Genetic analysis of *Chaerephon pumilus* (Chiroptera: Molossidae) from southern Africa

by

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Abstract

Chaerephon pumilus, the little free-tailed bat, (family: Molossidae) has a distribution throughout most of sub-Saharan Africa and the eastern region of Madagascar. The vast geographical distribution of this species is accompanied by considerable phenotypic variation, which may conceal cryptic species. The cytochrome *b* (845 nucleotides) and D-loop (314 nucleotides) regions of the mitochondrial DNA were sequenced to assess phylogenetic relationships within *C. pumilus* (southern Africa) and in relation to *Chaerephon* species from Madagascar (*C. pumilus, C. leucogaster*). Samples were obtained from KwaZulu-Natal, South Africa, and localities in Swaziland. The cytochrome *b* sample (n = 11) comprised four haplotypes, with a haplotype diversity of 0.6727, whilst the D-loop (n = 34) dataset comprised 13 haplotypes with a haplotype diversity of 0.8342. Neighbour joining, maximum parsimony and Bayesian analyses revealed congruent tree structures for both mtDNA regions. All *Chaerephon* taxa in this study formed a monophyletic clade with respect to the outgroup *Mops midas*. *Chaerephon pumilus* from the eastern side of Madagascar formed a well-supported monophyletic group, sister to a clade comprising *C. pumilus* (southern Africa) and *C. leucogaster*, and is suggested to comprise a separate species. Southern African *C. pumilus* formed two paraphyletic clades, A and B, separated by a genetic distance of 0.9 %. *Chaerephon leucogaster* formed a monophyletic group nested within southern African *C. pumilus*, suggesting conspecificity. However, the well-characterized morphology of *C. leucogaster* lends support to its specific status, and suggests the possible existence of cryptic species among southern African *C. pumilus*. Population genetic analysis suggests that two *C. pumilus* (southern African) clades have been expanding, one for between 2432 and 4639 years, and the other for the 11156 to 21280 years. A combined cytochrome *b* analysis, trimmed to 343 nucleotides, was carried out on the data from this study and that of Jacobs *et al.* (2004), also on southern African *C. pumilus*. Haplotypes from the Jacobs *et al.* (2004) study, which also identified two 0.9 % divergent clades (light- and dark-winged) were found to be identical or very similar to haplotypes from this study and were interspersed among southern African *C. pumilus* haplotypes in phylogenetic analyses. *Chaerephon pumilus* haplotypes from Zambia and Tanzania were found to be more closely related to those from southern Africa and to *C. leucogaster* than to *C. pumilus* (Madagascar), further indicating that this may be a separate species. Haplotypes from the light-winged clade of Jacobs *et al.* (2004) were identical to those of dark-winged samples from this study, suggesting that wing shade may not be diagnostic of the two clades.

Preface

The experimental work described in this dissertation/thesis was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Howard College and Westville Campus, Durban. This work was carried out from January 2006 to December 2008, under the supervision of Dr. Jennifer M. Lamb and Dr. Peter J. Taylor.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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Declaration 2 – Publications

Publication 1: Publication 1: Taylor, P. J., Lamb, J., Reddy, D., Naidoo, T., Ratrimomanarivo, F. and Goodman, S. M. (submitted) *African Zoology*.

Taylor, P. J., Ratrimomanarivo, F. H. and Goodman, S.M.: Field inventories, sample collection and morphological analysis

Taylor, P.J.: Ecological and morphological analysis

Reddy, D., Naidoo, T. and Lamb, J.: Genetics and molecular sequencing and analysis

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List of abbreviations

π - Nucleotide diversity

AIC - Akaike information criteria

AMOVA - Analysis of Molecular Variance

ESU - Evolutionary significant unit

EtBr - ethidium bromide

GTR - General Time Reversible

h - Haplotype diversity

hLRT - hierarchical likelihood ratio test

IUCN - International Union for Conservation of Nature

Iss – index of substitution saturation

Iss.c. - critical value of index of substitution saturation

LGM – Last glacial maximum

mtDNA - mitochondrial DNA

Ma - million years ago

NCBI - National Center for Biotechnology Information

nt - nucleotides

PCR - polymerase chain reaction

rg – Raggedness

S/d - Expansion co-efficient

TBE - Tris-borate/EDTA

Chapter 1: Introduction

1.1 *Chaerephon pumilus* (Cretzschmar, 1826)

1.1.1 Taxonomic review

The genus *Chaerephon* is part of the Family Molossidae (free-tailed bats), Suborder Vespertilioniformes (Wilson and Reeder, 1993; Nowak, 1994; Bouchard, 1998; Neuweiler, 2000; Eick *et al*., 2005; Taylor, 2005; Wilson and Reeder, 2005). Of the six molossid genera in the southern African subregion, *Chaerephon* is probably the most diverse (Taylor, 2005). The genus contains 13 species (Koopman, 1993; Bouchard, 1998; Bouchard, 2001; Taylor, 2005), five of which occur in the subregion; *C. bivittata*, *C. ansorgei*, *C. nigeriae*, *C. chapini* and *C. pumilus* (Taylor, 2005). *Chaerephon pumilus*, *'pumilus'* meaning "dwarf" in Latin (Bouchard, 1998), was initially classified as *Dysopes pumilus* (Cretzschmar 1826) using the type specimen from Massawa, Eritrea (Koopman, 1993; Bouchard, 1998; Simmons, 2005).

The numerous geographically-variable forms of *Chaerephon* were grouped into a single species complex, *C. pumilus*, which included dark- and lighter-winged forms (Hayman and Hill, 1971; Meester and Setzer, 1971). The lighter-winged form was more commonly known as *limbatus* Peters 1852 (Meester and Setzer, 1971; Taylor, 1999a). While some classified *limbatus* as a distinct species (Rosevear, 1965), others felt that *limbatus* was at least conspecific with *pumilus* (Meester and Setzer, 1971; Meester *et al.*, 1986; Jacobs *et al.*, 2004) or supported the view that *limbatus* should be maintained as a race of *pumilus* (Koopman, 1993).

Over the past century many authors have either included or excluded *Chaerephon* and its species from the genera *Mops* and *Tadarida* (Meester *et al.*, 1986; Freeman, 1981; Koopman, 1993; Koopman, 1994; Nowak, 1994; Peterson *et al.*, 1995; Kingdon, 1997). This appeared to be due to the morphological diversity of *Chaerephon*, whose species characters often overlap with those of *Mops* and *Tadarida*. These studies were based primarily on general morphological characters (e.g. pelage patterns, colouration, dentition and palatal emargination), the lengths of anatomical features (e.g. entire body, tail, hind foot, ear, tragus and forearm), body mass and the presence or absence of specific features such as the aural crest in the males of *C. pumilus* (Kingdon, 1974; Smithers, 1983; Meester *et al.*, 1986; Freeman, 1981; Koopman, 1993; Koopman, 1994; Nowak, 1994; Peterson *et al.*, 1995; Kingdon, 1997; Bouchard, 1998; Taylor, 1999a). Distinguishing characteristics of *Chaerephon* were; ears joined by a band of skin, an

elevated mandibular condyle, broader wing tips, reduced anterior palatal emargination and well-developed third commissures of the last upper molar (Freeman, 1981; Nowak, 1994; Bouchard, 1998; Taylor, 1999a). Koopman (1994) subsequently recognized 12 subspecies, including *pumilus* and *leucogaster*, in a single complex; however, these were poorly defined, resulting in a subspecific classification which was of little use (Simmons, 2005). Other authors divided *C. pumilus* into four species (*T. leucogaster, T. limbata*, *T. naivashae* and *T. pumila*), excluding some species that share characteristics with *Mops* (Peterson *et al.*, 1995). Although *Chaerephon* and *Mops* were previously included as subgenera of *Tadarida* (Meester *et al.*, 1986), they were separated into distinct genera by Freeman (1981) and Koopman (1993).

Mops and *Tadarida* have since been reported to be larger, on average, than *Chaerephon* and to lack both the prominent tuft of hair (aural crest) found on the foreheads of males and the longitudinal band of white hair found laterally along the body beneath the wings (Kingdon, 1997). Currently the genus *Chaerephon* has been retained, whilst the specific name *pumila* has been changed to *pumilus* to match the masculine generic name (Bouchard, 1998; Simmons, 2005).

Recent attempts at subspecific separation of *C. pumilus*, based on morphology, have been flawed (Taylor, 1999a). Taylor (1999a) found that Koopman's (1975) key was inadequate to classify southern African Molossidae, as a known colony of *C. pumilus* from Durban, South Africa was assigned to three distinct species based on this key. The reason was that southern African *Chaerephon* show considerable variation in pelage colour and certain diagnostic characters of the skeleton (Taylor, 1999a).

In studies investigating the possible existence of cryptic species of *C. pumilus*, Aspetsberger *et al.* (2003) compared individuals from Tanzania (Amani) and South Africa (Durban) on the basis of morphology, echolocation and diet, while Jacobs *et al.* (2004) compared morphology, echolocation and genetics of southern African individuals. *Chaerephon pumilus* specimens from Amani were found to differ from populations elsewhere in Africa in morphology, diet and echolocation frequency, a character which was previously used to identify cryptic species of *Pipstrellus* (Jones and Van Parijs, 1993; Barratt *et al.*, 1997). However, the difference in peak frequency between *C. pumilus* in Amani and Durban was found to be only 7 kHz, which was within the range of intraspecific flexibility reported for other species (Jacobs, 1999). Jacobs *et al.* (2004) found that the average intraspecific cytochrome *b* sequence divergence between the light and dark forms of southern African *C. pumilus* was 0.9 %, which was suggested to be

insufficient to define them as distinct species. Within the dark southern African form, individuals were found to be genetically indistinguishable, therefore eliminating the possibility of a cryptic species (Jacobs *et al.*, 2004).

Recent studies of the genus *Chaerephon* that have utilized morphological characters to define species include those of Goodman and Cardiff (2004), who reported a third *Chaerephon* species, *C. jobimena*, in Madagascar, and Goodman and Ratrimomanarivo (2007), who investigated the taxonomic status of *C. pumilus* from the western Seychelles. *Chaerephon jobimena* was found to differ morphologically from both *C. leucogaster* and *C. pumilus* present in Madagascar, as well as from similarly-sized African and Asian *Chaerephon* species, as it lacked the white wing patches characteristic of several members of the genus. *Chaerephon pusillus* was previously considered distinct, but was later synonomized with the widespread *C. pumilus* (Hayman and Hill, 1971). The samples from the Seychelles were found to be distinctly smaller, resulting in the resurrection of the name *C. pusillus* as an endemic species of the Seychelles (Goodman and Ratrimomanarivo, 2007).

1.1.2 Morphology

Chaerephon pumilus (Cretzschmar, 1826), the little free-tailed bat, is one of the smaller molossid bats (forearm length *c.* 36 - 38 mm; mass *c.* 10 - 14 g). The pelage of this species is characteristically highly variable throughout its distribution (Aspertsberger *et al.*, 2003; Jacobs *et al.*, 2004) and is typified by darker upper-parts and a lighter under-side (Taylor, 1999a; Taylor, 2000). Malagasy animals are dark brown, with a brown throat and white mid-ventral band (Garbutt, 1999). The upper-parts of southern African animals vary from a deep blackish-brown to a reddish-brown, whilst the under-side is a lighter shade and in certain individuals is grayish to white (Taylor, 1999a). The grayish-white region may vary to white, which is mainly found in the east African *C. limbata* form (Taylor, 1999a).

The head of *Chaerephon pumilus* is leathery, and has a broad muzzle and wrinkled upper lip which accommodate aerial feeding, resulting in a bulldog-like appearance (Nowak, 1994; Garbutt, 1999; Taylor, 2000; Taylor, 2005). The ears, which are large in comparison to the head, are rounded and attached by a flap of skin (Taylor, 2005). Mature adult males may be distinguished by the presence of an aural crest (Taylor, 2000). The lack of emargination in the palatal area of the skull was described as a diagnostic trait of this species (Taylor, 1999a). High variability was, however, found in this character among KwaZulu-Natal specimens, with some

individuals showing partial or complete emargination (Taylor, 1999b). The mean forearm length is usually between 36 and 38 mm, however a large form is found in Kenya (mean forearm length 43 mm) (Kingdon, 1974; Aspertsberger *et al.*, 2003; Jacobs *et al.*, 2004). The high aspect ratio (8.90) and wing loading (12.6 Nm^{-2}), along with a low wingtip shape index (1.2), are indications of low maneuverability and high speed during flight (Bouchard, 1998).

1.1.3 Distribution and habitat

Chaerephon pumilus is broadly distributed throughout most of sub-Saharan Africa, occurring from Senegal (western limit) to Yemen (eastern limit) in the north and, in the south, down the east coast of South Africa, as far as Oribi Gorge (Bouchard, 1998; Taylor, 1999a). This bat also occurs on the islands of Madagascar, Aldabra, Pemba and Zanzibar, and is usually found at altitudes of less than 1000 m (Meester and Setzer, 1971; Nowak, 1994; Bouchard, 1998; Taylor, 1999a; Taylor, 2000). In Madagascar the distribution is limited to the eastern rainforest region and the eastern band of the high plateau (between 500 m and 1100 m) (Nowak, 1994). *Chaerephon pumilus* is commonly found in humid environments, and inhabits open forests, savannahs and agricultural areas (Nowak, 1994). Roost sites include hollows and crevices in trees and rocky environments, as well as roof spaces of human dwellings (Bouchard, 1998; Goodman and Cardiff, 2004; Taylor, 2005).

1.1.4 Reproduction

Females are sexually mature between 5 and 12 months of age; a single hairless young (mass *c.* 3.2 g) is born after a gestation of 60 days (Taylor, 2005). Breeding seasons usually vary in relation to rainfall patterns (Bouchard, 1998); there are two breeding seasons in Kenya (Harrison, 1958), whilst breeding occurs year-round in Uganda and West Africa (Bouchard, 1998). In southern Africa pregnancies occur from August to April in association with spring rainfalls, however temperature plays a defining role as cooler temperature directly affect insect (food) abundance (van der Merwe *et al.*, 1986; van der Merwe, *et al.*, 1987; Bouchard, 1998). Females generally give birth three times a year, though the possibility of five births has been suggested (van der Merwe *et al.*, 1986)

1.1.5 Behaviour and ecology

Chaerephon pumilus is a gregarious species, roosting in colonies which vary in size from a few individuals (5 - 20) to hundreds (Kingdon, 1974; Bouchard, 1998). Colonies become very noisy and restless prior to leaving the roost just after sunset (Kingdon, 1974; Smithers, 1983). These bats forage singly, with flight being fast and erratic (Bouchard, 1998). Activity occurs throughout the night, but flight activity is greatest just after sunset and drops to a low around midnight (Bouchard, 1998).

1.1.6 Echolocation

Chaerephon pumilus uses long (12.4 ms) search-phase echolocation calls with a narrow bandwidth (15.7 kHz) and low peak frequency of 25.6 kHz (Taylor, 1999b); these calls are ideal for long-range detection of prey in open areas (Bouchard, 1998). A difference of 7 kHz in peak frequency has been reported between bats from Amani (Tanzania) and Durban (South Africa) (Aspertsberger *et al.,* 2003).

1.1.7 Food

The ability to fly fast and hunt above buildings and the forest canopy, at heights of over 70 m, allows the diet of *C. pumilus* to include a variety of small insects, including Coleoptera, Hemiptera, Lepidoptera, Hymenoptera and Diptera (Kingdon, 1974). Southern African and Kenyan *C. pumilus* generally prefer Coleoptera and Hemiptera (Aldridge and Rautenbach, 1987). Bats around Lake Naivasha in Kenya feed mainly on Diptera. Tanzanian bats from Amani, however, feed predominantly on Blattodea (60 %) (Aspertsberger *et al.,* 2003).

1.2 Previous studies

1.2.1 Phylogeography

The term phylogeography was coined in 1987 (Avise *et al.,* 1987) and refers to the principles and processes that regulate the geographical distribution of genealogical lineages (Avise, 1989; Avise, 1998). It is a bridge that links biogeography and population genetics, while emphasizing the historical aspects of the spatial distribution of gene lineages (Avise 1989; Avise, 1998; Bermingham and Moritz, 1998; Knowles and Maddison, 2002). The understanding of historical responses to changes in landscape and the identification of evolutionary isolated areas can be

used to inform and prioritize conservation strategies (Bermingham and Moritz, 1998). This has enabled phylogeographical analyses to play an important role in defining evolutionary significant units (Moritz, 1994).

Phylogeographic patterns can be inferred by various methods and programs, such as analysis of molecular variance (AMOVA) and nested clade analysis (NCA) (Ruedi and McCracken, 2006). Avise (2000) proposed four categories (I, II, III, and IV), which describe commonly-found phylogeographic patterns. Each category is based on the consideration of phenotypic divergence, mtDNA divergence and the geographic distribution of the variants, and is associated with a taxonomic interpretation (Avise, 2000; Ruedi and McCracken, 2006). Ruedi and McCraken (2006) made adjustments to Avise's original categories, by considering animals that are strongly divergent phenotypically, resulting in four additional categories (I*, II*, III*, and IV*). The interpretation of results through the use of these categories could possibly provide insight into the phylogeographic patterns of specific populations.

The phylogeographic patterns exhibited by bats are expected to differ from those of other small mammals that do not have the capacity to fly (Ditchfield 2000). Larger bats are usually reported to be more vagile (Nowak, 1994; Ratrimomanarivo *et al.*, 2007; Lamb *et al.*, 2008). Highly vagile bats usually exhibit poor genetic structure (Ratrimomanarivo *et al.*, 2007; Lamb *et al.*, 2008), while non-vagile, usually smaller, bats are often associated with high genetic structure (Ratrimomanarivo *et al.*, submitted (a)). Dispersal often influences phylogeographic patterns through ongoing gene flow, which can have profound effects on bat population diversity (Freeland 2005). Examples of this are provided in several studies by Russell *et al.* (2005, 2006, 2008), who made use of morphological and molecular analysis in their studies of dispersal capabilities and their effects on phylogeographic structuring. Russell *et al.* (2005) studied four groups of *Tadarida brasiliensis mexicana*, each with distinct migratory behaviour, and found no significant genetic structure either among previously-described migratory groups or between migratory and non-migratory groups. Russell *et al.* (2006) aimed to differentiate among conflicting hypotheses relating to the biogeographical history of Madagascar's *Triaenops* bats. The study led to the conclusion that two independent but unidirectional dispersal events from Africa to Madagascar were best supported by the data. Russell *et al.* (2008) readdressed the issue of the direction of dispersal between Madagascar and Africa and found their results to support multiple unidirectional dispersals from Africa to Madagascar, resulting in multiple independent Malagasy lineages.

In other phylogeographical studies, Castella *et al.* (2001) used molecular and nuclear approaches, while Miller-Butterworth *et al.* (2003) used morphology and population genetics to examine population structure and philopatry in bats. Castella *et al.* (2001) sampled thirteen nursery colonies of *Myotis myotis* from central Europe, and found that three evolutionary lineages are present, with strong haplotypic segregation, suggesting that breeding females are philopatric. Miller-Butterworth *et al.* (2003) evaluated the effects of the environment on the morphology and population genetics of *Miniopterus schreibersii natalensis*. The pattern of genetic differentiation obtained provided evidence of strong population substructure in this migratory bat species and philopatry in both sexes.

