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Comparative population structure of *Cynopterus* fruit bats in peninsular Malaysia and southern Thailand

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Abstract

The extent to which response to environmental change is mediated by species-specific ecology is an important aspect of the population histories of tropical taxa. During the Pleistocene glacial cycles and associated sea level fluctuations, the Sunda region in Southeast Asia experienced concurrent changes in landmass area and the ratio of forest to open habitat, providing an ideal setting to test the expectation that habitat associations played an important role in determining species' response to the opportunity for geographic expansion. We used mitochondrial control region sequences and six microsatellite loci to compare the phylogeographic structure and demographic histories of four broadly sympatric species of Old World fruit bats in the genus, *Cynopterus*. Two forest-associated species and two open-habitat generalists were sampled along a latitudinal transect in Singapore, peninsular Malaysia, and southern Thailand. Contrary to expectations based on habitat associations, the geographic scale of population structure was not concordant across ecologically similar species. We found evidence for long and relatively stable demographic history in one forest and one open-habitat species, and inferred non-coincident demographic expansions in the second forest and open-habitat species. Thus, while these results indicate that Pleistocene climate change did not have a single effect on population structure across species, a correlation between habitat association and response to environmental change was supported in only two of four species. We conclude that interactions between multiple factors, including historical and contemporary environmental change, species-specific ecology and interspecific interactions, have shaped the recent evolutionary histories of *Cynopterus* fruit bats in Southeast Asia.

Keywords: climate change, demographic expansion, forest, Old World tropics, speciation, Sunda shelf

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Introduction

Comparisons of genetic structure among codistributed species can provide significant insight into the extent to which extrinsic and intrinsic factors interact to influence the geographic scale of population differentiation (Bermingham & Moritz 1998; Avise 2000; Patton *et al*. 2000; Arbogast & Kenagy 2001). For example, ecologically and phylogenetically disparate taxa may exhibit striking concordance in phylogeographic structure across historical barriers to gene flow (Joseph *et al*. 1995; Schneider *et al*. 1998; Evans *et al*. 2003). Conversely, relatively minor differences in life history traits (Patton *et al*. 1996; Matocq *et al*. 2000) and ecology (Brouat *et al*. 2003) among closely related species may translate into significant differences in the degree and scale of population structure.

In phylogeographic studies of tropical taxa, the relationship between environmental history and ecology is particularly pertinent: late Tertiary and Quaternary climate fluctuations caused global cycles of contraction and expansion in the latitudinal range of tropical forests (Haffer 1987; Morley & Flenley 1987), simultaneously restricting the ranges of

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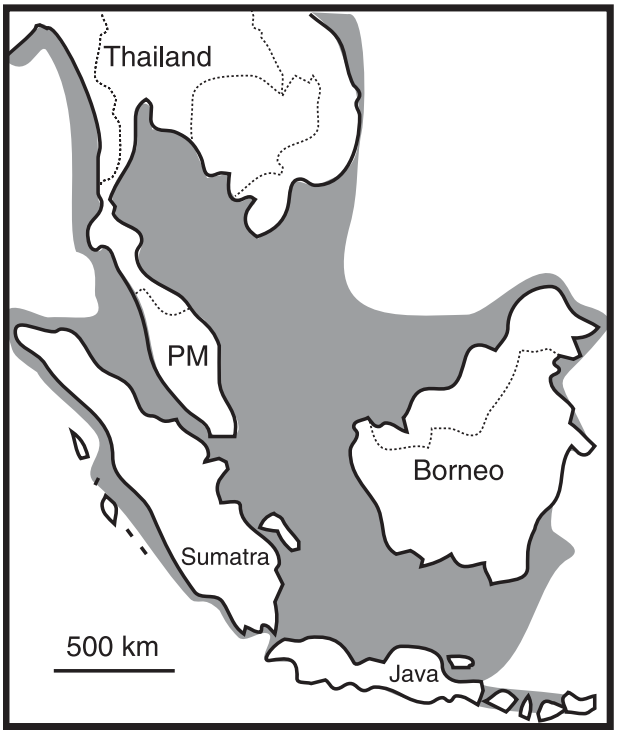

Fig. 1 Map of the Sunda region. Shading represents the additional 1.53 million km^2 of terrestrial habitat exposed when sea levels dropped to ≥120 m below present levels during Pleistocene glacial cycles (after Vorris 2000). PM, Peninsular Malaysia.

forest-dependent species and providing opportunity for range expansion in open-habitat-adapted species. However, while the potential importance of habitat association in mediating population response to environmental change is implicit to most phylogeographic studies in the tropics, relatively few explicit comparisons have been made between forest specialists and habitat generalists (Joseph & Moritz 1993; Stuart-Fox *et al*. 2001; Storz & Beaumont 2002; Heaney *et al*. 2005).

The Sunda region in Southeast Asia provides an ideal setting for comparative analysis of the population histories of codistributed ecologically differentiated species. Among tropical regions, the Quaternary history of this area is unique, in that reversals in the relative proportions of forest and open habitats were accompanied by major changes in the area and contiguity of all terrestrial habitats (Heaney 1991). Lowered sea levels associated with Pleistocene glacial cycles periodically united the Malay Peninsula and the continental islands of Borneo, Sumatra and Java in a single block, increasing contiguous terrestrial habitat by approximately 150% (Fig. 1; Vorris 2000).

In contrast to the well-documented Pleistocene fragmentation of rainforest blocks elsewhere in the Old World (e.g. Australian Wet Tropics: Kershaw 1994; Schneider *et al*. 1998; equatorial West Africa: Maley 1996; Quérouil *et al*. 2003), available data suggest that areas in the Sunda region such as the Malay Peninsula that remained land positive throughout the Pleistocene did not experience significant forest reduction (Whitmore 1987; Hope *et al*. 2004). Most sources of palaeoecological and palaeoclimatic data indicate that relatively arid conditions prevailed on the periodically exposed Sunda shelf, favouring open savannah grassland-pine and thorn scrub as the dominant habitat types (Adams & Faure 1997; Verstappen 1997; Morley 2000; Tamburini *et al*. 2003; Hope *et al*. 2004). Thus, while the area and connectivity of forest habitats remained relatively stable, periodic increases in the distribution of contiguous open habitat provided generalist species with extensive opportunities for geographic and demographic expansion.

In this study, we compare mitochondrial phylogeographic structure and spatial patterns of nuclear genetic diversity among four species of Southeast Asian fruit bats in the genus, *Cynopterus*. We take advantage of the well-established geological history of the Sunda region to ask whether simultaneous increases in the area of open habitat and the total area of terrestrial habitat evoked a common, or species-specific, response in taxa with different habitat affinities but comparable dispersal capacities.

Cynopterus is among the most diverse and broadly distributed genera of Old World fruit bats (Corbet & Hill 1992; Simmons 2005). Seven species are currently recognized (Simmons 2005), but the recent identification of six genetically distinct lineages within *Cynopterus brachyotis* indicates that several additional species remain to be described (Campbell *et al*. 2004). Here, we focus on the Malay peninsula and southern Thailand (Fig. 2) where four nominal species are broadly sympatric: *Cynopterus horsfieldi*, *Cynopterus sphinx*, and *C. brachyotis*, with the latter split into two ecologically, morphologically and genetically divergent lineages, referred to herein as *C. brachyotis* Sunda and *C. brachyotis* Forest (Campbell *et al*. 2004). Morphological differentiation among the species is not concordant with genetic distance. Based on 1266 bp of mitochondrial DNA (mtDNA) (partial control region and cytochrome *b* combined), the smallest species, *C. brachyotis* Forest is paraphyletic with respect to the largest, *C. horsfieldi* (4.2% divergence). In contrast, despite considerable overlap in size between *C. brachyotis* Forest and *C. brachyotis* Sunda, and between *C. brachyotis* Sunda and *C. sphinx*, genetic divergence between these species is 8.3% and 8.9%, respectively (Campbell *et al*. 2004).

