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Multiple colonisations of the western Indian Ocean by *Pteropus* fruit bats (Megachiroptera: Pteropodidae): The furthest islands were colonised first

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ABSTRACT

We investigate the genetic relationships between purported island species of *Pteropus* fruit bat (Megachiroptera) from the western Indian Ocean islands using mitochondrial DNA sequencing in order to infer the pattern of colonisation of this biogeographic region. Most significantly, our genetic data questions the current taxonomic assignment based on morphology of many of the island species and subspecies, suggesting instead that many of the western Indian Ocean islands harbour 'races' of *P. giganteus* from mainland India. Our results strongly argue against a single colonisation event from mainland Asia. Evidence is presented for three colonisation events; the first to the western-most extremity of their range (Comoros and Pemba Island), the second to Rodrigues Island; and a third giving rise to the remaining extant island taxa, the latter two events occurring relatively recently and rapidly.

1. Introduction

Fruit bats of the genus *Pteropus* are geographically distributed from the western Indian Ocean to the mid-Pacific archipelagos in primarily tropical and sub-tropical climates (Mickleburgh et al., 2002). On many island systems they are the only endemic mammals and play a vital role in island ecology acting as pollinators and seed-dispersers (Fujita and Tuttle, 1991). Nowak (1999) recognised 60 species based on the morphological taxonomy of Andersen (1912), classified into 17 species groups. Intriguingly, the species groups of Andersen (1912) and Nowak (1999) do not correspond to biogeographic regions, with group members exhibiting disjunct distributions and some taxa more closely related to geographically distant species than they are to species on adjacent islands. This suggests a complex colonisation process involving multiple dispersals or, alternatively, morphological convergence confounds phylogenetic inference in this genus.

Patterns of speciation and contemporary genetic variability among insular faunas are influenced greatly by colonisation or vicariant history and the degree of subsequent isolation, and information on these processes can be derived from phylogenetic studies. The paucity of fossil and sub-fossil material in Chiroptera (Teeling et al., 2005) has precluded extensive phylogenetic inferences based on morphology in this Order, and intra-familial relationships in particular remain poorly resolved (Jones et al., 2002). The application of molecular-based studies to bat systematics has the potential to circumvent this difficulty (Burland and Worthington-Wilmer, 2001), and progress has been made in unravelling the evolutionary history of some taxa (e.g. *Mystacina* spp., Teeling et al., 2003; *Cynopterus* spp., Campbell et al., 2004; *Artibeus* spp., Carstens et al., 2004; Family Natalidae, Dávalos, 2005; *Triaenops* spp., Russell et al., 2007). A number of hypotheses can be tested within a molecular phylogenetic framework given the assumptions that (i) genotypes that are more genetically divergent tend to be more geographically separated and (ii) the degree of genetic divergence is related to time and degree of subsequent isolation. Molecular phylogenetics has been used successfully to infer faunal evolutionary and colonisation history for many island systems (Galapagos, Sato et al. (2001); Seychelles, Gardner (1986); Hawaii, Shaw (2002); Caribbean Islands, Hedges et al. (1992) and Dávalos (2004); Canary Islands, Thorpe et al. (1994) and Juan et al. (2000);

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Madagascar, Yoder et al. (1996) and Yoder et al. (2003); western Indian Ocean, Groombridge et al. (2002) and Warren et al. (2005)).

Eight extant species of *Pteropus* are recognised from the western Indian Ocean (Fig. 4; Nowak (1999), but see Hill (1971) and Hutson (2004)), a discrete biogeographic region and a biodiversity hotspot (Brooks et al., 2002) under increasing environmental threat. In the Mascarene Islands *P. rodricensis* Dobson 1878 is restricted to Rodrigues Island, although recent sub-fossil bones have been found on Round Island north of Mauritius; *P. niger* Kerr 1792 is currently endemic to Mauritius although there is recent sub-fossil evidence from Rodrigues and historic evidence from Réunion; while the extinct *P. subniger* Kerr 1792 was sympatric with *P. niger* on both Réunion and Mauritius (Cheke and Dahl, 1981). *P. rufus* Geoffroy 1803 occurs only in Madagascar, while *P. voeltzkowi* Matschie 1909 is found only on Pemba Island. *P. livingstonii* Gray 1866 is confined to the islands of Anjouan and Moheli of the Comoros archipelago where it is sympatric with the Comoran subspecies of *P. seychellensis* Milne-Edwards 1877 (*P. s. comorensis*), a species that also occurs on Gran Comoro and Mayotte, and on Mafia Island 40 km east of the Tanzanian coast. The other subspecies of *P. seychellensis* inhabits the granitic Seychelle islands (*P. s. seychellensis).* The endemic fruit bat confined to Aldabra Island has been alternatively designated a full species (*P. aldabrensis* True 1893) (Andersen, 1912; Nowak, 1999; Hutson, 2004) or a subspecies of *P. seychellensis* (*P. s. aldabrensis*, Hill, 1971). For the purposes of this study, the original nomenclature giving full species designation to the Aldabra Island endemic is used here. A subspecies of *P. giganteus* (*P. g. ariel* Allen 1908) occurs on the Maldive Islands and a subspecies of *P. hypomelanus* (*P. h. maris* Allen 1936) is now thought to be extinct from Addu Atoll of this archipelago (Holmes et al., 1994).

To date, published studies of fruit bat taxonomy have generated poorly resolved or conflicting phylogenetic trees (Springer et al., 1995; Hollar and Springer, 1997; Álvarez et al., 1999; Juste et al., 1999; Bastian et al., 2002; Colgan and da Costa, 2002) possibly due to a rapid and recent diversification of the family Pteropodidae (Jones et al., 2002). In addition, such studies generally include only a limited number of species from represented genera (Hollar and Springer, 1997; Juste et al., 1999; Romagnoli and Springer, 2000). Furthermore, fruit bats of the genus *Pteropus* from the western Indian Ocean are either absent or under-represented in these phylogenies and, apart from those by Bastian et al. (2002) and Colgan and da Costa (2002), studies do not tend to focus on biogeographic regions, inhibiting phylogeographic analyses. Confident taxonomic assignments are of fundamental importance in effective conservation management in terms of preventing the extinction of evolutionary distinctive lineages, by facilitating accurate censuses of population size and for implementing and legislating conservation programmes (Goldstein et al., 2000). Extensive 'cryptic' diversity in many Chiropteran taxa has been uncovered using molecular techniques (Barratt et al., 1997; von Helversen et al., 2001; Campbell et al., 2004) and species assignments for some western Indian Ocean species remain equivocal (Bergmans, 1997). The World Conservation Union (IUCN) classifies three pteropid species *(P. aldabrensis, P. rodricensis* and *P. livingstonii)* from the region as Critically Endangered and three (*P. niger, P. rufus* and *P. voeltzkowi*) as Vulnerable (IUCN, 2006) and Mickleburgh et al. (1992) identified *P. rodricensis, P. livingstonii* and *P. voeltzkowi* as being in need of urgent conservation action.