Hogan *et al.* (1997) conducted a phylogeographic study on the species *Peromyscus maniculatus*. This morphologically-diverse rodent is found throughout North America, a situation which parallels that of the widely distributed and diverse *C. pumilus* in Africa. *Peromyscus maniculatus* is part of a polyphyletic group which includes *P. slevini, P. maniculatus, P. keeni, P. polionotus, P. sejugis,* and *P. melanotis*. Though the removal of *P. slevini* resolves the polyphyly, phylogenetic analyses reveal that a subspecies of *P. maniculatus* (*P. m.coolidgei*) is more closely related to *P. sejugis* (Hogan *et al.*, 1997). Further, this complex (*P. m.coolidgei / P. sejugis*) shows a sister-species relationship with *P. keeni*. These results point to the necessity to recognize some subspecies as distinct species (Hogan *et al.*, 1997).

1.2.2 Morphological and mitochondrial DNA analyses

The combination of molecular and morphological analysis has become a preferred approach to phylogenetic analysis and the definition of species. Studies on bats that have employed this approach include those of Miller-Butterworth *et al.* (2003) (*Miniopterus*), Teeling *et al.* (2003) (*Mystactina*), Jacobs *et al.* (2006) (*Scotophilus*) and Mayer *et al.* (2007) (Western Palaearctic vespertilionid bats). Ratrimomanarivo *et al.* (2007) assessed the specific status of *Mops midas* in Africa and Madagascar using both morphological and molecular approaches. African (*M. m. midas*) and Madagascan (*M. m. miarensis*) subspecies showed a low cytochrome *b* genetic distance (0.1 %); on this basis it was proposed that no subspecific variation be recognized in *Mops midas.*

Mitochondrial DNA has been used extensively for phylogenetic analysis (Avise *et al.*, 1987), and is still at the forefront of phylogeography studies. Rapid evolution in populations of higher animals, along with maternal transmission and the absence of recombination have contributed to

the utility of mtDNA sequencing in identifying and tracking the ancestry of higher organisms (Brown *et al.*, 1979; Avise *et al.*, 1987; Avise, 1998). Mitochondrial DNA possesses several regions that may be analysed for phylogenetic purposes, however the cytochrome *b* and D-loop regions have been most commonly-used. Phylogenetic studies based on the cytochrome *b* region include those of Ditchfield (2000) (*Artibeus*, *Carollia*, *Sturnira* and *Glossophaga*), Hoffmann and Baker (2001) (*Glossophaga*), Hoffmann and Baker (2003) (*Carollia*), Jacobs *et al.* (2004) (*Chaerephon*), Lim *et al.* (2004) (*Artibeus*), Stadelmann *et al.* (2004) (*Myotis*), Jacobs *et al.* (2006) (*Scotophilus*), Roberts (2006) (*Haplonycteris*), and Pulvers and Colgan (2007) (*Melonycteris*). Other studies have utilized the D-loop region, including those of Wilkinson and Chapman (1991) (*Nycticeius*), Kerth and Morf (2004) (*Myotis*), Salgueiro *et al.* (2004) (*Nyctalus*), Russell *et al.* (2005) (*Tadarida*), and Bilgin *et al.* (2006) (*Miniopterus*). Studies designed to answer questions on taxonomy, population genetics, biodiversity, conservation and phylogeography, based on both cytochrome *b* and D-loop regions sequences, include those of Kocher *et al.* (1989), Castella *et al.* (2001) (*Myotis*), and Goodman *et al.* (2006) (*Emballonura*).

Several recent studies on the genetic diversity of African Molossidae have utilized mtDNA sequencing. Lamb *et al.* (2008) utilized mitochondrial cytochrome *b* and D-loop sequences to determine phylogeographic structure and clade divergence in the African molossid genus, *Otomops.* Ratrimomanarivo *et al.* (submitted (b)) found Afro/Malagasy *Mops midas*, African *M. condylurus* and Malagasy *M. leucostigma* to be monophyletic. While *M. midas* was separated from *M. condylurus* and *M. leucostigma* by a mean cytochrome *b* genetic distance of 13.8 %, the reciprocally-monophyletic sister taxa *M. condylurus* and *M. leucostigma* were only 2.5 % divergent. A single *Mops leucostigma* clade included individuals from Madagascar and the Comoros, which were only 0.22 % divergent in cytochrome *b* sequences despite their separation by 480 km of ocean (Mayotte to Northern tip of Madagascar). Morphologically-variable *Chaerephon leucogaster* individuals from the western Indian Ocean islands of Madagascar, Mayotte and Pemba were found to be monophyletic and separated by a low cytochrome *b* genetic distance (0.00349 %) (Ratrimomanarivo *et al.*, submitted (a)).

Studies based on mtDNA sequences have revealed the importance of past climatic events such as Pleistocene glaciations in structuring populations of many organisms, including bats. Chen *et al.* (2006) examined the genetic structure and evolutionary origin of the Formosan lesser horseshoe bat (*Rhinolophus monoceros*) and found a high haplotype and nucleotide diversity. They estimated that populations had been expanding for the last 30 000 years and suggested that the taxon arose from a single period of colonization before the last glacial maximum.

1.2.3 Cryptic species

Morphologically-similar species that are reproductively or genetically isolated are referred to as cryptic species (Baker and Bradley, 2006). Species that are widely distributed and display morphological divergence may possibly consist of more than one cryptic species (Aspetsberger *et al.*, 2003; Jacobs *et al.*, 2004). Jacobs *et al.* (2006) found that light and dark forms of *Scotophilus dinganii* exhibited distinct peak echolocation frequencies. Analysis of cytochrome *b* and D-loop sequences indicated that the two phonic types were reciprocally monophyletic, implying that they are sibling species. Jones and Van Parijs (1993) found cryptic species within the common European bat, *Pipistrellus pipestrellus*. This was based on echolocation calls, which fell in two distinct frequency bands, and was later supported by Barrat *et al.* (1997), who found the two groups to be separated by a cytochrome *b* genetic distance of 11 %.

1.3 Defining conservation units

1.3.1 Species and species concepts

There are currently more than 22 definitions of the term species (Van Valen, 1976; Cracraft, 1983; Claridge *et al.*, 1997; Wiley and Mayden, 2000). Scientists generally follow a specific concept, although numerous species have been classified using a combination of species concepts (Ridley 1993; Bradley and Baker, 2001). Some of the more commonly used species concepts include the morphological species concept (Ruse, 1969; Mayr, 2000), biological species concept (Mayr, 1942; Simpson, 1961; Mayr, 1963; Mallet, 2001; Agapow *et al*., 2004; de Queiroz, 2005), recognition species concept (Paterson, 1985), ecological species concept (Van Valen, 1976; Mayr, 1982; Grant, 1992; de Queiroz, 2005), evolutionary species concept (Wiley, 1978; Mayr, 2000), cohesion species concept (Templeton, 1989; Mallet, 2001), phylogenetic species concept (Cracraft, 1983; Meffe and Carroll, 1997) and genetic species concept (Bradley and Baker, 2001; Baker and Bradley, 2006).

In the past, Chiroptera were generally classified according to the criteria of the morphological species concept, which groups individuals with similar morphological characters (Ruse, 1969; Mayr, 2000). Other concepts that have been widely applied include the biological species concept, which emphasizes reproductive isolation in the maintenance of the created gene pool (Mayr, 1942; Simpson, 1961; Mayr, 1963; Mallet, 2001; Agapow *et al*., 2004; de Queiroz, 2005). This is often difficult to assess in high-flying bat species, such as molossids, which are seldom caught in flight. The phenetic species concept is a mathematically-based version of the

morphological species concept (Sokal and Crovello, 1970). Genetic distance analyses and the construction of neighbour-joining (distance) trees fall under this concept and are included in this study.

The advancement of molecular phylogenetics has resulted in two species concepts that are arguably most applicable to this molecular study. These are the phylogenetic or cladistic species concept, which reflects the cladistic relationships among species or higher taxa (Cracraft, 1983; Meffe and Carroll, 1997), and the genetic species concept, which focuses on genetic isolation rather than reproductive isolation (Bradley and Baker, 2001; Baker and Bradley, 2006). The phylogenetic species concept is based on monophyly and groups organisms that diverge from a shared common ancestor. This species concept, however, does not recognize subspecies; populations are either a phylogenetic species or not. The result is taxonomic inflation, as divergent populations which share a common ancestor are regarded as separate species. According to the genetic species concept, mitochondrial cytochrome *b* genetic distance values, typical of population and intraspecific variation, are utilized in evaluating the status of populations that may be conspecific, sister species or even unrecognized species (Bradley and Baker, 2001; Baker and Bradley, 2006).

1.3.2 Evolutionary Significant Unit (ESU) Concepts

The term Evolutionary Significant Unit was coined by Ryder (1986) for the purpose of preserving adaptive genetic variance within species, by conserving unique population groups below the species level (Avise, 1989; Waples, 1991; Waples, 1995; Waples, 1998; Fraser and Bernatchez, 2001). With the rise of genetic techniques the ESU concept has evolved towards defining units on the basis of molecular genetic markers alone (Avise, 1994; Moritz, 1994; Crandall *et al.*, 2000). One problem with this is that an anomaly may be able to overturn the reciprocally-monophyletic status of a population or group of populations (Pennock and Dimmick, 1997; Fraser and Bernatchez, 2001).

1.4 Methods of studying genetic diversity

1.4.1 Mitochondrial DNA verses nuclear DNA

DNA sequencing is frequently used to infer evolutionary relationships within and among species. Nuclear DNA is diploid and biparentally-inherited, while mitochondrial DNA

(mtDNA) is haploid and inherited maternally in most organisms (Freeland, 2005). Though mtDNA is small in comparison with nuclear DNA, it is found in relative abundance within a cell (Ballard and Whitlock, 2004; Freeland, 2005). Analysis of mitochondrial DNA molecular markers has played a significant role in speciation, conservation and ecological studies. The availability of universal primers, a high mutation rate, lack of recombination and maternal inheritance has made mtDNA a favourable tool for the identification of lineages (Kocher *et al.*, 1989; Ballard and Whitlock, 2004; Freeland, 2005).

Studies on the sequence evolution of slower-evolving nuclear genes are more appropriate than those on mtDNA for the resolution of deeper phylogenetic divergences among major lineages of mammals (Kjer and Honeycutt, 2007). Microsatellites have been one of the most frequently-used nuclear molecular markers, however the variation of mutation rates among organisms along with ambiguous ancestral information limits the certainty with which genealogical patterns of relationships can be deduced (Zhang and Hewitt, 2003). Single nucleotide polymorphisms (SNPs) are currently frequently used as molecular markers (Zhang and Hewitt, 2003; Aitken *et al.*, 2004; Seddon *et al.*, 2005; Smith *et al.*, 2005). Ultimately the choice of marker must be matched to the type of study being undertaken, with mtDNA being more appropriate for genealogical and evolutionary studies of animal populations and microsatellites being more appropriate for inferring population genetic structure and dynamics (Zhang and Hewitt, 2003).

1.4.2 Genetic markers and their suitability for this study

The animal mitochondrial genome is a circular molecule that contains 37 genes and is approximately 16 kilobases in length (Anderson *et al.*, 1981; King and Low, 1987; Ballard and Whitlock, 2004). The two regions of interest in this study are the displacement-loop (D-loop) and cytochrome *b* gene regions.

The D-loop is the only non-coding segment of the mitochondrial genome, but is still of functional importance as the origin of heavy strand replication and two major transcriptional promoters are present in this region (Aquadro and Greenberg, 1983; King and Low, 1987). The D-loop or control region is the fastest-evolving region in the mitochondrial genome (Cann *et al.*, 1984; Baker and Marshall, 1997). It allows for reliable times of divergence to be estimated while recent and rapid evolutionary changes can be effectively tracked (Saccone *et al.*, 1991). In this study the 5’ hypervariable region of the D-loop was sequenced.

The cytochrome *b* gene is 1140 base pairs long in bats. The cytochrome *b* protein is involved in electron transport in the respiratory chain of mitochondria (Irwin *et al.*, 1991). Codon evolution in this gene is highly variable, with slow evolution of non-synonymous substitutions at codon positions 1 and 2 and rapid evolution in silent positions (codon position 3) (Irwin *et al.*, 1991; Farias *et al.*, 2001). This has allowed the cytochrome *b* region to become a useful universal marker that is variable enough for population level research and conservative enough for phylogenetic research (Farias *et al.*, 2001).

1.5 Aims and objectives

Chaerephon pumilus is broadly distributed throughout sub-Saharan Africa and exhibits high phenotypic variation. These attributes are typical of species that contain masked cryptic species (Barratt *et al.*, 1997; Aspetsberger *et al.*, 2003; Jacobs *et al.*, 2004). Previous studies by Aspetsberger *et al.* (2003) and Jacobs *et al.* (2004) aimed at demonstrating the existence of cryptic species in *C. pumilus* have identified distinct variation in echolocation calls, although still within the intraspecific range (Aspetsberger *et al.*, 2003).

Chaerephon pumilus populations in southern Africa alone have exhibited sufficient variation to suggest the possible existence of cryptic species, as they possess diagnostic morphological characters (Taylor, 1999a) and echolocation calls (Taylor, 1999b; Fenton *et al.*, 2004).

The aim of this study is to utilize molecular markers, similarly to Jacobs *et al.* (2004), but with a more complete sampling of the mitochondrial cytochrome *b* region (845 base pairs) and the inclusion of the faster-evolving D-loop region (314 base pairs) to assess the genetic diversity of southern African *C. pumilus*. Samples were taken from a wider geographic range along eastern South Africa and Swaziland than that used by Jacobs *et al.* (2004), and analysis included two Genbank sequences from the Kruger National Park. A reduced cytochrome *b* dataset (343 base pairs) including samples from Jacobs *et al*. (2004), obtained from KwaZulu-Natal, Tanzania and Zambia, was also included in the analysis.

(1) The main aim of this study was to investigate the possible existence of distinct genetic lineages within southern African *C. pumilus* populations, using cytochrome *b* and D-loop sequence analysis.

(2) *Chaerephon pumilus* and *C. leucogaster* samples from Madagascar were included as outgroups. The second aim was to establish phylogenetic relationships between southern African *C. pumilus* and Malagasy *C. pumilus* and *C. leucogaster*, and specifically to test whether the Malagasy *C. pumilus* samples fall into the same species group as *C. pumilus* (southern Africa).

(3) A third aim was to carry out population demographic analyses, using neutral D-loop data, in order to assess whether populations of *C. pumilus* are expanding or contracting.

(4) A fourth aim was to interpret the conservation status of *C. pumilus* in light of genetic estimates of diversity and phylogeny, and to make conservation recommendations, where appropriate.

(5) A fifth aim was to carry out a combined analysis of the cytochrome *b* dataset of Jacobs *et al.* (2004) and the experimental cytochrome *b* dataset, to integrate and compare results from these two independent studies. This was of interest as, although many of the samples in both studies came from KwaZulu-Natal and Swaziland, the Jacobs *et al.* (2004) study included samples not available in our study, from Zambia and Tanzania. Integration of the two datasets, although based on a reduced sequence length, as Jacobs *et al.* (2004) only sequenced the 5' 423 nucleotides of the cytochrome *b* gene, would allow a regional phylogeny to be constructed. The area covered would be Zambia in the north-east, to Madagascar in the north-west, and the KwaZulu-Natal coast in the south.

Chapter 2: Materials and method

2.1 Samples and collection

The genetic diversity of *Chaerephon pumilus* was assessed through the sequencing and analysis of the mitochondrial cytochrome *b* (n = 11) and D-loop (n = 34) regions (Table 2.1). Samples were obtained from several localities in KwaZulu-Natal, South Africa, as well as from Swaziland (Fig. 2.1). These were provided in the form of muscle, liver, heart or kidney tissue, preserved in 80 % ethanol, by Dr. Peter Taylor of the Durban Natural Science Museum, South Africa, in conjunction with members of the Bat Interest Group of KwaZulu-Natal. Sample localities included Durban (the broader metropolitan region), Lake St Lucia (Hell's Gate and Charters Creek) and uMkhuze Game Reserve in KwaZulu-Natal and Mlawula, Rosecraft and Wylesdale in Swaziland. All localities except Rosecraft and Wylesdale fall under the typical savannah biome and occur below 600 m altitude (Rutherford and Westphal, 1986; Nowak, 1994). Rosecraft and Wylesdale are just above 600 m in altitude, and may be classified in the Highveld biome (Rutherford and Westphal, 1986). *Chaerephon pumilus* samples were also retrieved from the NCBI Genbank for inclusion in the analysis. These included samples from the Kruger National Park in north eastern South Africa (Table 2.2), for which only D-loop sequences were available, and the cytochrome *b* sample set published by Jacobs *et al.* (2004) (Table 2.3), which contained samples from Zambia and Tanzania as well as locations within eastern South Africa. These sequences were shorter (423 nucleotides) than those sequenced as part of this project (830 nucleotides); these samples were included in a separate joint analysis (section 3.5). Also included for comparative purposes as outgroups were Malagasy samples, of *C. pumilus* and *C. leucogaster* , which were sequenced by fellow students with ongoing studies (Waheeda Buccas and Theshnie Naidoo) (Table 2.2). Malagasy samples were provided by Dr. Steven Goodman of Vahatra, Madagascar, and the Field Museum of Natural History, Chicago, USA. A molossid sample from a closely-related genus, *Mops midas*, was included as a more distant outgroup, on which to root the *Chaerephon* samples.

2.2 DNA extraction

DNA was extracted from 25 mg of muscle tissue using a Qiagen DNeasy® Tissue Kit 250 according to the protocol described in the DNeasy® Tissue Handbook. DNA samples were eluted into the buffer provided (Buffer AE). Samples were stored at -20^{o}C, whilst working stocks were stored at 4^{o}C.

