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Phylogeny and phylogeography of Old World fruit bats in the *Cynopterus brachyotis* complex

Polly Campbell[a,*], Christopher J. Schneider[a], Adura M. Adnan[b], Akbar Zubaid[b], Thomas H. Kunz[a]

[a] *Department of Biology, Boston University, Boston, MA 02215, USA*
[b] *Department of Zoology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia*

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Abstract

Taxonomic relationships within the Old World fruit bat genus, *Cynopterus*, have been equivocal for the better part of a century. While nomenclature has been revised multiple times on the basis of phenotypic characters, evolutionary relationships among taxa representing the entire geographic range of the genus have not been determined. We used mitochondrial DNA sequence data to infer phylogenetic relationships among the three most broadly distributed members of the genus: *C. brachyotis*, *C. horsfieldi*, and *C. sphinx*, and to assess whether *C. brachyotis* represents a single widespread species, or a complex of distinct lineages. Results clearly indicate that *C. brachyotis* is a complex of lineages. *C. sphinx* and *C. horsfieldi* haplotypes formed monophyletic groups nested within the *C. brachyotis* species complex. We identified six divergent mitochondrial lineages that are currently referred to *C. brachyotis*. Lineages from India, Myanmar, Sulawesi, and the Philippines are geographically well-defined, while in Malaysia two lineages, designated Sunda and Forest, are broadly sympatric and may be ecologically distinct. Demographic analyses of the Sunda and Forest lineages suggest strikingly different population histories, including a recent and rapid range expansion in the Sunda lineage, possibly associated with changes in sea levels during the Pleistocene. The resolution of the taxonomic issues raised in this study awaits combined analysis of morphometric characters and molecular data. However, since both the Indian and Malaysian Forest *C. brachyotis* lineages are apparently ecologically restricted to increasingly fragmented forest habitat, we suggest that reevaluation of the conservation status of populations in these regions should be an immediate goal.
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1. Introduction

The broad application of molecular techniques to phylogenetic reconstruction has revealed unrecognized levels of diversity in many vertebrate groups (Gleeson et al., 1999; Kizirian and Cole, 1999; Roca et al., 2001). In bats, the recent identification of morphologically cryptic, genetically divergent species, even within well-studied genera (Areletaz et al., 1997; Barratt et al., 1997; Kingston et al., 2001; Mayer and von Helversen, 2001; von Helversen et al., 2001), suggests that diversity within the order Chiroptera may be underestimated by current taxonomy. Likewise, the higher-level molecular systematics of Chiroptera have been the focus of numerous studies (Simmons, 1998; Simmons and Geisler, 1998; Teeling et al., 2000; Van Den Bussche and Hoofer, 2001), yet many interspecific phylogenetic

* Corresponding author. Fax: 1-617-353-6340.
E-mail address: pollyc@bu.edu (P. Campbell).

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relationships remain poorly characterized, particularly in tropical regions where diversity is highest (Jones et al., 2002).

The distribution of the Old World fruit bat genus *Cynopterus* (Fig. 1A), spans more than 40° of latitude and 60° of longitude in the Indomalayan region, and encompasses the continental and oceanic islands of the Sunda shelf (Fig. 1B) and the western portion of Wallacea (Corbet and Hill, 1992). Three species, *C. brachyotis*, *C. sphinx*, and *C. horsfieldi*, are widespread and share broad areas of sympatry in mainland Southeast Asia, the Malay peninsula, Borneo and Sumatra, and in India and Sri Lanka (*C. brachyotis* and *C. sphinx*). However, while *C. sphinx* and *C. horsfieldi* are thought to be continuously distributed throughout their respective ranges, *C. brachyotis* has a broad area of disjunction between populations in southwest India and Sri Lanka, and those in southern Myanmar, and extends to the islands of Sulawesi and the Philippines (Campbell and Kunz, 2004; Corbet and Hill, 1992).

Species-level boundaries within *Cynopterus* have been equivocal for the better part of a century and the genus has been subject to a number of revisions based on cranial, skeletal, and external characters (Andersen, 1912; Hill and Thonglongya, 1972; Hill, 1983; Kitchener and Maharadatunkamsi, 1991). Much of this taxonomic debate has focused on the apparent overlap in size between *C. brachyotis* and *C. sphinx* throughout the Southeast

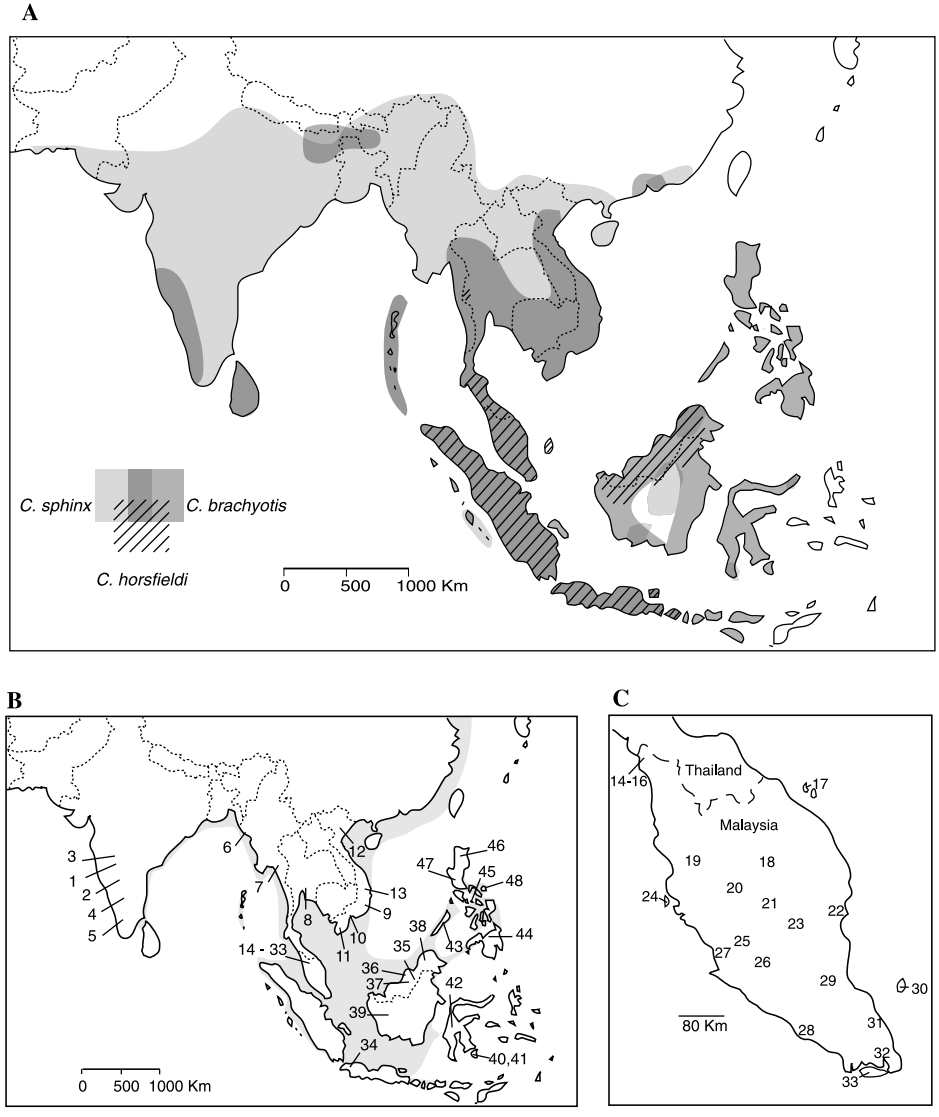

Fig. 1. (A) Map of the Indomalayan region showing the geographic distribution of *Cynopterus brachyotis*, *C. sphinx*, and *C. horsfieldi* (after Bates and Harrison, 1997; Corbet and Hill, 1992; Storz and Kunz, 1999). (B) Map of the Indomalayan region showing the approximate land area exposed when sea-levels dropped to >120 m below present levels during Pleistocene glacial cycles (after Brown and Lomolino, 1998). The Sunda shelf is defined as the area connecting the islands of Palawan, Borneo, Bali, Java, and Sumatra, with the Malay peninsula and the southern coasts of Thailand, Cambodia, and Vietnam. Numbered points refer to localities sampled in this study. (C) Sampling localities for *Cynopterus* in peninsular Malaysia and Singapore. See Appendix A for all locality data.

Asian portion of both species' ranges, and on the island of Sri Lanka (Andersen, 1912; Chasen, 1940; Hill, 1961). The problem of selecting appropriate morphological characters to differentiate the two species and to designate geographically defined subspecies, has been confounded by remarkably high morphological variation within *C. brachyotis*, both among geographic localities and, on the Malay peninsula and Borneo, across habitat types (Francis, 1990; P. Campbell, unpubl. data). Observed phenotypic variability within *C. brachyotis* has led to the suggestion that the taxa currently referred to this species may be in fact a complex of closely related races (Chasen, 1940; Francis, 1990), or diagnosable species (Kitchener and Maharadatunkamsi, 1991; Kofron, 1997).