Here, we examine the phylogenetic relationships between purported species from western Indian Ocean islands in order to test the hypothesis that the current biogeographic distribution of the genus in the western Indian Ocean can be explained by a single colonisation from the Asian mainland. We targeted mitochondrial DNA (mtDNA) sequence data from three loci for 17 extant species and subspecies of the genus *Pteropus*. We chose the 12S rRNA and cytochrome *b* loci to facilitate integration with available sequence data, while the control region was targeted to resolve terminal branches of the mitochondrial phylogeny.

2. Materials and methods

2.1. Sampling

Tissue biopsies were taken through the wing or tail membranes from all extant species of *Pteropus* in the western Indian Ocean using the non-lethal sampling procedure of Worthington-Wilmer and Barratt (1996). Exceptions include samples from the subspecies on the Maldive Islands (*Pteropus giganteus ariel*) and Mafia Island (*Pteropus seychellensis comorensis*) that were not available. Since, in most cases, only one species of *Pteropus* occurs on each island and, where sympatry occurs, each species is easily distinguishable, we are confident in species assignments. We included eight additional pteropid species from across the remainder of the genus range in this study. Altogether, we examined 17 *Pteropus* species and subspecies ($n = 72$ specimens), representing almost a quarter of the recognised species in the genus. We used *Mirimiri acrodonta* as an outgroup for individual and 12S+cyt*b* (see below) analyses based on a number of published phylogenies (Romagnoli and Springer, 2000; Colgan and da Costa, 2002; Jones et al., 2002). For subsequent ALL3 analysis, we used basal *Pteropus* lineages from these preliminary analyses as the outgroup (see below). The sources of specimen samples are given in Table 1. We downloaded additional sequences from GenBank (Accession Nos. *Rousettus amplexicaudatus* AB046329 and U93070, *Syconycteris australis* SAU93060, *Acerodon celebensis* U93071, *Ptenochirus jagori* AB046325, *Eonycteris spelaea* AB062476 and U93059, *Cynopterus brachyotis* AB046321 and U93068, *Epomophorus wahlbergi* U93064, *Macroglossus minimus* U93062, *Pteralopex atrata* PAU93069, *Pteropus scapulatus* AF321050, *Pteropus admiralitatum* PAU93072, *Pteropus dasymallus* AB042770, *Pteropus giganteus* AY012138, *Pteropus hypomelanus* U93073 and AB062472, *Pteropus pumilus* AB085732, *Pteropus rufus* AB085732, *Pteropus vampyrus* AB062475 and AB046326, *Pteropus speciosus* AB062474) to use as outgroup species and to broaden species sampling and geographic coverage.

2.2. DNA extraction, amplification and sequencing

We extracted total genomic DNA from tissue samples using the Qiagen DNeasy Tissue Kit according to manufacturer guidelines. We amplified the entire cytochrome *b* (1140 bp) and 12S rRNA (1002 bp) genes and a fragment of the control region (approximately 429 bp) using the primer combinations in Fig. 1 and detailed in Table 2. We carried out PCR amplifications using either standard 50 μl Taq Polymerase reactions [8.0 μl 10× buffer, 8.0 μl dNTP (2.5 mM of each nucleotide), 0.8 μl DNA polymerase (5 U/μl), 2.0 μl $MgCl^{2+}$ (50 mM), 2.0 μl (10 mM) of each locus-specific primer pair and 5.0 μl DNA extraction] or alternatively, using a MegaMix~BLUE (Microzone Ltd., UK) protocol with 3.0 μl DNA extraction, 1.0 μl each locus-specific primer and 45.0 μl MegaMix~BLUE combined in 50.0 μl reactions. Amplification was carried out using 35 cycles of PCR with an initial denaturing step at 94 °C for 3 min. Each PCR cycle consisted of a denaturing step at 94 °C for 45 s, annealing at 52 °C for 45 s and extension at 72 °C for 1 min. A final extension step at 72 °C for 10 min completed the amplification protocol. We sequenced purified PCR products in both directions at facilities in Yale University USA or Macrogen Inc. Korea.

2.3. Sequence alignments and analyses

We aligned cytochrome *b* sequences unambiguously by eye in Se-Al 2.0.3a, and confirmed the correct reading frame by translat-

Table 1
Origins and sources of specimens used in DNA sequencing.