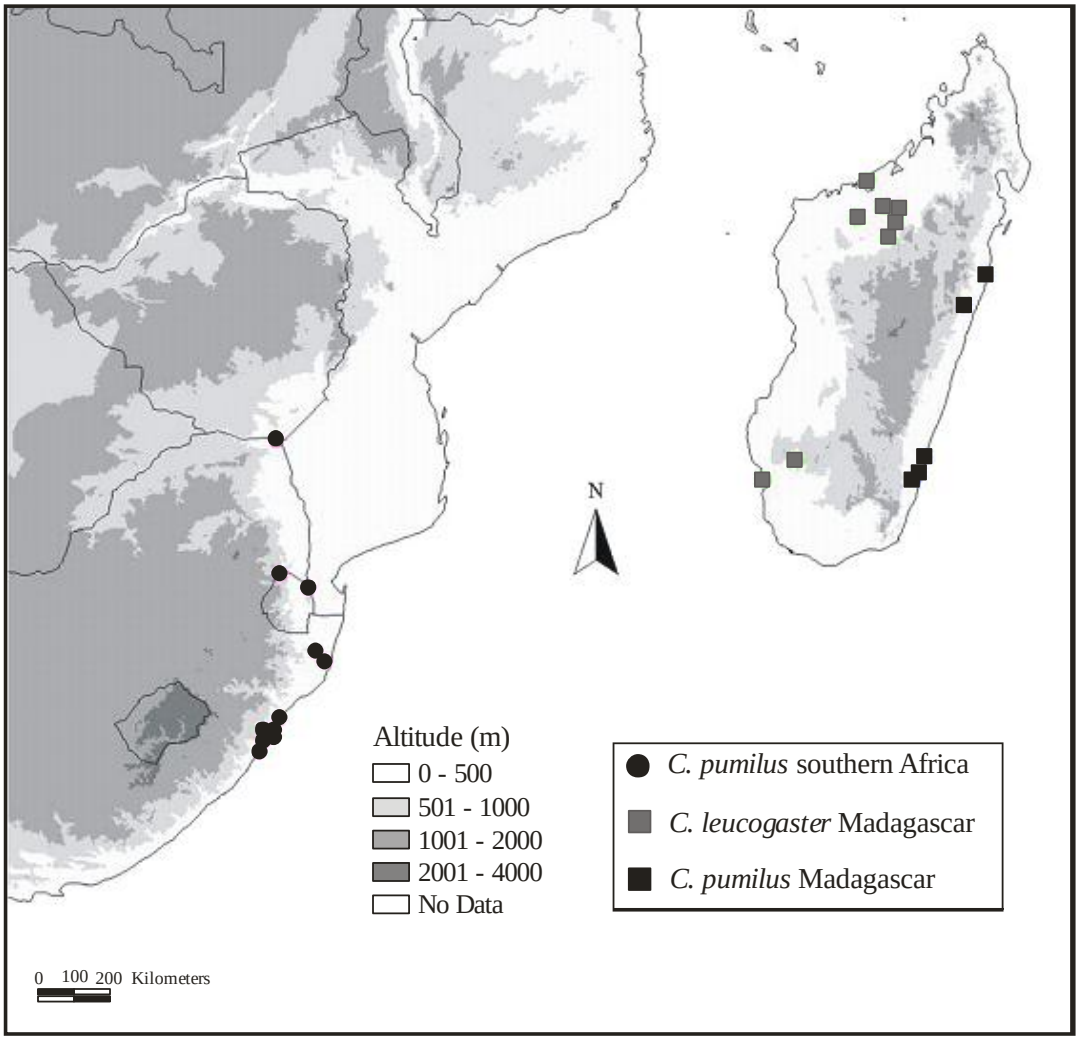

Figure 2.1. Map showing the capture localities of *C. pumilus* in southern Africa and *C. leucogaster* and *C. pumilus* in Madagascar.

Table 2.1. Specimen details, locality and Genbank accession numbers for *Chaerephon pumilus* samples. Cladal designations were defined by phylogenetic and phenetic analyses of cytochrome *b* and D-loop data.

Museum number	Field/ Lab Code	Locality	Latitude and longitude (decimal degrees)	Clade	Genbank number		Gender
					D-loop	cytochrome *b*	
DM7363	D1	Durban Int. Airport	-29.967 S; 30.942 E	A1	FJ415824	FJ415813	Male
DM7367	D2	Hell's Gate(Lake St Lucia)	-28.067 S; 32.421 E	A1	FJ415826	FJ415814	Female
DM7368	D3	Hell's Gate	-28.067 S; 32.421 E	A1	FJ415825	N/A	Male
DM7369	D4	Hell's Gate	-28.067 S; 32.421 E	A1	FJ415837	N/A	Female
DM7370	D5	Hell's Gate	-28.067 S; 32.421 E	A1	FJ415838	N/A	Female
DM7371	D6	Hell's Gate	-28.067 S; 32.421 E	A1	FJ415839	N/A	Female
DM7372	D7	Hell's Gate	-28.067 S; 32.421 E	A1	FJ415827	N/A	Male
DM7373	D8	Mkhuzi Game Reserve	-27.583 S; 32.217 E	A1	FJ415828	FJ415815	Female
DM7374	D9	Mkhuzi Game Reserve	-27.583 S; 32.217 E	A1	FJ415829	FJ415816	Male
DM7377	D10	Durban: Kissen Lane, Amanzimtoti	-30.050 S; 30.883 E	B1	FJ415846	N/A	Female
DM7378	D11	Durban: 13 Bunting Place, Amanzimtoti	-30.050 S; 30.833 E	A1	FJ415830	N/A	Male
DM7379	D12	Durban: Morningside	-29.833 S; 31.000 E	B1	FJ415848	FJ415817	Female
DM7380	D13	Durban: Yellowwood Park (CROW rehab. centre)	-29.917 S; 30.933 E	B1	FJ415849	FJ415818	Female
DM7381	D14	Hell's Gate (Captive born to DM 7382)	-28.067 S; 32.421 E	A1	FJ415841	N/A	Female
DM7382	D15	Hell's Gate	-28.067 S; 32.421 E	A1	FJ415831	N/A	Female
DM7383	D16	Durban: Yellowwood Park (CROW rehab. centre)	-29.917 S; 30.933 E	B1	FJ415850	N/A	Male
DM7384	D17	Hell's Gate	-28.067 S; 32.421 E	A1	FJ415832	N/A	Male
DM7385	D18	Durban: Bluff	-29.933 S; 31.017 E	A1	FJ415836	N/A	Female
DM7386	D19	Ballito: Westbrook (Captive born)	-29.533 S; 31.217 E	B1	FJ415847	N/A	Male
DM7387	D20	Durban: Bluff (Captive born to DM 7384)	-29.933 S; 31.017 E	A1	FJ415840	N/A	Male
DM7401	D22	Durban: Athlone Park	-30.050 S; 30.883 E	A1	FJ415843	N/A	Unknown
DM7525	D23	Charters Creek (Lake St. Lucia)	-28.200 S; 32.417 E	A1	N/A	FJ415819	Male
DM7851	D26	Durban: Umbilo	-29.833 S; 31.000 E	A2	FJ415844	N/A	Unknown
DM7905	D27	Durban: Athlone Park	-30.016 S; 30.917 E	B1	FJ415851	N/A	Unknown
DM7907	D28	Durban: Carrington Heights	-29.883 S; 30.967 E	B1	FJ415852	N/A	Male

Table 2.1 continued. Specimen details, locality and Genbank accession numbers for *Chaerephon pumilus* samples. Cladal designations were defined by phylogenetic and phenetic analyses of cytochrome *b* and D-loop data.

Museum number	Field/ Lab Code	Locality	Latitude and longitude (decimal degrees)	Clade	Genbank number		Gender
					D-loop	cytochrome *b*	
DM7910	D29	Pinetown, Underwood Rd	-29.817 S; 30.850 E	B1	FJ415853	N/A	Female
DM7913	D30	Durban: Illovo	-30.100 S; 30.833 E	A1	FJ415833	N/A	Female
DM7922	D31	Swaziland: Mlawula	-26.192 S; 32.005 E	A1	N/A	FJ415820	Unknown
DM8030	D34	Park Rynie, Ocean View Farm	-30.317 S; 30.733 E	B1	FJ415854	N/A	Male
DM8036	D35	Swaziland: Mlawula	-26.192 S; 32.005 E	A1	FJ415834	FJ415821	Male
DM8042	D36	Swaziland: Wylesdale	-25.819 S; 31.292 E	B2a	FJ415856	FJ415822	Female
DM8348	D37	Durban (City Hall)	-29.858 S; 31.025 E	B1	FJ415855	N/A	Male
DM8437	D38	Swaziland: Rosecraft	-26.632 S; 31.293 E	B2a	N/A	FJ415823	Unknown
DR01	D39	Durban	-29.867 S; 31.000 E	A1	FJ415842	N/A	Unknown
DR02	D40	Durban: Yellowwood Park	-29.917 S; 30.933 E	A2	FJ415845	N/A	Male
DR04	D42	Hell's Gate	-28.067 S; 32.421 E	A1	FJ415835	N/A	Male
DR05	D43	Durban	-29.867 S; 31.000 E	B2a	FJ415857	N/A	Unknown

Table 2.2. Specimen details for other *Chaerephon* samples and outgroups included in analyses. Cladal designations were defined by phylogenetic and phenetic analyses of cytochrome *b* and D-loop data.

Species	Reference/ GenBank number	Locality	Latitude and longitude (decimal degrees)	Clade	Region of mitochondrial DNA	Gender
Chaerephon leucogaster (Madagascar)	RHF 380	Mahajanga	-15.71 S; 46.31 E	B2b	D-loop	Male
	RHF 900	Ankijabe	-16.41 S; 46.76 E	B2b	D-loop	Female
	RHF 15065	Dzama		B2b	D-loop	Unknown
	RHF 786	Ambalanjanakomby	-16.07 S; 46.07 E	B2b	cytochrome *b*	Male
	RHF 909	Ankazomborona	-16.12 S; 46.76 E	B2b	cytochrome *b*	Female
	SMG 15265	NosyKomba		B2b	cytochrome *b*	Unknown
Chaerephon pumilus (Madagascar)	RHF 1061	Farafangana	-23.82 S; 47.83 E		D-loop	Male
	RHF 1444	Tamatave ville	-18.14 S; 49.38 E		D-loop	Female
	RHF 1652	Ranomafana/Ifanadiana	-21.26 S; 47.46 E		D-loop	Female
	RHF 453	Ambatondrazaka	-17.83 S; 48.42 E		cytochrome *b*	Female
	RHF 1299	Ifanadiana	-21.31 S; 47.64 E		cytochrome *b*	Female
	RHF 1475	Fanandrana	-18.25 S; 49.27 E		cytochrome *b*	Female
Chaerephon pumilus (Kruger National Park)	AY347954	Kruger National Park	-22.417 S; 31.3 E	B2a	D-loop	Unknown
	AY347955	Kruger National Park	-22.417 S; 31.3 E	B2a	D-loop	Unknown
Mops midas	RHF 263	Sakaraha	-22.91 S; 44.53 E	Outgroup	cyt *b* and D-loop	Male

Table 2.3. Specimen details for *Chaerephon pumilus* cytochrome *b* sequences of Jacobs *et al.* (2004).

Haplotype	Locality	Wing colour	GenBank number
JHap 1	Zambia	Light	AY500285
JHap 1	Zambia	Light	AY500286
JHap 1	Zambia	Light	AY500287
JHap 1	Zambia	Light	AY500289
JHap 1	Tanzania	Light	AY377955
JHap 2	Zambia	Light	AY500288
JHap 3	Unknown	Dark	AY377963
JHap 3	Durban, Glenwood	Dark	AY377962
JHap 3	Durban, Gillitts	Dark	AY377960
JHap 3	Durban, Waterfall	Dark	AY377959
JHap 3	Durban, Pinetown	Dark	AY377958
JHap 3	Durban, Glenwood	Dark	AY377957
JHap 3	Unknown	Dark	AY377956
JHap 3	Durban, Westville	Dark	AY377954
JHap 3	Durban, Carrington Heights	Dark	AY377953
JHap 3	Durban, Springfield	Dark	AY377952
JHap 4	Durban, Amanzimtoti	Dark	AY377951
JHap 4	Durban, Amanzimtoti	Dark	AY377939
JHap 4	Hell's Gate(Lake St Lucia)	Dark	AY377949
JHap 4	Hell's Gate	Dark	AY377945
JHap 4	Hell's Gate	Dark	AY377944
JHap 4	Hell's Gate	Dark	AY377943
JHap 4	Hell's Gate	Dark	AY377942
JHap 4	Hell's Gate	Dark	AY377941
JHap 4	Hell's Gate	Dark	AY377940
JHap 5	Durban, Yellowwood Park	Dark	AY377948

2.3 Quantification of DNA

2.3.1 Evaluation of DNA integrity

The integrity of the sample DNA was assessed by electrophoresis in agarose gels with reference to a standard DNA marker (O'GeneRuler^{TM} 100 bp DNA Ladder, Fermentas Life Sciences). Approximately 5 µl of each sample was mixed with 3 µl of loading dye (Appendix 1) before being loaded into a 1 % (w/v) agarose gel containing 200 µl of 0.5 µg.ml^{-1} ethidium bromide (Appendix 1). The gel was submerged in 0.5 × TBE running buffer (Appendix 1) and electrophoresed at 100 V for approximately 90 minutes. The presence of distinct high molecular weight bands and the absence of low molecular weight smear was used as an indication that the DNA was of high integrity and suitable as a template for PCR amplification.

2.3.2 Measurement of DNA concentration

The DNA concentration of samples was measured using a NanoDrop 1000 Spectrophotometer V3.7 with reference to a blank comprising 1 µl of buffer AE (Qiagen DNeasy® Tissue Kit 250).

2.4 PCR-Amplification of target fragments

The cytochrome *b* gene was PCR-amplified as two separate fragments owing to its relatively long length (1140 bp) (Saiki *et al.*, 1988). Details of the primers (Irwin *et al.*, 1991) are given in Table 2.4. The D-loop region was amplified using the primers described in Wilkinson and Chapman (1991) (Table 2.5). Amplification was carried out using either primers P and F or primers P and E, as F is nested within E.

For both cytochrome *b* and the D-loop, 25 µl reaction mixtures contained 9 µl genomic DNA solution (containing 30 ng DNA), 0.8 µl sterile water, 2.5 µl 10 X reaction buffer (Super-Therm), 4 µl $MgCl_2$ (25 mM) (Super-Therm), 0.5 µl dNTP mix (10 mM) (Roche Diagnostics), 0.2 µl *Taq* polymerase (5 U/µl) (Super-Therm) and 4 µl each of forward and reverse primer (6 µM). PCR reactions were performed in a Perkin Elmer GeneAmp PCR System 2400 thermocycler. The thermal cycling parameters for cytochrome *b* amplification consisted of an initial denaturation step at 94°C for 4 min, followed by 36 cycles of denaturation at 94°C for 60 s, annealing at 50°C for 90 s, extension at 72°C for 2 min, and a final extension step at 72°C for 10 min. The thermal cycling parameters for the D-loop consisted of an initial denaturation at 95°C for 60 s, followed by 40 cycles of denaturation at 95°C for 60 s, annealing at 55°C for 90 s, extension 72°C for 2 minutes, and a final extension step at 72°C for 7 min.

Table 2.4. Primers used for PCR amplification of the mitochondrial cytochrome *b* region.

Amplified region	Primer	Direction	Primer sequence (5’ to 3’)
cytochrome *b*	L14723 (L23)	Forward	ACCAATGCAATGAAAAATCATGGTT
5’ fragment	H15553 (H53)	Reverse	TAGGCAAATAGGAAATATCATTCTGGT
cytochrome *b*	L15146 (L46)	Forward	CAT GAG GAC AAA TAT CAT TCT GAG
3’ fragment	H15915 (H15)	Reverse	TCT CCA TTT CTG GTT TAC AAG AC

Table 2.5. Primers used for PCR amplification of the mitochondrial D-loop region.

Amplified region	Primer	Direction	Primer sequence (5' to 3')
D-loop	P	Forward	TCCTACCATCAGCACCCAAAGC
	F	Reverse	GTTGCTGGTTTCACGGAGGTAG
	E	Reverse	GGTTCAAGTCCCTCTATCCC

2.5 Purification of DNA fragments

PCR-amplified fragments were separated by electrophoresis in 1.5 % (w/v) agarose gels at 15 V overnight (as previously described in section 2.3.1). Appropriate bands, identified by their position relative to the marker, were excised and purified using the QIAquick® Gel Extraction Kit according to the protocol described in the DNeasy® Tissue Handbook. Concentrations of the purified products were checked using a NanoDrop 1000 Spectrophotometer V3.7 with reference to a blank comprising 1 µl of buffer AE (Qiagen DNeasy® Tissue Kit 250), before sequencing.

2.6 DNA sequencing

DNA was sequenced using the primers used in the initial amplifications. Sequencing was carried out by Inqaba Biotec (Hatfield, Pretoria, Gauteng, South Africa).

2.7 Data analysis

2.7.1 Molecular phylogenetics

Mitochondrial DNA sequences of single genes are commonly used in molecular phylogenetic studies (Cummings *et al.,* 1995). Closely related species usually differ by point mutations, with the third codon position having the fastest evolutionary rate owing to lower selective constraints. Slow-evolving genes that encode enzymes and structural proteins are often useful, as they maintain phylogenetically-informative sequence differences between distantly-related species (Brown *et al.,* 1979).

2.7.2 Construction alignment of consensus sequences

The construction of consensus sequences and their subsequent alignment were carried out using the BioEdit Sequence Alignment Editor (Version 7.0.0 for Windows 95/98/NT/2000/XP) (Hall, 1999). The homology of the forward and reverse electropherograms of each sample was inspected. Appropriate changes were made to eliminate inconsistencies so as to obtain a single

consensus sequence. Sequences were aligned using the Clustal W option (Thompson *et al*., 1994) in the BioEdit Sequence Alignment Editor and the alignment was corrected through visual inspection. The cytochrome *b* and D-loop sequences were trimmed to 830 and 345 nucleotides, respectively, to allow for uniform comparisons between individual samples. The combined cytochrome *b* dataset (this study and Jacobs *et al.*, 2004) was trimmed to a common length of 343 nucleotides.

2.7.3 Data saturation

Saturated data is unsuitable for analyses as it results in the underestimation of the accumulation of mutations over time (Xia, 2000). The program DAMBE (Data Analysis in Molecular Biology and Evolution) version 5.0.32 (Xia, 2000) was used to plot the number of transitions and transversions versus divergence, producing a graphic representation of substitution saturation. The HKY + I model, determined as most appropriate for both cytochrome *b* and the D-loop datasets (MrModeltest version 2.2, Nylander (2004), was not available in DAMBE therefore the F84 substitution model was used. The amount of substitution saturation was also assessed using the test of Xia *et al*. (2003). This test calculates the index of substitution saturation (Iss), which is compared to the Iss critical value (Iss.c) for both symmetrical and asymmetrical trees. An Iss value significantly less than the Iss.c value indicates little saturation.

2.7.4 Haplotype and sequence analysis

Haplotype and sequence analyses were performed using the program DnaSP (DNA Sequence Polymorphism) version 4.10.9 (Rozas *et al*., 2003) to determine the number of haplotypes for each data set, as well as the number of conserved, variable, parsimony informative and singleton sites.

2.7.5 Genetic distance models

Over time any two sequences will diverge from each another as a result of the evolutionary forces that act upon them. Genetic distance is a measure of the dissimilarity between two sequences, and enables the divergence to be quantified (Huelsenbeck and Rannala, 1997; Nei and Kumar, 2000; Strimmer and von Haeseler, 2003).

A quantitative measure of divergence between two sequences can be obtained by counting the number of substitutions in an alignment, and is referred to as the *p*-distance (Nei and Kumar, 2000; Huelsenbeck and Rannala, 1997; Strimmer and von Haeseler, 2003). At high evolutionary rates or large divergence times, the *p*-distance becomes saturated on account of multiple

substitutions at the same site, thus leading to an underestimation of the true genetic distance (Nei and Kumar, 2000; Strimmer and von Haeseler, 2003). Various mathematical models of substitution have been created to correct for the occurrence of multiple substitutions at the same site (Strimmer and von Haeseler, 2003). Some of the more commonly-used substitution models are described below.