All four species are nonmigratory, have low natal dispersal (P. Campbell, unpublished) and exhibit a harem-based mating system (Tan *et al*. 1997, 1999; Storz *et al*. 2000; Fletcher 2001). Thus, interspecific comparisons of genetic structure are not likely to be confounded by behaviourally mediated differences in gene flow among or within populations. There

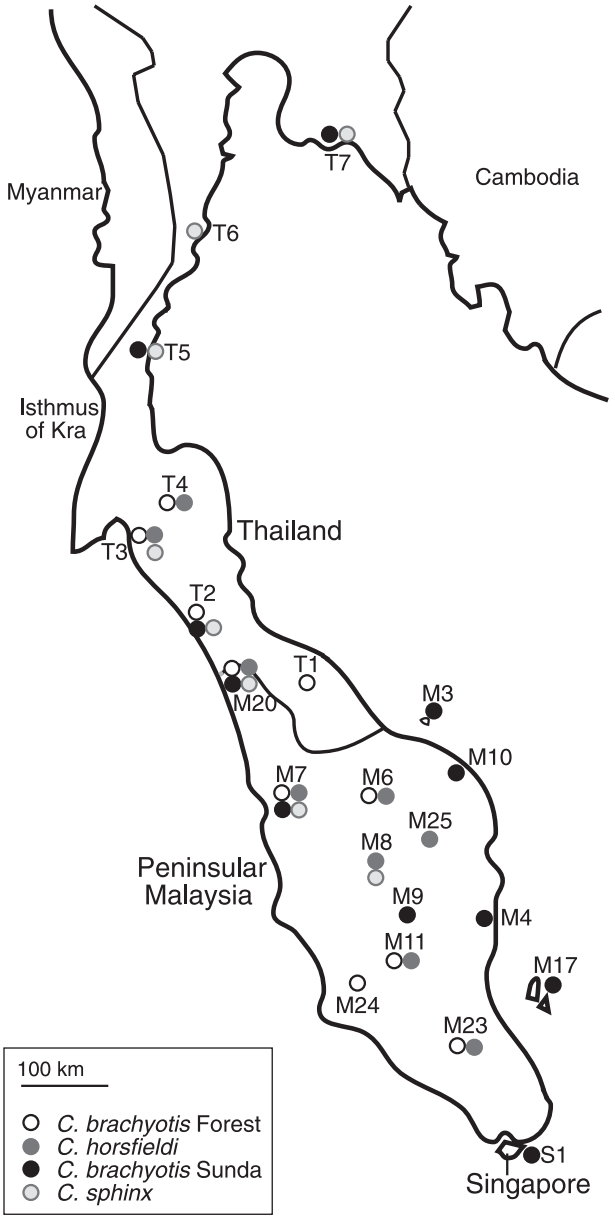

Fig. 2 Sampling localities for *Cynopterus* in Singapore, peninsular Malaysia and southern Thailand. Complete locality data provided in Table 1. One to four of the species were sampled at each site: white circles, *C. brachyotis* Forest; dark grey circles, *C. horsfieldi*; black circles, *C. brachyotis* Sunda; light grey circles, *C. sphinx*.

are, however, considerable interspecific differences in geographic range size. Whereas *C. sphinx* is continuously distributed throughout India and much of mainland Southeast Asia, *C. horsfieldi* is restricted to the Malay peninsula and the continental islands of Borneo, Sumatra, and Java (Corbet & Hill 1992). While the range limits of *C. brachyotis* Forest and *C. brachyotis* Sunda are currently poorly defined due to unresolved taxonomy, mitochondrial data indicate a high level of geographic overlap between *C. brachyotis* Forest and *C. horsfieldi* (Abdullah 2003; Campbell *et al*. 2004). *C. brachyotis* Sunda co-occurs with these two species, but extends farther north into Thailand and Vietnam (Campbell *et al*. 2004) and probably Cambodia and Laos.

At the local scale, the presence or absence of forest habitat is a strong predictor of species occurrence and abundance. *C. sphinx* and *C. brachyotis* Sunda are widespread in open suburban and agricultural habitats, whereas *C. horsfieldi* and *C. brachyotis* Forest are common in primary and mature secondary rainforest from 1° to 6°N in peninsular Malaysia, and in transitional rainforest/monsoon forest from 6° to 11°N in southern Thailand. While *C. brachyotis* Forest and *C. brachyotis* Sunda are almost completely allotopic and restricted to forest and open anthropogenic habitats, respectively, *C. sphinx* and *C. horsfieldi* co-occur across habitat types but at opposite abundances. Capture rates decline with distance from forest edge for *C. horsfieldi* and increase for *C. sphinx* (P. Campbell, unpublished).

Preliminary population genetic analyses of haplotype data for *C. brachyotis* Forest and Sunda suggested discordant demographic histories, featuring an expansion in the latter species (Campbell *et al*. 2004). However, without inclusion of nuclear markers, we could not exclude a selective sweep of the mitochondrial genome of *C. brachyotis* Sunda as an alternative explanation, nor could we conclusively demonstrate the evolutionary independence of the two putative species. Here, we build on this result, using highly polymorphic microsatellite markers and mtDNA sequence data to address two main goals. First, it was important to confirm that nuclear markers recover the same four lineages identified using mtDNA (Campbell *et al*. 2004). Second, we tested the prediction that the population histories of *Cynopterus* species on the Malay Peninsula have been shaped by species-specific ecology, not by shared geographic distribution in a region that has undergone major structural change in recent evolutionary time. Based on evidence that Pleistocene climate change dramatically increased the area of open habitat on the Sunda shelf but did not greatly impact the distribution of forest, we expected to find a strong genetic signature of demographic expansion in *C. brachyotis* Sunda, contrasted with that of long-term demographic stability and equilibrium population dynamics in *C. brachyotis* Forest. Because present-day populations of *C. sphinx* and *C. horsfieldi* are associated with both forest and open-habitat types, ecologically based predictions for these species were less clearly defined. However, the abundance of *C. sphinx* in deforested areas throughout an extensive geographic range implies a long-term association with open habitats, while recent shared ancestry with *C. brachyotis* Forest suggests a forest origin for *C. horsfieldi*. Accordingly, we expected concordance in genetic structure to be greatest between ecologically similar species pairs: *C. sphinx* and *C. brachyotis* Sunda, and *C. horsfieldi* and *C. brachyotis* Forest.

