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Genetic similarity amongst phenotypically diverse little free-tailed bats, *Chaerephon pumilus*

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The African molossid *Chaerephon pumilus* shows extensive variation in colour, size and echolocation across its wide distributional range with a light-winged form in north-eastern Africa and a dark form in southern Africa. There is also much variation in supposedly diagnostic characters (e.g., degree of palatial emargination) amongst the dark form of this species in southern Africa. These differences suggest that there may be cryptic species within *C. pumilus*. We used phylogenetic and phenetic analyses of sequence data from the mitochondrial cytochrome-*b* gene of a number of *C. pumilus* individuals to investigate the status of the light and dark-winged forms of this species and to evaluate the possibility of cryptic species within the dark-winged form of *C. pumilus* in southern Africa. We evaluated species status by comparing the level of sequence divergence amongst *C. pumilus* with the level of sequence divergence between known species in the genus. These included *C. ansorgei*, *C. chapini*, *C. nigeriae* and *C. jobensis*. Intrageneric sequence divergences among the *Chaerephon* spp. included here ranged from 6.51 to 11.18%, whereas the average sequence divergence between the light and dark forms was 0.9%. This suggests that these two forms are not distinct species. Individuals of the dark form of *C. pumilus* were genetically indistinguishable from each other having the same cytochrome *b* haplotype. We thus found no evidence of cryptic species in southern African *C. pumilus*.

Key words: *Chaerephon pumilus*, cytochrome *b*, genetics species concept

INTRODUCTION

There is much evidence for intraspecific variation in bat morphology (Bogdanowicz, 1990; Jacobs, 1996, 1999*a*; Aspetsberger *et al*., 2003; Miller-Butterworth *et al*., 2003), diet (Jacobs, 1999*b*; Brack and Whitaker, 2001; Aspetsberger *et al*., 2003) and echolocation calls (Buchler, 1980; Heller and von Helversen, 1989; Jones *et al*., 1992; Obrist, 1995; Betts, 1998; Jacobs, 1999*a*; Aspetsberger *et al*., 2003). Such variation can be small in magnitude and in some cases might mask cryptic species. For example, *Pipistrellus pipistrellus*, has been shown, on the basis of differences in echolocation calls (Jones and van Parijs, 1993), to consist of two distinct species despite a marked similarity in their morphology. Genetic divergence supports the reclassification of *P. pipistrellus* as two different species (Barratt *et al*., 1997; Mayer and von Helversen, 2001*a*). Similarly, large genetic differences in morphologically similar species of

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Plecotus spp. (Mayer and von Helversen, 2001*b*; Kiefer *et al*., 2002; Mucedda *et al*., 2002) and *Myotis* spp. (Mayer and von Helversen, 2001*b*; von Helversen *et al*., 2001) indicate the existence of cryptic species. Therefore, a widely distributed species that displays much morphological divergence might in fact consist of more than one species. However, very little genetic differences have been found between *Eptesicus serotinus* and *E. nilssonii*, two well-recognized species that differ in many morphological characteristics (Mayer and von Helversen, 2001*b*).

The African molossid *Chaerephon pumilus* (Cretzschmar, 1830; see Bouchard, 1998 for review of the species taxonomy) is a 10–14g insectivorous bat that is widespread in Africa south of the Sahara (Hayman and Hill, 1971; Skinner and Smithers, 1990; Bouchard, 1998; Jacobs and Fenton, 2001). This wide distribution is accompanied by considerable variation in colour and size. Populations of *C. pumilus* vary in the colour of wings and venter (Bouchard, 1998), with dark or light wings being the primary character to divide the forms. At Amani in Tanzania, the population consists of the light form which has pale almost transparent wings, white abdomens and white to yellow bands of fur on the wings running along the flank of the bats from armpit to leg, characters that had been associated with the subspecies *C. p. limbata* (Koopman, 1965). However, this subspecies has been dropped because sub-specific separation based on colour is not possible. Bats of different colour sometimes occupy the same roost (Hayman and Hill, 1971). The form, which is found in southern Africa, is dark in colour with dark wings and body. However, there is some variation within this group in the white colouring on the venter. Some do not have the white undersides or flank bands (Hayman and Hill, 1971; Aspetsberger *et al*., 2003) while others have the white flank bands with the white on the abdomen reduced, ranging from a tiny spot near the genitalia to a wide white stripe the whole length of the abdomen. Skinner and Smithers (1990) include both the dark and light forms in *Chaerephon pumilus*.