Taxonomic uncertainty notwithstanding, ecological studies of *C. brachyotis* in Southeast Asia typically describe this bat as a ubiquitous generalist, common in secondary forest and agricultural areas, and present at lower population densities in primary forest (Heideman and Heaney, 1989; Tan et al., 1997; Zubaid, 1993). Based on the abundance of *C. brachyotis* in anthropogenic habitats its conservation status is listed as not threatened and it is not considered vulnerable to the effects of deforestation (Mickleburgh et al., 1992). However, the assumption that *C. brachyotis* constitutes as single species, both across habitat types and among geographic localities, is questionable. Likewise, the evolutionary relationship of *C. brachyotis* to other members of the genus has not been investigated on a comprehensive geographic scale.

Underestimation of evolutionary distinctiveness (Moritz, 1994) and ecological specialization (Crandall et al., 2000) can lead to loss of diversity through mismanagement of geographically or ecologically restricted populations. However, identification of populations whose evolutionary independence or adaptive diversity warrants their treatment as distinct species or management units (Moritz, 1994) is challenging if interspecific relationships are unresolved. Thus, a robust phylogeny is an essential foundation on which fine-grained analyses of population-level diversity can be built (Dimmick et al., 1999).

In this study, we investigate two main questions: (1) is current taxonomy consistent with the phylogenetic relationships of *C. brachyotis*, *C. horsfieldi*, and *C. sphinx*; and (2) is *C. brachyotis* a single widespread species, or a complex of evolutionarily distinct lineages? We address these questions using mitochondrial (mt) DNA sequence data from the control region and cytochrome *b* gene sampled across the geographic range of each species. Because of the exceptionally broad distribution of *C. brachyotis* and *C. sphinx*, and because of the high degree of morphological variation reported for *C. brachyotis*, we sampled multiple localities within geographic regions and multiple individuals within locality (Figs. 1B and C; Appendix A). This approach allowed us to evaluate broad-scale phylogeographic structure within the genus, and to examine the impact of historic and recent environmental change on the distribution and demography of *C. brachyotis* in the Sunda Shelf region.

2. Materials and methods

2.1. Sample collection and species identification

In peninsular Malaysia, we sampled *Cynopterus brachyotis* at 18 localities, *C. sphinx* at 5 localities, and *C. horsfieldi* at 6 localities (Figs. 1B and C; Appendix A). *C. brachyotis* samples were also collected from a single locality in Singapore. Tissue biopsies were obtained from the wing membranes of live bats and preserved in 95% EtOH (Worthington Wilmer and Barratt, 1996). Because of the considerable overlap in forearm length reported in the literature across the three species (*C. brachyotis*, 57–68 mm, *C. sphinx*, 65–76 mm, and *C. horsfieldi*, 70–78 mm; Medway, 1983), field identifications were based on the following additional characters: presence of cusps on the last lower premolar and first lower molar = *C. horsfieldi* (Corbet and Hill, 1992; Payne et al., 1985); no cusps + ear length > 18 mm = *C. sphinx*; no cusps + ear length < 17 mm = *C. brachyotis* (Bates and Harrison, 1997; Bumrungsri, 2002).

Additional samples from India, Myanmar, Thailand, and Vietnam (*C. brachyotis* and *C. sphinx*), Borneo (*C. brachyotis* and *C. horsfieldi*), and peninsular Malaysia, Java, Sulawesi, and the Philippines (*C. brachyotis*) were obtained from museum specimens and individual researchers (see Appendix A). Collectively, these samples represent the majority of the current distribution of the three species (Figs. 1A and B). However, Sumatra and the Nusa Tengarra region of the Indonesian archipelago are not represented in this study.

2.2. DNA extraction, PCR amplification, and sequencing

Total genomic DNA was isolated from tissue samples using the DNAeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Initially, the entire mitochondrial control region was amplified for a subset of *C. brachyotis* samples using a species-specific forward primer located in the tRNA Proline gene (CYBR.tPro: 5′ CTCCACCATCAACACCCAAAG 3′) and a mammal-specific reverse primer (H1301.M: 5′ TAATAR AAAGGCYAGGACCAAAC 3′). The sequences obtained were used to design an internal reverse primer located ca. 274 base pairs (bp) downstream of the conserved F-block region (CYBR.dlo: 5′ TGAATG GTGCAATATAAGTCCAGC 3′). Using primers CYBR.tPro and CYBR.dlo, a ca. 640 bp segment of the 5′ end of the control region was amplified for 109 *C. brachyotis* from 41 localities, 27 *C. sphinx* from 13

localities, and 13 *C. horsfieldi* from 7 localities (see Appendix A; GenBank Accession Nos. AY629008–AY629148). Amplifications were carried out in a volume of 25 µl using 1.75 mM $MgCl_2$, 0.2 mM of each primer, 0.5 U Gold *Taq* DNA polymerase, and ca. 20 ng DNA template. The thermal profile (95 °C for 45 s; 55 °C for 45 s; and 72 °C for 45 s) was repeated for 35 cycles with an initial denaturation step at 95 °C for 10 min and a final extension at 72 °C for 5 min. Amplified products (20 µl) were electrophoresed on a 1% agarose (Promega) gel, excised using a sterile razor blade, and purified with a QIAquick Gel Extraction Kit (Qiagen) following manufacturer's instructions. Cycle-sequencing products were cleaned using Sephadex G-50 columns. Sequencing reaction products were run on an Applied Biosystems 377 automated sequencer. Sequences were edited in Sequence Navigator, Version 1.01.

Partial cytochrome *b* (690 bp) sequences were obtained for a subset of individuals (49 *C. brachyotis*, 27 *C. sphinx*, and 13 *C. horsfieldi*) using vertebrate forward primer, L14996 (5′ AAY ATY TCW GYH TGA TGA AAY TTY GG 3′) (Sorenson et al., 1999) and mammal-specific reverse primer, H15739.M (5′ CCK CCT AGT TTR TTD GGR ATD GAK CG 3′); GenBank Accession Nos. AY628916–AY629004. The ingroup samples used in this analysis were selected as representative of clades identified through preliminary phylogenetic analysis of control region sequences. PCR and sequencing protocols were the same as those used for the control region, with the exception of a PCR annealing temperature of 50 °C.

Both the control region and cytochrome *b* (cyt *b*) were sequenced for three outgroup taxa: *Megaerops ecaudatus*, *Aethalops alecto*, and *Chironax melanocephalus*. These were selected based on their hypothesized close relationship to *Cynopterus* (Jones et al., 2002). GenBank Accession Nos. for outgroup taxa are AY629149–AY629151 (control region) and AY629005–AY629007 (cyt *b*).

2.3. Sequence alignment

All sequences were initially aligned by eye in Se-Al (Version 2.0a8). A ca. 77 bp segment of the control region was removed from the dataset because all haplotypes in one of the major *C. brachyotis* clades (see Section 3; Fig. 2) had lost the segment in a single deletion event, making contiguous gap characters nonindependent when gaps are treated as a fifth character state. The large number of unique 1–6 bp insertions and deletions near the 5′ end of the control region fragment made manual alignment of this region ambiguous. Rather than discard potentially useful phylogenetic information (Giribet and Wheeler, 1999; Sorenson and Payne, 2001) and to avoid imposing a priori expectations of taxonomic relationships on the data, two fragments of the control region (designated, gap region 1, ca. 10 bp and gap region 2, ca. 79 bp) were aligned in POY (Gladstein and Wheeler, 1996), a program that uses a parsimony optimality criterion to simultaneously align sequences and estimate tree topology. Tree topologies were evaluated for the full dataset in POY, however, only gap regions 1 and 2 were treated as unaligned. Having obtained an implied alignment for these regions, we used this alignment for subsequent analyses.

2.4. Phylogenetic analysis

We reconstructed phylogenetic relationships based on maximum parsimony (MP) and likelihood criteria using the programs PAUP* (Version 4.0b10; Swofford, 2002) and MrBayes (Version 3.0; Huelsenbeck and Ronquist, 2001). Control region and cyt *b* sequences were combined in a dataset comprised of 89 ingroup taxa and three outgroup taxa. MP analyses were implemented under three transition/transversion weighting schemes (equal weights, 1:2 and 1:5), using full heuristic searches, tree-bisection-reconnection (TBR) branch-swapping and random stepwise addition (100 replicates), with gaps treated both as a fifth character state (all weighting schemes), and as missing data (equal weights only). Bootstrap values (full heuristic search, 1000 replicates, TBR branch swapping) were obtained as a measure of relative support for branch nodes identified on the most parsimonious trees.

We chose Bayesian inference over a branch-swapping maximum likelihood search because the Markov Chain Monte Carlo method (MCMC; Hastings, 1970; Metropolis et al., 1953) incorporated in MrBayes reduces the chance that the search will fix on local, rather than global optima (Huelsenbeck et al., 2002; Larget and Simon, 1999). Moreover, for a large data set, Bayesian inference is less computationally intensive than a full maximum likelihood analysis. Because trees are saved throughout the search at user-specified intervals, it is possible to obtain posterior probability estimates for clades in the consensus of sampled trees once the likelihoods have reached stationarity (the burnin period). These values reflect the proportion of sampled trees that contain a given clade. We ran four MCMC chains for 500,000 generations, saving one tree every 10 generations, and used trees from the last 250,000 generations (post-burnin) to estimate clade position probabilities. We selected a General Time-Reversible model of nucleotide substitution with the following parameters estimated during the course of the run: rate matrix, C–T = 30.77, C–G = 2.34, A–T = 2.28, A–G = 33.89, and A–C = 3.32; nucleotide frequencies, A = 0.32, C = 0.31, G = 0.12, and T = 0.25; gamma shape parameter, $\alpha = 0.63$; proportion invariant sites = 0.49.

