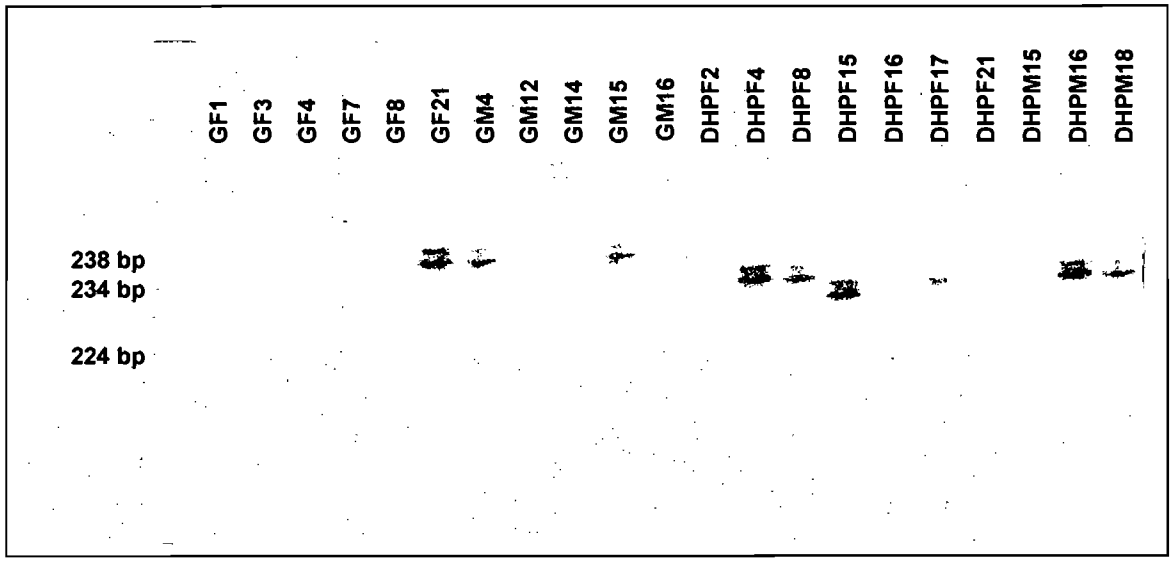

Figure A3.2. Example of a gel from the NCAM locus. Individuals from Grahamstown (GF1 – GM16) and De Hoop (DHPF2 – DHPM18) colonies are shown.

APPENDIX 4

BLAST SEARCH RESULTS for presumed *M. schreibersii* mtDNA control region sequence

BLASTN 2.2.1 [Apr-13-2001]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 996933533-8176-29364

Query= (534 letters)

Database: nt 928,293 sequences; 3,654,550,933 total letters

If you have any problems or questions with the results of this search please refer to the **BLAST FAQs**

Taxonomy reports

Sequences producing significant alignments:	Score (bits)	E Value
gi|2952042|gb|U95329.1|MSU95329 Miniopteros schreibersi mit...	192	2e-46 *
gi|14248904|gb|AF376830.1|AF376830 Miniopterus schreibersii...	176	1e-41 *
gi|11610804|ref|NC_002626.1| Chalinolobus tuberculatus mito...	123	1e-25 *
gi|11545689|gb|AF321051.1|AF321051 Chalinolobus tuberculatu...	123	1e-25 *
gi|11386118|ref|NC_002612.1| Pteropus dasymallus mitochondr...	100	2e-18 *
gi|11231066|dbj|AB042770.1|AB042770 Pteropus dasymallus mit...	100	2e-18 *
gi|4235294|gb|AF081038.1|AF081038 Eulemur rubriventer mitoc...	86	3e-14 *
gi|4235293|gb|AF081037.1|AF081037 Eulemur mongoz mitochondr...	86	3e-14 *
gi|4235292|gb|AF081036.1|AF081036 Eulemur macaco flavifrons...	86	3e-14 *
gi|4235291|gb|AF081035.1|AF081035 Eulemur macaco macaco mit...	86	3e-14 *
gi|4235289|gb|AF081033.1|AF081033 Eulemur fulvus rufus mito...	86	3e-14 *
gi|4235290|gb|AF081034.1|AF081034 Eulemur fulvus albifrons ...	82	5e-13 *
gi|7212513|ref|NC_002391.1| Talpa europaea mitochondrion, c...	80	2e-12 *
gi|6851065|emb|Y19192.1|TEUY19192 Talpa europaea complete m...	80	2e-12 *
gi|6651178|gb|AF142096.1|AF142096 Ceratotherium simum nucle...	78	7e-12 *
gi|4235288|gb|AF081032.1|AF081032 Eulemur fulvus collaris m...	78	7e-12
gi|13365537|dbj|AB048588.1|AB048588 Canis familiaris nuclea...	76	3e-11
gi|5835331|ref|NC_001779.1| Rhinoceros unicornis mitochondr...	74	1e-10
gi|4235283|gb|AF081027.1|AF081027 Propithecus tattersalli m...	74	1e-10
gi|2641302|gb|AF031767.1|AF031767 Peromyscus leucopus strai...	74	1e-10
gi|2641301|gb|AF031766.1|AF031766 Peromyscus leucopus strai...	74	1e-10
gi|2641300|gb|AF031765.1|AF031765 Peromyscus leucopus strai...	74	1e-10
gi|2641298|gb|AF031763.1|AF031763 Peromyscus leucopus strai...	74	1e-10
gi|2641297|gb|AF031762.1|AF031762 Peromyscus leucopus strai...	74	1e-10
gi|2641296|gb|AF031761.1|AF031761 Peromyscus leucopus strai...	74	1e-10
gi|2641295|gb|AF031760.1|AF031760 Peromyscus leucopus strai...	74	1e-10
gi|2641294|gb|AF031759.1|AF031759 Peromyscus leucopus strai...	74	1e-10
gi|7161840|emb|AJ287971.1|LCA287971 Lepus capensis mitochon...	74	1e-10
gi|1666193|emb|X97336.1|RUMTGENOM Rhinoceros unicornis comp...	74	1e-10
gi|13310075|gb|AF285451.1|AF285451 Propithecus verreauxi co...	72	4e-10
gi|5835401|ref|NC_001808.1| Ceratotherium simum mitochondri...	72	4e-10
gi|4235286|gb|AF081030.1|AF081030 Hapalemur griseus mitocho...	72	4e-10
gi|4235285|gb|AF081029.1|AF081029 Varecia variegata variega...	72	4e-10