Chaerephon pumilus is larger in Kenya (mean forearm = 42.8 mm; mass = 13.8 g; Smart and Clark, 1991) than elsewhere in Africa (mean forearm range = 36–38 mm; mean mass = 10.8 g; Marshall and Corbet, 1959; Kingdon, 1974; McWilliam, 1988; Aspetsberger *et al*., 2003). In Kenya *C. pumilus* has two breeding seasons (Harrison, 1958), but in Uganda and West Africa it breeds continuously (Marshall and Corbet, 1959; Bouchard, 1998). Similarly, although *C. pumilus* uses the same foraging habitat (i.e., open areas) in different parts of its range (Aldridge and Rautenbach, 1987; McWilliam, 1988), there is considerable variation in its diet. In the Kruger National Park, South Africa, this species eats mainly Coleoptera and Hemiptera (Aldridge and Rautenbach, 1987). In Kenya the diet varied greatly. Whitaker and Mumford (1978) found that *C. pumilus* eats mainly Hemiptera and Coleoptera with some Lepidoptera. However, at Lake Naivasha, also in Kenya, the diet consists of more than 50% Diptera with Coleoptera and Hemiptera eaten to a lesser extent (Clark and Smart, 1991). At Amani, Tanzania, Blattodea form a major component of the diet of *C. pumilus* with Hemiptera being the only other order to make up more than 10% of its diet (Aspetsberger *et al*., 2003). Variation in the echolocation of *C. pumilus* has not been as widely studied, but Aspetsberger *et al*. (2003) report that *C. pumilus* at Amani, Tanzania (the light form) used calls of lower frequency, shorter duration and longer interpulse interval (IPI) than *C. pumilus* in South Africa (the dark form). These morphological, dietary and echolocation differences between the dark and light forms of

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C. pumilus suggest cryptic species within *C. pumilus*. We investigated this possibility using mitochondrial cytochrome-*b* sequence data.

Furthermore, on the basis of morphological ambiguities (e.g., pronounced palatal emargination across a wide range of groups previously recognized as separate species), Taylor (1999) suggested a revision of Koopman's (1975) key for the genus. Using the revised key Taylor (1999) identified a number of *Chaerephon* spp. as *C. pumilus* that would have been classified as *C. nigeriae* or *C. ansorgei* using Koopman's (1975) key. We thus used tissue samples from some of the specimens used by Taylor (1999) for the revision of Koopman's (1975) key to determine if genetic data were consistent with Taylor's (1999) revised species key.

Materials and Methods

Sampling

We collected tail punches or heart tissue from *C. pumilus* from a variety of localities in South Africa, Zimbabwe, Zambia and Tanzania (Table 1). Tail punches were taken using a 3 mm diameter skin biopsy punch. Individuals of the dark form of *C. pumilus* were collected from the Durban metropolitan area (29°52'S, 30°59'E; 12 bats), Dalton (29°22'S, 30°37'E; one bat) and the 121 SA Infantry Battalion Training Base at Hell's Gate on the shores of Lake St. Lucia (28°03'S, 32°25'E; two bats) both sites in the KwaZulu-Natal Province of South Africa.

We collected tail punches from light forms of *C. pumilus* from Amani Nature Reserve (5°10'S, 38° 47'E; one bat), Tanzania and from near Kitwe, Zambia (12°56'S, 28°18'E; five bats – DSJZM101, 107, 109, 113, 114, University of Cape Town). We also used heart tissue from five specimens [Durban Natural Science Museum (DNSM): DM6283–DM6287] identified as *C. ansorgei* and four specimens (DM 6299–DM6302) identified as *C. pumilus* using Koopman's (1975) key. Taylor (1999) later identified all nine specimens as *C. pumilus* using his revised key. Tissue samples of known *C. ansorgei*, *C. nigeriae*, *C. chapini* and *C. jobensis* were used to determine the degree of sequence divergence between valid species in the genus *Chaerephon*. Vouchers of the first three species from which the tissue samples were taken reside in the Natural History Museum (NHM), Bulawayo, Zimbabwe. These were taken at the confluence of the Lutope and Ngolangola Rivers at Sebungwe (18°17'S, 28°5'E). FPD Cotterill of the NHM supplied the tissue samples (Table 1). The tissue sample from *C. jobensis* was obtained from the Australian Museum (EBU23959). *Cistugo seabrai* was included in this study as an outgroup taxon for *Chaerephon* given the sister-taxa status of Molossidae and Vespertilionidae (Teeling *et al*., 2002).