Alternative MP and Bayesian phylogenetic hypotheses were compared using the Kishino–Hasegawa paired-sites likelihood test with RELL resampling and 1000 replicates (Kishino and Hasegawa, 1989) and the

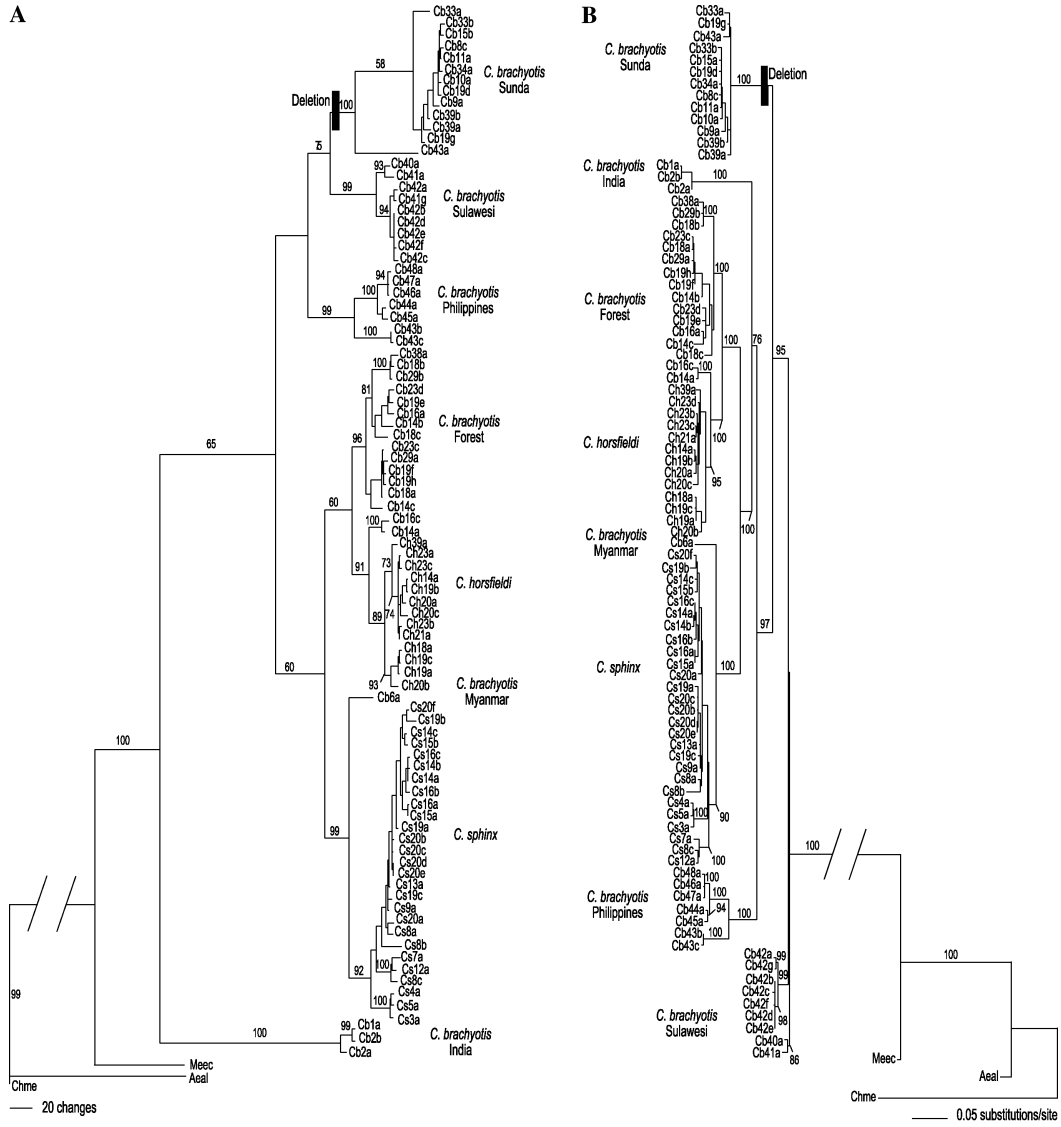

Fig. 2. Phylograms representing two alternative phylogenetic hypotheses for relationships among *Cynopterus* spp. based on 690 bp of cytochrome *b* and 576 bp (including gaps) of the control region. (A) One out of 1421 equally parsimonious trees found using equal weights parsimony criteria. Bootstrap support values >50 are shown above branches supporting major clades and subclades. (B) Tree with best likelihood score found using 500,000 generations of MCMC sampling in MrBayes. Posterior probabilities above branches supporting major clades and subclades are based on >50% consensus support for 25,000 trees saved during the final 250,000 MCMC generations. Major clades and subclades are identified by species based on current taxonomy, and in the case of *C. brachyotis*, by lineage based on the results of this study. Haplotypes are coded by species, and by locality number; no haplotypes for cyt *b* plus control region were shared among individuals. Deletion = ca. 77 bp control region deletion charaterizing all *C. brachyotis* Sunda haplotypes. Trees are rooted with outgroup taxa *Aethalops alecto* (Aeal), *Megaerops ecaudatus* (Meec), and *Chironax melanocephalus* (Chme).

Wilcoxon signed-ranks test (Templeton, 1983). Tests were implemented in PAUP*.

2.5. Demographic analyses

Phylogenetic analyses identified two *C. brachyotis* lineages that are broadly sympatric on the Malay peninsula and Borneo; these were designated Sunda and Forest (see Section 3; Fig. 2). We used two coalescent-based approaches to test for evidence of historic demographic expansion in the Sunda lineage, perhaps in response to Pleistocene climate changes, and to obtain a comparative estimate of differences in the demographic histories of the two lineages. Both data sets comprised ca. 565 bp of the control region, for 59 Sunda samples and 27 Forest samples.

Using the program ARLEQUIN (Version 2.0; Schneider et al., 2000), we compared the observed distribution of pairwise nucleotide differences among haplotypes within each lineage with the expectations of a sudden expansion model (Rogers, 1995; Rogers and Harpending, 1992). The distribution of pairwise differ-

ences ('mismatch distribution') between haplotypes drawn from populations at demographic equilibrium typically is multimodal, reflecting the stochastic process of lineage extinction via genetic drift. In contrast, a unimodal Poisson mismatch distribution is predicted for populations that have experienced a recent demographic expansion. This outcome results from the majority of lineage coalescence and extinction events occurring prior to the population expansion (Rogers and Harpending, 1992; Slatkin and Hudson, 1991).

In addition, we used the program FLUCTUATE (Kuhner et al., 1998) to obtain maximum likelihood estimates of the exponential rate of growth for each lineage. FLUCUTATE implements Metropolis–Hastings sampling of genealogies to evaluate the posterior probabilities of the observed data having been generated under different values of the population parameter g, where g is the exponential rate of growth, defined as $1/\mu$*generations, and μ is the mutation rate. We set the transition/transversion ratio to observed values (25:1 for Sunda and 4:1 for Forest) and allowed the program to estimate the base frequencies during the course of the run.

Finally, under a demographic expansion hypothesis, the observed pattern of sequence evolution is expected to be significantly different from that predicted under assumptions of selective neutrality and demographic equilibrium. Several test statistics have been developed to test for departures from a neutral model of evolution (reviewed in Ramos-Onsins and Rozas, 2002). We chose Fu's F_s (Fu, 1997) over the more widely used Tajima's D (Tajima, 1989), because it has been shown to out-perform this and other currently available neutrality tests in correctly detecting the signature of population expansion in simulated datasets (Ramos-Onsins and Rozas, 2002). F_s was calculated in ARLEQUIN.

3. Results

3.1. Phylogenetic relationships

Both parsimony and Bayesian analyses indicate that the taxon currently referred to as *C. brachyotis* is a complex of distinct evolutionary lineages, which include *C. horsfieldi* and *C. sphinx*. In the parsimony analysis, 775/1262 characters were constant, 93 were variable but parsimony uninformative, and 394 were variable and potentially informative. Tree topology was not sensitive to transition down-weighting, or differential treatment of gap characters. All MP searches identified >1000 equally parsimonious trees, however, consensus trees generated for each of the weighting schemes confirmed that both the position of the major clades and the segregation of taxa among clades, remained constant across all trees. One out of 1421 equal length trees found under equal weights parsimony with gaps treated as a fifth character state is shown in Fig. 2A.

In the Bayesian analysis, log likelihood values converged after ca. 25,000 generations but we chose 250,000 generations as the burnin time to ensure stationarity. A 50% majority-rule consensus tree representing the 25,000 trees saved from the final 250,000 MCMC generations was used to obtain the posterior probabilities for each clade. The tree with the best likelihood score is shown in Fig. 2B. Both trees in Fig. 2 are rooted with the three outgroup taxa: *Aethalops alecto*, *Megaerops ecaudatus*, and *Chironax melanocephalus*.

Taxa currently referred to *C. brachyotis* were split into six major lineages, which were well-supported under both parsimony and Bayesian criteria. *C. brachyotis* lineages from Sulawesi, the Philippines and India formed monophyletic, geographically well-defined clades. Identification of a single highly divergent *C. brachyotis* haplotype from Myanmar suggests that additional sampling in this region may confirm the presence of another distinct lineage within the *C. brachyotis* complex.