The Jukes-Cantor (JC69) model is one of the earliest models proposed and is probably the simplest model of sequence evolution, with only a single parameter, the overall rate of substitution (Jukes and Cantor, 1969; Strimmer and von Haeseler, 2003). The model is based on the somewhat unlikely assumption that the four bases have equal frequencies and that the rate of transitions is equal to the rate of transversions (Jukes and Cantor, 1969).

In the Kimura 2-parameter (K80) model the rates of transitions and transversions may differ, whereas the base frequencies are assumed to be equal, as in the JC69 model (Kimura, 1980; Nei and Kumar, 2000; Strimmer and von Haeseler, 2003). Variation in the base composition is another factor that may alter the frequency of nucleotide substitutions. The Felsenstein (F81) model accommodates this by allowing the frequencies for the four nucleotides to be unequal, but assumes that transitions and transversions occur at equal rates (Felsenstein, 1981; Felsenstien, 1985).

The Hasegawa, Kishino and Yano (HKY85) model is a combination of the K80 and F81 models (Nei and Kumar, 2000). The model allows for transitions and transversions to occur at different rates and for the base frequencies to vary (Nei and Kumar, 2000). The General time-reversible (GTR) model is the most comprehensive model; it allows unequal base frequencies, and for all six pairs of substitutions to have different rates (Tavaré, 1986; Hall, 2001; Zwickl and Holder, 2004). The reverse and forward rates for a specific pair are assumed to be the same.

2.7.6 Molecular model selection

An optimal substitution model was chosen using the program MrModeltest version 2.2 (Nylander, 2004). In MrModeltest version 2.2 statistical Akaike information criteria (AIC) and the hierarchical likelihood ratio test (hLRT) are used to select the appropriate model of evolution. The HKY + I model of evolution was selected for the analyses of cytochrome *b* and D-loop datasets.

2.7.7 Genetic distances

Genetic distances were calculated using the HKY + I model in PAUP* 4.0b10 (Swofford, 1993). Individual pairwise distances, mean distance within groups and mean distance between phylogenetically-defined groups were calculated.

2.7.8 Phylogenetic reconstruction

Phylogenetic trees may be constructed according to different criteria. Phenetic or neighbour-joining trees are based on a distance matrix of pairwise dissimilarities and the best tree is constructed using stepwise-clustering methods based on local topological relationships (Saitou and Imanishi, 1989; Harvey and Pagel, 1991; Rosenberg and Kumar, 2001; Van de Peer, 2003). Maximum parsimony and Bayesian trees are character-state methods in which the best phylogenetic tree may be selected using an exhaustive search method (Saitou and Nei, 1987; Saitou and Imanishi, 1989; Harvey and Pagel, 1991; Cummings *et al.*, 1995; Takezaki, 1998; Vandamme, 2003).

2.7.9 Neighbour-joining analysis

The neighbour-joining method is a phenetic method which creates tree topologies in a stepwise manner from a distance matrix (Saitou and Nei, 1987; Vandamme, 2003; Van de Peer, 2003). This method calculates the distances to internal nodes and attempts to reduce the entire length of the tree by grouping OTUs such that the result is the minimization of all internal branches (Saitou and Nei, 1987; Van de Peer, 2003). In neighbour-joining there is no assumption that all taxa are equidistant from the root. It thus attempts to obtain the "most parsimonious" tree through the minimization of overall genetic distances (Vandamme, 2003; Van de Peer, 2003). The reliability of nodes on a NJ tree maybe estimated by bootstrap resampling analysis (Felsenstein, 1985; Nei and Kumar, 2000).

Neighbour-joining phylograms based on the HKY + I model were constructed from cytochrome *b* and D-loop datasets using PAUP* 4.0b10 (Swofford, 1993) for *Chaerephon* populations and outgroups. Nodal support was estimated using bootstrap resampling analysis (cytochrome *b*, 100 replicates and D-loop, 1000 replicates) (Felsenstein, 1985).

2.7.10 Maximum parsimony analysis

Maximum parsimony is a cladistic method which aims to find the optimal tree topology for a set of aligned sequences. Of the numerous trees which may be generated, the best tree is regarded as that which requires the least number of evolutionary changes, but explains all the nodes at every sequence position (Vandamme, 2003). Maximum parsimony is a relatively fast method which does not require a substitution model, and is thus unable to correct for multiple substitutions at the same site (Gadagkar and Kumar, 2005). The reliability of nodes on a tree maybe estimated by bootstrap resampling analysis (Felsenstein, 1985; Nei and Kumar, 2000).

Cladograms were constructed from cytochrome *b* and D-loop data sets using PAUP* 4.0b10 (Swofford, 1993) to show the evolutionary relationships within and between *Chaerephon* populations and outgroups. The trees were based on heuristic searches with random additions. All characters were equally weighted, with starting trees obtained by stepwise addition. Bootstrap resampling analysis was carried out to infer the reliability of the tree nodes (cytochrome *b* 100 replicates and D-loop 1000 replicates) (Felsenstein, 1985).

2.7.11 Bayesian analysis of phylogeny

Bayesian analysis is a cladistic likelihood method which uses Markov Chain Monte Carlo statistics (Aldrich, 2002; Huelsenbeck *et al.*, 2002; Drummond *et al.*, 2005; Kelly, 2005). It is based on the posterior probability distribution of trees, and incorporates prior knowledge along with sample data, thus differentiating it from maximum likelihood methods (Huelsenbeck *et al.*, 2002; Ellison, 2004; Kelly, 2005). Bayesian analyses allow the incorporation of genetic distance models to correct for multiple substitutions at the same site. Nodal support is taken as the posterior probability of a branch (Aldrich, 2002; Huelsenbeck *et al.*, 2002; Kelly, 2005).

Bayesian analysis of cytochrome *b* and D-loop data sets was performed using MrBayes version 3.0B4 (Huelsenbeck and Ronquist, 2001). Analysis was based on the HKY + I model for both cytochrome *b* and D-loop data sets. For both cytochrome *b* and D-loop analyses four Markov chains were run for 5000000 generations with a burn-in value of 10000. The burn-in value was determined via visual inspection of probabilities. The priors for the active parameters were: transition/transversion ratio = Beta (1.00, 1.00); state frequency = dirichlet (1, 1, 1, 1); proportion of invariant sites = uniform (0.00, 1.00); topology = all topologies equally probable *a priori*; and branch lengths = unconstrained: exponential (10.0). A 50 % majority rule consensus tree was constructed. Posterior probabilities indicated the reliability of nodes.

2.7.12 Analysis of molecular variance (AMOVA)

An analysis of molecular variance was conducted for the D-loop dataset using the program Arlequin 3.11 (Excoffier *et al*., 2005). The analysis tested for significant molecular variance within and between the groups defined by phylogeographic analysis. These groups were Clade A1, Clade B1 and Clade B2a. Genetic structure (fixation) indices were calculated for individuals, populations and groups, and their significance was tested using non-parametric permutation approaches (Excoffier *et al*., 1992).

2.7.10 Diversity tests, neutrality tests and mismatch distribution analysis

Diversity tests, neutrality tests and a mismatch distribution analysis were performed on D-loop data using DnaSP version 4.10.9 (Rozas *et al*., 2003). These analyses were not carried out on the cytochrome *b* data as this coding region could not be regarded as selectively neutral. Population groups were based on the major clades defined in the phylogenetic analysis, Clade A1, Clade B1 and Clade B2a. The haplotype (*h*) and nucleotide (*Pi*) diversity values, neutrality tests (Fu's (1997) *FS* and Fu & Li's (1993) *D** and *F**) and mismatch distribution analysis were then used to estimate whether each population group was stationary or had undergone a historical population expansion. According to Peck and Congdon (2004), and Hull and Girman (2005) the following are indicators of a historical population expansion event: high *h* with low *Pi*; a unimodal pairwise difference distribution; non-significant D^* and F^* but significant *Fs*; or a high expansion co-efficient (*S*/*k*). The time since expansion for each population group was calculated based on the formula by Rogers and Harpending (1992), $\tau = 2ut$. Tau (τ) was calculated in the population analysis using DnaSP version 4.10.9, u = mutation rate (μ) × the sequence length (μ values were used from Rogers and Harpending (1992); a lower limit of 1.73×10^{-7} and an upper limit of 3.3×10^{-7} for D-loop). Generation time was taken as 2 years.

2.7.11 Haplotype networks

Haplotype networks were created for both data sets using TCS version 1.21 (Clement *et al*., 2000). This program implements the estimation of gene genealogies from DNA sequences as described by Templeton *et al*. (1992) to create a statistical parsimony network.

2.7.12 Comparison of cytochrome *b* haplotypes with those of Jacobs *et al.* (2004)

Maximum parsimony and Bayesian likelihood trees, based on 343 common nucleotides from the 5' end of the cytochrome *b* gene phenetic, were generated from *C. pumilus* haplotype sequences defined in this study and that of Jacobs *et al.* (2004).

Chapter 3: Results

3.1 Sequence analysis

3.1.1 Data saturation

The program DAMBE (Data Analysis in Molecular Biology and Evolution) version 5.0.32 (Xia, 2000) was used to measure the degree of substitution saturation in both cytochrome *b* and D-loop nucleotide sequences.

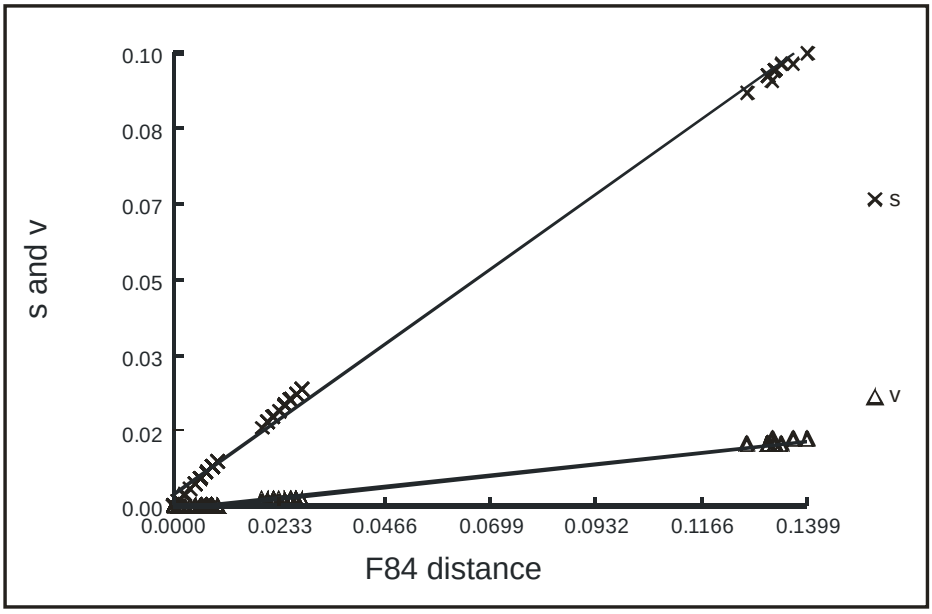

Figure 3.1. Graph representing transitions and transversions versus divergence for cytochrome *b* sequences of *C. pumilus* (southern Africa, n = 11), *C. pumilus* (Madagascar, n = 3), *C. leucogaster* (Madagascar, n = 3) and the outgroup *Mops midas* (n = 1). S = transitions, V = transversions. Solid lines are least-squares best-fit lines.

The saturation plot (Fig. 3.1) is linear for both transitions and transversions and indicates little saturation in the cytochrome *b* dataset. The overall lack of saturation is further supported by the results of the substitution saturation test by Xia *et al*. (2003). For cytochrome *b* the index of substitution saturation (Iss = 0.0292) was significantly lower than the critical values for symmetrical (0.7452) and asymmetrical (0.5335) trees (probability < 0.001). For the D-loop the index of substitution saturation (Iss = 0.3566) was also significantly lower than the critical values (Iss.c) for symmetrical (0.6943) and asymmetrical (0.4443) trees (probability < 0.001). Both results indicate little saturation in the respective datasets.

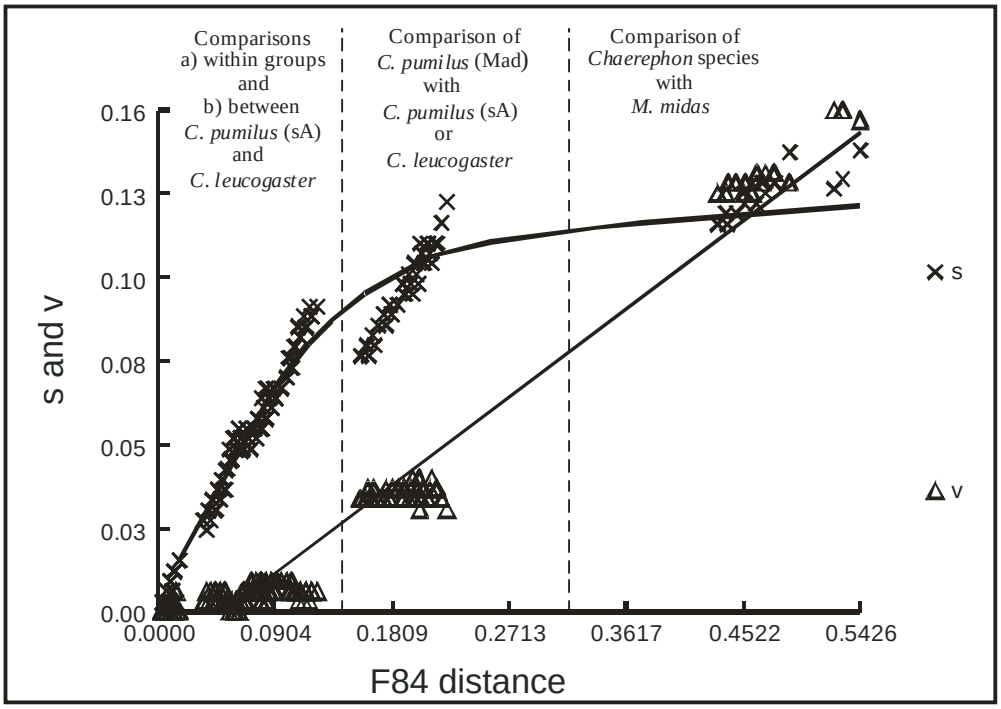

Figure 3.2. Graph representing transitions and transversions versus divergence for D-loop sequences of *C. pumilus* (southern Africa, n = 36), *C. pumilus* (Madagascar, n = 3), *C. leucogaster* (Madagascar, n = 3) and *Mops midas* (n = 1). S = transitions, V = transversions.

For the D-loop data, there appears to be some saturation in transitions, chiefly with respect to comparisons involving the outgroup, *Mops midas* (Fig. 3.2). There appears to be little saturation in transitions for the ingroup data, and no saturation in the transversion data.

3.1.2 Haplotypes

Haplotype and basic statistical analyses of the cytochrome *b* and D-loop datasets were carried out with DnaSP version 4.10.9 (Rozas *et al.*, 2003). Cytochrome *b* sequences were trimmed to a common length of 830 nucleotides. The ingroup dataset (*C. pumilus*, southern Africa) contained 820 conserved sites and 10 variable sites, of which 7 were parsimony-informative and 3 were singletons. There were 4 *C. pumilus* cytochrome *b* haplotypes, two of which comprised more than one sample (Table 3.1). The most common haplotype (H1) comprised 6 samples, whilst H3 comprised 3 samples. Haplotype diversity (h) was 0.673 (standard deviation 0.123). Nucleotide diversity (Pi) was 0.00478 (standard deviation 0.00097). The average number of nucleotide differences (k) was 3.96.

Table 3.1. Cytochrome *b* haplotypes of *C. pumilus* samples from southern Africa.

Haplotype number	Number of samples	Field/ lab code
1	6	D1, D2, D8, D23, D31, D35
2	1	D9
3	3	D12, D13, D38
4	1	D36

Table 3.2. D-loop haplotypes of *C. pumilus* samples from southern Africa.

Haplotype number	Number of samples	Field/ lab code
1	1	D1
2	13	D3, D4, D5, D6 D11, D14, D15, D17, D 18, D20, D30, D39, D42
3	2	D2, D7
4	2	D8, D9
5	2	D22, D35
6	2	D26, D40
7	2	D10, D19
8	1	D12
9	5	D13, D27, D28, D29, D37
10	1	D16
11	1	D34
12	1	D36
13	1	D43
14	2	AY347954, AY347955

The D-loop dataset of 36 samples included two samples from the Kruger National Park, Limpopo, South Africa, which were downloaded from the NCBI GenBank. D-loop sequences were trimmed to a common length of 314 nucleotides. The ingroup dataset (*C. pumilus*, southern Africa) contained 271 conserved sites and 43 variable sites, of which 39 were parsimony-informative and 4 were singletons. The 36 D-loop samples comprised 14 haplotypes, of which 8 contained more than one sample (Table 3.2). The most common haplotype (H2) comprised 13 samples. The haplotype diversity (h) was 0.851 (standard deviation 0.050). The nucleotide diversity (Pi) was 0.04144 (standard deviation 0.00373). The average number of nucleotide difference was 13.01.

3.2 Phylogenetic analysis

3.2.1 Maximum parsimony and Bayesian analyses

The maximum parsimony and Bayesian tree topologies were congruent and are presented as single figures for both the cytochrome *b* and D-loop datasets (Figs. 3.3 and 3.4). All *Chaerephon* samples formed a monophyletic clade with respect to the outgroup *Mops midas.*

For both cytochrome *b* and the D-loop, a strongly-supported (bootstrap 100 % / posterior probability 1.00) *C. pumilus* (Madagascar) clade was sister to a paraphyletic clade (91 % / 0.94, cytochrome *b* and 77 % / 0.70, D-loop) comprising *C. pumilus* (southern Africa) and *C. leucogaster* (Madagascar) samples. Within this clade are two major clades, A and B. Clade A comprises individuals from the greater Durban area, northern KwaZulu-Natal and Swaziland, and is well-supported in both cytochrome *b* (95 % / 1.00) and D-loop (93 % / 0.99) cladograms. In the D-loop dataset (Fig. 3.4), Clade A is further divided into two sister clades, A1 and A2 (Durban only individuals) (96 % / 0.99 and 100 % / 1.00 support respectively). Clade B is poorly- supported in both cladograms (< 50 % / 0.70, cytochrome *b* and 60 % / 0.63, D-loop), and comprises *C. pumilus* individuals from the greater Durban area, the Kruger National Park and Swaziland, as well as *C. leucogaster* from Madagascar. There are three subclades within Clade B, which have varying levels of support based on cytochrome *b* (Fig. 3.3) and D-loop (Fig. 3.4) data. Subclade B1 comprises individuals primarily from the Durban area, although one of the three cytochrome *b* samples is from Swaziland, and has strong (cytochrome *b*, 91 % / 1.00) to moderate (D-loop, 79 % / 0.89) support. Subclade B2a, which comprises individuals from Swaziland and the Kruger National Park, has poor (cytochrome *b*, - / 0.75) to strong (D-loop, 94 % / 0.99) support, whilst subclade B2b comprises *C. leucogaster* samples from Madagascar and has good (cytochrome *b*, 70 % / 0.97) to strong (D-loop, 100 % / 0.98) support.