Table 1 Locality data for *Cynopterus* species sampled in this study

Locality code	Country	Locality	Lat °N	Long °E	Species
S1	Singapore	Bukit Timah	01 22.0	103 46.6	*Cb*S
M23	Malaysia	Endau Rompin	02 32.0	103 24.5	*Cb*F, *Ch*
M17	Malaysia	P. Tioman	02 47.0	104 09.0	*Cb*S*
M24	Malaysia	Gombak	03 19.5	101 44.0	*Cb*F
M11	Malaysia	Krau	03 43.0	102 17.2	*Cb*F, *Ch*
M10	Malaysia	Cherating	04 07.6	103 24.0	*Cb*S
M9	Malaysia	Kuala Lipis	04 11.5	102 03.0	*Cb*S**
M25	Malaysia	Taman Negara	04 27.0	102 22.0	*Ch**
M8	Malaysia	Cameron Highlands	04 28.4	101 22.0	*Ch, Cs*
M7	Malaysia	Taiping	04 51.5	100 45.2	*Cb*F, *Ch, Cb*S, *Cs*
M6	Malaysia	Gua Musang	04 53.0	101 57.6	*Cb*F, *Ch, Cs**
M4	Malaysia	Merang	05 32.0	102 57.5	*Cb*S
M3	Malaysia	P. Perhentian Kecil	05 55.0	102 43.4	*Cb*S*
M20	Malaysia	Perlis State Park	06 40.0	100 11.0	*Cb*F, *Ch, Cb*S, *Cs*
T1	Thailand	Yala	06 28.0	101 16.4	*Cb*F, *Ch**
T2	Thailand	Trang	07 44.5	099 41.5	*Cb*F, *Cb*S, *Cs**
T3	Thailand	Krabi	08 09.0	098 50.6	*Cb*F, *Ch, Cs*
T4	Thailand	Surat Thani	08 54.2	098 31.4	*Cb*F*, *Ch**, *Cs***
T5	Thailand	Chumphon	10 37.0	099 13.0	*Cb*S, *Cs*
T6	Thailand	Prachuap Khiri Khan	12 05.4	099 42.4	*Cs*
T7	Thailand	Si-Yad	13 30.0	102 05.0	*Cb*S, *Cs*

*Cb*F, *C. brachyotis* Forest; *Ch*, *C. horsfieldi*; *Cb*S, *C. brachyotis* Sunda; *Cs*, *C. sphinx*.
*denotes mitochondrial data only, **denotes nuclear microsatellite data only.

Methods

Sampling and species identification

Cynopterus were sampled along a 1370 km latitudinal transect on the Malay peninsula from Singapore (1°N, 103°E) to Si-Yad (13°N, 101°E) in southeast Thailand (Fig. 2; Table 1). Samples from three sites were obtained from the collections of other researchers (Si-Yad, Thailand) and the Malaysian Department of Wildlife and National Parks (Taman Negara and Tioman Island, Malaysia). All other samples were collected during the course of this study (June 2002–January 2004) in habitats ranging from primary forest to suburban gardens. Field discrimination between *Cynopterus sphinx*, *Cynopterus horsfieldi* and *Cynopterus brachyotis* was based on a suite of morphological characters described in Payne *et al*. (1985) and Bates & Harrison (1997). The two *Cynopterus brachyotis* lineages were discriminated based on forearm length (Forest, mean = 59.5 mm ± SD 1.7, $n = 52$; Sunda mean = 63.8 ± SD 1.6, $n = 57$), coloration of nuchal collar (Forest = dark orange–red; Sunda = pale orange–yellow) and habitat associations (P. Campbell, unpublished). Our survey data and that of other researchers suggest that *C. sphinx* does not occur on the peninsula south of *c*. 4°N (Hodgkison *et al*. 2004), and that *C. horsfieldi* and *C. brachyotis* Forest are rare or absent north of the Isthmus of Kra in Thailand (11°N). Due to these regional differences in distribution, along with local differences in the availability of suitable habitat, one or more species was absent from the majority of sites.

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from wing membrane tissue preserved in 95% ethanol using the DNeasy Tissue Kit (QIAGEN). Approximately 640 base pairs (bp) of the 5′ end of the mitochondrial (mtDNA) control region were sequenced for 55 *C. brachyotis* Sunda, 101 *C. brachyotis* Forest, 51 *C. horsfieldi* and 47 *C. sphinx*. Seventy-seven of these were used in a previous study, 177 are new to this study (see Fig. 3 legend for GenBank Accession nos). Primers, polymerase chain reaction (PCR) conditions and sequencing reaction protocols are reported in Campbell *et al*. (2004). Sequencing reaction products were run on an Applied Biosystems 3100 automated sequencer. Sequences were edited in SEQUENCE NAVIGATOR (version 1.01) and aligned by eye in SE-AL (version 2.0a8).

Six microsatellite loci, originally isolated in *C. sphinx* (CSP-1, CSP-3, CSP-4, CSP-5, CSP-6, CSP-7, CSP-9; Storz 2000), amplified reliably and were polymorphic in all four species, with the exception of CSP-4 which was monomorphic in *C. brachyotis* Sunda. PCR amplifications were carried out in a volume of 16 μL using 1.9 mM $MgCl_2$, 0.75 mM dNTP's, 0.4 mM of each primer, and 0.5 U of *Taq* Gold DNA