Species	Museum ref. ID	Local ID	Tissue type	Origin	Source
Pteropus conspicillatus	538	PC012	Wing punch	Australia	Lubee Foundation[a]
	539	PC009	Wing punch	Australia	Lubee Foundation[a]
	540	PC008	Wing punch	Australia	Lubee Foundation[a]
	988	PC010	Wing punch	Australia	Lubee Foundation[a]
Pteropus giganteus	1170	929811	Wing punch	N/A	Lubee Foundation[a]
	1171	929837	Wing punch	Captive born	Lubee Foundation[a]
Pteropus hypomelanus	541	LUB016	Wing punch	N/A	Lubee Foundation[a]
	542	LUB017	Wing punch	N/A	Lubee Foundation[a]
Pteropus livingstonii[i]	544	PL001	Wing punch	Anjouan Island	P. Racey[b]
	545	PL003	Wing punch	Anjouan Island	P. Racey[b]
Pteropus niger[i]	Nig1	—	Wing punch	Mauritius Island	MWF[c]
	Nig3	—	Wing punch	Mauritius Island	MWF[c]
	Nig4	—	Wing punch	Mauritius Island	MWF[c]
	Nig6	—	Wing punch	Mauritius Island	MWF[c]
Pteropus poliocephalus	1185	—	Wing punch	Australia	Lubee Foundation[a]
	Pol2	—	Liver	Australia	A. Sanchez[d]
Pteropus pumilus	547	LUB002	Wing punch	N/A	Lubee Foundation[a]
	548	LUB003	Wing punch	N/A	Lubee Foundation[a]
	549	LUB008	Wing punch	N/A	Lubee Foundation[a]
Pteropus rodricensis[i]	550	LUB031	Wing punch	Captive born	Lubee Foundation[a]
	551	LUB030	Wing punch	Captive born	Lubee Foundation[a]
	Rod23	—	Wing punch	Solitude, Rodrigues Is.	V. Powell[f]
	Rod26	—	Wing punch	Solitude, Rodrigues Is.	V. Powell[f]
Pteropus rufus[i]	999	PR002	Wing punch	Madagascar	P. Racey[b]
	1001	PR004	Wing punch	Madagascar	P. Racey[b]
Pteropus scapulatus	Sca1	—	Liver	Australia	A. Sanchez[d]
Pteropus aldabrensis[i]	Ald2	—	Wing punch	Picard Island, Aldabra	J. Gerlach[h]
Pteropus seychellensis comorensis[i]	560	PSC001	Wing punch	Grande Comore	P. Racey[b]
	561	PSC002	Wing punch	Grande Comore	P. Racey[b]
	562	PSC003	Wing punch	Grande Comore	P. Racey[b]
	563	MT1B	Wing punch	Mayotte Island	P. Racey[b]
	564	MT2B	Wing punch	Mayotte Island	P. Racey[b]
Pteropus seychellensis seychellensis[i]	Sey1	—	Ear punch	Silhouette Island, Seychelles	J. Gerlach[h]
	Sey3	—	Ear punch	Silhouette Island, Seychelles	J. Gerlach[h]
Pteropus vampyrus	553	LUB040	Wing punch	N/A	Lubee Foundation[a]
	554	LUB041	Wing punch	N/A	Lubee Foundation[a]
	555	LUB042	Wing punch	N/A	Lubee Foundation[a]
Pteropus voeltzkowi[i]	Voe1	—	Wing punch	Ngezi, Pemba Island	DCCFF, Zanzibar[g]
	Voe5	—	Wing punch	Mgogoni, Pemba Island	DCCFF, Zanzibar[g]
Mirimiri acrodonta	—	EBU9655	Liver	Fiji	Australian Museum[e]

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[d] A. Sanchez, Facultad de Ciencias Experimentales, Jaen, Spain.
[e] Australia Museum, Sydney, Australia.
[f] V. Powell, Manchester Metropolitan University, UK.
[g] Department of Commercial Crops, Fruits & Forestry, Zanzibar.
[h] J. Gerlach, Nature Preservation Trust Seychelles, Seychelle Islands.
[i] Taxa constrained to be monophyletic in topological tests of monophyly (see text) N/A, information not available.

ing the sequences in MacClade v.3.0.4 (Maddison and Maddison, 1992). We manually conducted the 12S rRNA alignment based on a secondary structure model to account for stems and loops (modified from Springer and Douzery, 1996 and Olson et al., 2005). We excluded ambiguous regions in the control region and 12S rRNA from analyses and treated remaining gaps in the control region and 12S rRNA alignments as missing data. We assessed nucleotide compositions and uncorrected genetic '*p*' distances in the program MEGA v.2.1 (Rosenberg and Kumar, 2001). We applied the tree topology methodology of de Queiroz (1993) to test the suitability of individual loci for concatenation. This method is based on the assumption that if strong support for conflicting clades does not occur in individual gene trees, the sequences from which these trees are derived can be concatenated, provided they have not undergone vastly different evolutionary histories. Initially, we carried out analysis on a concatenation of the 12S rRNA and cytochrome *b* (12S+cyt*b*) datasets. Subsequently, we incorporated partial control region sequences from a subset of specimens (ALL3) to resolve terminal branches more clearly, but at the expense of taxon sampling.

We conducted phylogenetic analysis under the maximum parsimony (MP) and maximum likelihood (ML) criteria using PAUP*. For MP tree searches, we weighted characters equally. Likelihood searches employed models of nucleotide substitution estimated separately for the 12S+cyt*b* dataset and the ALL3 dataset under the Akaike Information Criterion as implemented in ModelTest (v.3.7; Posada and Crandall, 1998; optimal models and parameter values are available in the data matrix provided as Supplementary oneline material). Under both criteria, tree searches included 20 heuristic replicates using the tree bisection-reconnection (TBR)

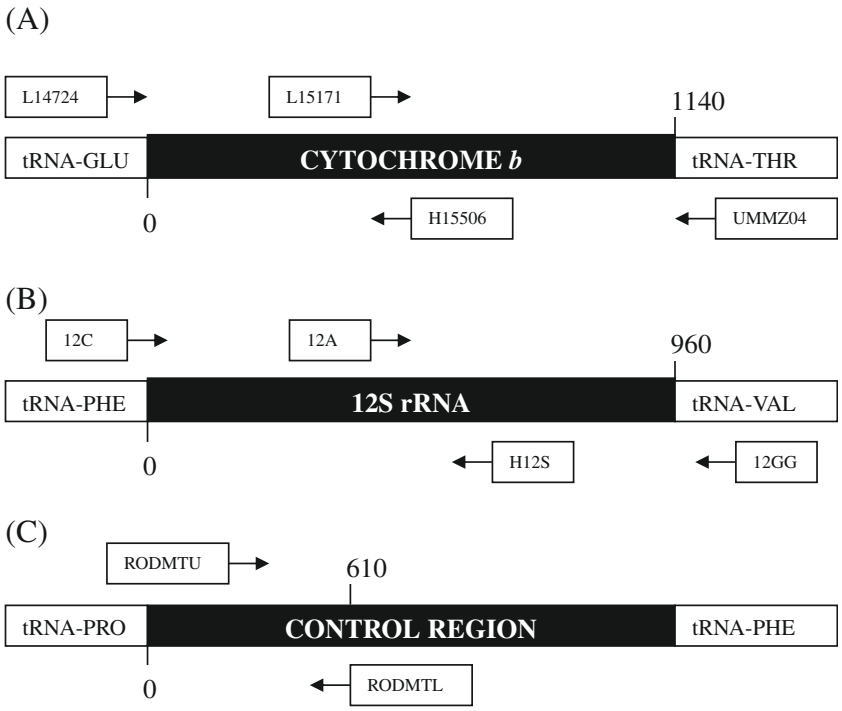

Fig. 1. Position and orientation of primers used to amplify mDNA for (A) cytochrome *b*, (B) 12S rRNA and (C) control region.

Table 2
Primer details for mtDNA sequencing. *T* = annealing temperature.