In the Sunda Shelf region we identified two distinct lineages (Sunda and Forest) that are broadly sympatric in peninsular Malaysia, and on Borneo. Mean divergence between the two lineages was 8.3% (Table 1). With the exception of a single divergent haplotype from the Philippine island of Palawan, no geographic structure was evident in the Sunda lineage and haplotypes from Thailand, Vietnam, peninsular Malaysia, Singapore, Java, and Borneo were separated by a low number of

Table 1
Measures of mean within-lineage (on diagonal) and among-lineage (below diagonal) genetic distances, based on 1266 bp of mitochondrial DNA (partial cytochrome *b* and control region combined)

	Sunda	India	Forest	Philippines	Sulawesi	*C. horsfieldi*	*C. sphinx*
Sunda	0.011						
India	0.122	0.01					
Forest	0.083	0.1	0.025				
Philippines	0.086	0.11	0.089	0.029			
Sulawesi	0.07	0.11	0.08	0.078	0.011		
C. horsfieldi	0.084	0.11	0.042	0.08	0.083	0.013	
C. sphinx	0.089	0.09	0.075	0.086	0.088	0.069	0.017

Distances were obtained in the program MEGA (Kumar et al., 1993), using the substitution model of Tamura and Nei (1993) and a specified gamma shape parameter of $\alpha = 0.6$, estimated in MrBayes.

mutational steps (mean within-lineage divergence, 1.1%; Table 1). The ca. 78 bp deletion near the 5′ end of control region was exclusive to the Sunda lineage and characterized all haplotypes in this clade (Fig. 2).

In contrast, the Forest *C. brachyotis* lineage was characterized by deep among-haplotype divergence (mean within-lineage divergence, 2.5%; Table 1); both MP and Bayesian analyses revealed subclades within this larger clade, one of which was paraphyletic with respect to the *C. horsfieldi* lineage. Based on our sampling, the geographic range of the Forest *C. brachyotis* lineage is contained within that of the widespread Sunda lineage, and may be restricted to peninsular Malaysia and Borneo. Although morphological and ecological data were not formally analyzed in this study, we found that all individuals with Forest haplotypes were consistently small (adult forearm length <63 mm) and were captured only in primary and mature secondary forest. In contrast, bats with Sunda haplotypes were larger (adult forearm length >64 mm) and were abundant in highly disturbed habitat (i.e., agricultural, landscaped, and suburban areas) and absent from closed forest.

Mean observed sequence divergence between *C. horsfieldi* and Forest *C. brachyotis* was 4.2%, a value lower than that obtained for all other ingroup comparisons (Table 1). This surprisingly shallow split between phenotypically distinct taxa was consistently well supported in all analyses and cannot be explained by misidentification of either species. At our sampling localities in peninsular Malaysia, *C. horsfieldi* was readily distinguished from all other sympatric congeners by distinctive dental morphology and by consistently large body size (adult forearm length >68 mm).

All *C. sphinx* haplotypes grouped in a well-supported monophyletic clade that was nested within the *C. brachyotis* complex. A sister relationship between *C. sphinx* and the Forest *C. brachyotis*/*C. horsfieldi* clade was strongly supported on all trees. With the exception of three Indian haplotypes, little geographic structure was evident within *C. sphinx* and haplotypes from Myanmar, Thailand, Vietnam, and peninsular Malaysia were separated by a low number of mutational steps.

The basal relationships within the *C. brachyotis* species complex were not concordant between MP and Bayesian

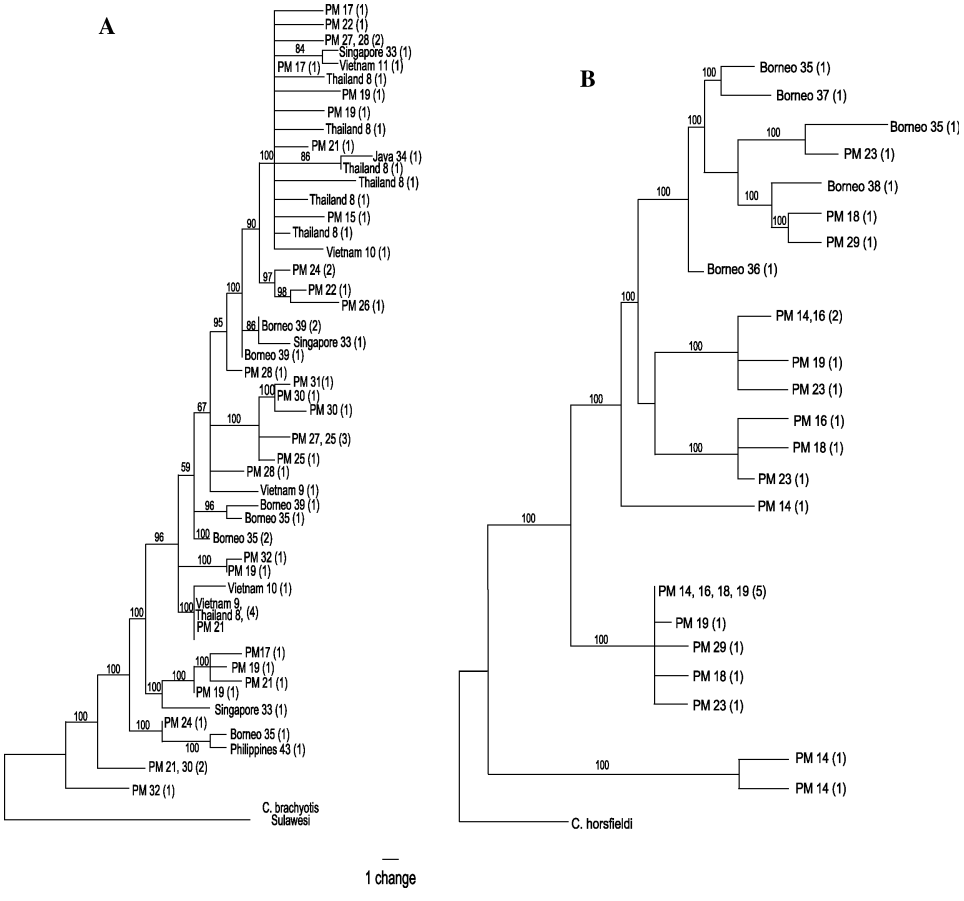

Fig. 3. Phylograms of (A) Sunda and (B) Forest *Cynopterus brachyotis* haplotypes based on ca. 565 bp of the mitochondrial control region. Trees are strict consensus trees of 706,300 equally parsimonious trees for the Sunda group and eight equally parsimonious trees for the Forest group. Branch lengths are drawn to represent observed number of substitutions among haplotypes. Tips are labeled with region (PM, Peninsular Malaysia), followed by the locality number corresponding to Fig. 1 and Appendix A, and the number of individuals displaying that haplotype (in parentheses). Trees are rooted with haplotypes from sister taxa identified in this study: (a) Sulawesi *C. brachyotis* and (b) *C. horsfieldi*.

analyses (Fig. 2). The basal placement of the Indian *C. brachyotis* clade was consistent across all most parsimonious solutions but received low support (bootstrap = 65). Likewise, the position of the Philippine *C. brachyotis* clade as sister to Sunda plus Sulawesi *C. brachyotis* was poorly supported on the MP tree (bootstrap <50). On the Bayesian tree, the placement of the Sulawesi *C. brachyotis* clade as sister to all other ingroup taxa was well supported (posterior probability = 95%). However, the position of the Indian clade as sister to the clade containing Forest *C. brachyotis*, *C. horsfieldi*, Myanmnar *C. brachyotis* and *C. sphinx* was weakly supported (posterior probability = 76%). Tests of the relative robustness of the two topologies under likelihood and parsimony criteria yielded conflicting results. The Kishino–Hasegawa test was non-significant ($p = 0.3$), although the Bayesian tree received a higher likelihood score. Conversely, the Wilcoxon signed-ranks test suggested that the MP tree was a better explanation of the data than the Bayesian tree under a parsimony criterion ($p = 0.03$).

3.2. Historical demography of the Sunda and Forest lineages

Based on the broad geographic distribution and short terminal branch lengths of the Sunda *C. brachyotis* clade (Fig. 3A), we hypothesized that this lineage had undergone an evolutionarily recent demographic expansion. In contrast, the long terminal branch lengths characterizing the Forest *C. brachyotis* lineage suggested a historically large and stable effective population size (Fig. 3B). However, the results of our demographic analyses suggest that both lineages have undergone significant fluctuations in population size at different times in the past.

The observed mismatch distribution of the Sunda *C. brachyotis* lineage was not strictly unimodal, but was consistent with population growth in the recent past (Fig. 4A). Statistical comparison of the observed distribution of pairwise differences with that expected under a population expansion hypothesis indicated a good fit between the data and the sudden expansion model ($P_{SSD} = 0.28$). The highly significant negative value of Fu's F_s ($F_s = -24.881$, $p < 0.0001$) and the large positive value for the maximum likelihood estimate of the exponential growth rate ($g = 482.072$, SD = 29.709) also provided strong support for the hypothesis of rapid demographic expansion.

While the shape of the observed mismatch distribution for the Forest lineage was multimodal (Fig. 4B), it did not conform to the expectations for a historically stable population at demographic equilibrium (Rogers and Harpending, 1992; Slatkin and Hudson, 1991), and we were not able to reject the expansion hypothesis for this lineage ($P_{SSD} = 0.27$). Likewise, the value of F_s bordered on significance ($F_s = -4.981$, $p = 0.05$) and the value of g was smaller, but still positive ($g = 104.475$, SD = 15.720).