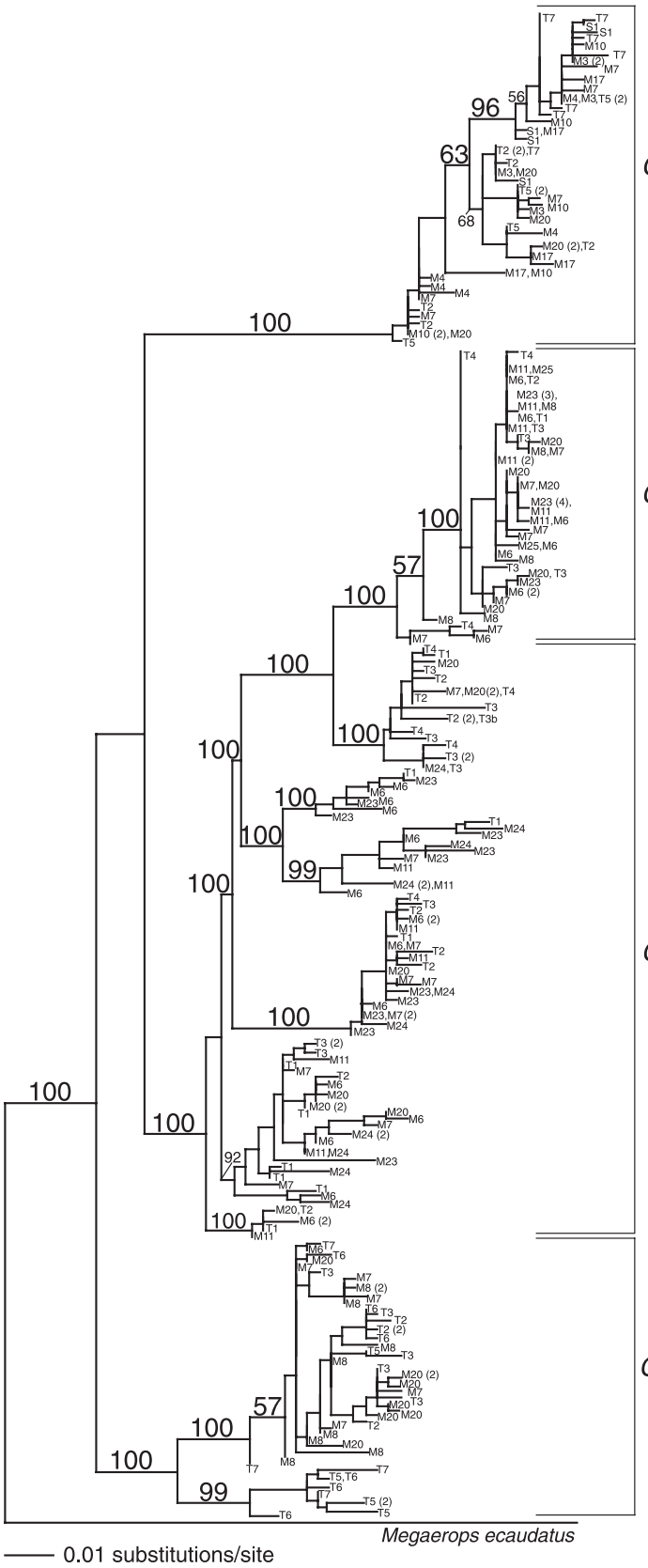

Fig. 3 Phylogram representing the tree with the best likelihood score, found using 1 000 000 generations of MCMC sampling in MRBAYES (Huelsenbeck & Ronquist 2001) under a general time-reversible model of nucleotide substitution with the following parameters estimated during the course of the run: rate matrix, C–T = 39.16, C–G = 1.02, A–T = 3.82, A–G = 36.62, A–C = 3.74; nucleotide frequencies, A = 0.25, C = 0.30, G = 0.14, T = 0.31; gamma shape parameter, $\alpha = 0.65$; proportion invariant sites = 0.71. Numbers above branches are posterior probabilities of clades represented as percent occurrence in 50% majority rule consensus tree constructed from the 12 000 trees saved post-burn-in (600 000 MCMC generations). Tree based on *c.* 567 bp of the mitochondrial control region. Tip labels correspond to sampling localities in Fig. 2; see Table 1 for full locality data. Numbers in parentheses denote number of haplotypes shared within locality. GenBank Accession nos: *C. sphinx*, AY629127, AY629131–146, AY974524–558; *C. horsfieldi*, AY629109–117, AY629119–121, AY974480–523; *C. brachyotis*, AY629009–017, AY629019–024, AY629029–040, AY629043–044, AY629046–048, AY629058, AY629064–065, AY629067, AY629074–081, AY629085–092, AY629108, AY629107, AY974360–479.

polymerase (Applied Biosystems). For *C. sphinx* ($n = 111$), *C. horsfieldi* ($n = 93$) and *C. brachyotis* Forest ($n = 133$), the following thermal profile was repeated for 35 cycles with an initial denaturation step at 95 °C for 9 min and a final extension at 72 °C for 5 min: 94 °C for 30 s, annealing at 55–57 °C for 45 s, and 72 °C for 50 s. For *C. brachyotis* Sunda ($n = 99$), we used the following touchdown PCR profile (Don *et al*. 1991): 95 °C for 9 min, 16 cycles of 94 °C for 20 s, annealing at 59–51 °C for 20 s (temperature reduced 1 °C every other cycle), and 72 °C for 1 min, followed by 19 cycles of 94 °C for 20 s, 51 °C for 20 s, 72 °C for 1 min, with a final extension at 72 °C for 5 min. Amplified products were run on an Applied Biosystems 3100 automated sequencer; allele size was quantified in GENEMAPPER (version 3.7) and edited by eye.

Data analysis

Mitochondrial DNA. Phylogenetic methods were used to confirm proposed evolutionary relationships among the four species (Campbell *et al*. 2004) and as a first approximation of intraspecific genetic structure. An approximately 77-bp segment corresponding to a single deletion event in all *Cynopterus brachyotis* Sunda control region sequences was trimmed from the data set, leaving *c.* 567 bp with multiple single-base indels included in the alignment. Phylogenetic hypotheses were evaluated in MRBAYES (version 3.0b4; Huelsenbeck & Ronquist 2001). Four MCMC chains were run for 1 000 000 generations with one tree saved every 50 generations. The first 400 000 generations were discarded as burn-in and a consensus tree was generated in PAUP* (version 4.0b10; Swofford 2002) from the 12 000 trees saved after likelihood scores reached stationarity. The tree was rooted with *Megaerops ecaudatus* (GenBank Accession no. AY629151), a putative sister taxon to *Cynopterus* (Jones *et al*. 2002).

Intraspecific haplotype diversity (h), nucleotide diversity (π) and population pairwise F_{ST} and Φ_{ST} values were calculated in ARLEQUIN (version 2.0; Schneider *et al*. 2000) for all populations with sample sizes ≥ 4. We used the Akaike information criterion in the program MODELTEST (version 3.06; Posada & Crandall 1998) to determine the best-fit model of sequence evolution for each species. Based on the results, Φ_{ST} was calculated using genetic distances estimated under the Tamura model with a specified gamma shape parameter for *Cynopterus sphinx* ($\alpha = 0.3$), *C. brachyotis* Sunda ($\alpha = 0.4$), and *C. brachyotis* Forest ($\alpha = 0.6$), and under the Kimura 2-parameter model for *Cynopterus horsfieldi*. Because of the high proportion of unique haplotypes in the control region data set, estimates of population differentiation based on pairwise distances among haplotypes (Φ_{ST}) were more informative than differentiation estimates calculated from haplotype frequency (F_{ST}) (Hedrick 1999; Sefc *et al*. 2005). For this reason, we report Φ_{ST} values only.

The genetic structure of populations at migration–drift equilibrium is expected to exhibit an effect of isolation by distance (Slatkin 1993). Correlation between geographic and genetic distance was tested using the ISOLDE program in GENEPOP (version 3.4, Raymond & Rousset 1995). Linearized Φ_{ST} was regressed on straight-line distances between populations as proposed by Rousset (1997) for F_{ST}.

The distribution of pairwise nucleotide differences, or mismatch distribution (Rogers & Harpending 1992; Rogers 1995), for each species was evaluated in ARLEQUIN. For populations experiencing long-term demographic stability, the stochastic process of lineage extinction via genetic drift produces a ragged multimodal distribution. Conversely, in a recently expanded population, the majority of lineage coalescence events are expected to postdate the expansion, producing a smooth unimodal poisson distribution, reflecting the starlike phylogeny of alleles due to the accumulation of low frequency mutations since the expansion (Slatkin & Hudson 1991; Rogers & Harpending 1992).

Tests for departures from a neutral model of evolution are used widely to infer past demographic expansions or contractions (Ramos-Onsins & Rozas 2002). If genetic polymorphism is selectively neutral, significant departures from neutral expectations may be interpreted as evidence for historic demographic nonequilibrium. Because we were interested in discriminating between demographic expansion and contraction, we chose two test statistics, each with particular sensitivity to one demographic scenario. Fu and Li's D^* is designed to detect an excess of old mutations, characteristic of a population that has experienced a historical reduction in effective population size (Fu & Li 1993; Fu 1996). In contrast, Fu's F_S is sensitive to an excess of recent mutations, a pattern typical of both a demographic expansion and a selective sweep (Fu 1997; Ramos-Onsins & Rozas 2002). D^* was calculated in DNASP (version 4.0; Rozas *et al*. 2003), F_S in ARLEQUIN.

Microsatellites. Intraspecific allelic richness and heterozygosity were calculated in FSTAT (version 2.9.3.2; Goudet 1995). Deviations from Hardy–Weinberg equilibrium, heterozygote deficits and linkage equilibrium were tested in GENEPOP. F_{ST} values were calculated in GENEPOP and isolation-by-distance tests were implemented as for the mtDNA data sets, with linearized F_{ST} regressed on geographic distance. We did not use R_{ST} (Slatkin 1995), as variation did not conform to a stepwise-mutation model at all loci (Storz 2000).

STRUCTURE (version 2; Pritchard *et al*. 2000) was used to investigate the level of interspecific differentiation, the potential for interspecific gene flow, and the degree of intraspecific population structure. STRUCTURE uses a Bayesian clustering approach to assign individuals to K populations, where the posterior probabilities of observing the data given alternative values of K can be estimated with

or without prior assumptions based on geographic sampling or species membership (Pritchard *et al*. 2000). We found that a burn-in period of 5×10^3 iterations followed by a run of 10^6 iterations gave consistent results across taxa. Subsequently, each value of *K* was evaluated over a minimum of three runs to confirm the consistency of ln-likelihood scores. In the first set of analyses, the four species were pooled and *K* values from 1 to 7 were evaluated without specifying prior population information (in this case species ID), and with species identity incorporated for $K = 4$ only.