Primer	*T*	Orientation	Primer Sequence	Source
12A	52	Forward	AAACTGGGATTAGATACCCCACTAT	Kocher et al. (1989)
12C	52	Forward	AAAGCAAARCACTGAAAATG	Springer et al. (1995)
12GG	52	Reverse	TRGGTGTARGCTRRRTGCTTT	Kirsch et al. (1995)
H12S	52	Reverse	TTACAGAACAGGCTCCTCTAG	Kirsch et al. (1995)
L14724	52	Forward	CGAAGCTTGATATGAAAAACCATCGTTG	Kocher et al. (1989)
L15171	52	Forward	CATGAGGACAAATATCATTCTGAGG	Kocher et al. (1989)
H15506	52	Reverse	AGTGGRTTRGCTGGTGTRTARTTGTC	Kocher et al. (1989)
UMMZ04	52	Reverse	TCTTCATTTYWGGTTTACAAGAC	Jansa et al. (1999)
RODMTU	52	Forward	GCTGAGGTTCTACTTAAACT	McCracken (unpubl.)
RODMTL	52	Reverse	GAGATGTCTTATTTAAGGGG	McCracken (unpubl.)

branch-swapping algorithm, with starting trees obtained via stepwise addition. We estimated bootstrap support based on 1000 pseudoreplicates using either the TBR (MP) or NNI (ML) algorithm on starting trees obtained by stepwise addition, while Bayesian posterior probabilities were estimated using MrBayes (v.3.1.2; Ronquist and Huelsenbeck, 2003). We specified five partitions for the 12S+cyt*b* data set (12S rRNA pairing and non-pairing, as well as each codon position in cyt*b*) and a sixth partition (d-loop) was specified in the ALL3 analysis. For the 12S rRNA pairing partition, the doublet model, which is appropriate for modeling pairing positions of ribosomal genes (Ronquist and Huelsenbeck, 2003), was specified. We used a model with six categories of base substitution for all remaining partitions, with a gamma-distributed rate parameter and a proportion of invariant sites. We estimated, separately, parameter distributions for each partition within a partitioned data set ("unlinked"). In all MrBayes analyses, we allowed two simultaneous independent runs to proceed for 20 million generations, with chains sampled every 1000 generations. We include settings used in all MrBayes analyses at the end of each matrix in the Supplementary online material. We imported the resulting trees into PAUP* and, after discarding the first 10% as burn-in, combined them in a majority-rule consensus tree to obtain posterior probabilities. In the 12S+cyt*b* analyses, we rooted trees with *Mirimiri acrodonta*. Because the d-loop sequences of *Mirimiri* and *Pteropus scapulatus* were characterised by extensive insertion–deletion events not present in the remaining taxa, we excluded them from the ALL3 analyses, and instead rooted them with *Pteropus conspicillatus*, *P. hypomelanus*, and *P. poliocephalus* based on the 12S+cyt*b* topology.

We tested the hypothesis that western Indian Ocean species derived from a single colonisation event by constraining the concatenated (ALL3) tree topology so that all Indian Ocean taxa (denoted with an asterisk in Table 1) formed a monophyletic group. We used PAML (v.4.1; Yang, 2007) to calculate site-wise likelihood scores for the unconstrained maximum likelihood (ML) tree, the constrained ML tree, and a random tree generated in MacClade (Maddison and Maddison, 1992). Alternative tree topologies were compared with the most likely topology using Shimodaira's (2002) approximately unbiased (AU) test in Consel (v.0.1i; Shimodaira and Hasegawa, 2001).

3. Results

3.1. Individual loci

Sequencing of cytochrome *b* generated a fragment of 1140 bp (455 variable and 348 parsimony informative sites). There was

significant evidence of bias in base frequency in favour of thymine in position 2 (*G*-test, G = 7.98, df = 3, $P = 0.05$) and adenine and cytosine in position 3 (*G*-test, G = 25.9, df = 3, $P < 0.05$). The 12S alignment was 905 bp after alignment-ambiguous positions were excluded (stems 476 bp; loops 429 bp). There was no evidence of base frequency bias between species (*G*-test: stems $G = 0.0001$, df = 3, $P = 1.000$; loops $G = 0.0521$, df = 3, $P = 0.9969$). The control region had an aligned length of 394 bp, once alignment-ambiguous positions were excluded. Nucleotide composition analysis indicated a significant bias against guanine (*G*-test, G = 10.363, df = 3, $P < 0.05$). All novel sequences have been submitted to GenBank (Accession Nos. cytochrome *b* FJ561376–FJ561405; 12S rRNA FJ588879–FJ588908; control region FJ548573–FJ548609).

3.2. Concatenated datasets

Applying the de Queiroz (1993) topological congruence method indicated that the 12S and cytochrome *b* sequences could be concatenated (results not shown), yielding an overall aligned length of 2045 bp. An MP analysis of 32 concatenated 12S+cyt*b* haplotypes yielded 12 equally most parsimonious trees of length 861 (CI = 0.656, RI = 0.812, RC = 0.533). The single optimal tree recovered under the likelihood criterion had a $-\ln L$ score of 7224.78. A summary phylogenetic tree of MP, ML and Bayesian analysis is presented in Fig. 2.

The reduced dataset incorporating control region sequences (ALL3, 2439 bp), generated three equally most parsimonious trees (length = 1020, CI = 0.588, RI = 0.771, RC = 0.454), and the single optimal ML tree had a $-\ln L$ score of 8676.28. A summary tree of the ALL3 dataset is presented in Fig. 3.

A single most likely tree was generated with the enforced constraint making all Indian Ocean taxa monophyletic and requiring the exclusion of *P. dasymallus*, *P. pumilus*, *P. giganteus* and *P. vampyrus* from the Indian Ocean clade. Both this constrained tree and a random tree were significantly different in their topologies from the observed ML tree generated without constraints (AU test: constrained tree $P = 0.0005$, random tree $P = 4 \times 10^{-43}$).