Genetic Analysis

Total genomic DNA was extracted from tail punches or tissue preserved in 95% ethanol using a standard phenol-chloroform extraction procedure (Sambrook *et al*., 1989). A 604 bp fragment of the mitochondrial cytochrome *b* gene was amplified using primers L14724 and H15275 (Irwin *et al*., 1991). Polymerase chain reactions (PCRs) were performed in 25 l reaction volumes containing the following reagents: 0.2 U BIOTAQ DNA polymerase (Bioline, Whitehead Scientific), 1 X Bioline reaction buffer, 0.2 mM dNTPs, 3 mM $MgCl_2$, 0.4 M forward and reverse primer and approximately 100 ng of template DNA. PCR reaction mixtures were covered with a layer of mineral oil before thermal cycling on an MJ Research Inc. PTC-100™ thermal cycler. The cycling profile consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 50°C for 45 seconds and extension at 72°C for 45 seconds, followed by a final extension step at 72°C for 10 minutes. PCR products were sequenced directly following automated sequencing protocols (ABI Prism® BigDye™ Cycle Sequencing Ready Reaction Protocol manual). Cycle sequencing products were purified by ethanol precipitation and analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, U.S.A.). Sequences were trimmed to 423 bases to facilitate comparisons amongst all individuals sequenced, and aligned using Clustal X (Thompson *et al*., 1997). All sequences generated in this study have been deposited in Genbank (Table 1). Sequence data was analysed using both phylogenetic (parsimony) and phenetic (genetic distance) methods in PAUP* (Swofford, 2002). In the parsimony analysis, all nucleotide characters were given equal weight. Nodal support was evaluated using bootstrap values (Felsenstein, 1985) generated from 1000 pseudoreplicate data sets. For purposes of comparison with previously published papers, genetic distances were calculated using the Kimura 2-parameter model of nucleotide substitution (Kimura, 1980) under the criterion of minimum evolution.

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Results

Five haplotypes (A–E) were identified among the 30 *C. pumilus* sampled in southern Africa (Table 1). When included in a parsimony analysis, these five haplotypes formed a highly supported monophyletic group that was also recovered in a phenetic analysis of the data (Fig. 1). The *Chaerephon* spp. from Hell's Gate identified as *C. ansorgei* using Koopman's (1975) key, had the same haplotype as *C. pumilus* individuals sampled at the same site (Table 1) but differed from the known *C. ansorgei* by 10.62% (Table 1). The dark form of *C. pumilus* in South Africa displayed slightly more variability represented by three haplotypes (A, B and E; Table 1) with the sequence divergence among these haplotypes ranging from 0.47 to 1.68% (Table 2). Intrageneric species divergences in *Chaerephon* based on the species included in this

Table 1. List of *Chaerephon* specimens. Dark (D) or Light (L) winged forms of *C. pumilus* are indicated in parentheses after the species name. * — bats identified using Koopman's (1975) key

Species	Accession No.	Collection Locality	Haplotype	Genbank No.
**C. ansorgei* (D)	DM6287	Hell's Gate, South Africa	B	AY377940
**C. ansorgei* (D)	DM6285	Hell's Gate, South Africa	B	AY377941
**C. ansorgei* (D)	DM6284	Hell's Gate, South Africa	B	AY377942
**C. ansorgei* (D)	DM6286	Hell's Gate, South Africa	B	AY377947
**C. ansorgei* (D)	DM6283	Hell's Gate, South Africa	B	AY377966
C. ansorgei	FWC4783	Sebungwe, Zimbabwe	n.a.	AY377967
C. chapini	FWC3664	Zambia	n.a.	AY591329
C. jobensis	EBU23959	Solomon Islands	n.a.	AY591331
C. nigeriae	FWC3824	Zambia	n.a.	AY591330
C. pumilus (D)	–	Durban, Amanzimtoti	B	AY377939
**C. pumilus* (D)	DM6301	Hell's Gate	B	AY377943
**C. pumilus* (D)	DM6299	Hell's Gate	B	AY377944
**C. pumilus* (D)	DM6302	Hell's Gate	B	AY377945
**C. pumilus* (D)	DM6300	Hell's Gate	B	AY377946
C. pumilus (D)	–	Durban, Yellowwood Park	A	AY377948
C. pumilus (D)	–	Hell's Gate	B	AY377949
C. pumilus (D)	–	Hell's Gate	B	AY377950
C. pumilus (D)	–	Durban, Amanzimtoti	B	AY377951
C. pumilus (D)	–	Durban, Springfield	E	AY377952
C. pumilus (D)	–	Durban, Carrington Heights	E	AY377953
C. pumilus (D)	–	Durban, Westville	E	AY377954
C. pumilus (L)	–	Tanzania	C	AY377955
C. pumilus (D)	–	Durban, Glenwood	E	AY377957
C. pumilus (D)	–	Durban, Pinetown	E	AY377958
C. pumilus (D)	–	Durban, Waterfall	E	AY377959
C. pumilus (D)	–	Durban, Gillitts	E	AY377960
C. pumilus (D)	–	Durban, Glenmore	E	AY377962
C. pumilus (D)	–	Dalton	E	AY377964
C. pumilus (D)	–	Durban, Newlands	E	AY377965
C. pumilus (L)	DSJZM114	Zambia	C	AY500285
C. pumilus (L)	DSJZM107	Zambia	C	AY500286
C. pumilus (L)	DSJZM109	Zambia	C	AY500287
C. pumilus (L)	DSJZM113	Zambia	D	AY500288
C. pumilus (L)	DSJZM101	Zambia	C	AY500289
Cistugo seabrai	M977	Northern Cape, S. Africa	n.a.	AY591332