* Alignments shown on pages A7-A11

gi|4235284|gb|AF081028.1|AF081028	Varecia variegata rubra m...	72	4e-10
gi|3169646|gb|AF065088.1|AF065088	Rhinolophus megaphyllus i...	72	4e-10
gi|3169645|gb|AF065087.1|AF065087	Rhinolophus megaphyllus i...	72	4e-10
gi|3169644|gb|AF065086.1|AF065086	Rhinolophus megaphyllus i...	72	4e-10
gi|3169643|gb|AF065085.1|AF065085	Rhinolophus megaphyllus i...	72	4e-10
gi|3169642|gb|AF065084.1|AF065084	Rhinolophus megaphyllus i...	72	4e-10
gi|3169641|gb|AF065083.1|AF065083	Rhinolophus megaphyllus i...	72	4e-10
gi|3169640|gb|AF065082.1|AF065082	Rhinolophus megaphyllus i...	72	4e-10
gi|3169639|gb|AF065081.1|AF065081	Rhinolophus megaphyllus i...	72	4e-10
gi|3169638|gb|AF065080.1|AF065080	Rhinolophus megaphyllus i...	72	4e-10
gi|3169637|gb|AF065079.1|AF065079	Rhinolophus megaphyllus i...	72	4e-10
gi|3169636|gb|AF065078.1|AF065078	Rhinolophus megaphyllus i...	72	4e-10
gi|3169635|gb|AF065077.1|AF065077	Rhinolophus megaphyllus i...	72	4e-10
gi|3169634|gb|AF065076.1|AF065076	Rhinolophus philippinensi...	72	4e-10
gi|3169632|gb|AF065074.1|AF065074	Rhinolophus philippinensi...	72	4e-10
gi|3169631|gb|AF065073.1|AF065073	Rhinolophus philippinensi...	72	4e-10
gi|3169629|gb|AF065071.1|AF065071	Rhinolophus philippinensi...	72	4e-10
gi|3169628|gb|AF065070.1|AF065070	Rhinolophus philippinensi...	72	4e-10
gi|3169627|gb|AF065069.1|AF065069	Rhinolophus philippinensi...	72	4e-10
gi|2052151|emb|Y07726.1|MTCSXX	Ceratotherium simum complete...	72	4e-10
gi|2952056|gb|U95343.1|NHU95343	Nycticeius humeralis mitoch...	70	2e-09
gi|2952054|gb|U95341.1|MAU95341	Myotis adversus tRNA-Thr an...	70	2e-09
gi|3169630|gb|AF065072.1|AF065072	Rhinolophus philippinensi...	68	7e-09
gi|7161851|emb|AJ287982.1|LSI287982	Lepus sinensis mitochon...	68	7e-09
gi|7161839|emb|AJ287970.1|LCA287970	Lepus capensis mitochon...	68	7e-09
gi|7161835|emb|AJ287966.1|LCA287966	Lepus capensis mitochon...	68	7e-09
gi|7161834|emb|AJ287965.1|LCA287965	Lepus capensis mitochon...	68	7e-09
gi|7161833|emb|AJ287964.1|LCA287964	Lepus capensis mitochon...	68	7e-09
gi|7161827|emb|AJ287958.1|LCA287958	Lepus capensis mitochon...	68	7e-09
gi|11602891|ref|NC_002619.1|	Pteropus scapulatus mitochondr...	66	3e-08
gi|11545675|gb|AF321050.1|AF321050	Pteropus scapulatus mito...	66	3e-08
gi|13310113|gb|AF285489.1|AF285489	Microcebus murinus YLE19...	66	3e-08
gi|13310112|gb|AF285488.1|AF285488	Microcebus murinus 00-01...	66	3e-08
gi|13310110|gb|AF285486.1|AF285486	Microcebus murinus Jorg ...	66	3e-08
gi|7677258|gb|AF213966.1|AF213966	Brachyteles arachnoides h...	66	3e-08
gi|7677257|gb|AF213965.1|AF213965	Lagothrix lagotricha hype...	66	3e-08
gi|7677256|gb|AF213964.1|AF213964	Alouatta palliata hyperva...	66	3e-08
gi|2641342|gb|AF031807.1|AF031807	Peromyscus gossypinus str...	66	3e-08
gi|2641340|gb|AF031805.1|AF031805	Peromyscus leucopus strai...	66	3e-08
gi|1398932|gb|U59753.1|MSU59753	Monachus schauinslandi mito...	66	3e-08
gi|14599763|gb|AF348080.1|AF348080	Ochotona collaris mitoch...	64	1e-07
gi|14599791|gb|AF348082.1|AF348082	Volemys kikuchii mitocho...	64	1e-07
gi|5835345|ref|NC_001788.1|	Equus asinus mitochondrion, com...	64	1e-07
gi|12006030|gb|AF267284.1|AF267284	Microtus nivalis tRNA-Pr...	64	1e-07
gi|13310115|gb|AF285491.1|AF285491	Microcebus griseorufus Y...	64	1e-07