Simulations have shown that the value of g tends to provide an upward biased estimate of growth for single loci data sets (Kuhner et al., 1998). However, in this context g provides a useful comparative measure of the difference in timing of inferred demographic fluctuations in the two lineages. The substantially larger value of g obtained for the Sunda lineage suggests that, all else being equal, the inferred expansion of this lineage was much more recent than that of the Forest lineage.

4. Discussion

4.1. Phylogeny and taxonomy

Phylogenetic analysis of mtDNA sequence data clearly indicates that *C. brachyotis* does not constitute a single monophyletic species. This conclusion is supported both by the nesting of *C. sphinx* and *C. horsfieldi* within the *C. brachyotis* complex, and by the identification of six highly distinct mitochondrial lineages, all of which are currently referred to *C. brachyotis* (Fig. 2).

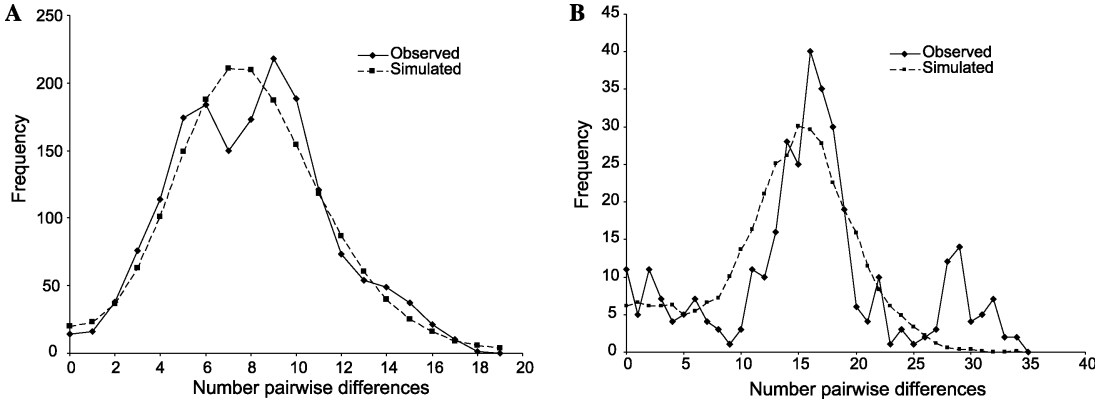

Fig. 4. Distribution of the number of pairwise nucleotide differences among (A) Sunda *Cynopterus brachyotis* ($n = 59$; $P_{SSD} = 0.28$) and (B) Forest *C. brachyotis* ($n = 27$; $P_{SSD} = 0.27$), based on ca. 565 bp of the control region. Solid lines represent the observed data, dashed lines represent the line fitted to the data under the expectations of the sudden expansion model, based on 1000 simulated samples in ARLEQUIN.

Historically, taxonomic issues raised by the highly variable morphology of *C. brachyotis* were resolved by adding (Andersen, 1912), subtracting (Hill and Thonglongya, 1972), and shuffling (Phillips, 1934, 1980) subspecies among *C. brachyotis* and *C. sphinx*. However, the results of this study provide strong evidence that taxa currently referred to *C. brachyotis* in the Sunda shelf region are split into two phylogenetically, ecologically and morphologically diagnosable lineages, neither of which is interdigitated with *C. sphinx*. Notably, while the range in forearm length for individuals with Sunda haplotypes does overlap with that of *C. sphinx* and the two taxa are co-distributed on the Southeast Asian mainland, mean divergence between the *C. sphinx* and Sunda *C. brachyotis* lineages is 8.9% (Table 1).

Based on the level of divergence (8.3% observed sequence difference in the combined data; Table 1) and the broadly sympatric distribution of the Sunda and Forest *C. brachyotis*, we suggest that species status is warranted for both lineages. However, which lineage should be renamed is not readily apparent, since the results of this study suggest that both lineages may occur at the type locality for *C. brachyotis* in south central Borneo (Müller, 1838). Forearm length is uninformative as the range in forearm length (60–63.5 mm) reported by Andersen (1912) for a series of 10 cotypes of *C. brachyotis* falls within that of both the Sunda and Forest lineages (Sunda, 60–70 mm, $n = 58$; Forest, 54.3–63.7 mm, $n = 26$; P. Campbell, unpubl. data). Detailed morphological analyses of both the type material and representatives of the mtDNA clades are needed to clarify the taxonomy.

The issue of nomenclature is further confounded by the reclassification of smaller *C. brachyotis* specimens from Borneo, Java, Sumatra, and Sulawesi as *C. minutus* (Kitchener and Maharadatunkamsi, 1991), a subspecies described by Miller (1906) as restricted to Nias Island off the west coast of Sumatra. The range in forearm length for *C. minutus* as described by Kitchener and Maharadatunkamsi (1991, 52.9–61.9, $n = 41$) suggests that this nominal species may correspond to the Forest lineage. However, such a designation is not warranted without direct comparison between the specimens from the present study and those examined by Kitchener and Maharadatunkamsi (1991).

Based on morphometric analyses, Kitchener and Maharadatunkamsi (1991) also restricted the eastern limits of the range of *C. brachyotis* to Bali and Borneo, proposing replacement in the Indonesian islands east of Bali by a new species, *C. nusatenggara*, and in the Philippines and Sulawesi by *C. luzoniensis*. Differentiation of *C. nusatenggara* and *C. luzoniensis* from *C. brachyotis* and from *C. sphinx* was supported by morphometric (Kitchener and Maharadatunkamsi, 1991) and allozyme data (Schmitt et al., 1995). However, the taxonomic revisions proposed by Kitchener and Maharadatunkamsi (1991) are not consistently accepted (Corbet and Hill, 1992; Koopman, 1993, 1994; but see Simmons, in press).

The connection between the results of the present study and the taxonomic and evolutionary relationships proposed by Kitchener and Maharadatunkamsi (1991) and Schmitt et al. (1995) is somewhat tenuous, since the samples used in these earlier studies were not available for comparison. However, since the taxon described as *C. luzoniensis* by Schmitt et al. (1995) was from Sulawesi, and our analysis suggests the presence of a single *Cynopterus* lineage on the island, it is likely that *C. luzoniensis* (sensu Kitchener and Maharadatunkamsi, 1991) is represented in this study by the Sulawesi lineage. Based on mtDNA sequence data, this lineage is clearly geographically and evolutionarily distinct from the Philippine endemic lineage. Since the type locality for *C. luzoniensis* is Luzon in the Philippines (Peters, 1861), we suggest that if one or both lineages were to be raised to species status, the name *C. luzoniensis* should be applied exclusively to Philippine populations.

In Sri Lanka, a recent investigation of the systematic relationship between *C. brachyotis* and *C. sphinx* identified two divergent mitochondrial lineages on the island (Mapatuna et al., 2002). Individuals from the *C. brachyotis* lineage were morphologically invariant, and were captured only in primary forest, whereas the *C. sphinx* lineage was phenotypically variable and occurred in a variety of habitats (Mapatuna et al., 2002). We downloaded sequences generated by Mapatuna et al. from GenBank (Accession Nos. AY009889–AY009895) and confirmed that Sri Lankan *C. brachyotis* and *C. sphinx* haplotypes grouped, respectively, with *C. brachyotis* and *C. sphinx* haplotypes from India (results not shown). The deep divergence between the Indian/Sri Lankan *C. brachyotis* lineage and all other ingroup taxa (Table 1) strongly suggests that this lineage represents an additional unrecognized species within the genus. Since the geographic range of the Indian/Sri Lankan lineage is well defined and corresponds to the range of *C. b. ceylonensis* (Campbell and Kunz, 2004; Corbet and Hill, 1992), it may be appropriate to raise this subspecies to species status.

In contrast to *C. sphinx* and *C. brachyotis*, the taxonomic status of *C. horsfieldi* has not been questioned. Andersen (1912) grouped this species separately from *C. sphinx* and *C. brachyotis* based on a suite of morphological characters and this distinction has been consistently acknowledged (Corbet and Hill, 1992; Koopman, 1993; Simmons, in press). However, while taxa with *C. horsfieldi* and Forest *C. brachyotis* haplotypes are at opposite extremes of the continuum in body size described by the bats sampled in this study, genetic divergence between the two is 4.2%, a value lower than that estimated for all other ingroup comparisons (Table 1). These observations suggest that the morphological

differentiation of these two lineages has taken place on a remarkably brief evolutionary timescale.

We summarize our taxonomic recommendations for the *C. brachyotis* lineages identified in this analysis as follows: the name *C. ceylonensis* is available for the Indian/Sri Lankan lineage. The Philippine lineage may be referred to *C. luzoniensis*. The Forest and Sunda lineages should be treated as distinct species, but which should retain the name *C. brachyotis* will not be apparent until additional analyses can be conducted. The Sulawesi lineage may warrant species status, however, no name already associated with *C. brachyotis* is applicable as the apparent range of this lineage does not correspond with that of any subspecies. Resolution of the relationship between the Myanmar lineage and the rest of the species complex will require additional sampling in that region. Despite nesting within the *C. brachyotis* complex, the status of *C. sphinx* and *C. horsfieldi* should not be revised. These species are morphologically distinct from sympatric *C. brachyotis* lineages (Bates and Harrison, 1997; Payne et al., 1985) and, in this analysis, haplotypes from *C. sphinx* and *C. horsfieldi* were not interdigitated with those of *C. brachyotis*.