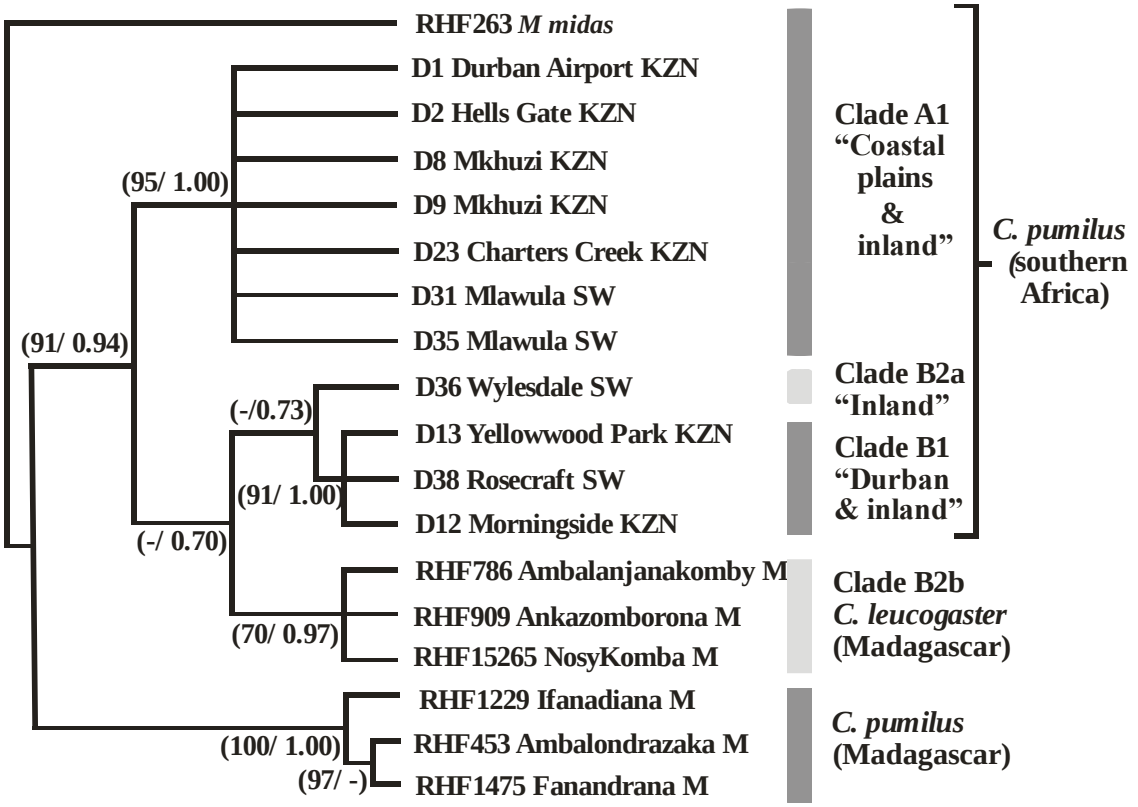

Figure 3.3. Cladogram based on 830 nucleotides of the mitochondrial cytochrome *b* gene, representing the results of maximum parsimony (100 bootstrap replicates) and Bayesian (ngen = 5000000, burnin = 10000, HKY85 model) analyses. Bootstrap values and posterior probabilities (within parenthesis) indicate nodal support. KZN = KwaZulu-Natal, South Africa. SW = Swaziland. M = Madagascar.

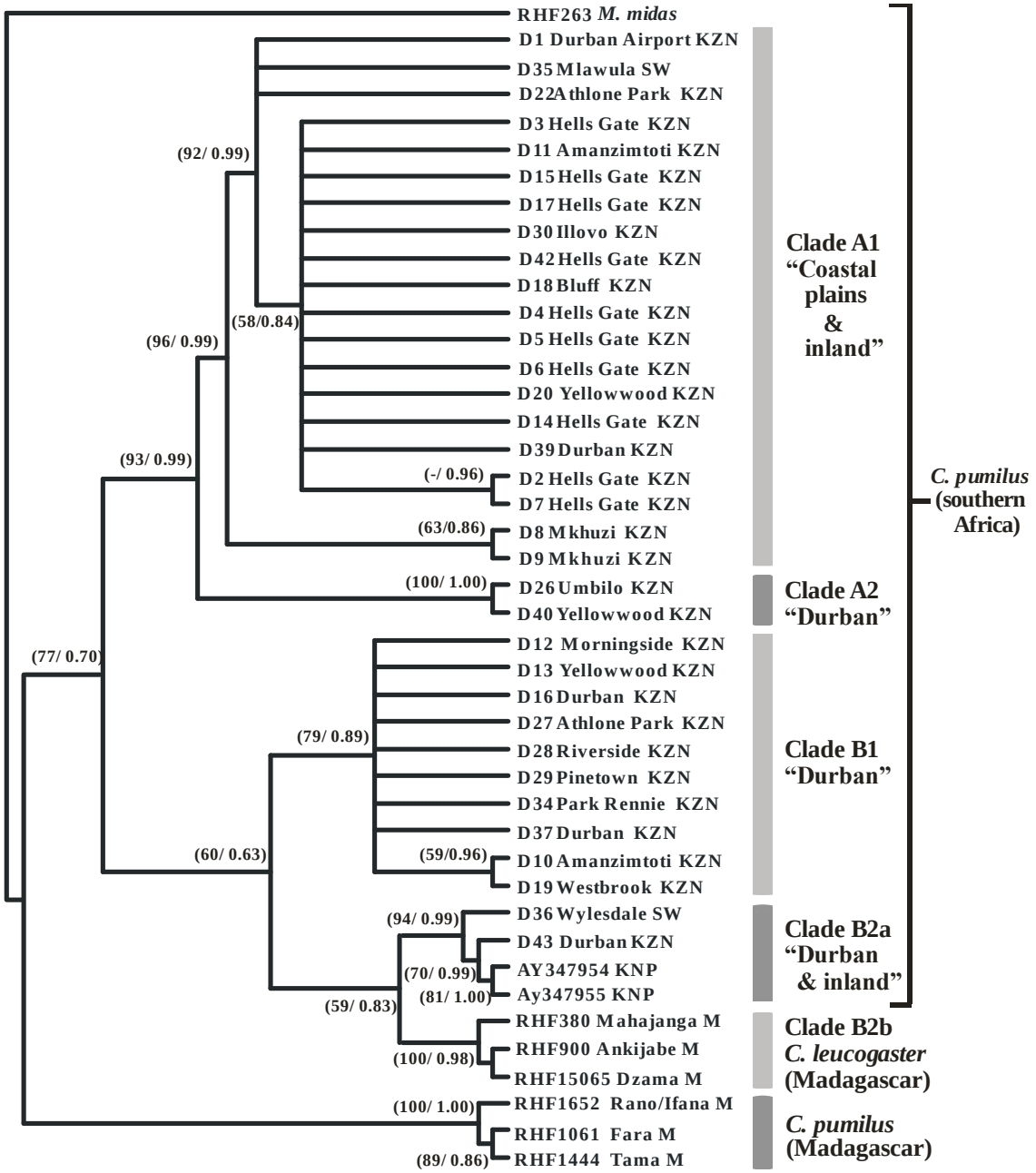

Figure 3.4. Cladogram based on 345 nucleotides of the mitochondrial D-loop, representing the results of maximum parsimony (1000 bootstrap replicates) and Bayesian (ngen = 5000000, burnin = 10000, HKY85 model) analyses. Bootstrap values and posterior probabilities (within parenthesis) indicate nodal support. KZN = KwaZulu-Natal, South Africa. SW = Swaziland. M = Madagascar.

3.3 Phenetic analysis

3.3.1 Genetic distances

Phenetic analysis was implemented with the HKY85 (1.5.2) model, selected by MrModeltest 2.2 (Nylander, 2004) as best fitting both the cytochrome *b* and D-loop datasets. Genetic

distances were calculated using PAUP* 4.0b10 (Swofford, 1993). Individual pairwise cytochrome *b* genetic distances are presented in Tables 3.3, 3.4 and 3.5. Divergence was calculated between the two major clades (A and B), of southern African *C. pumilus* defined by phylogenetic analysis (Figs. 3.3 – 3.4), in relation to Malagasy *Chaerephon* (Tables 3.6 and 3.7).

Table 3.3. Individual pairwise HKY85 cytochrome *b* genetic distances for samples 1 to 6 (units are substitutions per site).

Samples	1	2	3	4	5	6
1. RHF263 *M. midas*	-					
2. Durban Airport D1	0.1185	-				
3. Hells Gate D2	0.1185	0.0000	-			
4. Mkhuzi D8	0.1185	0.0000	0.0000	-		
5. Mkhuzi D9	0.1201	0.0012	0.0012	0.0012	-	
6. Morningside D12	0.1201	0.0085	0.0085	0.0085	0.0097	-
7. Yellowwood Park D13	0.1201	0.0085	0.0085	0.0085	0.0097	0.0000
8. Charters Creek D23	0.1185	0.0000	0.0000	0.0000	0.0012	0.0085
9. Mlawula D31	0.1185	0.0000	0.0000	0.0000	0.0012	0.0085
10. Mlawula D35	0.1185	0.0000	0.0000	0.0000	0.0012	0.0085
11. Wylesdale D36	0.1140	0.0085	0.0085	0.0085	0.0097	0.0048
12. Rosecraft D38	0.1201	0.0085	0.0085	0.0085	0.0097	0.0000
13. RHF786 Ambala	0.1201	0.0061	0.0061	0.0061	0.0073	0.0048
14. RHF909 Ankazomb	0.1200	0.0085	0.0085	0.0085	0.0097	0.0073
15. SMG15265 NosyKom	0.1216	0.0073	0.0073	0.0073	0.0085	0.0061
16. RHF1229 Ifanadiana	0.1261	0.0260	0.0260	0.0260	0.0273	0.0273
17. RHF453 Ambaton	0.1230	0.0235	0.0235	0.0235	0.0247	0.0247
18. RHF1475 Fanandrana	0.1184	0.0197	0.0197	0.0197	0.0209	0.0209

Table 3.4. Individual pairwise HKY85 cytochrome *b* genetic distances for samples 7 to 12 (units are substitutions per site).

Samples	7	8	9	10	11	12
7. Yellowwood Park D13	-					
8. Charters Creek D23	0.0085	-				
9. Mlawula D31	0.0085	0.0000	-			
10. Mlawula D35	0.0085	0.0000	0.0000	-		
11. Wylesdale D36	0.0048	0.0085	0.0085	0.0085	-	
12. Rosecraft D38	0.0000	0.0085	0.0085	0.0085	0.0048	-
13. RHF786 Ambala	0.0048	0.0061	0.0061	0.0061	0.0048	0.0048
14. RHF909 Ankazomb	0.0073	0.0085	0.0085	0.0085	0.0073	0.0073
15. SMG15265 NosyKom	0.0061	0.0073	0.0073	0.0073	0.0061	0.0061
16. RHF1229 Ifanadiana	0.0273	0.0260	0.0260	0.0260	0.0247	0.0273
17. RHF453 Ambaton	0.0247	0.0235	0.0235	0.0235	0.0222	0.0247
18. RHF1475 Fanandrana	0.0209	0.0197	0.0197	0.0197	0.0184	0.0209

Table 3.5. Individual pairwise HKY85 cytochrome *b* genetic distances for samples 13 to 18 (units are substitutions per site).

Samples	13	14	15	16	17	18
13. RHF786 Ambala	-					
14. RHF909 Ankazomb	0.0024	-				
15. SMG15265 NosyKom	0.0012	0.0036	-			
16. RHF1229 Ifanadiana	0.0247	0.0273	0.0260	-		
17. RHF453 Ambaton	0.0222	0.0247	0.0235	0.0024	-	
18. RHF1475 Fanandrana	0.0184	0.0209	0.0197	0.0061	0.0036	-

The mean cytochrome *b* genetic distance between samples of *C. pumilus* (southern Africa) was 0.0050 (range 0.0000 to 0.0097). The mean distances between *C. pumilus* (southern Africa) and the outgroups were 0.0070 (vs. *C. leucogaster*), 0.0230 (vs. *C. pumilus*, Madagascar) and 0.1180 (vs. *M. midas*).

Table 3.6. Mean HKY85 cytochrome *b* genetic distances between phylogenetically-defined groups.

	Groups	1	2	3	4	5
(1)	Clade A (*C. pumilus*)	-				
(2)	Clade B (*C. pumilus*)	0.0090	-			
(3)	*C. leucogaster* (Madagascar)	0.0070	0.0060	-		
(4)	*C. pumilus* (Madagascar)	0.0230	0.0240	0.0230	-	
(5)	*M. midas*	0.1180	0.1180	0.1200	0.1210	-

The mean cytochrome *b* genetic distance between members of Clades A and B was 0.0090. The distances from Clades A and B to outgroups were, respectively, 0.0070 and 0.0060 to *C. leucogaster*, and 0.0230 and 0.0240 to *C. pumilus* (Madagascar) (Table 3.6).

The mean D-loop genetic distance within *C. pumilus* (southern Africa) was 0.0430 (range 0.0000 to 0.1016). The mean distances between *C. pumilus* (southern Africa) and the outgroups were 0.0850 (vs. *C. leucogaster*), 0.1620 (vs. *C. pumilus*, Madagascar) and 0.3260 (vs. *M. midas*). The D-loop genetic distance between members of Clades A and B was 0.0710 (Table 3.7). The distances from Clades A and B to the outgroups were, respectively, 0.1020 and 0.0590 to *C. leucogaster*, and 0.1720 and 0.1480 to *C. pumilus* (Madagascar).

Table 3.7. Mean HKY85 genetic distance between phylogenetically-defined groups based on D-loop data.

	Groups	1	2	3	4	5
(1)	Clade A (*C. pumilus*)	-				
(2)	Clade B (*C. pumilus*)	0.0710	-			
(3)	*C. leucogaster* (Madagascar)	0.1020	0.0590	-		
(4)	*C. pumilus* (Madagascar)	0.1720	0.1480	0.1540	-	
(5)	*M. midas*	0.3380	0.3090	0.3210	0.3440	-

3.3.2 Neighbour-joining analysis

Cladal designations were assigned on the basis of congruent phylogenetic and phenetic analyses (Figs. 3.3 – 3.6). Neighbour-joining analysis for both cytochrome *b* (Fig. 3.5) and the D-loop (Fig. 3.6) revealed that *Chaerephon* formed an exclusive group with respect to outgroup, *M. midas*. The *C. pumilus* group from Madagascar was well-supported (100 %) and sister to a mixed group containing *C. pumilus* (southern Africa) and *C. leucogaster* (79 % and 67 % support). A single sample (D36, Wylesdale, Swaziland) fell outside this mixed group in the cytochrome *b* analysis. The southern African cytochrome *b* group contained a well-supported (99 %) cluster of individuals exclusively from southern Africa, which was phylogenetically defined as Clade A1 (Figs. 3.3 and 3.4). Sister to this were two clusters, one containing individuals from southern Africa (90 % support) (Clade B1), and another containing *C. leucogaster* individuals from Madagascar (75 % support) (Clade B2). The D-loop analysis was congruent with this, in that the *C. pumilus* cluster contained three sister-groups. Two of these comprised South Africa only samples, and had 98 % (Clades A1 and A2) and 95 % (Clade B1) bootstrap support respectively. The third was essentially unsupported (53 %) and comprised strongly-supported southern African (97 %) (Clade B2a) and *C. leucogaster* (Madagascar) (100 %) (Clade B2b) subgroups.

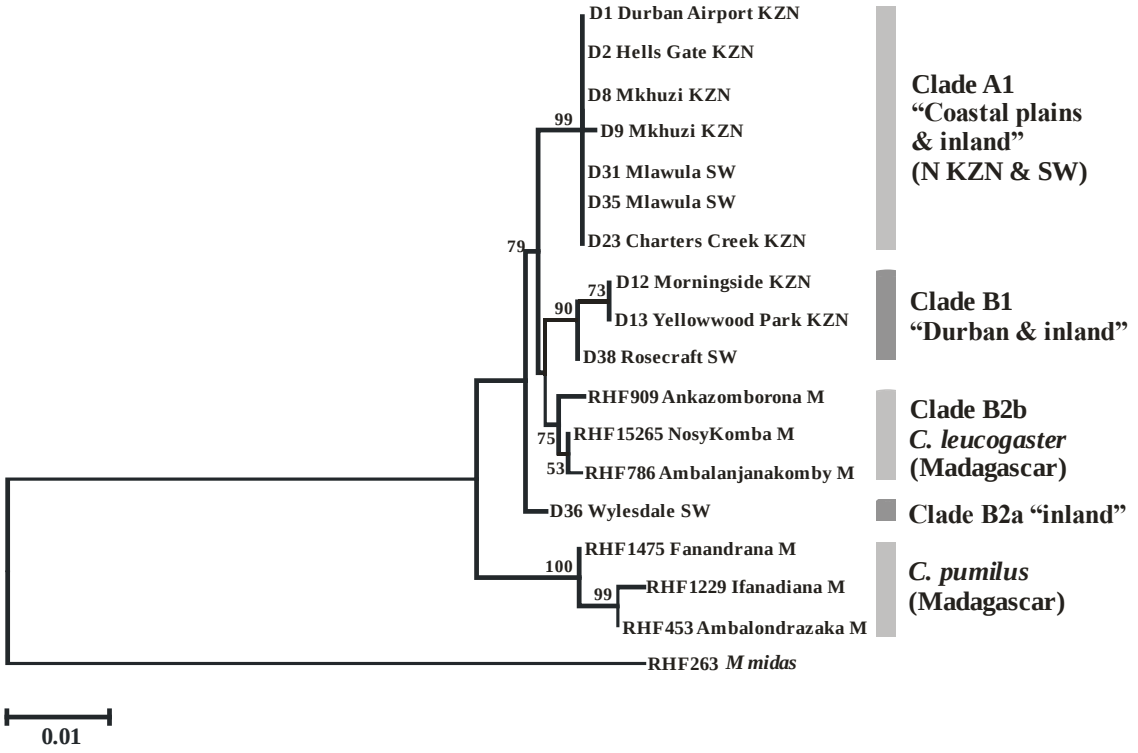

Figure 3.5. Neighbour-joining phylogram using the HKY85 distance model and based on 830 nt of the mitochondrial cytochrome *b* gene, showing genetic distance relationships between *C. pumilus* (southern Africa) and the outgroups, *C. pumilus* (Madagascar), *C. leucogaster* (Madagascar) and *Mops midas*. Bootstrap values below 50% have been omitted. Cladal designation is on the basis of congruent phylogenetic analyses (Figs 3.3 and 3.4). KZN = KwaZulu-Natal, South Africa. SW = Swaziland. M = Madagascar.