A second set of analyses was run for each species separately with no geographic data input, assuming priors for *K* from 1 to 4. Finally, because microsatellite locus, CSP-6, exhibited strong departures from Hardy–Weinberg equilibrium in seven *C. brachyotis* Forest populations, we re-ran the intraspecific analysis for *C. brachyotis* Forest, and all interspecific analyses, with CSP-6 removed from the data set.

The program BOTTLENECK (version 1.2.02; Cornuet & Luikart 1996) was used to test for evidence of demographic expansion/contraction in each species. Analyses can be run assuming an infinite alleles model (IAM), a stepwise-mutation model (SMM) or a two-phase model (TPM), which incorporates a user-specified proportion of the SMM into a multistep mutation model. We ran analyses under both the IAM and TPM (with 50% SMM). Significant departures from heterozygosity expectations estimated under a given mutation model reject a null hypothesis of mutation–drift equilibrium. A significant excess or deficit in heterozygosity is interpreted as evidence for a demographic contraction or expansion, respectively (Cornuet & Luikart 1996). The rationale for these expectations is that following a significant reduction in effective population size, the observed number of alleles in a population will be less than that expected from the observed heterozygosity. Conversely, following a significant increase in effective population size, the observed number of alleles is expected to exceed that predicted from observed heterozygosity (Nei *et al*. 1975; Maruyama & Fuerst 1984, 1985; Cornuet & Luikart 1996).

Results

Summary statistics and diversity indices

For mitochondrial control region sequences, haplotype diversity was uniformly high across species (0.982–0.995; Table 2a). Nucleotide diversity was 1.8–3 times higher in *Cynopterus brachyotis* Forest and *Cynopterus sphinx* than in *Cynopterus horsfieldi* and *Cynopterus brachyotis* Sunda (Table 2a).

No linkage disequilibrium was detected among any of the nuclear microsatellite loci in any species. Several departures from Hardy–Weinberg equilibrium were detected, mainly at CSP-6 in *C. brachyotis* Forest (Supplementary material, Table S1). Observed heterozygosity was highest in *C. sphinx* and lowest in *C. brachyotis* Sunda (Table 2b). Alleles per polymorphic locus ranged from 5 to 36 within species, and 13–46 among species. Overall allelic richness was highest in *C. brachyotis* Forest and lowest in *C. horsfieldi*

Table 2 Sample sizes (*n*), number of sampling localities (in parentheses) and diversity indices for the four *Cynopterus* species analysed in this study. (a) Number of haplotypes, number of polymorphic sites, nucleotide (π) and haplotype diversity (*h*) for mitochondrial control region sequences. (b) Total number of alleles per species (*k*), mean allelic richness per species corrected for sample size (A_R), number of private alleles per species (A_P), frequency of private alleles within species (f_P), expected heterozygosity (H_E) and observed heterozygosity (H_O) based on six nuclear microsatellite loci

(a)

Species	*n*	No. of haplotypes	No. of polymorphic sites/bp sequenced	π	*h*
C. brachyotis Forest	101 (10)	84	116/641	23.6 ± 11.26	0.995 ± 0.0023
C. brachyotis Sunda	55 (10)	41	41/566	7.9 ± 3.80	0.982 ± 0.0003
C. horsfieldi	51 (8)	42	52/642	10.1 ± 4.85	0.992 ± 0.0052
C. sphinx	47 (8)	42	94/644	18.4 ± 8.88	0.995 ± 0.0054

(b)

Species	*n*	*k*	A_R	A_P	f_P	H_E	H_O
C. brachyotis Forest	133 (9)	102	17	26	25.5	0.684	0.609
C. brachyotis Sunda	99 (9)	65	10.5	11	16.9	0.480	0.444
C. horsfieldi	93 (8)	59	9.8	5	8.5	0.499	0.490
C. sphinx	111 (8)	84	13.9	17	20.2	0.756	0.735

(Table 2b). Within species, the observed frequency of private alleles was highest in *C. brachyotis* Forest, followed by *C. sphinx*, *C. brachyotis* Sunda, and *C. horsfieldi* (Table 2b); 18.4% of all alleles were observed in all four species.

Interspecific relationships

Bayesian phylogenetic analysis of mtDNA sequences recovered the same species-level clades identified in Campbell *et al*. (2004) (Fig. 3). As in the previous study, haplotypes from *C. brachyotis* Forest were paraphyletic with respect to *C. horsfieldi*, and *C. brachyotis* Sunda was clearly differentiated. However, inferences of sister-group relationships were not the same as in Campbell *et al*. (2004) because *C. brachyotis* lineages from the Philippines and Sulawesi were not included in the current analysis.

Bayesian clustering analysis of the interspecific microsatellite data set with no prior species assignments identified between four and six genetically distinct groups. Likelihood scores improved slightly from $K = 4$ to $K = 6$, and were substantially worse at $K = 3$ and $K = 7$ (Fig. 4). For $K = 4$, all *C. brachyotis* Sunda genotypes and the majority of *C. sphinx* (100/107), *C. horsfieldi* (92/93) and *C. brachyotis* Forest (123/133) genotypes were clearly assigned to exclusive genetic groups. Misassignments were not consistent among species pairs: misassigned *C. sphinx* and *C. brachyotis* Forest genotypes were assigned to two and three other species, respectively. Mitochondrial DNA sequence data placed all 18 misassigned individuals in the species to which they were identified using morphological characters in the field.

For $K > 4$, group assignments for *C. brachyotis* Sunda, *C. sphinx* and *C. horsfieldi* remained unchanged; *C. brachyotis* Forest genotypes were approximately equally distributed among the additional groups. Although observed structure in the *C. brachyotis* Forest lineage was consistent across runs, it did not correspond to sampling localities or to broad geographic structure. Likewise, we found no clear correspondence between *C. brachyotis* Forest genotype clusters and mtDNA haplotype subclades. Removing CSP-6 from the data set improved likelihood scores overall but did not greatly alter group assignments within *C. brachyotis* Forest. However, with species identity incorporated for $K = 4$, all individuals were assigned to the assumed species.

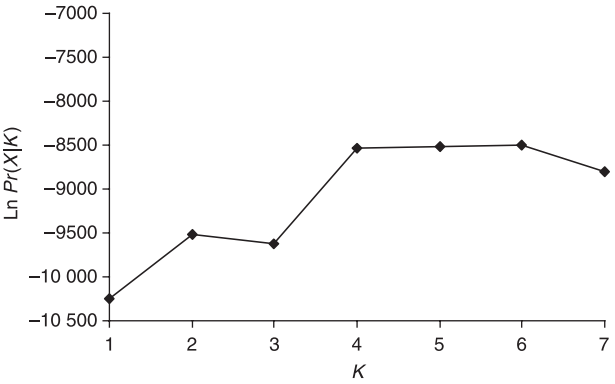

Fig. 4 Comparison of ln-likelihood scores for the interspecific microsatellite data set with all loci included and prior assumptions of 1–7 genotypic clusters (K). Probabilities of observing the data given each value of K, log $Pr(X \mid K)$, were averaged across three independent runs in the program STRUCTURE (Pritchard *et al*. 2000).