The lack of agreement and low level of support for basal relationships in the MP and Bayesian trees precludes inference of the biogeographic origins of the *C. brachyotis* species complex. Statistical comparison of the two alternative topologies did not resolve this question: the MP tree was significantly better than the Bayesian tree in a parsimony framework, but was marginally worse under likelihood. Moreover, the high levels of among-clade divergence suggest that additional evolutionarily distinct lineages may persist in geographically isolated regions that are not well represented in this study, such as the Indonesian islands. If present, these lineages may affect biogeographic inferences. Additional taxon sampling, and character sampling from multiple loci, may help to resolve basal relationships. However, if, as suggested by Schmitt et al. (1995), the initial radiation of *Cynopterus* took place over an evolutionarily brief timespan, the geographic origin of extant lineages may prove difficult to reconstruct, regardless of the addition of taxa or loci. It is clear, however, that the taxonomy of the genus *Cynopterus* requires substantial revision to recognize the evolutionary diversity that exists within the *C. brachyotis* complex.

4.2. Phylogeographic and demographic inference

While the mechanisms of diversification in bats remain poorly understood, evidence from both population genetic analyses (reviewed in Burland and Worthington Wilmer, 2001) and studies combining molecular and phenotypic data (Barratt et al., 1997; Kingston et al., 2001) suggest that ecological specialization may promote intraspecific divergence, and in some cases, lead to speciation. Likewise, several phylogeographic studies of broadly distributed species have found that even relatively minor disjunctions in the distribution of suitable habitat may act as significant barriers to gene flow (Castella et al., 2000; Ditchfield, 2000). These findings suggest that for bats, as for many other taxa (e.g., Schluter, 2001), the effects on divergence of extrinsic factors such as vicariance and geological change may be mediated by ecological selection.

The contrasting phylogeographic structure and ecological affinities of the *Cynopterus* lineages identified in this study are notable in that they provide evidence for the differential effects of geography and ecology on the recent evolutionary history of a complex of closely related taxa. The absence of phylogeographic and demographic concordance among the Sunda and Forest lineages is particularly striking because it suggests that recent geological and environmental changes in the Sunda Shelf region have had very different impacts on the genetic structure and distribution of these two lineages. Although we did find evidence for demographic expansion in both lineages, the inferred expansion of the Sunda clade was more extreme and more recent.

During Pleistocene glacial maxima, global sea levels dropped by as much as 120–200 m (Gascoyne et al., 1979; Peltier, 2002; Verstappen, 1975). During these periods, large portions of the Sunda Shelf were exposed, uniting the major islands of Borneo, Palawan, Java, Sumatra, and Bali with mainland Southeast Asia, and significantly reducing sea crossing distances between Sulawesi and Borneo, and between Palawan and the rest of the Philippines (Fig. 1B; Heaney, 1991; Voris, 2000).

The observed geographic distribution of the Sunda *C. brachyotis* lineage tracks the regions of the Sunda Shelf that were periodically connected by land during the Pleistocene (Fig. 1B). While the capacity for flight clearly increases the dispersal potential of bats relative to similar sized terrestrial mammals, the wing morphology of *Cynopterus* is ill-suited to long distance flight (Hodgkison et al., 2004), suggesting that the current sea-crossing distances between Borneo and the Asian mainland greatly exceed the dispersal capacity of bats in this genus. Thus, the occurrence of the Sunda *C. brachyotis* lineage on both Borneo and Palawan strongly supports colonization during periods when the Sunda Shelf was exposed. The substantial genetic distance between the widespread Sunda lineage and the Philippine-restricted lineage (8.6%; Table 1) suggests that the present day geographic overlap of the two lineages on Palawan is the consequence of independent colonization events from distinctly different geographic centers of origin.

The broad geographic distribution of Sunda *C. brachyotis* haplotypes, low within-lineage divergence and absence of spatial genetic structure is concordant with expectations for populations whose recent evolu-

tionary history has been characterized by rapid demographic expansion (Rogers and Harpending, 1992). The sudden expansion hypothesis was supported for this lineage by a roughly unimodal mismatch distribution, highly significant departure from the expectations of selective neutrality and demographic equilibrium, and a large positive value for the estimated rate of exponential growth. However, we cannot determine whether the absence of genetic structure in the Sunda lineage is solely a consequence of the effect expected when source populations undergo rapid demographic expansion, or if variation has been further reduced by fixation of a positively selected mitochondrial haplotype via a selective sweep (Maruyama and Birky, 1991). Thus, without comparison of nuclear markers, we cannot rule out the possibility that this lineage may have a significantly longer and more complex evolutionary history than that inferred from mitochondrial haplotype data.

While the broad scale pattern of geographic distribution observed for the Sunda lineage strongly suggests that the range expansion inferred in this study was associated with increased dispersal potential during the Pleistocene, the present day ecology of this lineage suggests that the signature of historic range expansion may be overlaid on a finer geographic scale by recent changes in demography and distribution due to human factors. The absence of this lineage from closed forest habitat and concomitant high abundance in agricultural and suburban areas (P. Campbell, unpubl. data), indicate that this bat is successful at exploiting anthropogenic environments for both food and roosts. A long-term study of the diet and roosting behavior of *C. brachyotis* in landscaped parks adjacent to secondary forest found a high proportion of roosts in ornamental palms in areas with high human traffic (Tan et al., 1997). Likewise, while the bats in this study fed in the forest during seasonal fruiting cycles, the fruits of cultivated and ornamental plant species were exploited year round (Tan et al., 1998).

In contrast, the distribution of the Forest *C. brachyotis* lineage is apparently closely associated with relatively undisturbed forest (P. Campbell, unpubl. data). Based on the high levels of divergence, both within and among localities, we expected to find evidence of historic demographic equilibrium for this lineage (Rogers and Harpending, 1992). However, while the ragged shape of the mismatch distribution supported this expectation, we were not able to reject the sudden expansion hypothesis. Likewise, while we did not expect analysis of mtDNA to detect geographic structure among adjacent sites within peninsular Malaysia, the lack of differentiation between peninsular Malaysia and Borneo was surprising.

A possible explanation for the non-equilibrium dynamics of the Forest lineage is provided by the historic fluctuations in climate and associated instability of forest habitat in the Sunda Shelf region. Paeleoecological data suggest that the distribution of tropical forest on the Malay peninsula and Borneo, has been characterized by cycles of contraction and expansion (Flenley, 1984; Morley, 1981; Sun et al., 2002). Dramatic changes in the distribution of this forest type on the Sunda Shelf almost certainly occurred in conjunction with the major changes in humidity and land mass area associated with Pleistocene glacial cycles (Heaney, 1991; Morley and Flenley, 1987; Voris, 2000). Thus, although the current distribution of Forest *C. brachyotis* strongly suggests that this lineage is restricted both geographically and ecologically, the signature of demographic fluctuation in response to historic changes in forest cover may be retained in the genetic structure of this group.

5. Implications for conservation

From a practical perspective, the insular distribution and apparent restriction of the Forest lineage to habitat that is increasingly fragmented and reduced by human activity, suggests that its recognition as an evolutionarily significant unit (Crandall et al., 2000; Moritz, 1994) is an essential first step towards development of an appropriate management plan for this taxon. While we found no evidence for loss of genetic diversity in this lineage, mitochondrial markers are not expected to bear the signature of very recent changes in effective population size or barriers to gene flow. Whether present day gene flow between Forest *C. brachyotis* populations is restricted due to anthropogenic habitat fragmentation and reduction is a question we are currently addressing with population-level analyses.

Likewise, the Indian/Sri Lankan *C. brachyotis* lineage is spatially and evolutionarily diagnosable as distinct from all other lineages identified in this study, and is apparently restricted to primary forest (Mapatuna et al., 2002; Storz and Beaumont, 2002). These factors, combined with evidence for an evolutionarily recent demographic contraction in Indian populations (Storz and Beaumont, 2002), suggest that the conservation status of Indian/Sri Lankan *C. brachyotis* may also warrant reevaluation.

6. Conclusions

Clarification of the taxonomic issues raised in this study clearly awaits analysis of morphometric characters in conjunction with additional molecular data, and the comparison of type specimens. The high levels of among-clade divergence suggest that other evolutionarily distinct lineages within the *C. brachyotis* species complex may persist in geographically isolated regions

such as the Indonesian islands and Myanmar. If this is the case, then sampling at additional sites may be required before taxonomic issues can be fully resolved. While the lack of observed concordance between morphological and molecular divergence in the lineages identified in this study suggests that selecting appropriate and consistent criteria to delimit species within the *C. brachyotis* complex may prove challenging, the recognition of these taxa as independent evolutionary lineages lays an essential foundation to which ecological and morphological data can be added.