* Alignments (only top 15 shown)

```
>gi|2952042|gb|U95329.1|MSU95329 Miniopteros schreibersi mitochondrial D-loop, partial sequence
          Length = 302
 Score =  192 bits (97), Expect = 2e-46
 Identities = 206/240 (85%), Gaps = 2/240 (0%)
 Strand = Plus / Plus
Query: 292 ctatgtacatcgtgcattaactttatatccccatgaatatgtagcatgtacatttatgat 351
           |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
Sbjct: 22  ctatgtacatcgtgcattaactttatttccccatgaatatgtagcatgtacatttatgat 81

Query: 352 catagattacatgagtacataatattatttatcgtacatagaccatgatatgtgacaaat 411
           | |  ||||||||||||||| ||||||| ||||||| ||| |||||   |||||| ||||
Sbjct: 82  cttgtattacatgagtacattatattatatatcgtanataaaccatctgatgtgataaat 141

Query: 412 tctagtcagcatgactatcccacaggtattgttggtttaacagactcaccatcctccgtg 471
           || ||||| ||||||||| |  || | | ||||||||||  |  || ||||||||||||
Sbjct: 142 tccagtcaacatgactattctgcaagcactgttggtttattaatct-accatcctccgtg 200

Query: 472 aaaccagcaacccgcccacaagcgtgttacccttctcgccccgggcccattaaccgtggg 531
           ||||| || |||||||||| | |||||  | ||||||||||||||||||| || ||||||
Sbjct: 201 aaacccgccacccgcccacca-cgtgtccctcttctcgccccgggcccataaatcgtggg 259


>gi|14248904|gb|AF376830.1|AF376830 Miniopterus schreibersii cytochrome b (cytb) gene, complete cds;
          mitochondrial gene for mitochondrial product
   Length = 1140
 Score =  176 bits (89), Expect = 1e-41
 Identities = 110/117 (94%)
```

```
Query: 4     ccagtagaacatccctatgttatcatcggccaactagcatctattctttacttttaggatc 63
              |||||||||||||||||| |||| || |||||||| |||||||| |||||||||| ||||
Sbjct: 1024 ccagtagaacatccctatattattattggccaactggcatctatcctttacttttatgatc 1083

Query: 64    attcttgttctcataccacttatcagcattatagaaaaccatctactcaagtgaaaga 120
              ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1084 attctagttctcataccacttatcagcattatagaaaaccatctactcaagtgaaaga 1140


>gi|11610804|ref|NC_002626.1| Chalinolobus tuberculatus mitochondrion, complete genome
          Length = 16818
 Score =  123 bits (62), Expect = 1e-25
 Identities = 158/190 (83%)
 Strand = Plus / Plus

Query: 4     ccagtagaacatccctatgttatcatcggccaactagcatctattctttacttttaggatc 63
              ||||| ||||| || || ||||||||||||||||||||||| || || |||||    ||
Sbjct: 15175 ccagtcgaacacccatacgttatcatcggccaactagcatcaatcctgtacttcctaatt 15234

Query: 64    attcttgttctcataccacttatcagcattatagaaaaccatctactcaagtgaagagtc 123
              ||| |||| || ||||| || | |||  | ||||||||||||| || | || |||||||||
Sbjct: 15235 attattgtactaataccccctaaccagtctcatagaaaaccatttattgaaatgaagagtc 15294

Query: 124   tatgtagtatacttattacactggtcttgtaaaccagaaatgaggaaaacaattcctcca 183
              ||||||||||||   |||||||||||||||||||||||||| |||||| | ||||||||||
Sbjct: 15295 tatgtagtatatcaattacactggtcttgtaaaccagaaaagaggaagaaaattcctcca 15354

Query: 184   gagactcaag 193
               |||||||||
Sbjct: 15355 aagactcaag 15364

 Score = 67.9 bits (34), Expect = 7e-09
 Identities = 53/58 (91%), Gaps = 1/58 (1%)
 Strand = Plus / Plus

Query: 464   cctccgtgaaaccagcaacccgcccacaagcgtgttacccttctcgccccgggcccat 521
              |||||||||||||| |||||||||| || ||| | ||||||||||||||||||||
Sbjct: 15776 cctccgtgaaaccaacaacccgccca-aaacgtatcacccttctcgccccgggcccat 15832


>gi|11545689|gb|AF321051.1|AF321051 Chalinolobus tuberculatus mitochondrial DNA, complete genome
          Length = 16818
 Score =  123 bits (62), Expect = 1e-25
 Identities = 158/190 (83%) Strand = Plus / Plus
Query: 4     ccagtagaacatccctatgttatcatcggccaactagcatctattctttacttttaggatc 63
              ||||| ||||| || || ||||||||||||||||||||||| || || |||||    ||
Sbjct: 15175 ccagtcgaacacccatacgttatcatcggccaactagcatcaatcctgtacttcctaatt 15234

Query: 64    attcttgttctcataccacttatcagcattatagaaaaccatctactcaagtgaagagtc 123
              ||| |||| || ||||| || | |||  | ||||||||||||| || | || |||||||||
Sbjct: 15235 attattgtactaataccccctaaccagtctcatagaaaaccatttattgaaatgaagagtc 15294

Query: 124   tatgtagtatacttattacactggtcttgtaaaccagaaatgaggaaaacaattcctcca 183
              ||||||||||||   |||||||||||||||||||||||||| |||||| | ||||||||||
Sbjct: 15295 tatgtagtatatcaattacactggtcttgtaaaccagaaaagaggaagaaaattcctcca 15354
Query: 184   gagactcaag 193
               |||||||||
Sbjct: 15355 aagactcaag 15364

 Score = 67.9 bits (34), Expect = 7e-09
 Identities = 53/58 (91%), Gaps = 1/58 (1%)
 Strand = Plus / Plus

Query: 464   cctccgtgaaaccagcaacccgcccacaagcgtgttacccttctcgccccgggcccat 521
              |||||||||||||| |||||||||| || ||| | ||||||||||||||||||||
Sbjct: 15776 cctccgtgaaaccaacaacccgccca-aaacgtatcacccttctcgccccgggcccat 15832


>gi|11386118|ref|NC_002612.1| Pteropus dasymallus mitochondrion, complete genome
          Length = 16705
 Score = 99.6 bits (50), Expect = 2e-18
 Identities = 200/246 (81%), Gaps = 3/246 (1%)
 Strand = Plus / Plus

Query: 10    gaacatccctatgttatcatcggccaactagcatctattctttacttttaggatcattctt 69
              |||||||| | | |||||||||||||||||||| || || || ||    ||||||||
Sbjct: 15211 gaacatccatttattatcatcggccaactagcatccatcctatatttccttatcattcta 15270
```

```
Query: 70    gttctcataccacttatcagcattatagaaaaccatctactcaagtgaagagtctatgta 129
             ||||| |||||  | |  |||||| |||||||||||||| || || ||||| |||||||||
Sbjct: 15271 gttctgatacccatcacaagcattgtagaaaaccatctcctaaaatgaag-gtctatgta 15329

Query: 130   gtatacttattacactggtcttgtaaaccagaaatgagga-aaacaattcctccagagac 188
             |||||   ||||||||||||||||||||||||||| | |||  ||| | ||| ||||||||
Sbjct: 15330 gtatataaattacactggtcttgtaaaccagaaaaggggagcaacca-tcccccagagac 15388

Query: 189   tcaaggaaagagcatgagccctaccgtcagcacccaaagctgaaattctacttaaactac 248
             ||||||||  | |   ||  | ||| ||| ||||||||||||| ||||||||||||||
Sbjct: 15389 tcaaggaagaaactcaagttccaccatcaacacccaaagctgatattctacttaaactat 15448

Query: 249   tccttg 254
             ||||||
Sbjct: 15449 tccttg 15454
```

>gi|11231066|dbj|AB042770.1|AB042770 Pteropus dasymallus mitochondrial DNA, complete genome
Length = 16705
Score = 99.6 bits (50), Expect = 2e-18
Identities = 200/246 (81%), Gaps = 3/246 (1%)
Strand = Plus / Plus

```
Query: 10    gaacatccctatgttatcatcggccaactagcatctattctttactttaggatcattctt 69
             |||||||| | | ||||||||||||||||||||| || || || ||    ||||||||
Sbjct: 15211 gaacatccatttattatcatcggccaactagcatccatcctatatttccttatcattcta 15270

Query: 70    gttctcataccacttatcagcattatagaaaaccatctactcaagtgaagagtctatgta 129
             ||||| |||||  | |  |||||| |||||||||||||| || || ||||| |||||||||
Sbjct: 15271 gttctgatacccatcacaagcattgtagaaaaccatctcctaaaatgaag-gtctatgta 15329

Query: 130   gtatacttattacactggtcttgtaaaccagaaatgagga-aaacaattcctccagagac 188
             |||||   ||||||||||||||||||||||||||| | |||  ||| | ||| ||||||||
Sbjct: 15330 gtatataaattacactggtcttgtaaaccagaaaaggggagcaacca-tcccccagagac 15388

Query: 189   tcaaggaaagagcatgagccctaccgtcagcacccaaagctgaaattctacttaaactac 248
             ||||||||  | |   ||  | ||| ||| ||||||||||||| ||||||||||||||
Sbjct: 15389 tcaaggaagaaactcaagttccaccatcaacacccaaagctgatattctacttaaactat 15448

Query: 249   tccttg 254
             ||||||
Sbjct: 15449 tccttg 15454
```