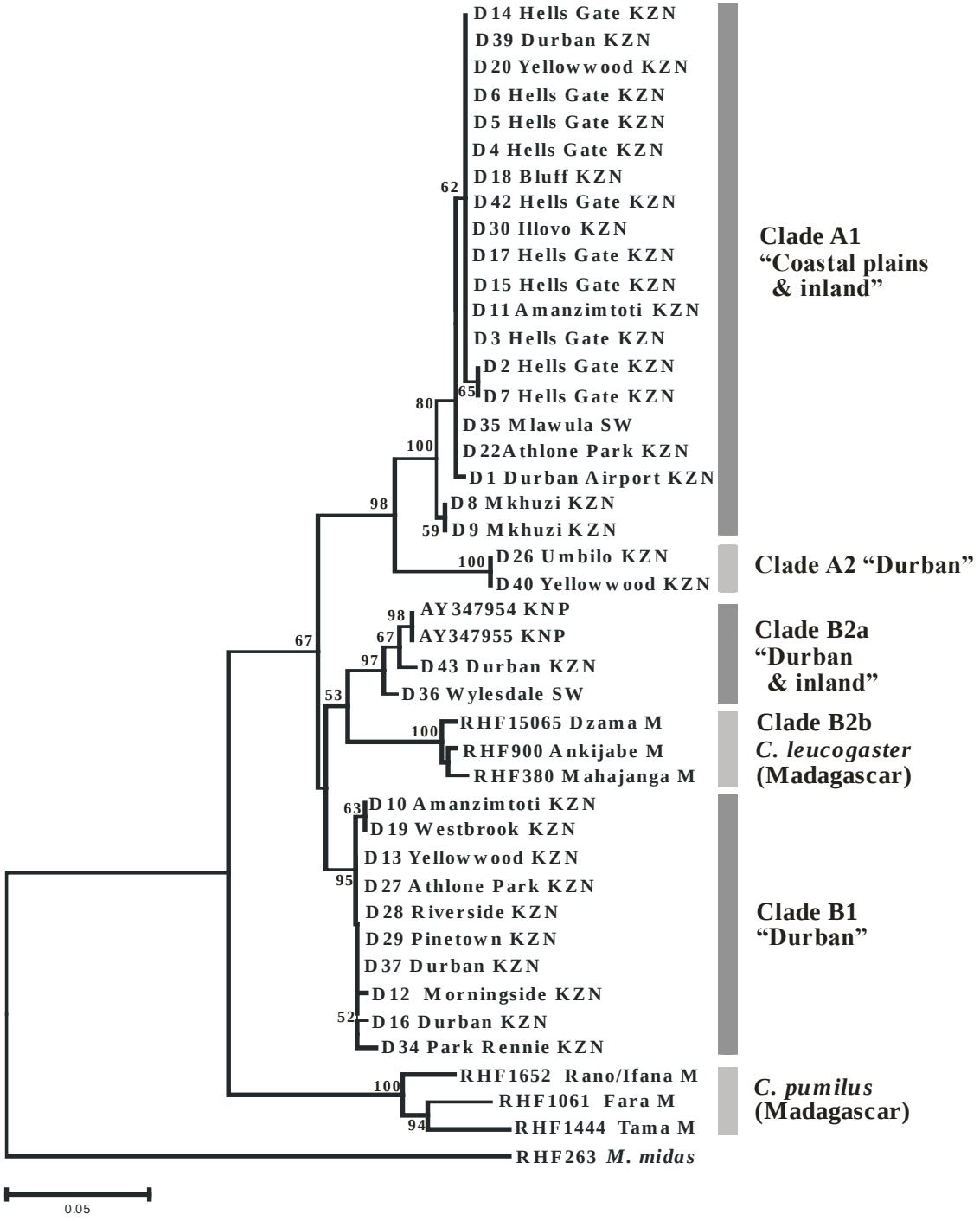

Figure 3.6. Neighbour-joining phylogram using the HKY85 distance model and based on 345 nt of the mitochondrial D-loop, showing genetic distances between *C. pumilus* (southern Africa) and the outgroups, *C. pumilus* (Madagascar), *C. leucogaster* (Madagascar) and *Mops midas*. Bootstrap values below 50 % have been omitted. Cladal designation is on the basis of congruent phylogenetic analyses (Figs 3.3 and 3.4). KZN = KwaZulu-Natal, South Africa. SW = Swaziland. M = Madagascar.

3.3.3 Analysis of Molecular Variance (AMOVA)

AMOVA was performed on the D-loop dataset using the clades defined in the phylogenetic analysis. The 36 D-loop samples were grouped according to membership of clades A1, A2, B1 and B2a. The percentage of variance distributed among groups (15.41 %) was not significant (P-value = 0.21212 ± 0.01225) (Table 3.8). The percentage of variance among populations within groups (31.90 %) was significant (P-value = 0.00002 ± 0.00000) (Table 3.8), as was the molecular variance (52.69 %) that occurred within populations (P-value = 0.00002 ± 0.00000) (Table 3.8).

Table 3.8. Results of Analysis of Molecular Variance (AMOVA) for the D-loop dataset.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variance
Among groups	2	3.665	0.07391 Va	15.41
Among populations within groups	5	3.529	0.15303 Vb	31.90
Within populations	26	6.571	0.25275 Vc	52.69
Total	33	13.765	0.47969	

Fixation indices	
FSC	0.37713
FST	0.47310
FCT	0.15408

Significance tests (1023 permutations)	
Vc and FST	P (random value < observed value) = 0.00001
	P (random value = observed value) = 0.00001
	P (random value ≤ observed value) = 0.00002 ± 0.00000
Vb and FSC	P (random value > observed value) = 0.00001
	P (random value = observed value) = 0.00001
	P (random value ≥ observed value) = 0.00002 ± 0.00000
Va and FCT	P (random value > observed value) = 0.20821
	P (random value = observed value) = 0.00391
	P (random value ≥ observed value) = 0.21212 ± 0.01225

3.4 Population genetics

3.4.1 Diversity tests, neutrality tests and mismatch distribution analyses

Chaerephon pumilus D-loop data were analysed using diversity tests, neutrality tests and mismatch distribution analyses implemented in DnaSP version 4.10.9. These analyses were not carried out on the cytochrome *b* dataset as it is coding and cannot be regarded as selectively

neutral. The analyses were based on the three major clades that were defined in phylogenetic and phenetic analysis, Clade A1, Clade B1 and Clade B2a (Table 3.9).

Table 3.9. Diversity tests, neutrality tests and mismatch distribution analysis for three defined clades of southern African *C. pumilus*, based on 314 nucleotides of the mitochondrial D-loop.

	Clade A1 (n=20)	Clade B1 (n=10)	Clade B2a (n=4)	Expectation#
Nucleotide diversity (Pi)	0.00399	0.00368	0.01062	Low
Haplotype diversity (*h*)	0.574⊥	0.756	0.833	High
Expansion Co-efficient (*S/k*)	4.789	4.325	1.800⊥	High
Fu & Li's (1993) F*	0.18541	-1.52186	0.17272	Not significant
Fu & Li's (1993) D*	0.54727	-1.34803	0.17969	Not significant
Fu & Li's (1993) Fs	-0.540	-1.896	0.888⊥	Significant
Raggedness (*r*)	0.0913	0.1151	0.3056	Not significant
Mismatch distribution	Unimodal	Unimodal	Multimodal⊥	Unimodal
Tau (τ)	0.252	1.156	2.968	-
Time since expansion (yr BP)**	2432 - 4639	11156 - 21280	-	-

Expected trends for a model of demographic population expansion (Peck and Congdon, 2004)

⊥ Does not satisfy requirements for population expansion.

** The value is obtained from the formula $\tau = 2ut$ (Rogers & Harpending, 1992), where u is the product of mutation rate (μ) per generation and sequence length (314 bp) and t is the time (in generations) since expansion (generation time taken as 2 years). The mutation rate (μ) for the D-loop was from Rogers & Harpending 1992: 17.3 % divergence per million years, or $\mu = 1.73 \times 10^{-7}$ mutations per site per generation (upper limit), and 33.0 % divergence or 3.3 mutations per site per generation $\times 10^{-7}$ (lower limit).

Clade B1 shows a good overall fit to the expected trend for a model of demographic population expansion. Clade A1 fits most of the expectations of an expanding population, although the haplotype diversity is lower than would be expected according to Peck and Congdon (2004). Clade B2a does not show evidence of a historical population expansion, as the Fs test (0.888, $p > 0.10$) was not significant and the mismatch distribution was multimodal (Table 3.9). The time since expansion for Clade A1 was calculated as 2432 – 4639 years, while that for Clade B1 was 11156 – 21280 years (Table 3.9).

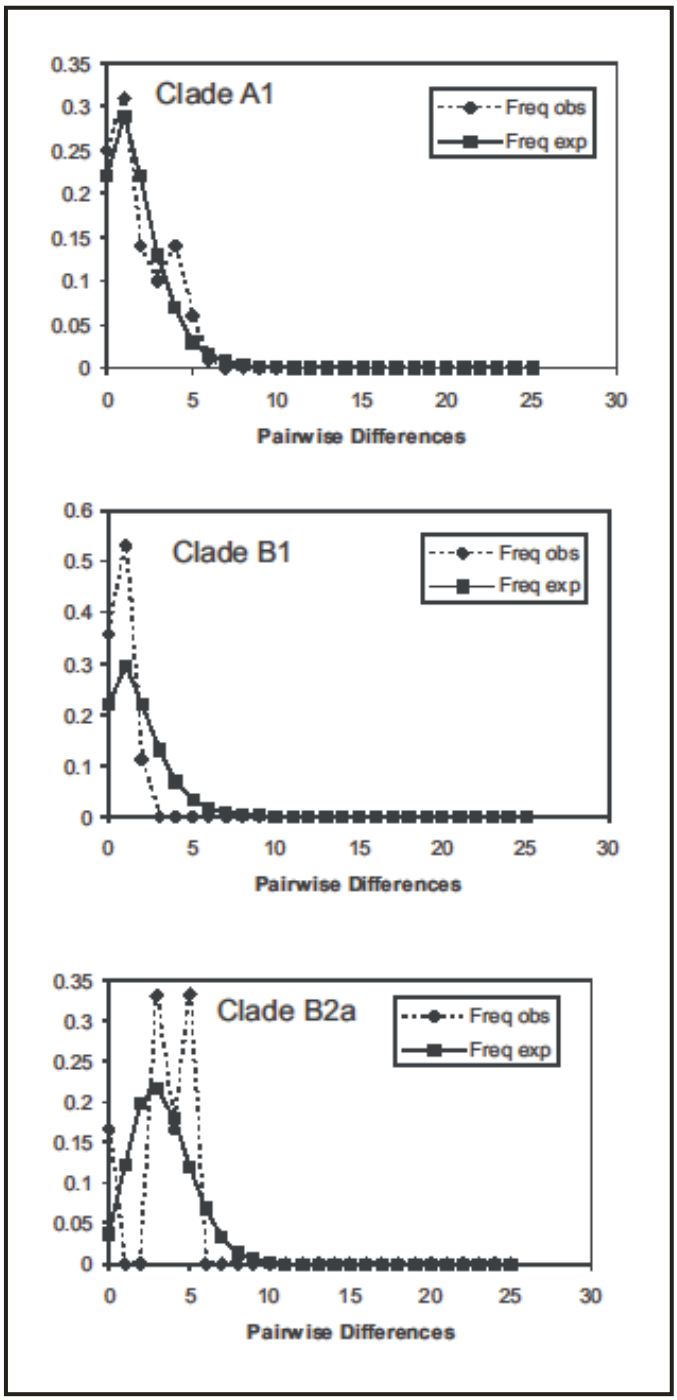

Figure 3.7. Mismatch distribution plots for the D-loop dataset (Clade A1, Clade B1 and Clade B2a).

3.4.2 Haplotype networks

Statistical parsimony analysis was carried out using TCS version 1.2.1 on both the cytochrome *b* and D-loop data. The cytochrome *b* analysis was based on 11 *C. pumilus* individuals with reference to *C. leucogaster* (Madagascar) and *C. pumilus* (Madagascar) (Fig. 3.8A), and only *C. leucogaster* (Madagascar) (Figs. 3.8B, C and D). Two haplotype networks were generated when connections were set at a 95 % confidence limit. *Chaerephon pumilus* (Madagascar) formed a separate network from the network which included all *C. pumilus* (southern Africa) and *C. leucogaster* samples. A single haplotype network was generated when the tolerance was set to 100 mutational steps. *Chaerephon pumilus* (Madagascar) formed an exclusive clade within this network, which was separated from a separate southern African *C. pumilus* and Malagasy *C. leucogaster* clade by a minimum of 19 mutational steps (Fig. 3.8A). The clades referred to are those defined in the phylogenetic analysis (Figs. 3.3 and 3.4). There are 5 steps between Clade A1 and Clade B2b (*C. leucogaster*) and 7 steps between Clade A1 and the combination of Clade B1 and Clade B2a. Clade B2b is separated by 4 steps from Clade B1 and Clade B2a. Most Clade B samples were female, whilst Clade A comprised both males and females (Fig 3.8B). *Chaerephon pumilus* (southern Africa) individuals were generally found below 600 m (Fig. 3.8C), with the exception of two individuals from the highlands of Swaziland. Clade A haplotypes appeared to be found in areas with lower rainfall (<1000 mm per year) than Clade B haplotypes (700 - >1000 mm per year) (Fig. 3.8D).

D-loop analysis was based on 36 *C. pumilus* individuals with reference to *C. leucogaster* (Madagascar) and *C. pumilus* (Madagascar) (Fig. 3.9), and only *C. leucogaster* (Madagascar) (Fig 3.10). At a 95 % connection limit, six haplotype networks were generated and contained clades congruent to those in the maximum parsimony (Fig. 3.4) and Bayesian (Fig. 3.6) trees. A single haplotype network was generated when the tolerance was set to 100 mutational steps (Fig. 3.9). A haplotype network with overlays of sex, altitude and rainfall is presented in Figure 3.10. The minimum number of mutational steps between southern African *C. pumilus* and Malagasy *C. pumilus* is 48, while the minimum number of steps between southern African *C. pumilus* and *C. leucogaster* (Madagascar) is 16. The Malagasy *Chaerephon* (*pumilus* and *leucogaster*) are separated by a minimum of 52 steps. Clades B1 and Clade B2a are separated by a minimum of 10 mutational steps. Clade A2 is separated from both Clade B2a and B2b (*C. leucogaster*) by a minimum of 33 steps. Clade B2a and Clade B2b are separated by a minimum of 26 steps. The major clade (A1) is separated by a minimum of 28 steps from both Clade B2a and Clade B2b. The two major clades, A1 and B1, are separated by a minimum of 18 steps. In

Figure 3.9 Clade B2a individuals are found across the widest latitude range (22^{o}S – 30^{o}S), whilst Clades A2 and B1 are found exclusively in the Durban area (29^{o}S – 30^{o}S). There is no apparent relationship between haplotype structure and sex (Fig 3.10A). *Chaerephon pumilus* individuals were generally found almost entirely below 600 m (Fig. 3.10B) where the annual rainfall is >900 mm per year (Fig. 3.10C). A single individual (D36, Wylesdale, Swaziland) was found above 600 m (Fig. 3.10B), where the annual rainfall is > 1000 mm per year (Fig. 3.10C). Some individuals in Clades A1 and B2a were also found in areas where the annual rainfall is <700 mm per year (Fig. 3.10C).

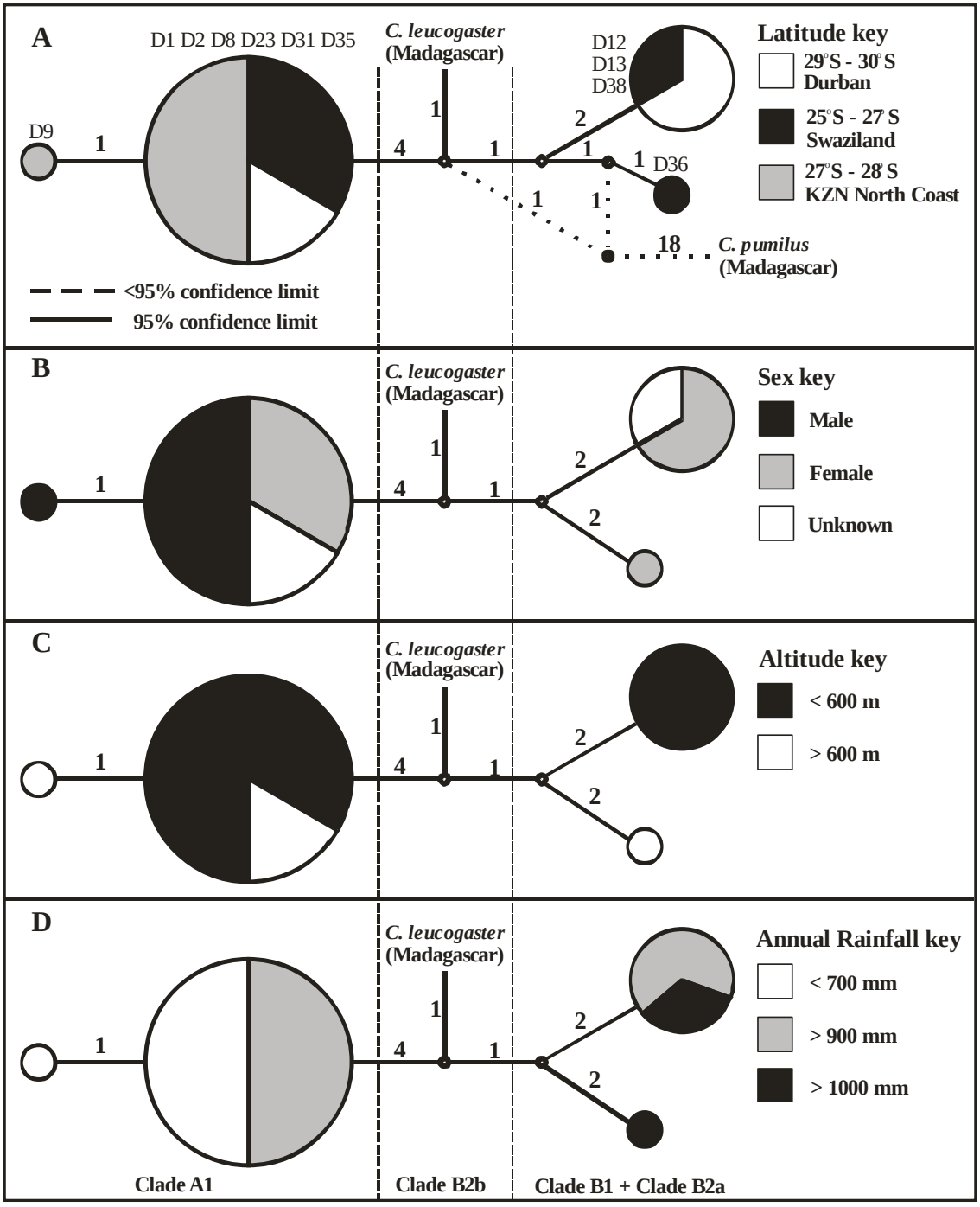

Figure 3.8. Haplotype networks for the cytochrome *b* data with overlays representing: A) latitude; B) sex; C) altitude and D) rainfall. Networks represent the relationships of 11 *C. pumilus* samples from southern Africa and 3 *C. leucogaster* samples from Madagascar.