As molecular markers are increasingly used for phylogenetic inference, the incongruence between established taxonomy and inferred evolutionary relationships has become more apparent (Patton and Smith, 1994). While molecular phylogenies can recover the patterns of ancestry and descent that gave rise to the species or lineages sampled in the present, ecological data are often essential to unraveling the degree to which closely related taxa function as biologically distinct species in sympatry (Schluter, 1998). The phylogenetic relationships among the *Cynopterus* lineages that co-occur in Malaysia suggest a complex pattern of divergence, in which ecological selection may have played an important role. Phylogenetic inference in combination with preliminary ecological and morphological data strongly suggests that, despite phenotypic similarity substantial enough to lead several generations of taxonomists to treat *C. brachyotis* as a single taxon (Andersen, 1912; Koopman, 1993), the Sunda and Forest lineages may in fact be two ecologically distinct species. Likewise, despite strong evidence for recent common ancestry, morphological divergence between the Forest *C. brachyotis* lineage and *C. horsfieldi* suggests that these two lineages also occupy distinctly different niches. These hypotheses are currently being addressed at the population level through combined analysis of molecular, ecological, and morphometric data.

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Appendix A

Locality data for ingroup taxa sampled in this study

Locality No.	Locality	Species	Lineage	Haplotype	Data	Collector ID	Museum Cat. No.
	India						
1	Hosabale, Karnataka	*C. brachyotis*	India	Cb1a	CR, cyt *b*		
2	Appangala, Karnataka	*C. brachyotis*	India	Cb2a	CR, cyt *b*		
2	Appangala, Karnataka	*C. brachyotis*	India	Cb2b	CR, cyt *b*		
3	Belgaum, Karnataka	*C. sphinx*	*C. sphinx*	Cs3a	CR, cyt *b*		
4	Metapalayam, Tamil Nadu	*C. sphinx*	*C. sphinx*	Cs4a	CR, cyt *b*		
5	Nagercoil, Tamil Nadu	*C. sphinx*	*C. sphinx*	Cs5a	CR, cyt *b*		
	Myanmar						
6	Sitwe, Rakhine State	*C. brachyotis*	Myanmar	Cb6a	CR, cyt *b*		HZM MiMi41
7	Kyaik-Kha-Mi, Mon State	*C. sphinx*	*C. sphinx*	Cs7a	CR, cyt *b*		HZM.46.35037
	Thailand						
8	Si-Yad, Chanthaburi Prov.	*C. brachyotis*	Sunda	Cb8a	CR		
8	Si-Yad, Chanthaburi Prov.	*C. brachyotis*	Sunda	Cb8b	CR		
8	Si-Yad, Chanthaburi Prov.	*C. brachyotis*	Sunda	Cb8c	CR, cyt *b*		
8	Si-Yad, Chanthaburi Prov.	*C. brachyotis*	Sunda	Cb8d	CR		
8	Si-Yad, Chanthaburi Prov.	*C. brachyotis*	Sunda	Cb8e	CR		
8	Si-Yad, Chanthaburi Prov.	*C. brachyotis*	Sunda	Cb8f	CR		
8	Si-Yad, Chanthaburi Prov.	*C. brachyotis*	Sunda	Cb8g	CR		
8	Si-Yad, Chanthaburi Prov.	*C. brachyotis*	Sunda	Cb8h	CR		
8	Si-Yad, Chanthaburi Prov.	*C. sphinx*	*C. sphinx*	Cs8a	CR, cyt *b*		
8	Si-Yad, Chanthaburi Prov.	*C. sphinx*	*C. sphinx*	Cs8b	CR, cyt *b*		
	Vietnam						
9	Dong Nai, Tan Phu	*C. brachyotis*	Sunda	Cb9a	CR, cyt *b*		ROM110924
9	Dong Nai, Tan Phu	*C. brachyotis*	Sunda	Cb9b	CR		ROM110917
9	Dong Nai, Tan Phu	*C. sphinx*	*C. sphinx*	Cs9a	CR, cyt *b*		ROM110927
10	Thanh Pho Ho, Can Gio	*C. brachyotis*	Sunda	Cb10a	CR, cyt *b*		ROM111005
10	Thanh Pho Ho, Can Gio	*C. brachyotis*	Sunda	Cb10b	CR		ROM111002
11	Soc Trang	*C. brachyotis*	Sunda	Cb11a	CR, cyt *b*		ROM110966
12	Tuyen Quang, Na Hang	*C. sphinx*	*C. sphinx*	Cs12a	CR, cyt *b*		ROM107663
13	Quang Nam, Nuoc Xa	*C. sphinx*	*C. sphinx*	Cs13a	CR, cyt *b*		ROM111266
	Peninsular Malaysia						
14	Perlis State Park, Perlis	*C. brachyotis*	Forest	Cb14a	CR, cyt *b*		
14	Perlis State Park, Perlis	*C. brachyotis*	Forest	Cb14b	CR, cyt *b*		
14	Perlis State Park, Perlis	*C. brachyotis*	Forest	Cb14c	CR, cyt *b*		
14	Perlis State Park, Perlis	*C. brachyotis*	Forest	Cb14d	CR		
14	Perlis State Park, Perlis	*C. brachyotis*	Forest	Cb14e	CR		
14	Perlis State Park, Perlis	*C. sphinx*	*C. sphinx*	Cs14a	CR, cyt *b*	Ms070	
14	Perlis State Park, Perlis	*C. sphinx*	*C. sphinx*	Cs14b	CR, cyt *b*		
14	Perlis State Park, Perlis	*C. sphinx*	*C. sphinx*	Cs14c	CR, cyt *b*		
14	Perlis State Park, Perlis	*C. horsfieldi*	*C. horsfieldi*	Ch14a	CR, cyt *b*	Mh038	

15	Kangar, Perlis	*C. brachyotis*	Sunda	Cb15a	CR, cyt *b*	
15	Kangar, Perlis	*C. sphinx*	*C. sphinx*	Cs15a	CR, cyt *b*	
15	Kangar, Perlis	*C. sphinx*	*C. sphinx*	Cs15b	CR, cyt *b*	
16	Kuala Perlis, Perlis	*C. brachyotis*	Forest	Cb16a	CR, cyt *b*	
16	Kuala Perlis, Perlis	*C. brachyotis*	Forest	Cb16b	CR	
16	Kuala Perlis, Perlis	*C. brachyotis*	Forest	Cb16c	CR, cyt *b*	
16	Kuala Perlis, Perlis	*C. brachyotis*	Forest	Cb16d	CR	
16	Kuala Perlis, Perlis	*C. brachyotis*	Forest	Cb16e	CR	
16	Kuala Perlis, Perlis	*C. sphinx*	*C. sphinx*	Cs16a	CR, cyt *b*	
16	Kuala Perlis, Perlis	*C. sphinx*	*C. sphinx*	Cs16b	CR, cyt *b*	
16	Kuala Perlis, Perlis	*C. sphinx*	*C. sphinx*	Cs16c	CR, cyt *b*	
17	Pulau Perhanitian Kecil, Terengganu	*C. brachyotis*	Sunda	Cb17a	CR	
17	Pulau Perhanitian Kecil, Terengganu	*C. brachyotis*	Sunda	Cb17b	CR	
17	Pulau Perhanitian Kecil, Terengganu	*C. brachyotis*	Sunda	Cb17c	CR	
18	Gua Musang, Kelantan	*C. brachyotis*	Forest	Cb18a	CR, cyt *b*	
18	Gua Musang, Kelantan	*C. brachyotis*	Forest	Cb18b	CR, cyt *b*	
18	Gua Musang, Kelantan	*C. brachyotis*	Forest	Cb18c	CR, cyt *b*	
18	Gua Musang, Kelantan	*C. brachyotis*	Forest	Cb18d	CR	
18	Gua Musang, Kelantan	*C. horsfieldi*	*C. horsfieldi*	Ch18a	CR, cyt *b*	
19	Taiping, Perak	*C. brachyotis*	Sunda	Cb19a	CR	
19	Taiping, Perak	*C. brachyotis*	Sunda	Cb19b	CR	
19	Taiping, Perak	*C. brachyotis*	Forest	Cb19e	CR, cyt *b*	
19	Taiping, Perak	*C. brachyotis*	Forest	Cb19f	CR, cyt *b*	
19	Taiping, Perak	*C. sphinx*	*C. sphinx*	Cs19a	CR, cyt *b*	Ms131
19	Taiping, Perak	*C. sphinx*	*C. sphinx*	Cs19b	CR, cyt *b*	Ms146
19	Taiping, Perak	*C. sphinx*	*C. sphinx*	Cs19c	CR, cyt *b*	
19	Taiping, Perak	*C. horsfieldi*	*C. horsfieldi*	Ch19a	CR, cyt *b*	
19	Taiping, Perak	*C. horsfieldi*	*C. horsfieldi*	Ch19b	CR, cyt *b*	
19	Taiping, Perak	*C. horsfieldi*	*C. horsfieldi*	Ch19c	CR, cyt *b*	Mh089
20	Cameron Highlands, Pahang	*C. sphinx*	*C. sphinx*	Cs20a	CR, cyt *b*	CYSP20
20	Cameron Highlands, Pahang	*C. sphinx*	*C. sphinx*	Cs20b	CR, cyt *b*	CYSP21
20	Cameron Highlands, Pahang	*C. sphinx*	*C. sphinx*	Cs20c	CR, cyt *b*	
20	Cameron Highlands, Pahang	*C. sphinx*	*C. sphinx*	Cs20d	CR, cyt *b*	
20	Cameron Highlands, Pahang	*C. sphinx*	*C. sphinx*	Cs20e	CR, cyt *b*	
20	Cameron Highlands, Pahang	*C. sphinx*	*C. sphinx*	Cs20f	CR, cyt *b*	
20	Cameron Highlands, Pahang	*C. horsfieldi*	*C. horsfieldi*	Ch20a	CR, cyt *b*	
20	Cameron Highlands, Pahang	*C. horsfieldi*	*C. horsfieldi*	Ch20b	CR, cyt *b*	
20	Cameron Highlands, Pahang	*C. horsfieldi*	*C. horsfieldi*	Ch20c	CR, cyt *b*	
21	Kuala Lipis, Pahang	*C. brachyotis*	Sunda	Cb21a	CR	
21	Kuala Lipis, Pahang	*C. brachyotis*	Sunda	Cb21b	CR	
21	Kuala Lipis, Pahang	*C. brachyotis*	Sunda	Cb21c	CR	
21	Kuala Lipis, Pahang	*C. brachyotis*	Sunda	Cb21d	CR	
21	Kuala Lipis, Pahang	*C. horsfieldi*	*C. horsfieldi*	Ch21a	CR, cyt *b*	
22	Cherating, Pahang	*C. brachyotis*	Sunda	Cb22a	CR	
22	Cherating, Pahang	*C. brachyotis*	Sunda	Cb22b	CR	
23	Krau Forest Reserve, Pahang	*C. brachyotis*	Forest	Cb23a	CR	M051
23	Krau Forest Reserve, Pahang	*C. brachyotis*	Forest	Cb23b	CR	M055