>gi|4235294|gb|AF081038.1|AF081038 Eulemur rubriventer mitochondrial control region, partial sequence
Length = 464
Score = 85.7 bits (43), Expect = 3e-14
Identities = 49/51 (96%)
Strand = Plus / Plus

```
Query: 205 agccctaccgtcagcacccaaagctgaaattctacttaaactactccttgc 255
           ||||| ||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11  agccccaccttcagcacccaaagctgaaattctacttaaactactccttgc 61
```

Score = 60.0 bits (30), Expect = 2e-06
Identities = 30/30 (100%)
Strand = Plus / Plus

```
Query: 459 accatcctccgtgaaaccagcaacccgccc 488
           ||||||||||||||||||||||||||||||
Sbjct: 404 accatcctccgtgaaaccagcaacccgccc 433
```

>gi|4235293|gb|AF081037.1|AF081037 Eulemur mongoz mitochondrial control region, partial sequence
Length = 466
Score = 85.7 bits (43), Expect = 3e-14
Identities = 49/51 (96%)
Strand = Plus / Plus

```
Query: 205 agccctaccgtcagcacccaaagctgaaattctacttaaactactccttgc 255
           ||||| ||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11  agccccaccttcagcacccaaagctgaaattctacttaaactactccttgc 61
```

Score = 63.9 bits (32), Expect = 1e-07
Identities = 32/32 (100%)
Strand = Plus / Plus

```
Query: 459 accatcctccgtgaaaccagcaacccgcccac 490
           ||||||||||||||||||||||||||||||
Sbjct: 406 accatcctccgtgaaaccagcaacccgcccac 437
```

>gi|4235292|gb|AF081036.1|AF081036 Eulemur macaco flavifrons mitochondrial control region, partial sequence

```
          Length = 466
 Score = 85.7 bits (43), Expect = 3e-14
 Identities = 49/51 (96%)
 Strand = Plus / Plus

Query: 205 agccctaccgtcagcacccaaagctgaaattctacttaaactactccttgc 255
           ||||| ||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11  agccccaccctcagcacccaaagctgaaattctacttaaactactccttgc 61

 Score = 60.0 bits (30), Expect = 2e-06
 Identities = 30/30 (100%)
 Strand = Plus / Plus

Query: 459 accatcctccgtgaaaccagcaacccgccc 488
           ||||||||||||||||||||||||||||||
Sbjct: 406 accatcctccgtgaaaccagcaacccgccc 435
```

>gi|4235291|gb|AF081035.1|AF081035 Eulemur macaco macaco mitochondrial control region, partial sequence

```
          Length = 467
 Score = 85.7 bits (43), Expect = 3e-14
 Identities = 49/51 (96%)
 Strand = Plus / Plus

Query: 205 agccctaccgtcagcacccaaagctgaaattctacttaaactactccttgc 255
           ||||| ||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11  agccccaccttcagcacccaaagctgaaattctacttaaactactccttgc 61

 Score = 60.0 bits (30), Expect = 2e-06
 Identities = 30/30 (100%)
 Strand = Plus / Plus

Query: 459 accatcctccgtgaaaccagcaacccgccc 488
           ||||||||||||||||||||||||||||||
Sbjct: 407 accatcctccgtgaaaccagcaacccgccc 436
```

>gi|4235289|gb|AF081033.1|AF081033 Eulemur fulvus rufus mitochondrial control region, partial sequence

```
          Length = 467
 Score = 85.7 bits (43), Expect = 3e-14
 Identities = 49/51 (96%)
 Strand = Plus / Plus

Query: 205 agccctaccgtcagcacccaaagctgaaattctacttaaactactccttgc 255
           ||||| ||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 11  agccccaccttcagcacccaaagctgaaattctacttaaactactccttgc 61
 Score = 60.0 bits (30), Expect = 2e-06
 Identities = 30/30 (100%)
 Strand = Plus / Plus

Query: 459 accatcctccgtgaaaccagcaacccgccc 488
           ||||||||||||||||||||||||||||||
Sbjct: 407 accatcctccgtgaaaccagcaacccgccc 436
```

>gi|4235290|gb|AF081034.1|AF081034 Eulemur fulvus albifrons mitochondrial control region, partial sequence

```
          Length = 467
 Score = 81.8 bits (41), Expect = 5e-13
 Identities = 41/41 (100%)
 Strand = Plus / Plus

Query: 215 tcagcacccaaagctgaaattctacttaaactactccttgc 255
           |||||||||||||||||||||||||||||||||||||||||
Sbjct: 21  tcagcacccaaagctgaaattctacttaaactactccttgc 61

 Score = 60.0 bits (30), Expect = 2e-06
 Identities = 30/30 (100%)
 Strand = Plus / Plus
```

```
Query: 459 accatcctccgtgaaaccagcaacccgccc 488
           ||||||||||||||||||||||||||||||
Sbjct: 407 accatcctccgtgaaaccagcaacccgccc 436


>gi|7212513|ref|NC_002391.1| Talpa europaea mitochondrion, complete genome
          Length = 16884
 Score = 79.8 bits (40), Expect = 2e-12
 Identities = 64/72 (88%)
 Strand = Plus / Plus

Query: 94    atagaaaaccatctactcaagtgaagagtctatgtagtatacttattacactggtcttgt 153
              ||||||||| | |||||||| |||||||||| |||||||||  |||||| ||||||||||
Sbjct: 15298 atagaaaacaacctactcaaatgaagagtctttgtagtataactattactctggtcttgt 15357

Query: 154   aaaccagaaatg 165
              ||||||| ||||
Sbjct: 15358 aaaccagcaatg 15369


>gi|6851065|emb|Y19192.1|TEUY19192 Talpa europaea complete mitochondrial genome
          Length = 16884
 Score = 79.8 bits (40), Expect = 2e-12
 Identities = 64/72 (88%)
 Strand = Plus / Plus

Query: 94    atagaaaaccatctactcaagtgaagagtctatgtagtatacttattacactggtcttgt 153
              ||||||||| | |||||||| |||||||||| |||||||||  |||||| ||||||||||
Sbjct: 15298 atagaaaacaacctactcaaatgaagagtctttgtagtataactattactctggtcttgt 15357

Query: 154   aaaccagaaatg 165
              ||||||| ||||
Sbjct: 15358 aaaccagcaatg 15369


>gi|6651178|gb|AF142096.1|AF142096 Ceratotherium simum nuclear cytb, tRNA-Thr and tRNA-Pro
pseudogenes, mitochondrial control region, and tRNA-Phe pseudogene, complete sequence; and 12S
ribosomal RNA pseudogene, partial sequence
          Length = 2808
 Score = 77.8 bits (39), Expect = 7e-12
 Identities = 66/75 (88%)
 Strand = Plus / Plus

Query: 89   gcattatagaaaaccatctactcaagtgaagagtctatgtagtatacttattacactggt 148
             ||||||| |||||| | |||||||| |||||||||| |||||||||   ||||| |||||
Sbjct: 1109 gcattattgaaaacaacctactcaaatgaagagtctttgtagtatataaattaccctggt 1168

Query: 149  cttgtaaaccagaaa 163
             |||||||||||||||
Sbjct: 1169 cttgtaaaccagaaa 1183

 Score = 75.8 bits (38), Expect = 3e-11
 Identities = 47/50 (94%)
 Strand = Plus / Plus

Query: 205  agccctaccgtcagcacccaaagctgaaattctacttaaactactccttg 254
             ||||| ||| ||| ||||||||||||||||||||||||||||||||||
Sbjct: 1227 agccccaccatcaacacccaaagctgaaattctacttaaactactccttg 1276

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  Number of letters in database: -640,416,359
  Number of sequences in database:  928,293

Lambda     K      H
    1.37    0.711     1.31
Gapped
Lambda     K      H
    1.37    0.711     1.31
Matrix: blastn matrix:1 -3
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 1,014,911
Number of Sequences: 928293
Number of extensions: 1014911
Number of successful extensions: 32926
Number of sequences better than 10.0: 4488
length of query: 534
length of database: 3,654,550,933
effective HSP length: 20
effective length of query: 514
effective length of database: 3,635,985,073
effective search space: 1868896327522
effective search space used: 1868896327522
T: 0
A: 30
X1: 6 (11.9 bits)
X2: 15 (29.7 bits)
S1: 12 (24.3 bits)
S2: 19 (38.2 bits)
```