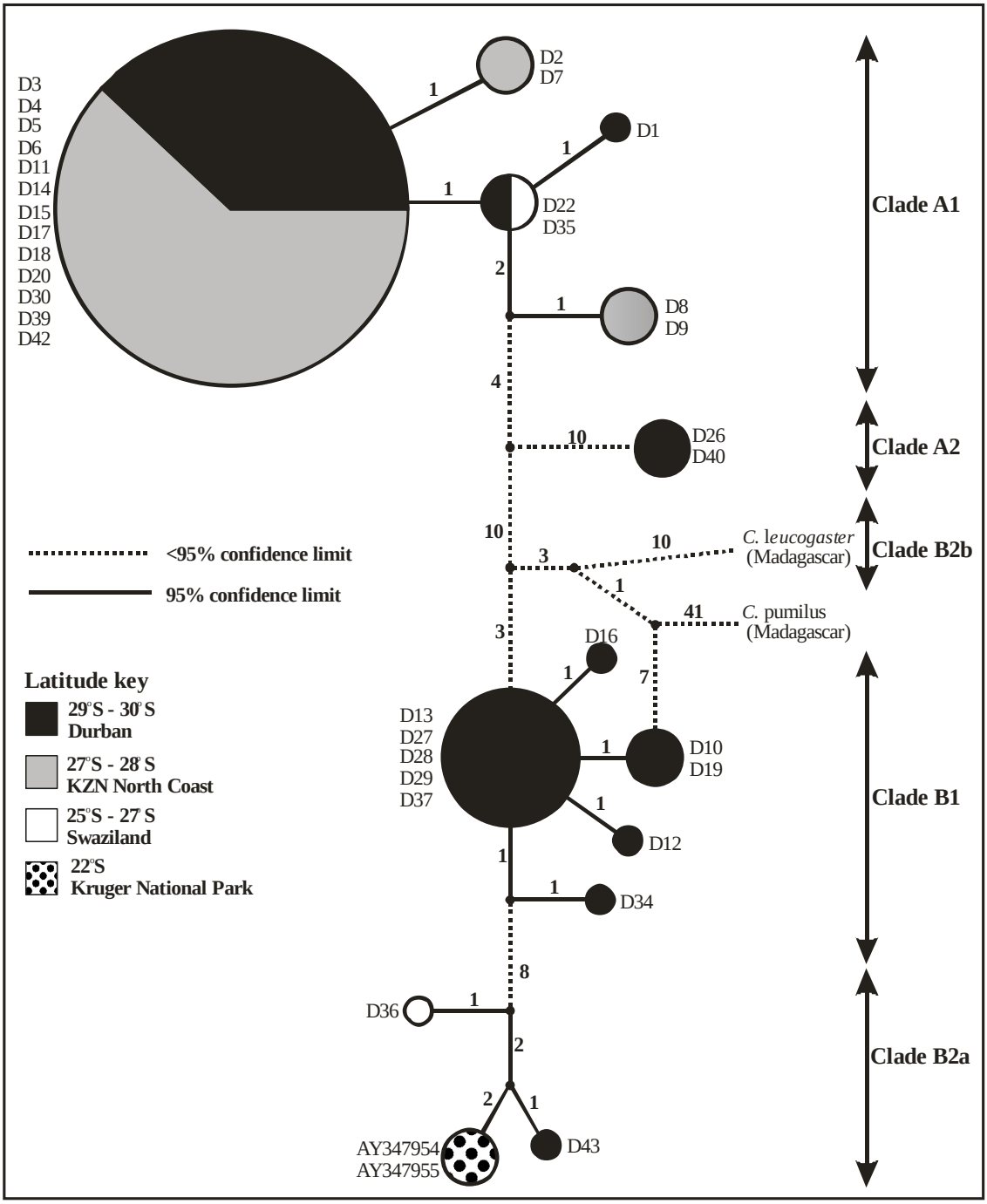

Figure 3.9. Haplotype network for the D-loop data illustrating the relationships between 36 *C. pumilus* samples from southern Africa and 3 *C. leucogaster* samples from Madagascar. Information regarding latitude has been superimposed onto the illustration.

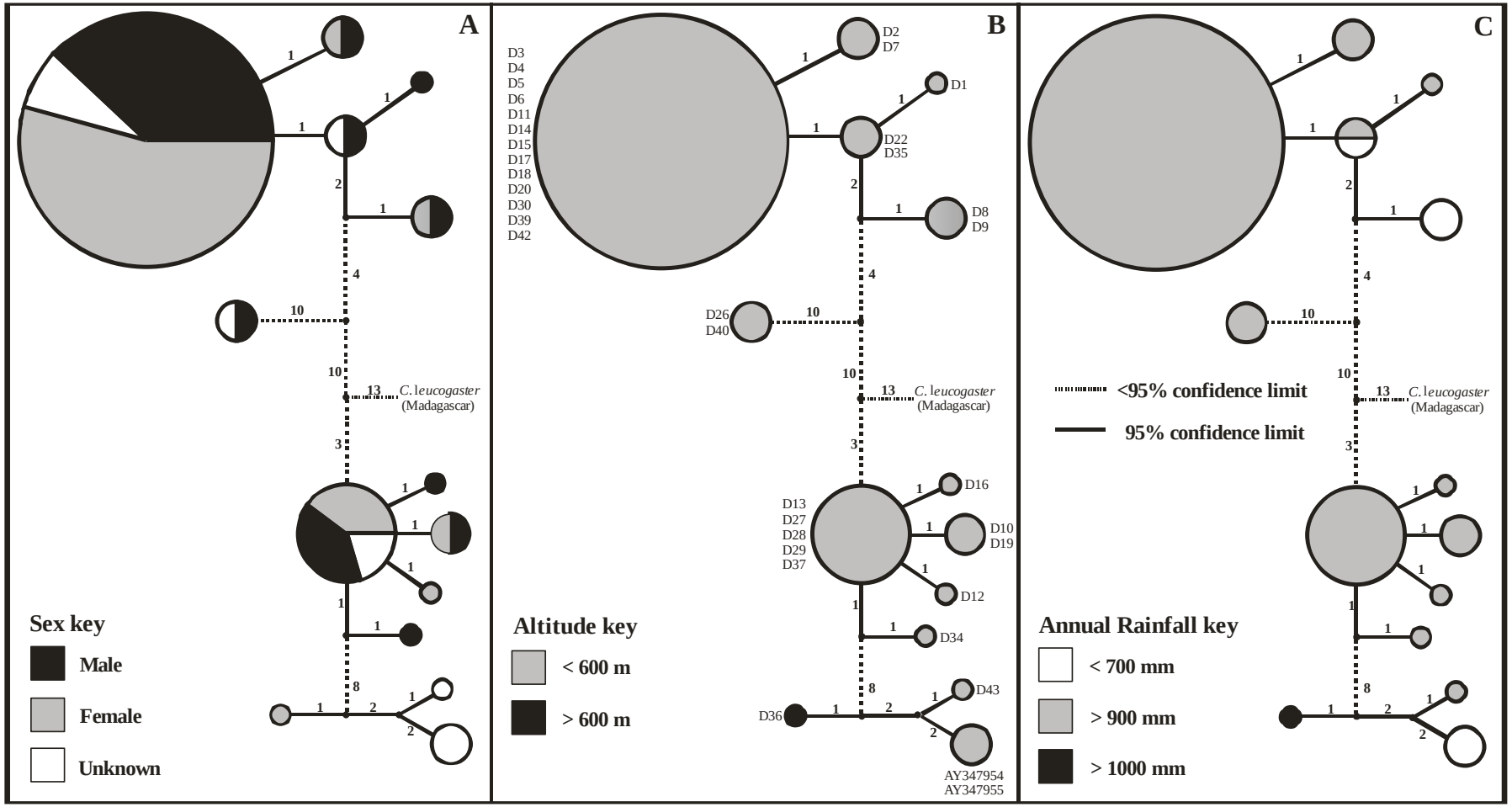

Figure 3.10. Haplotype networks for the D-loop data with overlays representing: A) sex; B) altitude and C) rainfall. The networks include 36 *C. pumilus* samples from southern Africa and 3 *C. leucogaster* samples from Madagascar.

3.5 Comparison of cytochrome *b* haplotypes with those of Jacobs *et al.* (2004)

A combined analysis was carried out in order to assess relationships between the 10 cytochrome *b* haplotypes defined in this study (4 *C. pumilus* (southern Africa), 3 *C. leucogaster* (Madagascar) and 3 *C. pumilus* (Madagascar)) and 5 haplotypes from a published study on southern African *C. pumilus* (Jacobs *et al.*, 2004). Sequences from Jacobs *et al.* (2004) were 423 nucleotides long, whilst those from this study were 830 nucleotides. The combined dataset was trimmed to 343 common nucleotides. Details of haplotypes are given in Table 3.10.

Table 3.10. Haplotype details for a combined *Chaerephon* dataset based on 343 nucleotides of the mitochondrial cytochrome *b* gene. Haplotypes 1 – 10, from this study, are those defined on the basis of 830 nucleotides of the cytochrome *b* gene (see Table 3.1), and trimmed to 343 nucleotides. JHaps 1 – 5 are from the study of Jacobs *et al.* (2004), originally based on 423 nucleotides, and trimmed to 343 nucleotides.

Species	Haplotype Reference	Number of Samples	Sample Names
C. pumilus (southern Africa)	Hap 1	6	D1 (Dbn Airport); D2 (Hell's Gate); D8 (Mhkuzi); D23 (Charters Creek); D31 & D35 (Both Mlawula, Swaziland)
	Hap 2	1	D9 (Mhkuzi)
	Hap 3	3	D12 (Dbn, Amanzimtoti); D13 (Dbn, Morningside); D38 (Rosecraft, Swaziland)
	Hap 4	1	D36 (Wylesdale, Swaziland)
C. leucogaster	Hap 5	1	RHF786 (Ambalanjanakomby)
	Hap 6	1	RHF909 (Ankazomborona)
	Hap 7	1	SMG15265 (NosyKomba)
C. pumilus (Madagascar)	Hap 8	1	RHF1229 (Ifanadiana)
	Hap 9	1	RHF1475 (Fanandrana)
	Hap 10	1	RHF453 (Ambatondrazaka)
C. pumilus (Jacobs *et al.*, 2004)	JHap 1	5	AY500285, AY500286, AY500287, AY500289 (all Zambia); AY377955 (Tanzania)
	JHap 2	1	AY500288 (Zambia)
	JHap 3	10	AY377963 (Unknown); AY377962 (Dbn, Glenwood); AY377960 (Dbn, Gillitts); AY377959 (Dbn, Waterfall); AY377958 (Dbn, Pinetown); AY377957 (Dbn, Glenwood); AY377956 (Unknown); AY377954 (Dbn, Westville); AY377953 (Dbn, Carrington Heights); AY377952 (Dbn, Springfield)
	JHap 4	9	AY377951, AY377939 (Both Dbn, Amanzimtoti); AY377949, AY377945, AY377944, AY377943, AY377942, AY377941, AY377940 (All Hell's Gate)
	JHap 5	1	AY377948 (Dbn, Yellowwood Park)

Three of the haplotypes of Jacobs *et al.* (2004) were identical to haplotypes from this study (JHap 1 = Hap 7, JHap 3 = Hap 3 and JHap 4 = Hap 1), whilst the other two differed by a minimum of one mutation (out of 343 nucleotides) (genetic distance 0.0029) from haplotypes in this study (JHap 2: Hap 5 / 7 and JHap 5: Hap 1) (Table 3.11). Because Haps 1 – 10 (this study) were originally defined on the basis of 830 nucleotides (Table 3.1) and then trimmed to 340 nucleotides, two of these haplotypes are identical (Haps 5 and 7)

Table 3.11a. Individual pairwise HKY85 cytochrome *b* genetic distances for samples 1 to 7 (units are substitutions per site).

Samples	1	2	3	4	5	6	7
1. Hap 1	-						
2. Hap 2	0.0029	-					
3. Hap 3	0.0088	0.0118	-				
4. Hap 4	0.0059	0.0088	0.0088	-			
5. Hap 5	0.0029	0.0059	0.0059	0.0029	-		
6. Hap 6	0.0059	0.0088	0.0088	0.0059	0.0029	-	
7. Hap 7	0.0029	0.0059	0.0059	0.0029	0.0000	0.0029	-
8. Hap 8	0.0208	0.0238	0.0239	0.0208	0.0178	0.0208	0.0178
9. Hap 9	0.0208	0.0238	0.0239	0.0208	0.0178	0.0208	0.0178
10. Hap 10	0.0148	0.0178	0.0178	0.0148	0.0112	0.0148	0.0112
11. JHap 1	0.0029	0.0059	0.0059	0.0029	0.0000	0.0029	0.0000
12. JHap 2	0.0059	0.0088	0.0088	0.0059	0.0029	0.0059	0.0029
13. JHap 3	0.0088	0.0118	0.0000	0.0088	0.0059	0.0088	0.0059
14. JHap 4	0.0000	0.0029	0.0089	0.0029	0.0029	0.0059	0.0029
15. JHap 5	0.0029	0.0059	0.0118	0.0088	0.0059	0.0088	0.0059

Haps 1 – 10 are from this study

JHaps 1 – 5 are from Jacobs *et al.* (2004)

Table 3.11b. Individual pairwise HKY85 cytochrome *b* genetic distances for samples 8 to 14 (units are substitutions per site).

Samples	8	9	10	11	12	13	14
8. Hap 8	-						
9. Hap 9	0.0000	-					
10. Hap 10	0.0059	0.0059	-				
11. JHap 1	0.0178	0.0178	0.0118	-			
12. JHap 2	0.0208	0.0208	0.0148	0.0029	-		
13. JHap 3	0.0239	0.0239	0.0178	0.0059	0.0088	-	
14. JHap 4	0.0209	0.0209	0.0149	0.0029	0.0059	0.0089	-
15. JHap 5	0.0239	0.0239	0.0178	0.0059	0.0088	0.0118	0.0029

The cytochrome *b* genetic distance within the southern African *C. pumilus* samples from this study (0.008) and that of Jacobs *et al.* (2004) (0.006), and between these groups (0.006) were

similar. Both groups were separated by a greater genetic distance from *C. pumilus* (Madagascar) (0.020 and 0.019) (Table 3.12) than they were from each other.

Table 3.12. HKY85 cytochrome *b* genetic distances within and between haplotypes groups from this study and that of Jacobs *et al.* (2004).

Haplotype groups	Genetic distance
within Haps 1 – 4 (*C. pumilus*, southern Africa) (this study)	0.008
within JHaps 1 – 5 (*C. pumilus*) (Jacobs *et al.*, 2004)	0.006
between Haps 1 – 4 and JHaps 1 – 5	0.006
between Haps 8 – 10 (*C. pumilus* Madagascar) and Haps 1 – 4	0.020
between Haps 8 – 10 and JHaps 1 – 5)	0.019

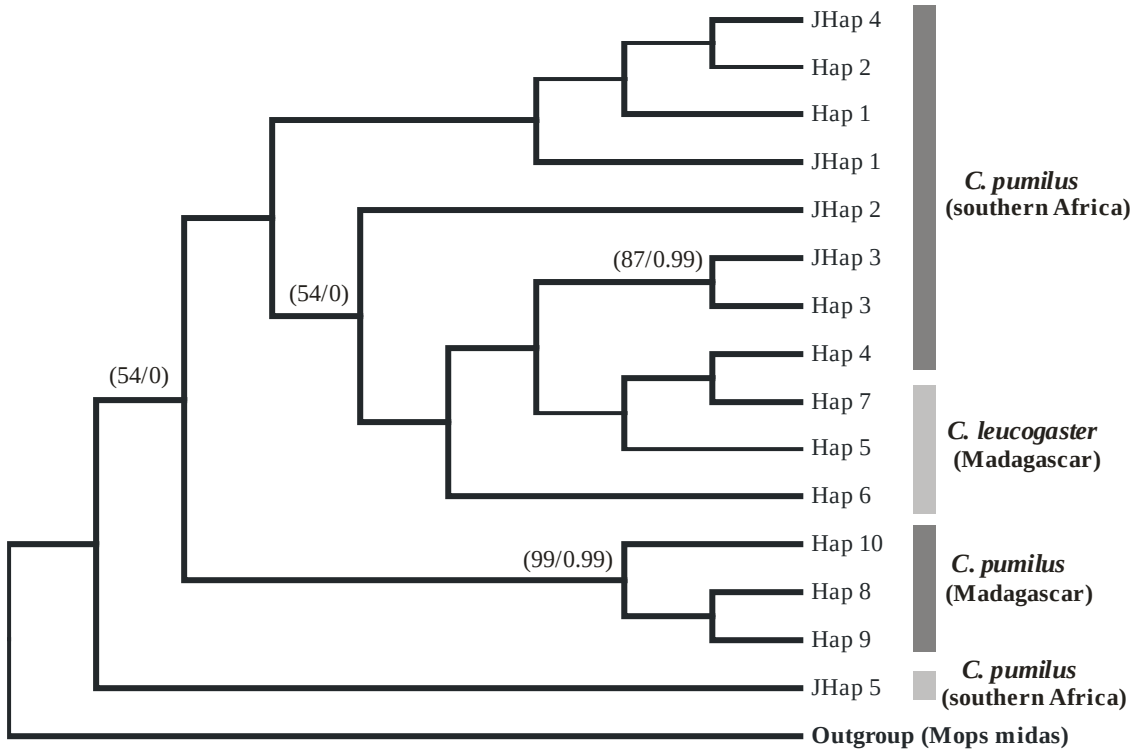

Figure 3.11. Cladogram representing maximum parsimony (100 replicates) and Bayesian (ngen = 5000000, burnin = 10000) analyses of relationships between haplotypes from Jacobs *et al.* (2004), *C. pumilus* (southern Africa), *C. pumilus* (Madagascar), and *C. leucogaster* (Madagascar) with reference to the outgroup *Mops midas*. Bootstrap values and posterior probabilities (within parenthesis) indicate nodal support. Bootstrap values below 50 % have been omitted. Haps 1- 10 are from this study. JHaps 1-5 are from Jacobs *et al.* (2004).

Chaerephon individuals formed an exclusive group with respect to the outgroup *M. midas*. Haplotypes from the Jacobs *et al.* (2004) study were interspersed with *C. pumilus* (southern Africa) haplotypes from this study. There were two well-supported clades. One comprised *C.*

pumilus (Madagascar) haplotypes 8, 9 and 10 (bootstrap 99 %, posterior probability 0.99) (Fig. 3.11). The other comprised haplotype 3 from this study and haplotype 3 from the Jacobs *et al.* (2004) study (bootstrap 84 %, posterior probability 0.99), which, as previously mentioned, were identical. Haplotypes from Tanzania and Zambia (JHaps 1 and 2) were interspersed with haplotypes from KwaZulu-Natal and Swaziland (Haps 1, 2, 3 and 4, and JHaps 3 and 4) (Fig. 3.11).