4. Discussion

4.1. Molecular phylogenetic relationships between species and subspecies

Genetic data are presented for the first time for five purported species and subspecies of *Pteropus* fruit bats (*P. aldabrensis, P. livingstonii, P. voeltzkowi, P. s. seychellensis, P. s. comorensis*). In total, 17 species and subspecies of the genus *Pteropus* are included in this study. Individually, the mitochondrial loci chosen for this study did not generate high power for phylogenetic analysis. However, the 12S+cyt*b* concatenated dataset yielded a phylogenetic tree with strong clade support for most lineages. In this dataset *P. scapulatus* (an Australian species) resolved as the most basal pteropid lineage. Examination of the genetic '*p*' distances between *P. scapulatus* and all other *Pteropus* species suggest that it is one of the oldest lineages of *Pteropus* bat, although this may only be confirmed by fully sampling all species of the genus. In fact, the uncorrected '*p*' distance between *P. scapulatus* and *Acerodon celebensis* for the 12S rRNA gene (4.52%) is less than that between *P. scapulatus* and other *Pteropus* fruit bats (range 5.04–7.14%) suggesting that *Pteropus* may be paraphyletic, as has also been proposed by Giannini et al. (2008).

The remaining taxa resolve into two well-supported clades (i) one (the Australasian clade) consisting of Australian species (*P. poliocephalus* and *P. conspicillatus*) and *P. hypomelanus* from throughout SE Asia and parts of Australia and (ii) a second (the Indian Ocean clade) incorporating all the western Indian Ocean species and representatives from across the Asian tropical regions.

To enhance the phylogenetic signal from this second Indian Ocean clade, a subset of partial control region sequences were added to the initial 12S+cyt*b* dataset to generate a smaller (in terms of taxon sampling) concatenated dataset (ALL3). Both the 12S+cyt*b* and ALL3 datasets indicate that western Indian Ocean species are not monophyletic. *P. livingstonii* (Comoros Islands) and *P. voeltzkowi* (Pemba Island) are strongly supported as sister taxa and are the oldest lineages in the western Indian Ocean, but are separated from allopatric species by lineages from Ryuku Island (*P. dasymallus*) and the Philippines (*P. pumilus*). The placement of *P. pumilus* and *P. dasymallus* within the Indian Ocean clade indicates that western Indian Ocean species do not have a single recent common ancestor in Southeast Asia. These two East Asian species resolve as the closest relatives to *P. rodricensis* in this dataset, although their disjunct geographic distributions suggest that the true closest relative of *P. rodricensis* may not have been sampled in this study. The remaining species sampled in our study are characterised by short inter-branch nodes and, in some cases, poor branch support, thus rendering taxonomic hypotheses problematic. Clearly, it is a recently derived group, with *P. vampyrus* possibly representing the oldest of these lineages. *P. giganteus* undoubtedly also descended from the ancestor of *P. aldabrensis*, *P. rufus*, *P. niger* and the two purported subspecies of *P. seychellensis*. In fact, the polytomy comprising these taxa and the extremely short branch lengths recovered in the ML analysis suggest that this divergence (if it can be considered divergence) was a relatively recent and quite rapid event. Sequence differences between *P. giganteus* and the taxa in this polytomy are <1.7%, <1.1% and <10.3% for the cytochrome *b*, 12S rRNA and control region, respectively (Node A, Figs. 2 and 3). Bastian et al. (2002) described a mean sequence divergence of 1.87% between complete cytochrome *b* sequences of *P. vampyrus* populations collected from two sites in the Philippines.

Significantly, therefore, the partitioning of western Indian Ocean island forms into species and subspecies based on morphology is not supported by the genetic data presented here. Despite *P. niger* having phenotypically distinctive characters including extremely small ears, long fur, pelage colour (a darkly-coloured ventral surface) and thickly-haired tibia (Andersen, 1912) that differentiate it from *P. s. seychellensis*, mean sequence divergences between these two taxa are 0.5%, 0.2% and 3.0% for the cytochrome *b*, 12S rRNA and control region, respectively (Node D, Figs. 2 and 3). Andersen (1912) considered *P. rufus* to be "sharply differentiated" from *P. s. comorensis* based on the smaller size and reduced ear lobes of the latter taxon, and *P. aldabrensis* is markedly smaller than all other island forms in the region. But mean sequence divergences between these taxa are <0.2%, <0.2% and <4.0% for the cytochrome *b*, 12S rRNA and control region, respectively.

The limited DNA sequence divergence between purported species documented here is in marked contrast to phenotypic differentiation, on which current taxonomy is based, thereby highlighting the potential for over-emphasising morphological characters (particularly those that are non-discrete) and/or under-valuing genetic divergence in systematic studies. The incongruities between these two methodologies underline the limitations of current species concepts. Based on our phylogenies, body size and ear reduction are not synapomorphies (see Fig. 3), but have confounded morphological inference through the use of small sample sizes and over-reliance on continuous phylogenetic characters. In fact, although clearly sister taxa, *P. livingstonii* and *P. voeltzkowi* have very different ear morphologies: those of *P. livingstonii* being large and semi-rounded, while those of *P. voeltzkowi* are small and sub-acutely pointed (Andersen, 1912). Additionally, pelage differences are most likely attributable to founder events followed by rapid