Intraspecific population structure

Cynopterus brachyotis *Forest*. Neither mitochondrial nor nuclear microsatellite data provided evidence for vicariant fragmentation or major range expansion in *Cynopterus brachyotis* Forest. A highly significant correlation between geographic and genetic distance was inferred from both types of markers ($P < 0.0001$ Fig. 5a, b), supporting current genetic equilibrium and historically uninterrupted gene flow among neighbouring populations. Pairwise F_{ST} values for microsatellites ranged from 0.001 to 0.125 with 20/36 significantly differentiated population pairs (Table 3a). Given the extremely high allelic diversity in this species, the level of population differentiation inferred from microsatellite data were notable, since the high intrapopulation heterozygosity associated with hypervariable genetic markers tends to decrease the magnitude of F_{ST} estimates (Hedrick 1999). The Φ_{ST} values for mtDNA data ranged from zero to 0.35 with 17/44 significantly differentiated population pairs (Table 3a). All of these were also significantly differentiated in the microsatellite data set.

The shape of the mismatch distribution derived from the mtDNA data were ragged and multimodal, suggesting long-term demographic stability (Fig. 6a). Likewise, nonsignificant values for both Fu's F_S, and Fu and Li's D^* supported sequence evolution consistent with the expectations of selective neutrality and stable demographic history. Despite high interpopulation differentiation, results of the test for demographic fluctuation based on microsatellite heterozygosities were nonsignificant under both the IAM and TPM (Table 4).

Both the Bayesian clustering analysis of microsatellite genotypes and the topology of the Bayesian mitochondrial haplotype tree (Fig. 3) identified differentiated groups within *C. brachyotis* Forest. These did not correspond to the geographic distribution of sampling localities, nor was group membership concordant across nuclear and mitochondrial markers. The clustering analysis of microsatellite data obtained comparably high likelihood scores for $K = 1$ to $K = 3$. However, when CSP-6 was excluded $K = 1$ provided a marginally better fit to the data.

Cynopterus horsfieldi. Contrary to our expectations of demographic stability for *Cynopterus horsfieldi*, based upon

Table 3 Pairwise population differentiation values for (a) *Cynopterus brachyotis* Forest, (b) *Cynopterus horsfieldi*, (c) *Cynopterus brachyotis* Sunda (d) *Cynopterus sphinx*. Mitochondrial Φ_{ST} values are above the diagonal, microsatellite F_{ST} values are below. Because of occasional nonamplification of samples from some sites either for mitochondrial, or for nuclear markers, 1–3 localities per species are represented in the mtDNA or the microsatellite data set only

(a)

Population	M23 (11)	M11 (7)	M24 (12)	M6 (16)	M7 (10)	M20 (9)	T1 (10)	T2 (9)	T3 (11)	T4 (5)
M23 (16)		0.022	0.031	−0.005	−0.006	0.175**	0.069	0.172***	0.298***	0.351**
M11 (10)	0.011		−0.072	−0.028	−0.026	0.074	−0.026	0.109	0.228**	0.321**
M24 (14)	0.009	0.008		0.006	0.008	0.051	−0.036	0.105*	0.185*	0.268**
M6 (15)	0.022*	0.005	0.008		0.026	0.111*	0.002	0.151**	0.273***	0.342**
M7 (16)	0.019*	0.001	0.011	0.003		0.071	0.026	0.057	0.198*	0.229*
M20 (19)	0.043***	0.014	0.012	0.018*	0.015*		−0.008	−0.007	0.054	0.111
T1 (12)	0.033**	0.005	0.021	0.024	0.015	0.027*		0.079	0.163*	0.273**
T2 (16)	0.064***	0.035**	0.034**	0.040**	0.020*	0.002	0.036*		0.012	0.002
T3 (15)	0.125***	0.087***	0.077***	0.078***	0.064***	0.027**	0.085***	0.01		−0.037
T4 (0)	nd	nd	nd	nd	nd	nd	nd	nd	nd	

(b)

Population	M23 (8)	M11 (7)	M8 (5)	M25 (0)	M6 (8)	M7 (7)	M20 (6)	T3 (4)	T4 (4)
M23 (15)		−0.015	0.099	nd	0.096	0.128	0.111	0.114	0.302*
M11 (19)	0.201***		0.087	nd	0.064	0.105	0.088	0.12	0.319*
M8 (5)	0.219***	0.017		nd	0.014	−0.037	0.016	−0.061	−0.047
M25 (7)	0.227***	0.004	0.020*		nd	nd	nd	nd	nd
M6 (15)	0.031*	0.122***	0.134**	0.155***		−0.057	−0.045	−0.121	0.024
M7 (16)	0.125***	0.070***	0.044*	0.096***	0.049**		−0.036	−0.091	−0.074
M20 (12)	0.153***	0.021*	−0.02	0.025	0.077***	0.015		−0.104	0.112
T3 (4)	0.133**	0.013	0.001	−0.016	0.058	0.031	−0.018		−0.059
T4 (0)	nd	nd	nd	nd	nd	nd	nd	nd	

(c)

Population	S1 (5)	M17 (5)	M9 (0)	M10 (6)	M7 (5)	M4 (5)	M3 (5)	M20 (5)	T2 (5)	T5 (6)	T7 (8)
S1 (15)		0.006	nd	0.057	0.069	0.173*	0.123	0.18	0.257*	0.038	0.057
M17 (0)	nd		nd	0.011	0.04	0.091	0.024	0.035	0.12	0.037	0.113
M9 (5)	0.042	nd		nd	nd	nd	nd	nd	nd	nd	nd
M10 (9)	0.018	nd	0.028		0.113	0.054	0.029	0.006	0.007	0.077	0.142*
M7 (14)	0.002	nd	0.052*	0.024		0.06	0.015	0.02	0.027	0.109	0.174
M4 (10)	0.017	nd	0.011	−0.023	0.024		0.136	0.004	0.045	0.017	0.248*
M3 (0)	nd	nd	nd	nd	nd	nd		0.132	0.201	0.061	0.064
M20 (13)	0	nd	0.028	0.033	0.006	0.024	nd		0.128	0.042	0.239***
T2 (9)	−0.012	nd	0.012	−0.021	−0.004	−0.01	nd	0.006		0.051	0.291***
T5 (9)	0.022	nd	0.092*	0.04	0.011	0.044	nd	0.016	0.012		0.123*
T7 (15)	0.025	nd	0.080*	0.083***	0.022	0.075***	nd	0.028*	0.044*	0.01	

(d)

Population	M8 (9)	M7 (5)	M20 (8)	T2 (4)	T3 (5)	T4 (0)	T5 (5)	T6 (6)	T7 (4)
M8 (16)		−0.069	0.275**	0.279**	0.142*	nd	0.516***	0.267**	0.341**
M7 (15)	0.037***		0.163*	0.211*	0.041	nd	0.455**	0.201	0.259**
M20 (14)	0.080***	0.041**		0.190*	0.008	nd	0.566***	0.343**	0.448**
T2 (0)	nd	nd	nd		−0.024	nd	0.568**	0.256	0.439*
T3 (14)	−0.004	0.048**	0.069***	nd		nd	0.489*	0.208	0.338**
T4 (15)	0.019*	0.009	0.043***	nd	0.006		nd	nd	nd
T5 (9)	0.072**	0.073**	0.031*	nd	0.042*	0.043*		0.058	−0.008
T6 (15)	0.014	0.017	0.058***	nd	0.006	−0.002	0.019		−0.057
T7 (13)	0.098***	0.057***	0.069***	nd	0.082***	0.042**	0.051**	0.046**	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; S, Singapore; M, Malaysia; T, Thailand; numbers correspond to localities in Fig. 2, see Table 1 for complete locality data, sample sizes are in parentheses; nd, no data.