APPENDIX 5

mtDNA SEQUENCE ALIGNMENTS

```
                                                                                                                                  80
DHP1  CAACCAGTAG  AACATCCCTA  TATTATCATC  GGTCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
DHP2  CAACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
DHL1  CAACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
DHL2  GCACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTTATACC
SKK   CACCCAGTAG  AACATCCCTA  TGTTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
KB    CACCCAGTAG  AACATCCCTA  TGTTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTAGG  ATCATTCTTG  TTCTCATACC
G1    CAACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
G2    CAACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
MM1   CAACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
MM2   GCACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
SHD   CAACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
JD1   CAACCAGTAG  AACATCCCTA  TATCATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
JD2   GCACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
PRB   CAACCAGTAG  AACATCCCTA  TATCATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
PC    CAACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
SW1   CAACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
SW2   CAACCAGTAG  AACACCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
SW3   CAACCAGTAG  AACATCCCTA  TATTATCATC  GGCCAACTAG  CATCTATTCT  TTACTTTATG  ATCATTCTTG  TTCTCATACC
MD    CAACCAGTAG  AACATCCCTA  TATCATTATT  GGCCAACTAG  CATCTATCCT  TTACTTTATG  ATCATTCTTA  TTCTCATACC
I     CAACCAGTAG  AACACCCCTA  TATTATTATT  GGCCAACTAG  CATCTATCCT  TTACTTTATG  ATCATTCTAG  TTCTCATACC
AB    CAACCAGTGG  AACACCCCTA  CATCATCATC  GGCCAACTAG  CATCCATCCT  TTACTTTATG  ATCATTCTTA  TTCTCATACC
AO    CAACCAGTGG  AACACCCCTA  TATCATCATC  GGCCAACTAG  CATCCATCCT  TTACTTTATG  ATCATTCTTA  TTCTCATACC
MF1   CAACCAGTAG  AACACCCCTA  CATCATTATT  GGACAGCTAG  CATCCATCCT  TTACTTTATG  ATCATTCTTA  TTCTCATACC
MF2   CAACCAGTAG  AACACCCCTA  CATCATTATT  GGACAACTAG  CATCCATCCT  TTACTTTATG  ATCATTCTTA  TTCTCATACC
MF3   CACCCAGTGG  AACACCCCTA  CATCATTATA  GGACAACTAG  CATCCATCCT  TTACTTTATG  ATCATTCTTA  TTCTCATACC
MF4   GCACCAGTAG  AACACCCCTA  CATCATTATT  GGACAACTAG  CATCCATCCT  TTACTTTATG  ATCATTCTTA  TTCTCATACC
MF5   CAACCAGTAG  AACACCCCTA  CATCATTATT  GGACAACTAG  CATCCATCCT  TTACTTTATG  ATCATTCTTA  TTCTCATACC

                                                                                                                                 160
DHP1  ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
DHP2  ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
DHL1  ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
DHL2  ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
SKK   ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
KB    ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
G1    ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
G2    ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
MM1   ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
MM2   ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
SHD   ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
JD1   ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
JD2   ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
PRB   ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
PC    ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
SW1   ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
SW2   ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
SW3   ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
MD    ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
I     ACTTATCAGC  ATTATAGAAA  ACCATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACCCATT  ACACTGGTCT  TGTAAACCAG
AB    ACTCATCAGC  ATTATAGAAA  ATCACCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTCATT  ACACTGGTCT  TGTAAACCAG
AO    ACTCATCAGC  ATTATAGAAA  ATCACCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTCATT  ACACTGGTCT  TGTAAACCAG
MF1   ACTTGTCAGC  ATTATAGAAA  ACTATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
MF2   ACTTGTCAGC  ATTATAGAAA  ACTATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
MF3   ACTTGTCAGC  ATTATAGAAA  ACTATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTACNCCAG
MF4   ACTTGTCAGC  ATTATAGAAA  ACTATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
MF5   ACTTGTCAGC  ATTATAGAAA  ACTATCTACT  CAAGTGAAGA  GTCTATGTAG  TATACTTATT  ACACTGGTCT  TGTAAACCAG
```