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analysis range from 6.51–11.18%, with *C. ansorgei* the most basal and genetically divergent from the other *Chaerephon* species (Table 2, Fig. 1). Sequence divergence between the genus *Chaerephon* and *Cistugo* ranged from 22.86% to 25.31% (Table 2). The divergence between the five *pumilus* haplotypes ranged from 0.47% to 1.68% (Table 2). The light-winged form (haplotypes C and D) differed by an average of 0.9% from the dark-winged *C. pumilus* (haplotypes A, B and E).

Discussion

Relationships within *C. pumilus* identified using genetic distances were identical to those recovered under the parsimony criterion. This suggests that our sequence divergence values are likely to be good indicators of species status (Bradley and Baker, 2001) in the genus *Chaerephon*. Despite the marked morphological, ecological and echolocation differences between the light and dark forms of *C. pumilus* (Aspetsberger *et al*., 2003) the low average genetic divergence (0.9%) between them suggests that they are all one species. This is also true for the dark form in South African. Not only do all the *C. pumilus* fall within a strongly supported monophyletic clade (Fig. 1) but the genetic divergence between them (including dark southern African forms and the light form from

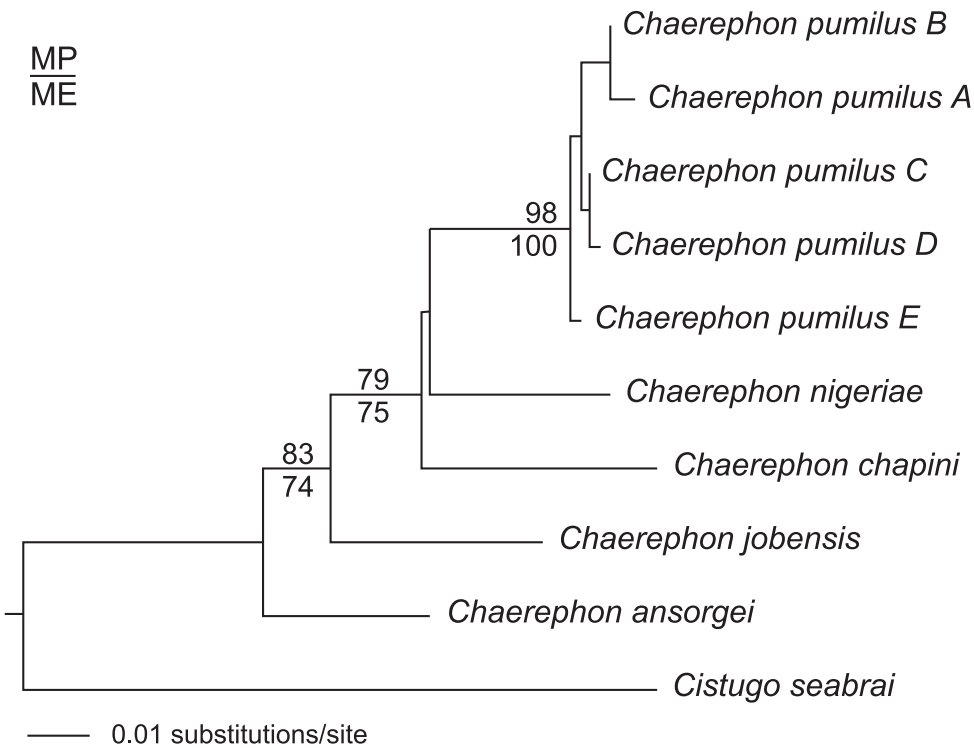


Fig. 1. Minimum evolution tree constructed using Kimura 2-parameter genetic distances for members of the bat genus *Chaerephon*. Maximum parsimony (MP) bootstrap values are indicated above nodes, and minimum evolution (ME) bootstrap values below nodes. Branch lengths are proportional to the number of substitutions per site as indicated by the scale bar

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Tanzania and Zambia) was much lower than that between known species in the genus (0.47–1.68% cf. 6.51–11.18%, Table 2). Furthermore, the range of divergence among the different forms of *C. pumilus* falls below the 2% criterion identified by Bradley and Baker (2001) for intraspecific variation.