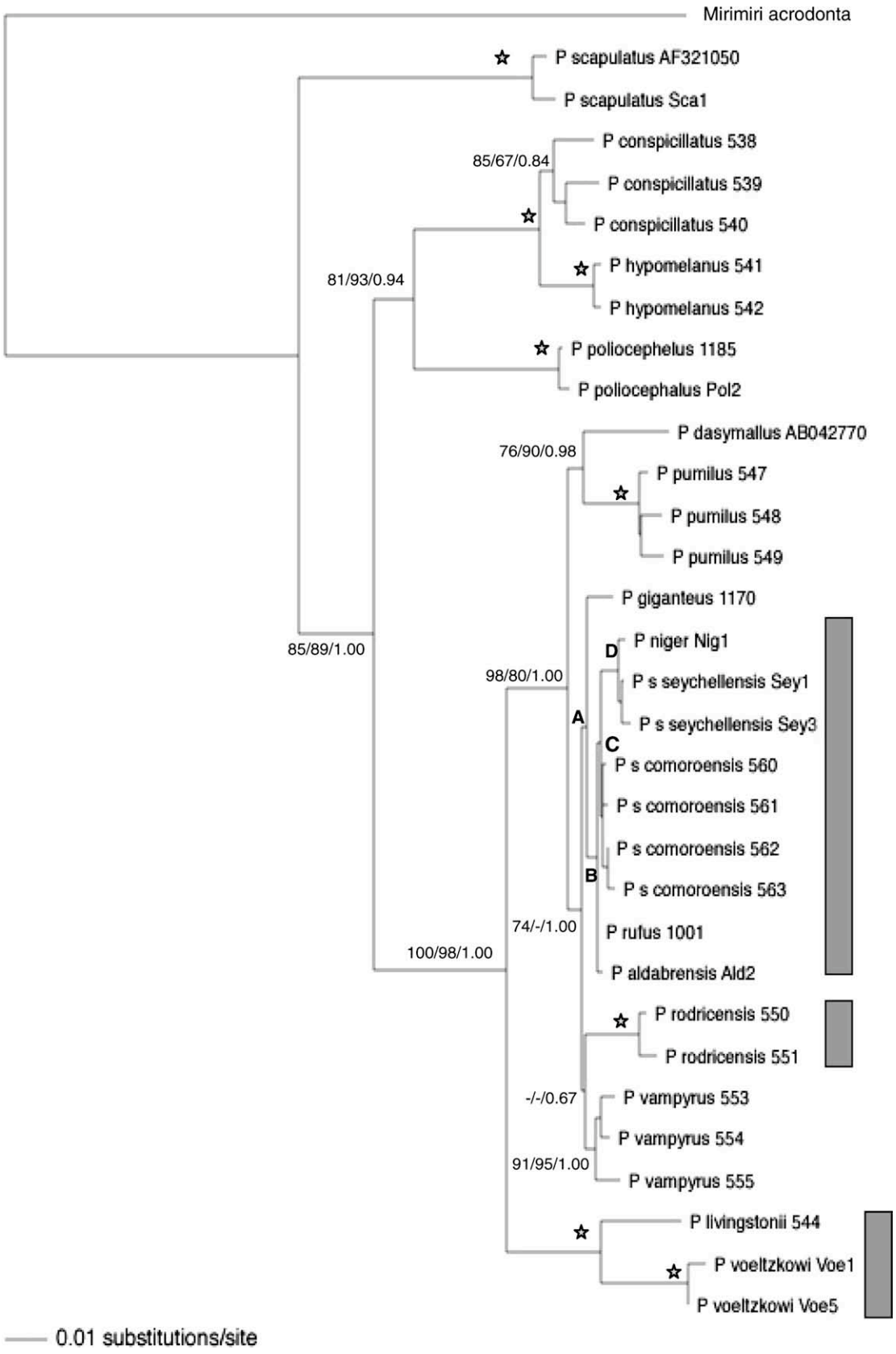

Fig. 2. Summarised phylogenetic tree for the 12S+cyt*b* dataset incorporating MP, ML and Bayesian results (MP/ML/Bayesian). Nodes marked with * have bootstrap support of 100 for MP and ML and posterior probability of 1.00 for Bayesian analyses. Letters refer to nodes discussed in text (bootstrap support: A = —/—/96; B = 90/83/100; C = 59/53/97; D = 98/96/100). Western Indian Ocean species are highlighted with an adjacent grey box.