Figure A5.1. Alignment of mitochondrial DNA control region sequences from *Miniopterus schreibersii* and *M. fraterculus*. Explanations of haplotype names are given in Table 5.2, Chapter 5.

```
                                                                                                   240
DHP1  AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
DHP2  AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
DHL1  AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
DHL2  AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGAAGCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
SKK   AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAGAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
KB    AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
G1    AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
G2    AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
MM1   AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
MM2   AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
SHD   AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
JD1   AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
JD2   AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
PRB   AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
PC    AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
SW1   AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
SW2   AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
SW3   AAATGAGGAA -AACAATTCC TCCAGAGACT CAAGGAAAGA GCATGA-GCC CTACCGTCAG CACCCAAAGC TGAAATTCTA
MD    AAATGAGGAA GAACAATTCC TCCAAAGACT CAAGGAAAGA GCATGA-GCC CTACCATCAG CACCCAAAGC TGAGATTCTA
I     AGACGAGGAA TAGTAGTTCC TCCAAAGACT CAAGGAAAGA GCATAA-GCT CTACCATCAG CACCCAAAGC TGAAATTCTA
AB    AGACGAGGAA TAGCAATTCC TCCAAAGACT CAAGGAAAGA GCATAA-GCC CTACCATCAG CACCCAAAGC TGAAATTCTA
AO    AGACGAGGAA TAGCAATTCC TCCAAAGACT CAAGGAAAGA GCATAA-GCC CTACCATCAG CACCCAAAGC TGAAATTCTA
MF1   AAATGAGGAA AAATAGTTCC TCCAAAGACT CAAGGAAAGA GCATTA-GCC CTACCATCAG CACCCAAAGC TGAAATTCTA
MF2   AAATGAGGAA AAATAGTTCC TCCAAAGACT CAAGGAAAGA GCATTA-GCC CTACCATCAG CACCCAAAGC TGAAATTCTA
MF3   AAATGAGGAA AAATAGTTCC TCCAAAGACT CAAGGAAAGA GCATTA-GCC CTACCATCAG CACCCAAAGC TGAAATTCTA
MF4   AAATGAGGAA AAATAGTTCC TCCAAAGACT CAAGGAAAGA GCATTA-GCC CTACCATCAG CACCCAAAGC TGAAATTCTA
MF5   AAATGAGGAA AAATAGTTCC TCCAAAGACT CAAGGAAAGA GCATTA-GCC CTACCATCAG CACCCAAAGC TGAAATTCTA

                                                                                                   320
DHP1  CTTAAACTAC TCCTTGCGAG TGTACGCAAT AAATGTTCTG TCTCATAATA ATGCTATGTA CGTCGTGCAT TAACTTTATA
DHP2  CTTAAACTAC TCCTTGCGAG TGTACGCAAT AAATGTTCTG TCTCATAATA ATGCTATGTA CGTCGTGCAT TAACTTTATA
DHL1  CTTAAACTAC TCCTTGCGAG TGTACGCAAT AAATGTTCTG TCTCATAATA ATGCTATGTA CGTCGTGCAT TAACTTTATA
DHL2  CTTAAACTAC TCCTTGCGAG TGTACGCAAG AAATGTTCTG TCTCATAATA ATGCTATGTA CGTCGTGCAT TAACTTTATA
SKK   CTTAAACTAC TCCTTGCAAG CGTACGCAAT AAATGTTCTG TTCTATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
KB    CTTAAACTAC TCCTTGCAAG CGTACGCAAT AAATGTTCTG TTCTATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
G1    CTTAAACTAC TCCTTGCAAG TATACATAAT AAATGTTCTG TCTCATAATA ATGCTATGTA CGTCGTGCAT TAACTTTATA
G2    CTTAAACTAC TCCTTGCAAG TATACATAAT AAATGTTCTG TCTCATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
MM1   CTTAAACTAC TCCTTGCAAG TATACATAAT AAATGTTCTG TCTCATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
MM2   CTTAAACTAC TCCTTGCAAG TATACATAAT AAATGTTCTG TCTCATAATA ATGCTATGTA CGTCGTGCAT TAACTTTATA
SHD   CTTAAACTAC TCCTTGCAAG TATACATAAT AAATGTTCTG TCTCATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
JD1   CTTAAACTAC TCCTTGCAAG TATACATAAT AAATGTTCTG TCTCATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
JD2   CTTAAACTAC TCCTTGCAAG TATACATAAT AAATGTTCTG TCTCATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
PRB   CTTAAACTAC TCCTTGCAAG TATACATAAT AAATGTTCTG TCTCATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
PC    CTTAAACTAC TCCTTGCAAG TATATATAAT AAATGTTCTG TCCCATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
SW1   CTTAAACTAC TCCTTGCAAG TATACATAAT AAATGTTCTG TCCCATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
SW2   CTTAAACTAC TCCTTGCAAG TATACATAAT AAATGTTCTG TCCCATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
SW3   CTTAAACTAC TCCTTGCAAG TATATATAAT AAATGTTCTG TCCCATAATA ATGCTATGTA CATCGTGCAT TAACTTTATA
MD    CTTAAACTAC TCCTTGCGAA TGTATACGAT AGATGTGCTG TCCCATAGTA ATGCTATGTA CATCGTGCAT TAACTTTATA
I     CTTAAACTAT TCCTTGCAAG CACATATGCT GGACATA-TA TCCTATAGTT ATACTATGTA CATCGTGCAT TAACTTTATT
AB    TTTAAACTAC TCCTTGCAGG TGTA------ --ATGTACCG C----TATTA ATGCTATGTA CATCGTGCAT TAACTTTATT
AO    TTTAAACTAT TCCTTGCAGG CGCA------ --ATGTACTG C----TATTA ATGCTATGTA CATCGTGCAT TAACTTTATT
MF1   CTTAAACTAC TCCTTGCAAG CATAC----- --------TG TTTCGTAACA ACCCTATGTA CATCGTGCAT TAACTTTATA
MF2   CTTAAACTAC TCCTTGCAAG CATAC----- --------TG TTTCGTAACA ACCCTATGTA CATCGTGCAT TAACTTTATA
MF3   CTTAAACTAC TCCTTGCAAG CATAC----- --------TG TATCGTAACA ACCCTATGTA CATCGTGCAT TAACTTTATA
MF4   CTTAAACTAC TCCTTGCAAG CATAC----- --------TG TTTCGTAACA ACCCTATGTA CATCGTGCAT TAACTTTATA
MF5   CTTAAACTAC TCCTTGCAAG CATAC----- --------TG TTTCGTAACA ACCCTATGTA CATCGTGCAT TAACTTTATA

                                                                                                   400
DHP1  TCCCCATGAA TA-TGCAGCA TGTACATTTA TGATCATAGA TTACATGAGT ACATAATATT ATTTATCGTA CATAGACCAT
DHP2  TCCCCATGAA TA-TGCAGCA TGTACATTTA TGATCATAGA TTACATGAGT ACATAATATT ATTTATCGTA CATAGACCAT
DHL1  TCCCCATGAA TA-TGCAGCA TGTACATTTA TGATCATAGA TTACATGAGT ACATAATATT ATTTATCGTA CATAGACCAT
DHL2  TCCCCATGAA TA-TGTAGCA TGTACATTTA TGATCATAGA TTACATGAGT ACATAATATT ATTTATCGTA CATAGACCAT
SKK   TCCCCATGAA TA-TGTAGCA TGTACATTTA TGATCATAGA TTACATGAGT ACATAATATT ATTTATCGTA CATAGACCAT
KB    TCCCCATGAA TA-TGTAGCA TGTACATTTA TGATCATAGA TTACATGAGT ACATAATATT ATTTATCGTA CATAGACCAT
G1    TCCCCATGAA TA-TGTAGCA TGTACATTTA TGATCATACA TTACATGAGT ACATAATATT ATTTATCGTA CATAAACCAT
G2    TCCCCATGAA TA-TGTAGCA TGTACATTTA TGATCATACA TTACATGAGT ACATAATATT ATTTATCGTA CATAAACCAT
MM1   TCCCCATGAA TA-TGTAGCA TGTACATTTA TGATCATACA TTACATGAGT ACATAATATT ATTTATCGTA CATAAACCAT
MM2   TCCCCATGAA TA-TGTAGCA TGTACATTTA TGATCATAGA TTACATGAGT ACATAATATT ATTTATCGTA CATAGACCAT
SHD   TCCCCATGAA TA-TGTAGCA TGTACATTTA TGATCATAGA TTACATGAGT ACATAATATT ATTTATCGTA CATAAACCAT
JD1   TCCCCATGAA TA-TGTAGCA TGTACATTTA TGATCATAGA TTACATGAGT ACATAATATT ATTTATCGTA CATAGACCAT
JD2   TCCCCATGAA TA-TGTAGCA TGTACATTTA TGATCATAGA TTACATGAGT ACATAATATT ATTTATCGTA CATAAACCAT
PRB   TCCCCATGAA TA-TGTAGCA TGTACATTTA TGATCATAGA TTACATGAGT ACATAATATT ATTTATCGTA CATAAACCAT
```