Chapter 4: Discussion

4.1 Taxonomy of Chaerephon pumilus

The taxonomy of *Chaerephon pumilus* has been controversial as this taxon exhibits considerable phenotypic variation over its range on mainland Africa. It might be expected that considerable genetic variation would accompany this morphological variation, possibly sufficient to support the definition of cryptic species. Variation in echolocation frequency has been evident among the dark- and light-winged forms of *C. pumilus*, however this variation fell within the limits of intraspecific flexibility reported by Aspetsberger *et al.* (2003). Subsequent research by Jacobs *et al.* (2004), based on 423 nucleotides of the cytochrome *b* gene, found the sequence divergence between the dark- and light-winged forms to be 0.9 %. Both these studies suggest that these two forms are not sufficiently distinct to warrant designation as separate species (Aspertsberger *et al.*, 2003; Jacobs *et al.*, 2004).

In this study phenetic analysis was based on phylogenetic cladal designation. Two prominent clades (A and B), defined on the basis of congruent cytochrome *b* and D-loop analyses, were present within southern African *C. pumilus*. Phenetic analysis based on 830 nucleotides of the cytochrome *b* gene revealed them to be separated by a genetic distance of 0.9 %, which corresponds to the intercladal distance reported by Jacobs *et al.* (2004), and suggests that both studies are identifying the same two clades of southern African *C. pumilus*. Clades A and B of *C. pumilus* (southern Africa) and *C. leucogaster* were separated from *C. pumilus* (Madagascar) by similar cytochrome *b* genetic distances (2.3 % and 2.4 %, respectively) (Table 3.6). *Chaerephon pumilus* (southern Africa) appeared more similar to *C. leucogaster* (0.7 % and 0.6 % divergence) than to *C. pumilus* (Madagascar). According to Bradley and Baker (2001), a genetic distance of less than 2 % is typical of population and intraspecific variation in mammals. Baker and Bradley (2006) reported ranges of bat cytochrome *b* genetic distances that could be used to define species according to the genetic species concept. Their study, based on sequences of twelve bat genera, excluding Molossidae, revealed within-species differences of 0.6 % to 2.3 % and species level differences of between 3.3 % and 14.7 %. Taken together, the above suggests that *C. leucogaster* and *C. pumilus* (southern Africa) may be conspecific, and that *C. pumilus* (Madagascar), which falls at the high end of the within-species range, may also be conspecific with them, or may be a different, possibly new, subspecies or species. Baker and Bradley (2006) have noted that a cytochrome *b* genetic distance of less than 2 % does not

always identify conspecific populations, as cytochrome *b* genetic distances are a single metric which should be used in combination with other characters and methods to define species.

The low level of divergence (0.9 %) between *C. pumilus* (southern Africa) populations may be indicative of recently-diverged species. This is in accordance with previous studies by Ratrimomanrivo *et al.* (2007), on *Mops midas*, and Lamb *et al.* (2008), on *Otomops* species, as both suggest that the evolution of molossid bats may occur at relatively low cytochrome *b* mutation rates. Similarly, Ditchfield (2000) found that cytochrome *b* sequences of Phyllostomidae showed much lower genetic variation than those of other small mammals and that the rate of molecular evolution is generally slower in bats. Thus good species may be separated by cytochrome *b* genetic distances below the mammalian or bat average values reported by Baker and Bradley (2006). The mitochondrial ND1 gene, like the mitochondrial cytochrome *b* gene, is protein-coding and both regions are known to evolve at similar rates (Mayer *et al.*, 2007). Mayer and von Helverson (2001) reported that *Eptesicus serotinus* and *E. nilssonii* are separated by a ND1 distance of 0.7 % – 1.7 %, whilst *Myotis myotis* and *M. blythii* are separated by 0.25 % – 2.6 %. While the genetic distances separating these two morphologically-similar pairs of species are low, they are regarded as valid species. This is congruent with the definition of *C. pumilus* (southern Africa) and *C. leucogaster*, which are morphologically distinct, although separated by a low average sequence divergence of 0.7 %, as distinct species, and would imply, on genetic grounds, that *C. pumilus* (Madagascar) is an as-yet undefined cryptic species (morphologically similar to *C. pumilus* (southern Africa)).

4.2 Phylogeny and phylogeography

Phylogenetic analysis of cytochrome *b* and D-loop sequences revealed the presence of two major clades (A and B) of southern African *C. pumilus* (Fig. 3.3 – Fig. 3.4). Clade A haplotypes appeared to be found in areas with lower rainfall (< 1000 mm per year) than Clade B haplotypes (700 - > 1000 mm per year) (Fig. 3.8D). Clade A is further separated into subclades A1 and A2. Clade A1 includes individuals from the greater Durban area as well as eastern Swaziland, the coastal plain and adjacent lowlands of northern KwaZulu-Natal. Populations of Clade A generally lie in overlapping or adjacent areas along the east coast of southern Africa, with Clade A2 exclusively from the greater Durban area. Clade B includes three subclades, B1, B2a and B2b. Clade B1 is mainly from the greater Durban area, while Clade B2a is shared mainly between inland localities in the Kruger National Park and the highlands of Swaziland. Clade B2b, sister to B2a, comprises Malagasy *C. leucogaster*. Two hypotheses may explain the

nesting of *C. leucogaster* within southern African *C. pumilus*; either i) *C. leucogaster* is conspecific with *C. pumilus*, or ii) the major clades of southern African *C. pumilus* are cryptic species.

The most appropriate phylogeographic category (Avise, 2000) for the two *C. pumilus* clades (A and B) from southern Africa would be Category IV, which includes populations that have a weak phenotypic divergence, shallow mtDNA divergence and are sympatric with regards to geographic distribution. These characteristics are "typical of local, conspecific populations exchanging migrants" (Ruedi and McCracken, 2006). Though different species, the relationship between *C. pumilus* (southern Africa) and *C. leucogaster* would correspond to Category III*; strong phenotypic divergence (Goodman and Cardiff, 2004), shallow mtDNA divergence and allopatric populations. Such populations may be the result of a recent divergence and are likely to be subspecies (Ruedi and McCracken, 2006).

Chaerephon leucogaster, however, is a well-characterized morphospecies (Ratrimomanarivo *et al*., submitted (a)). Acceptance of the status of *C. leucogaster* as a separate species implies that Clades A and B, and possibly their subclades, may be cryptic species. However, as mentioned earlier, Jacobs *et al.* (2004) suggested that the two forms of *C. pumilus* are not distinct species.

The results from this study, based on 830 nucleotides rather than the 423 nucleotides of Jacobs *et al.* (2004), are in agreement with those of Jacobs *et al.* (2004) in identifying two groups separated by a mean cytochrome *b* divergence of 0.9 %. It is interesting to consider whether the groups defined in this study (Clades A and B) are congruent with those defined by Jacobs *et al.* (2004) (light- and dark-winged). A combined analysis was carried out, including sequences from both studies, trimmed to 343 common nucleotides. As might be expected if the two studies are identifying the same groups, haplotypes from Jacobs *et al.* (2004) were either identical to or different by one substitution from haplotypes from this study, although the sample animals were different. Phylogenetic analysis, however, showed the light- (JHaps 1 and 2) and dark- (JHaps 3, 4 and 5) winged forms of the Jacobs *et al.* (2004) study to be interspersed among the haplotypes of this study, which are all dark-winged (Fig 3.11). Thus, if the groups are the same, wing shade, as indicated by Jacobs *et al.* (2004), may not be a diagnostic characteristic. It is notable that *C. pumilus* haplotypes from Zambia and Tanzania are more similar to those from KwaZulu-Natal and Swaziland and to *C. leucogaster* from Madagascar than they are to *C. pumilus* from Madagascar. The combined phylogenetic analysis provided support for the distinctness of *C. pumilus* from Madagascar from the rest of the *Chaerephon* samples, but

contained no well-supported clades indicative of relationships between Clades A and B from this study and the light- and dark-winged clades of Jacobs *et al.* (2004), and should not be over-interpreted owing to the shortness of the sequence (343 nucleotides) on which it was based.

Analyses of D-loop data, indicate that Clades A and B are 7.1 % divergent, which is lower than the 10.2 % separating Clade A and *C. leucogaster* but higher than the 5.9 % separating Clade B and *C. leucogaster*. Haplotype networks (Figs. 3.9 and 3. 10) revealed that the major southern African clades are separated by minimum of 8 and a maximum of 33 mutational steps. This is considerably higher than the within-clade numbers of steps; Clade A1 (1.2), Clade B1 (1) and Clade B2a (2). These result indicated the possible existence of cryptic species or at least subspecies. Clade B2b, *C. leucogaster* is separated from Clades A1 and A2 by 28 and 33 steps respectively. This, combined with the 10.2 % D-loop divergence between Clade A and *C. leucogaster*, suggests that at least Clade A may be a distinct species.

4.3 Population genetics and historical demography

Analysis of molecular variance showed significant geographic structuring of southern African *C. pumilus* populations (clades A1, A2, B1 and B2a) (Fst 0.473, $P < 0.001$). Another small molossid, *C. leucogaster*, also showed significant geographic structuring within its range in Madagascar (Fst 0.792, $P < 0.001$; Ratrimomanarivo *et al.*, submitted (a)). In contrast, larger molossids that are presumably more vagile, *M. midas* (Fst 0.14; Ratrimomanarivo *et al.*, 2007), *M. leucostigma* (Fst 0.2; Ratrimomanarivo *et al.*, submitted (b)) and *Otomops madagascariensis* (Fst 0.05; Lamb *et al.*, 2008) show very little structuring on the same geographic scale. *Chaerephon pumilus* is a vagile species with a wing structure adapted to fast flight in open areas, but with low levels of maneuverability (Bouchard, 1998). It does not seem likely, however, that the smaller size of *C. pumilus* relative to *Mops* and *Otomops* species, significantly limits flight distance and therefore distribution range, as haplotypes from Jacobs *et al.*, (2004) are identical to haplotypes from this study, despite separation by up to 1700 km. The geographic structure shown by *C. pumilus* sampled in this study is more likely to be the result of other factors such as male or female philopatry, which should be investigated using techniques such as microsatellites.

Population genetic analyses such as, diversity tests, neutrality statistics and mismatch distributions provided evidence of historical expansions in certain clades (Table 3.12). Both Clade A1 and Clade B1 fulfilled the expectations of Peck and Congdon's (2004) model of a

population demographic expansion. The time since expansion was calculated to be between 2432 and 4639 years ago for Clade A1, whilst the expansion of Clade B1 appeared to begin earlier, around 11156 – 21280 years ago. Clade B2a appeared to represent a stable population, as it had high nucleotide diversity, an insignificant Fs test and a multimodal mismatch distribution.

A recent study by Lawes *et al.* (2007) addressed the effects of palaeoclimatic change on resilient forest faunal communities in South Africa. The article pointed out that scarp forests along the eastern seaboard (300 to 500 m above sea level and 10 – 15 km inland) may have acted as major refugia during the last glacial maximum (LGM), around 18000 years ago. The LGM was proposed to be followed by a recolonisation event during which community patterns suggested a southward expansion of tropical fauna. The expansion of the tropical fauna coincided particularly with the expansion of the subtropical Indian Ocean coastal belt forests approximately 8000 years ago. This may have been followed by secondary contact between the southward-expanding tropical fauna and the northward-retreating Afrotemperate fauna that occupied scarp forest relicts. The sequence of these events corresponds to the estimated dates for historical expansion of *C. pumilus* populations. It is therefore possible that climatic events have had an impact on shaping the community structure of forest-associated *C. pumilus* populations.

Populations that remain close to refugia following recolonisation are expected to show higher levels of genetic diversity than those that have dispersed further away (Freeland, 2005). This is based on the assumption that the refugia contain numerous populations of the same species, which may not disperse during recolonisation, thus allowing for the generation and maintenance of a high genetic diversity. Haplotype diversities were relatively high for all three southern African *C. pumilus* clades; Clade A1 (0.574), Clade B1 (0.756) and Clade B2a (0.833). Clade B2a, which appears to be a stable population (Table 3.12), is a predominantly inland clade and has the highest haplotype diversity of the three lineages, consistent with a population that may have originated from stable populations that survived the LGM by breeding and nestling in relic scarp forests.

The localities of both Clade A1 and Clade B1 along the east coast of KwaZulu-Natal my have been associated with recolonisation events after the LGM and more specifically the southward expansion of the subtropical Indian Ocean coastal belt forest. Various theories can be put forward to explain the establishment of these populations. Clade B1 may have been associated

with the initial southward expansion from the refugia around 18000 years ago, as its calculated time of expansion is between 11156 and 21280 years ago, while Clade A1 could owe its origin to the expansion of the subtropical Indian Ocean coastal belt forest in conjunction with the secondary contact of tropical and temperate faunas, assuming that the event occurred approximately 2432 – 4639 years ago. The higher haplotype diversity of Clade B1 in comparison to Clade A1, however, is more typical of a population formed due to a secondary contact. The LGM may have served as a vicariant event, resulting in separate refugia that served as isolation pockets, however according to Lawes *et al.* (2007), the scarp forest was a continuous band rather than the typical pockets of isolation usually associated with refugia. The high diversity of both Clade A1 and Clade B1 could be due to hybridization between lineages that came together in refugia. Populations, however, may have been in isolated pockets within the refugia, and contact of these populations after the LGM may also have resulted in high haplotype diversity (Freeland, 2005). A hypothesis explaining the genetic distinctiveness of Clades A1 and B1 could be that separate southward invasions occurred since the last LGM, with Clade B1 resulting from the initial invasion, and A1 from a more recent invasion.

The small sample size of the clades ($n \leq 20$) may have had an influence on the diversity analysis. As Clades B1 and B2a are both members of Clade B, they may therefore have occupied the same refugium, expanding simultaneously with the expansion of the subtropical Indian Ocean coastal belt forest. The genetic distinctiveness of Clades A1 from B and its diversity may have been due to secondary contact between different populations from the scarp forest refugium.

In southern Africa, and more specifically the greater Durban area, *C. pumilus* are most typically associated with synanthropic roosts, although natural roosts also occur in tree crevices and exposed rocky formations (Taylor, 2000). Prior to the LGM sea level changes would have submerged much of the low-lying Indian Ocean plain, resulting in the extinction of lowland populations and the survival of populations in relict scarp forest refugia. The distribution of species such as *C. pumilus* since the LGM would have been highly dependent on the availability of natural roosts. These would have been provided by the escarpment of eastern southern Africa, where natural crevices in exposed rock formations and natural holes in mature trees, associated with scarp forests, are abundant. *Chaerephon pumilus* is likely to have re-populated the existing habitats following the southward expansion of Indian Ocean forests from about 8000 years ago.

Since expansion is directly associated with habitat availability, the current typical association of *C. pumilus* with synanthropic roosts could link the post LGM expansion of *C. pumilus* with humans. The relevant area was occupied by hominines that dwelt mainly in caves and natural shelters, 1.5 Ma). Iron Age man is known to have entered the area only around 1500 to 2000 years ago, and to have occupied large villages (Laband, 1995). These, however, were in the form of primitive huts that may not have been suitable for the roosting of *C. pumilus*. Current typical synanthropic roosts only became available with the arrival of European settlers in the early nineteenth century. Thus it appears that the post-LGM expansion of *C. pumilus* populations may not have been linked to human habitation, at least initially.

4.4 Conservation implications and management

Chaerephon pumilus is an abundant species with a broad distribution. According to the 2008 IUCN (The World Conservation Union) Red List of Threatened Species, *C. pumilus* has been classified as "least concern" (Mickleburgh *et al*., 2008). There are no major threats to this species; however, it may be regarded as a pest as it commonly roosts in the roofs of houses. The possible existence of cryptic species means that the conservation status would need to be assessed if cryptic species are revealed.

4.5 Conclusions

The possible existence of cryptic species within southern African *C. pumilus* was resurrected with the resolution of three divergent clades that occur sympatrically in the greater Durban area. These clades were found to be separated by a high number of mutational steps in comparison to the number of within-clade mutational steps. Results also showed that *C. leucogaster* lies within the paraphyletic southern African *C. pumilus* clade; as part of Clade B, it is separated from Clade A by a low cytochrome *b* genetic distance (0.6 %).

Chaerephon pumilus (southern Africa and Madagascar) formed a paraphyletic clade within which *C. leucogaster* lies. This may imply that the *C. pumilus* (Madagascar) and *C. pumilus* (southern Africa) are two different species. Complete resolution of this question and the relationship of *C. leucogaster* to these two *C. pumilus* populations may only be achieved with the inclusion of data relating to the type specimen of *C. pumilus*, from Eritrea.

It appears likely that *C. pumilus* formed part of the mammalian faunal community in relic scarp forest refugia during the LGM, and that the formation of distinct clades was due to southward expansion after the LGM and secondary contact between populations that had been separated in refugia or with northward-retreating Afrotemperate taxa. In order to resolve these questions, a much wider genomic and geographic sampling is required, combined with the use of other, possibly nuclear, genetic markers. According to Springer *et al.* (2001) the value of mitochondrial sequences in phylogenetic analyses is further enhanced when collected in tandem with nuclear sequences, as they provide an independent biparental estimate of phylogenetic relationships. Nuclear genes and more specifically microsatellites will provide information on phylogenetic and population structures to complement the present study, based on maternally-inherited mitochondrial DNA.

Currently *C. pumilus* is abundant and considered to be of least concern, with regards to conservation. However, if cryptic species were to be revealed, their conservation status would need to be assessed separately.

A combined analysis which included samples from this study and that of Jacobs *et al.* (2004) showed *C. pumilus* haplotypes from both studies to be identical or similar, and to be interspersed in phylogenetic trees. Both studies identified two clades, separated by a genetic distance of 0.9 %. The light-winged clade of Jacobs *et al.* (2004) was found to contain haplotypes identical to dark-winged forms from this study, which comprised only dark-winged animals. This casts doubt on the use of wing shade as a diagnostic character for these two clades, if they are indeed the same.

Chapter 5: References

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Appendix 1

Agarose Gel Electrophoresis stock solutions

10 x TBE (Tris-borate/ EDTA) Stock Solution

To make 500 ml 10 X TBE:

Tris: 0.89 x 0.5 x 121.1 (MW) = 53.89 g

Boric acid: 0.89 x 0.5 x 56.08 (MW) = 24.96 g (powder)

EDTA: 0.01 x 0.5 x 372.2 (MW) = 1.861 g

Make up to 500 ml with deionised water, adjust pH to 8.3 with NaOH or HCL.

0.5 x TBE

10 x TBE

Distilled water

Mix TBE and water in a 1:19 dilution.

Ethidium bromide stock (10 $mg.ml^{-1}$ EtBr)

10 mg EtBr

1 ml distilled water

0.05 $mg.ml^{-1}$ EtBr (1:200 dilution)

0.1 ml EtBr (10$mg.ml^{-1}$)

19.9 ml distilled water

Loading dye solution

0.1 % (w/v) Bromophenol blue

0.02 % (w/v) Xylene Cyanol FF

15 % (w/v) Ficoll (Type 400, Pharamacia) in water