The four Hell's Gate individuals identified as *C. pumilus* and the five individuals from the same site identified as *C. ansorgei* using Koopman's (1975) key were genetically indistinguishable. In addition, sequence divergence between the Hell's Gate individuals and other *C. pumilus* ranged from 0.47–1.19% (Table 2), which is well within the range of intraspecific variation characterizing other bat species (Johns and Avise, 1998; Ditchfield, 2000; Bradley and Baker, 2001; Ruedi and Mayer, 2001). This suggests that all the Hell's Gate individuals are in fact *C. pumilus*. This is supported by the large sequence divergence between known *C. ansorgei* and the '*C. ansorgei*' from Hell's Gate (10.62%, Table 1) and the basal relationship of *C. ansorgei* to the highly supported *C. pumilus* clade (Fig. 1). This finding is consistent with Taylor's (1999) identification of the Hell's Gate specimens as *C. pumilus* based on his revision of Koopman's (1975) key. However, the genetic similarity between the different forms of *C. pumilus* does not support Taylor's (1999) suggestion of the existence of cryptic species.

The divergence we found between known *Chaerephon* species (6.51–11.18%) overlaps the range (7–11%) suggested by Bradley and Baker (2001) to be that of valid species and where the burden of proof should shift to the documentation that only a single species is involved. We are therefore confident that the low sequence divergence

TABLE 2. Percentage pairwise sequence divergence values for the taxa included in the study under a Kimura 2-parameter model of evolution. The species and haplotype names are abbreviated in the column titles as Cp A = *C. pumilus* haplotype A (for example), Ca = *C. ansorgei*, Cn = *C. nigeriae*, Cc = *C. chapini*, Cj = *C. jobensis* and Ciss = *Cistugo seabrai*

Species/Haplotype	Cp B	Cp A	Cp E	Cp C	Cp D	Ca	Cn	Cc	Cj	Ciss
Chaerephon pumilus B	–									
C. pumilus A	0.47	–								
C. pumilus E	1.19	1.68	–							
C. pumilus C	0.71	1.19	0.48	–						
C. pumilus D	0.95	1.43	0.71	0.24	–					
C. ansorgei	10.62	11.18	10.31	10.31	10.60	–				
C. nigeriae	7.10	7.67	6.51	6.79	7.09	10.94	–			
C. chapini	8.14	8.68	8.13	7.85	8.13	11.13	9.11	–		
C. jobensis	9.81	9.79	9.50	10.07	10.36	8.16	8.23	10.60	–	
Cistugo seabrai	24.19	24.86	22.86	23.20	22.86	20.89	24.86	25.31	23.81	–

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between light and dark forms of *C. pumilus* is indicative of intraspecific variation despite the remote possibility that in some cases speciation may be completed at < 2% genetic differentiation (Bradley and Baker 2001; Mayer and von Helversen, 2001*b*). However, genetic sequence divergence for some taxa can be overestimated as a result of poor sampling. It is possible that our sequence divergence between known *Chaerephon* species may not be an accurate reflection of sequence divergence within this genus because we included only five of the 18 recognised species of *Chaerephon* (Simmons, In press). The possibility exists that some valid species within this genus could be separated by lower levels of sequence divergence. However, Bradley and Baker (2001) did not find < 2% sequence divergence in mitochondrial cytochrome *b* between recognized species in any of the 21 species pairs they tested, ten of which were bat species pairs. We therefore consider it unlikely that our interpretation of the low sequence divergence between the different forms of *C. pumilus* is incorrect.

In conclusion, the existence of cryptic species where there is little morphological variation (Barratt *et al*., 1997; Mayer and von Helversen, 2001*a*, 2001*b*; von Helversen *et al*., 2001; Kiefer *et al*., 2002) and the absence of cryptic species (this study) or genetic divergence (Mayer and von Helversen, 2001*b*) where there is much morphological variation is indicative of the complexity and unpredictability of the process of speciation.

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