Figure A5.1 cont. Alignment of mitochondrial DNA control region sequences from *Miniopterus schreibersii* and *M. fraterculus*.

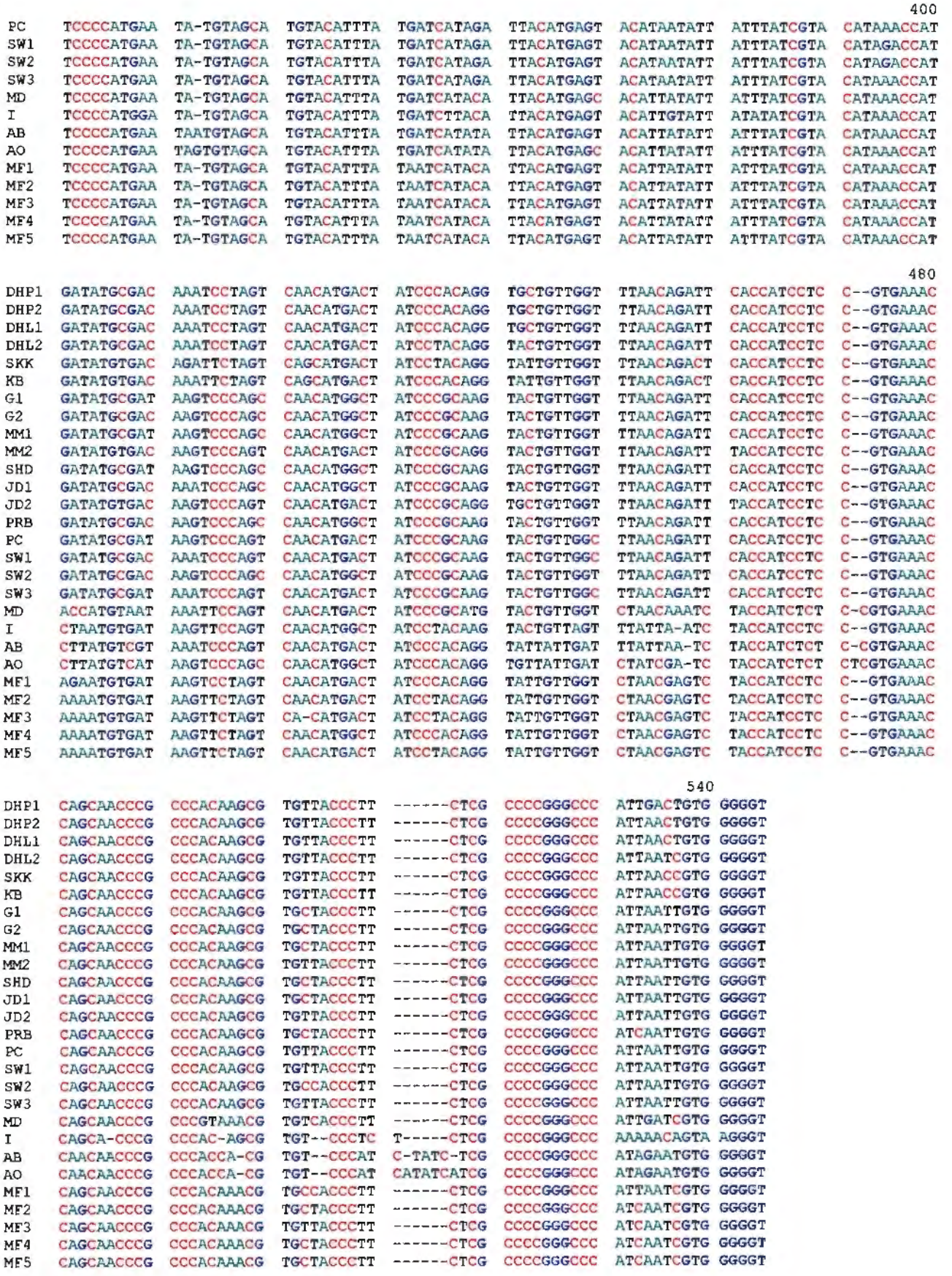

Figure A5.1 cont. Alignment of mitochondrial DNA control region sequences from *Miniopterus schreibersii* and *M. fraterculus*.