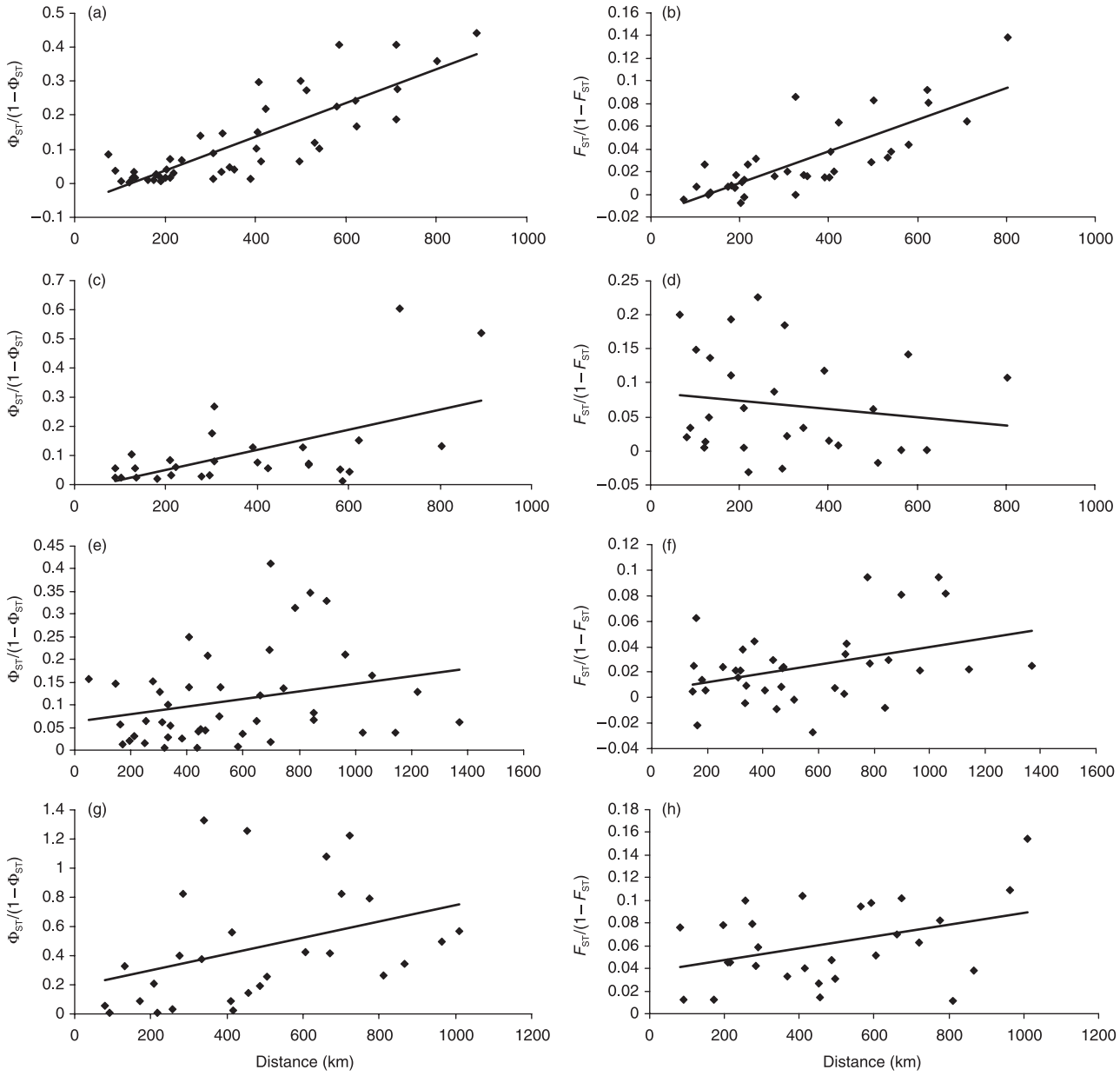

Fig. 5 Mantel tests of isolation by distance with $\Phi_{ST}/1 - \Phi_{ST}$ (mtDNA) and $F_{ST}/1 - F_{ST}$ (microsatellites) as a function of straight-line distances between populations. *Cynopterus brachyotis* Forest (a) mtDNA: $P < 0.0001$, $R^2 = 0.70$, (b) microsatellites: $P < 0.0001$, $R^2 = 0.63$. *C. horsfieldi*, (c) mtDNA: $P = 0.06$, $R^2 = 0.32$, (d) microsatellites: $P = 0.8$, $R^2 = 0.02$. *C. brachyotis* Sunda, (e) mtDNA: $P = 0.03$, $R^2 = 0.07$, (f) microsatellites: $P = 0.1$, $R^2 = 0.14$. *C. sphinx*, (g) mtDNA: $P = 0.02$, $R^2 = 0.14$, (h) microsatellites: $P = 0.1$, $R^2 = 0.15$.

its association with forest habitat on the Malay peninsula, both mitochondrial and nuclear results indicated nonequilibrium dynamics in this species. Haplotype, nucleotide and allelic diversity were generally lower in *C. horsfieldi* than in any other species (Table 2), suggesting either a historical reduction in genetic diversity due to a strong demographic bottleneck or, if divergence from *C. brachyotis* Forest is evolutionarily recent, relatively small effective population size at speciation and subsequent demographic expansion. The second scenario is supported by the fact that 80% (47/59) of all alleles observed in *C. horsfieldi* were also observed in *C. brachyotis* Forest. No evidence of population structure was detected in the Bayesian clustering analysis; likelihood scores were highest for $K = 1$. Likewise, no phylogeographic structure was recovered in the phylogenetic analysis of mtDNA (Fig. 3).

A weak effect of isolation by distance detected in mtDNA ($P = 0.06$; Fig. 5c) was largely due to two outlying comparisons