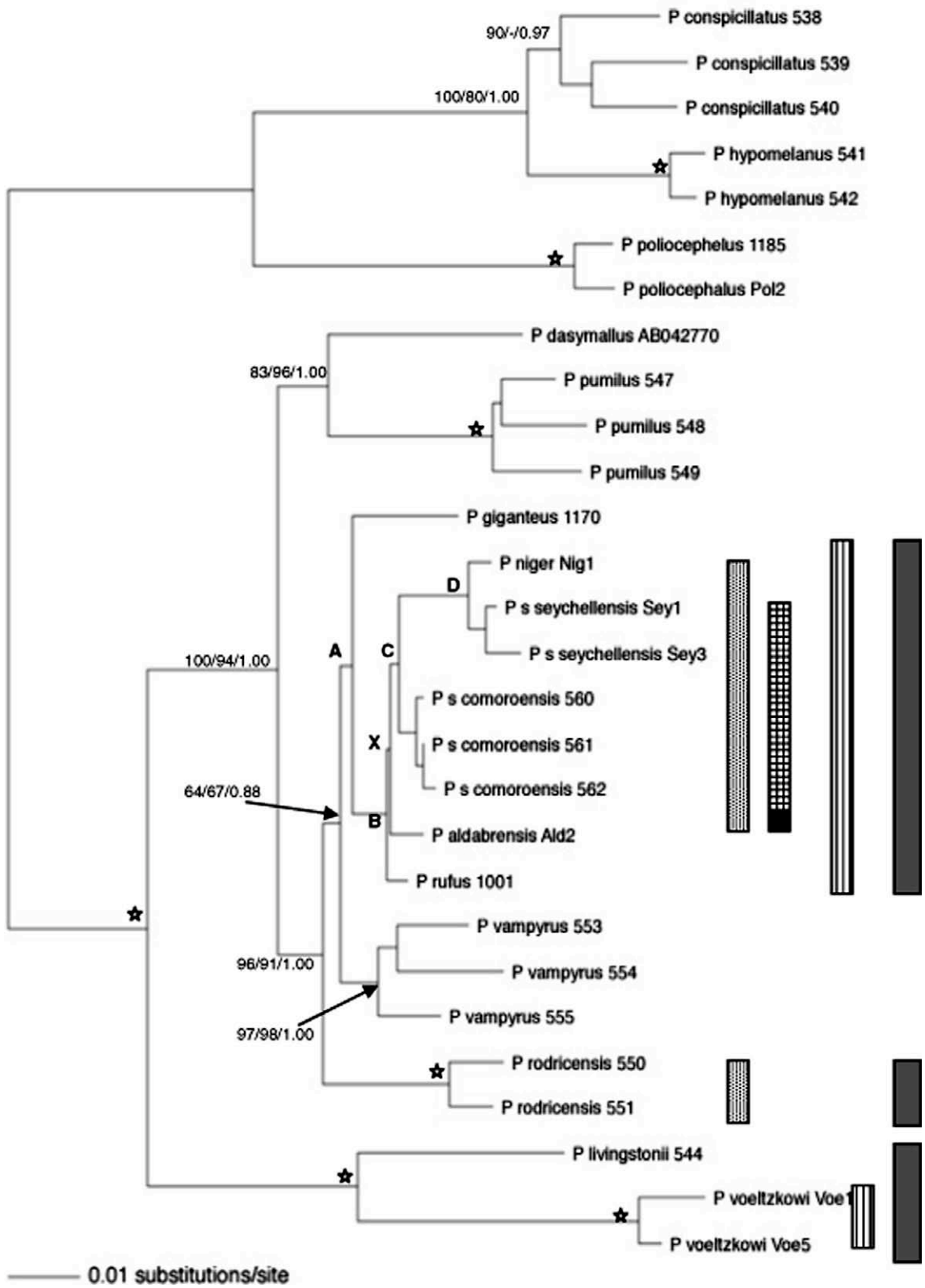

Fig. 3. Summarised phylogenetic tree for the ALL3 dataset incorporating MP, ML and Bayesian results (MP/ML/Bayesian). Nodes marked with * have bootstrap support of 100 for MP and ML and posterior probability of 1.00 for Bayesian analyses. Letters refer to nodes discussed in text (bootstrap support: A = 50/74/0.95; B = 92/94/1.00; C = 53/—/0.95; D = 100/100/1.00; X = —/—/0.65). Western Indian Ocean species are highlighted with an adjacent grey box. The species included in the Andersen (1912) western Indian Ocean 'Rufus' group are highlighted with a striped box. Stippled box adjacent to species possessing reduced ear lobes; hatched box adjacent to species displaying relatively reduced body size (note that *P. aldabrensis* is markedly smaller than other Indian Ocean species).

genetic drift, since Cheke and Dahl (1981) describe individual variation in coat colour in *P. rodricensis*. Andersen (1912) suggested that sampling across the entire range of *P. vampyrus* and *P. giganteus* would show a gradual transition from one species to another and even described a species of intermediate form (*P. intermedius*) in the contact zone, suggesting that these two species may be a complex of races with a contiguous geographic distribution, which may be supported by our study (1.9%, 0.7% and 10.8% mean sequence divergence for cytochrome *b*, 12S rRNA and control region, respectively). However, it is important to note that the specimens of these two species included here are captive specimens of unknown provenance. Our study suggests that this complex of races may extend into the western Indian Ocean islands. Interestingly, glandular neck tufts that are present in *P. giganteus* are absent from all derived western Indian Ocean species (Andersen, 1912). Cranial anatomy is possibly of more utility for morphological species identification in this genus than external characters. Andersen (1912) used cranial structure, among other anatomical features, in his seminal study of the genus to generate his species 'groupings'. In many cases, it is the cranial structure that appears to unify the groupings, but external characteristics that differentiate the species. Thus, the morphology and genetic data generated here may not be in conflict, the conflict solely arising from the use of external characters that are highly variable. For example, Maharadatunkamsi et al. (2003) described a relationship between body size and island area, but also a confounding trend for body size to increase from east to west on Indonesian island populations of a fruit bat (*Eonycteris spelaea*), and in both cases there were some populations that did not fit either trend.

We propose that the taxonomic nomenclature of the 'species' referred to as *P. aldabrensis, P. niger, P. rufus* and *P. seychellensis* may need to be revised down to races of *P. giganteus* based on the International Code for Zoological Nomenclature (ICZN, 1999). However, we recognise that further sampling is required for each of the island lineages in order to demonstrate reciprocal monophyly and to ascertain evolutionary 'uniqueness' before any change in taxonomic nomenclature can be accepted.

4.2. Phylogeography

Since the Indian Ocean species are not monophyletic, they do not have a single recent common ancestor from Southeast Asia and so their current distribution in the Indian Ocean cannot be described by a single colonisation event into the region. The results of the topological constraint test confirms the improbability of Indian Ocean monophyly. The phylogeny presented here suggests that there have been at least three colonisation events into the Indian Ocean. The extinct species (*P. subniger*) from the Mascarene Island of Réunion was not considered in this study. However, its distinct morphology and life history characteristics (Cheke and Dahl, 1981) suggest that this species may also be the result of an independent colonisation event, possibly associated with the extinct subspecies *P. hypomelanus maris* of the southern Maldive Islands. A map of the most plausible colonisation routes considered in this study is presented in Fig. 4. Examination of the phylogenetic trees indicates an initial colonisation to the western Indian Ocean giving rise to *P. voeltzkowi* and *P. livingstonii* at the western extremity of the distribution of the genus. The route of colonisation is unclear from our analysis. Meirte (1984) suggested that the Comoros Islands are the origin of the genus in the western Indian Ocean and that seasonal monsoon winds might have assisted in bridging the distance from Pakistan and India. If this is the true route of colonisation, it is all the more remarkable that the genus has never colonised the African mainland. An alternative, although unlikely, possibility is that the western Indian Ocean islands were used as stepping-stones, and that ancestral species became extinct on the intervening islands through stochastic changes or competitive exclusion by subsequent colonisers. The fact that such local extinctions take place regularly is highlighted by the extinction of *P. hypomelanus maris* from the southern Maldive Islands (Holmes et al., 1994), *P. subniger* from Mauritius and Réunion Islands, *P. niger* from Rodrigues and *P. rodricensis* from Round Island (Cheke and Dahl, 1981). However, there is no sub-fossil evidence to support the use of island stepping-stones in the colonisation of the westernmost extremity of the Indian Ocean, nor to suggest that the phylogeny we have presented here may have arisen through back-colonisation of the Asian mainland. The absence of a generalised east-to-west dispersal pattern would also appear to discount the use of stepping-stones or back-colonisation. The ancestral form that first colonised the western Indian Ocean cannot be inferred from our data and greater sampling coverage of SE Asia will be required to resolve this question. Disjunct distributions for other western Indian Ocean fauna have been described e.g. pigeons of the genus *Alectroenas* represented in the Comores, Seychelles and Aldabra Islands have their closest relatives in Southeast Asia (Benson, 1984) and the green-backed heron (*Butorides striatus*) from Aldabra has a direct Asian origin (Benson and Penny, 1971).

The morphological differentiation of *P. rodricensis* from other western Indian Ocean species is confirmed by genetic data and supports a separate colonisation event for this species. Morphologically, the closest relative to *P. rodricensis* is *P. lombocensis* (not sampled in this study) from the Lesser Sunda Islands in Southeast Asia (Andersen, 1912). The phylogenies presented here suggest that *P. rodricensis* is derived from an ancestral type similar to that of *P. vampyrus* and that both species may have arisen from a single species radiation. This ancestral form may have colonised Rodrigues Island directly and the rest of the western Indian Ocean by a more circuitous route through Southeast Asia and India, giving rise sequentially to *P. vampyrus*, *P. giganteus*, and the Indian Ocean complex (*P. rufus*, *P. aldabrensis*, *P. seychellensis*, *P. niger*). Examination of branch lengths and genetic '*p*' distances seem to suggest that this may have occurred in a similar pattern to the initial colonisation of the Comoros and Pemba Island, using the monsoon winds to colonise Madagascar initially, with subsequent dispersal to the remaining islands. This tentative hypothesis remains to be tested with more intensive analysis of polymorphic DNA markers. Given the close genetic distance between these taxa based on mitochondrial DNA sequencing, better resolution of inter-taxon relationships may be generated using microsatellites, amplified fragment length polymorphisms (AFLPs) or single nucleotide polymorphisms (SNPs), which are more appropriate for very fine-scale phylogenetic analyses.