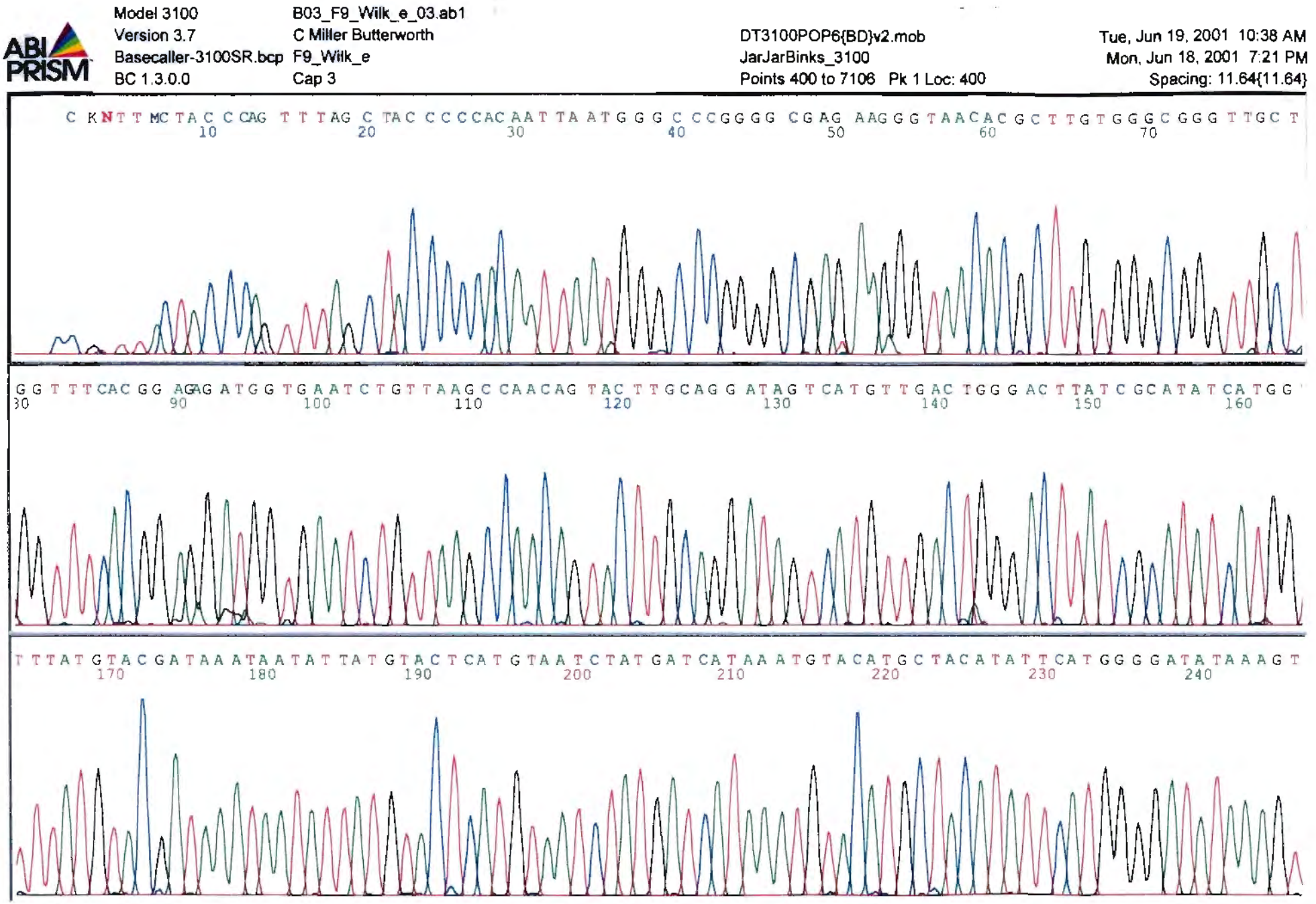

Figure A5.2. Example of an *M. schreibersii* mtDNA control region sequence, from Sudwala Female 9 (SWF9). The sequence was obtained using a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems), and was run on an ABI Prism 3100 Genetic DNA Analyzer.

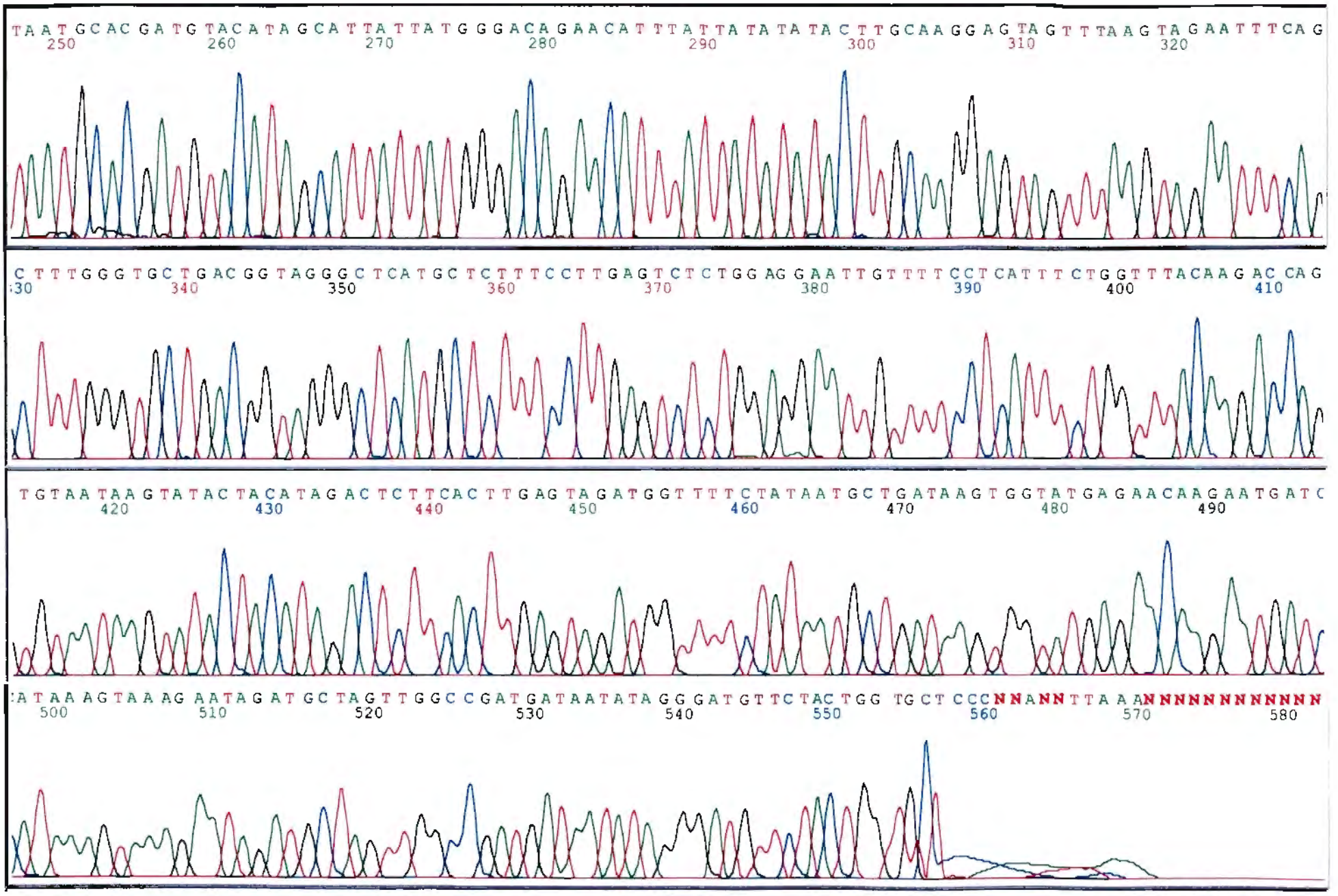

Figure A5.2 cont. Example of an *M. schreibersii* mtDNA control region sequence, from SWF9.