(*continued on next page*)

Appendix A (*continued*)

Locality No.	Locality	Species	Lineage	Haplotype	Data	Collector ID	Museum Cat. No.
23	Krau Forest Reserve, Pahang	*C. brachyotis*	Forest	Cb23c	CR, cyt *b*		
23	Krau Forest Reserve, Pahang	*C. brachyotis*	Forest	Cb23d	CR, cyt *b*		
23	Krau Forest Reserve, Pahang	*C. horsfieldi*	*C. horsfieldi*	Ch23a	CR, cyt *b*		
23	Krau Forest Reserve, Pahang	*C. horsfieldi*	*C. horsfieldi*	Ch23b	CR, cyt *b*		
23	Krau Forest Reserve, Pahang	*C. horsfieldi*	*C. horsfieldi*	Ch23c	CR, cyt *b*		
24	Pulau Pankor, Perak	*C. brachyotis*	Sunda	Cb24a	CR		
24	Pulau Pankor, Perak	*C. brachyotis*	Sunda	Cb24b	CR		
24	Pulau Pankor, Perak	*C. brachyotis*	Sunda	Cb24c	CR		
25	Sungai Dusun, Selangor	*C. brachyotis*	Sunda	Cb25a	CR	M002	
25	Sungai Dusun, Selangor	*C. brachyotis*	Sunda	Cb25b	CR	M003	
25	Sungai Dusun, Selangor	*C. brachyotis*	Sunda	Cb25c	CR	M004	
26	Taman Pantun, Selangor	*C. brachyotis*	Sunda	Cb26a	CR	M021	
27	Kampung Kuantan, Selangor	*C. brachyotis*	Sunda	Cb27a	CR		
27	Kampung Kuantan, Selangor	*C. brachyotis*	Sunda	Cb27b	CR		
28	Melaka Town, Melaka	*C. brachyotis*	Sunda	Cb28a	CR		
29	Endau Rompin, Johore	*C. brachyotis*	Forest	Cb29a	CR, cyt *b*	M342	
29	Endau Rompin, Johore	*C. brachyotis*	Forest	Cb29b	CR, cyt *b*	M344	
30	Pulau Tioman, Pahang	*C. brachyotis*	Sunda	Cb30a	CR	M158	
30	Pulau Tioman, Pahang	*C. brachyotis*	Sunda	Cb30b	CR	M160	
30	Pulau Tioman, Pahang	*C. brachyotis*	Sunda	Cb30c	CR	M161	
30	Pulau Tioman, Pahang	*C. brachyotis*	Sunda	Cb30d	CR		
31	Mersing, Johore	*C. brachyotis*	Sunda	Cb31a	CR		
32	Kota Tinggi, Johor	*C. brachyotis*	Sunda	Cb32a	CR		
32	Kota Tinggi, Johor	*C. brachyotis*	Sunda	Cb32b	CR		
	Singapore						
33	Bukit Timah	*C. brachyotis*	Sunda	Cb33a	CR, cyt *b*		
33	Bukit Timah	*C. brachyotis*	Sunda	Cb33b	CR, cyt *b*		
33	Bukit Timah	*C. brachyotis*	Sunda	Cb33c	CR		
	Java						
34	Jakarta	*C. brachyotis*	Sunda	Cb34a	CR, cyt *b*		
	Borneo						
35	Bario, Sarawak	*C. brachyotis*	Sunda	Cb35a	CR	TA-0083	
35	Bario, Sarawak	*C. brachyotis*	Forest	Cb35b	CR	TA-0064	
35	Bario, Sarawak	*C. brachyotis*	Sunda	Cb35c	CR	TA-0081	
35	Bario, Sarawak	*C. brachyotis*	Forest	Cb35d	CR	TA-0057	
35	Bario, Sarawak	*C. brachyotis*	Sunda	Cb35e	CR	TA-1022	
35	Bario, Sarawak	*C. brachyotis*	Sunda	Cb35f	CR	TA-1023	
36	Lambir Hill, Miri, Sarawak	*C. brachyotis*	Forest	Cb36a	CR	TA-0241	
37	Padawan, Sarawak	*C. brachyotis*	Forest	Cb37a	CR	TA-0251	
38	Mt. Kinabalu, Sabah	*C. brachyotis*	Forest	Cb38a	CR, cyt *b*		FMNH159016
39	Gunung Palung National Park, West Kalimantan	*C. brachyotis*	Sunda	Cb39a	CR, cyt *b*	AJG276	
39	Gunung Palung National Park, West Kalimantan	*C. brachyotis*	Sunda	Cb39b	CR, cyt *b*	AJG278	
39	Gunung Palung National Park, West Kalimantan	*C. brachyotis*	Sunda	Cb39c	CR	AJG300	
39	Gunung Palung National Park, West Kalimantan	*C. brachyotis*	Sunda	Cb39d	CR	AJG302	
39	Gunung Palung National Park, West Kalimantan	*C. horsfieldi*	*C. horsfieldi*	Ch39a	CR, cyt *b*	AJG272	

	Sulawesi						
40	Lambundo Bundo, Buton Island	*C. brachyotis*	Sulawesi	Cb40a	CR, cyt *b*		
41	Kabena Island	*C. brachyotis*	Sulawesi	Cb41a	CR, cyt *b*		
42	Donggala, Kulawi, Tomado, Lore Lindu N.P.	*C. brachyotis*	Sulawesi	Cb42a	CR, cyt *b*	PSUT25	
42	Donggala, Kulawi, Tomado, Lore Lindu N.P.	*C. brachyotis*	Sulawesi	Cb42b	CR, cyt *b*	PSUT43	
42	Donggala, Kulawi, Tomado, Lore Lindu N.P.	*C. brachyotis*	Sulawesi	Cb42c	CR, cyt *b*	PSUT56	
42	Donggala, Kulawi, Tomado, Lore Lindu N.P.	*C. brachyotis*	Sulawesi	Cb42d	CR, cyt *b*	PSUT195	
42	Donggala, Kulawi, Tomado, Lore Lindu N.P.	*C. brachyotis*	Sulawesi	Cb42e	CR, cyt *b*	PSUT196	
42	Donggala, Kulawi, Tomado, Lore Lindu N.P.	*C. brachyotis*	Sulawesi	Cb42f	CR, cyt *b*	PDX45	
42	Donggala, Kulawi, Tomado, Lore Lindu N.P.	*C. brachyotis*	Sulawesi	Cb42g	CR, cyt *b*	PDX49	
	Philippines						
43	Palawan, Puerto Princesa Municipality	*C. brachyotis*	Sunda	Cb43a	CR, cyt *b*		FMNH168876
43	Palawan, Puerto Princesa Municipality	*C. brachyotis*	Philippines	Cb43b	CR, cyt *b*		FMNH168877
43	Palawan, Puerto Princesa Municipality	*C. brachyotis*	Philippines	Cb43c	CR, cyt *b*		FMNH168879
44	Mindanao, Bukidnon Prov., Mt. Katanlad Range	*C. brachyotis*	Philippines	Cb44a	CR, cyt *b*		FMNH146602
45	Sibuyan, Romblon Prov., Magdiwang	*C. brachyotis*	Philippines	Cb45a	CR, cyt *b*		FMNH136947
46	Luzon, Isabela Prov., San Mariano	*C. brachyotis*	Philippines	Cb46a	CR, cyt *b*		FMNH140605
47	Luzon, Camarines Sur Prov., Mt. Isarog	*C. brachyotis*	Philippines	Cb47a	CR, cyt *b*		LRH4653*
48	Catanduanes, Catanduanes Prov.	*C. brachyotis*	Philippines	Cb48a	CR, cyt *b*		LRH3859*

Species names follow current taxonomy (Corbet and Hill, 1992; Koopman, 1993). Lineages are defined in this study. CR, control region; cyt *b*, cytochrome *b*. Samples obtained from museum specimens are identified by institution and catalogue number, or *, collector ID; HZM, Harrison Zoological Museum; ROM, Royal Ontario Museum; FMNH, Field Museum of Natural History. All other samples were collected by individual researchers; collector IDs are provided for samples from vouchers not currently deposited in museum collections. Information on sample/voucher availability available upon request from first author.

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