The short branch lengths of the maximum likelihood tree presented in Fig. 3 imply a rapid and recent diversification of the genus in the western Indian Ocean. Short branch lengths complicate the estimation of divergence times between taxa. Standardised molecular clocks are not applicable here because there are no reliably dated fossil or sub-fossil data that can be used for calibration. Furthermore, the use of geological events such as island formation, as has been used for other Indian Ocean taxa (Groombridge et al., 2002; Warren et al., 2005), is likely to grossly overestimate divergence times for island colonisation by fruit bats. The only calibration point that can be used with confidence in this dataset is the most recent emergence above sea level of Aldabra Island approximately 125,000 years ago (Warren et al., 2005). Thus, the lineage that gives rise to the endemic Aldabran species (*P. aldabrensis*) must have arisen subsequent to this date. Many other nodes in the ML tree arise at a similar point as that of *P. aldabrensis*, suggesting that many of the western Indian Ocean species diverged around this time. However, using a single calibration point to infer divergence dates at nodes throughout a tree is prone to significant margins of error, especially if the calibration point occurs at a

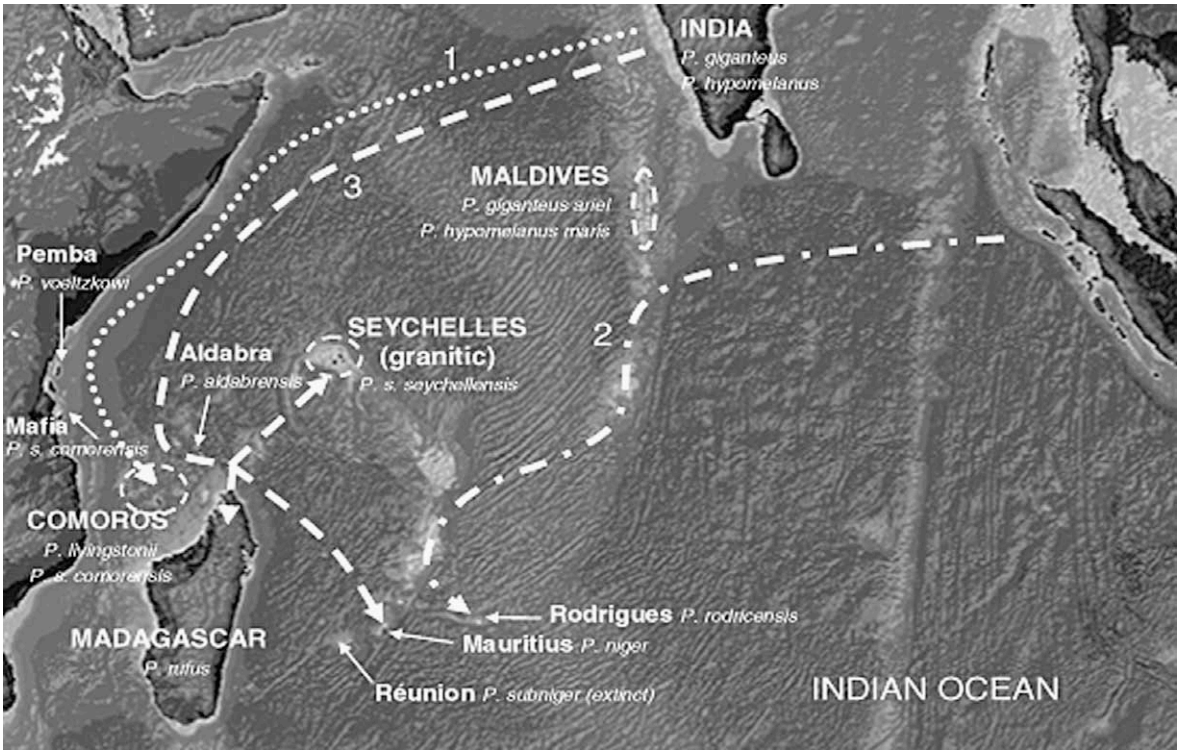

Fig. 4. Map of distribution and most plausible colonisation routes for *Pteropus* sp. fruit bats into the western Indian Ocean. Dotted line (1) is first colonisation event to the Comoros Islands (*P. livingstonii*) and Pemba Island (*P. voeltzkowi*). Dashed/dotted line (2) is second colonisation to Rodrigues Island (*P. rodricensis*). Dashed line (3) is third colonisation to Seychelles (*P. s. seychellensis*) and Mauritius (*P. niger*), and to Madagascar (*P. rufus*), Comoros (*P. s. comorensis*) and Aldabra (*P. aldabrensis*).

terminal node (Yoder and Yang, 2004). Until reliably dated sub-fossil material or more accurate statistical methodologies for assigning divergence times to recent speciation events are developed, it is impossible to estimate the timing of divergence events for the western Indian Ocean taxa with confidence.

5. Conclusion

The genetic data presented here reject the hypothesis that the western Indian Ocean was colonised once from mainland India by *Pteropus* fruit bats. Instead, evidence is presented for at least three colonisation events many of which have occurred relatively recently and rapidly. The hypothesized routes of colonisation require long-distance dispersal, a characteristic of the genus revealed by its colonisation of many remote Pacific Ocean islands. Although not documented in this study, hybridisation is more likely between taxa with strong dispersal abilities and more detailed population-level studies may reveal evidence of genetic introgression between island 'races'. The ambiguities in this study highlight the need for a detailed systematic study of the genus, incorporating multiple specimens across the entire range of all purported taxa and including representatives of the genus *Acerodon* and the recently re-erected genus *Desmalopex* to ascertain the basal relationships of *Pteropus* and these other genera (see Esselstyn et al., 2008). For the purposes of a conservation management plan for the genus in the region, we suggest that island races should be managed as separate evolutionary units (Fraser and Bernatchez, 2001) until a more intensive investigation of reciprocal monophyly can be carried out.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.02.010.

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