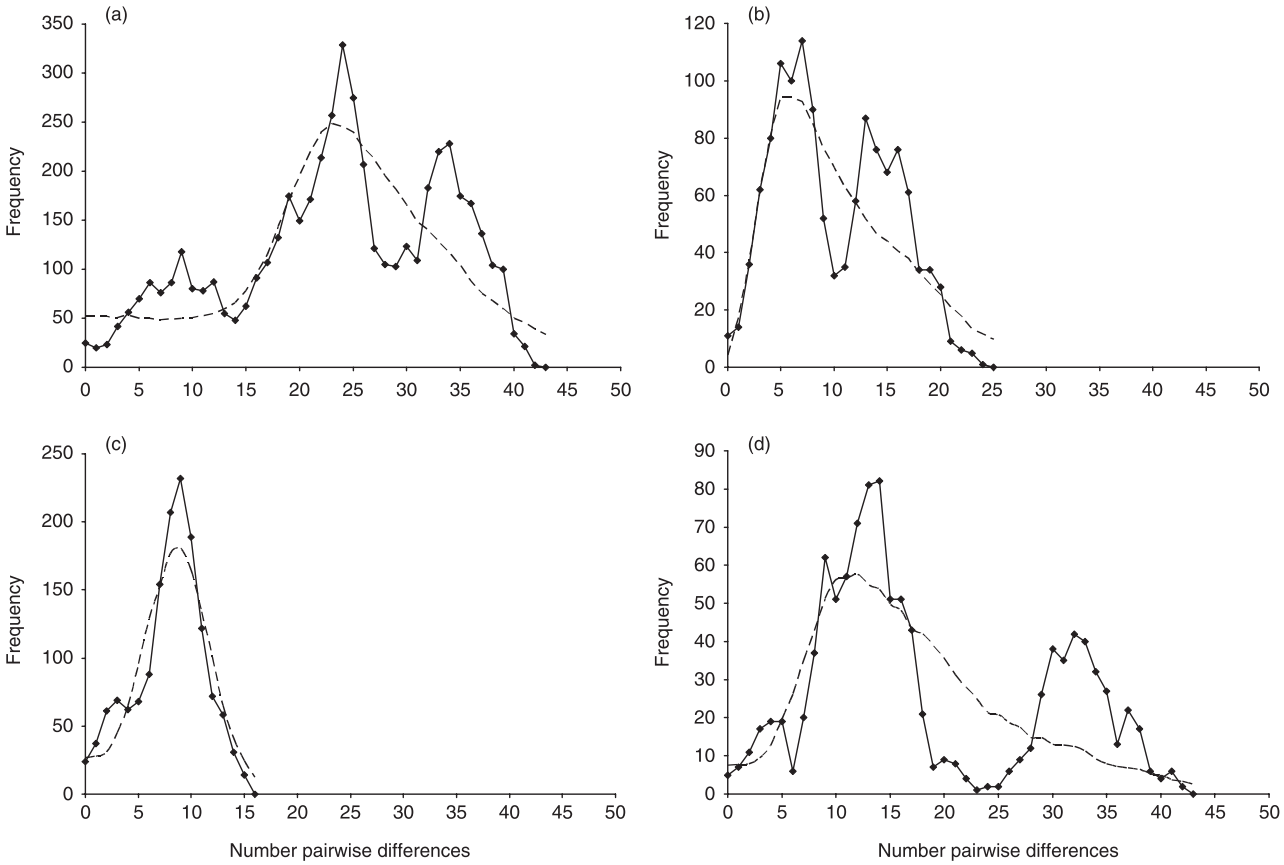

Fig. 6 Frequency distributions of pairwise nucleotide differences obtained from mitochondrial control region sequences for (a) *Cynopterus brachyotis* Forest (641 bp), (b) *C. horsfieldi* (642 bp), (c) *C. brachyotis* Sunda (566 bp), (d) *C. sphinx* (644 bp). Solid lines represent the observed data, dashed lines represent the line fitted to the data under the expectations of the sudden expansion model. Based on 10 000 simulated samples in ARLEQUIN (Schneider *et al*. 2000).

Table 4 Results of tests for heterozygosity excess/deficit under an infinite alleles model (IAM), and a two-phase model (TPM) with 50% stepwise mutation model incorporated. Significance values were obtained with a two-tailed Wilcoxon test

	Model			
	IAM		TPM	
	Heterozygosity	*P*	Heterozygosity	*P*
Species				
C. brachyotis Forest	excess	0.58	deficit	0.08
C. brachyotis Sunda	deficit	0.08	deficit	0.08
C. horsfieldi	deficit	0.06	deficit	0.04
C. sphinx	excess	0.02	unity	0.6

between the northernmost site, Surat Thani (T4), and the two southernmost sites, Endau Rompin (M23) and Krau (M11) (Fig. 2). The Φ_{ST} values ranged from zero to 0.32 with 2/28 significantly differentiated population pairs (Table 3b). No relationship between genetic and geographic distance was found for microsatellites ($P = 0.81$; Fig. 3d). Nuclear F_{ST} values ranged from zero to 0.23 with 16/28 significant population comparisons, seven of which included the Endau Rompin population (Table 3b). Notably, population differentiation estimates for *C. horsfieldi* were generally higher for nuclear than for mitochondrial markers, a trend that was reversed in the other three species.

Fu's F_S test detected highly significant departures from the neutral/equilibrium expectations ($P = 0.0004$) and Fu and Li's D^* was nonsignificant, both implicating demographic expansion. Marginally significant heterozygote deficits under both the IAM ($P = 0.06$) and TPM ($P = 0.04$) also suggested a recent expansion (Table 4). While the mismatch

distribution for *C. horsfieldi* was not unimodal, the accumulation of low-frequency mutations was characteristic of nonequilibrium population dynamics (Fig. 6b). Notably, the number of pairwise differences at the peak of the first 'wave' in the distribution was less than that in any other species, including *Cynopterus brachyotis* Sunda (Fig. 6c), suggesting more recent demographic instability in *C. horsfieldi*.

Cynopterus brachyotis *Sunda*. We found strong support for a demographic expansion in *C. brachyotis* Sunda. General concordance between nuclear and mtDNA allowed us to rule out a selective sweep of the mitochondrial genome as an alternative explanation for lack of phylogeographic structure and minimal differentiation among haplotypes. No structure was detected in microsatellite genotypes ($K = 1$). Likewise, mitochondrial haplotypes were not geographically structured on the Bayesian tree (Fig. 3).

Population differentiation indices for *C. brachyotis* Sunda were generally lower than in any other species. Mitochondrial Φ_{ST} values ranged from 0.004 to 0.29, with 7/45 significantly differentiated population pairs; nuclear F_{ST} values ranged from zero to 0.092 with 7/36 significant population comparisons (Table 3c). The four population pairs that were significantly differentiated in both data sets all included the northernmost locality (Si-Yad; T7), which was 410 km from the nearest locality (Chumphon; T5) by straight line distance, and substantially more following the present-day coast line. A marginally significant pattern of isolation by distance was detected in mitochondrial ($P = 0.03$; Fig. 5e) but not nuclear ($P = 0.1$; Fig. 5f) genetic structure, suggesting that sufficient time has elapsed since the inferred expansion for *C. brachyotis* Sunda populations on the peninsula to approach migration–drift equilibrium in the mitochondrial genome only. This result is consistent with expectations of approximately fourfold faster coalescence times in mitochondrial vs. nuclear DNA, given equivalent breeding sex ratios (Birky *et al*. 1989; Avise 2000).

The smooth unimodal shape of the mismatch distribution provided a good visual fit with the expectations of the sudden expansion model (Fig. 6c). Likewise, a highly significant value of Fu's F_S ($P = 0.003$), a nonsignificant value of Fu and Li's D^* supported demographic expansion. A heterozygote deficit under both the IAM and the TPM (both, $P = 0.08$) also suggested an expansion (Table 4). When we reran the BOTTLENECK test with the differentiated Si-Yad population excluded, the inferred heterozygote deficit was nonsignificant under the IAM ($P = 0.31$) and significant under the TPM ($P = 0.05$).

Cynopterus sphinx. Based on overlap in current habitat associations, we expected to find demographic concordance between *Cynopterus sphinx* and *C. brachyotis* Sunda. In fact, demographic inferences for *C. sphinx* were generally more comparable to those for *Cynopterus brachyotis* Forest, suggesting a long history, characterized by high gene flow and without demographic fluctuations on a scale sufficient to erase prior population history.

Like *C. brachyotis* Sunda, *C. sphinx* exhibited a weak pattern of isolation by distance for mitochondrial haplotypes ($P = 0.02$; Fig. 5g), contrasted with no relationship between geographic distance and nuclear differentiation ($P = 0.1$; Fig. 5h). However, the greatest distance between *C. sphinx* sampling localities was 360 km less than for *C. brachyotis* Sunda (Fig. 2). Likewise, differentiation among sampling localities was generally much higher in *C. sphinx*: F_{ST} values ranged from zero to 0.10 with 20/28 significantly differentiated populations, and Φ_{ST} values ranged from zero to 0.49 with 18/28 significantly differentiated population comparisons, 17 of which were concordant with the nuclear data set (Table 3d).

The shape of the mismatch distribution for *C. sphinx* was ragged and multimodal, supporting long-term demographic equilibrium (Fig. 6d). However, a highly significant value for Fu's F_S ($P = 0.001$), but not Fu and Li's D^*, suggested an expansion, while a significant heterozygote excess in microsatellite alleles under the IAM only ($P = 0.02$; Table 4), suggested a historical contraction.

No structure was identified in the Bayesian clustering analysis ($K = 1$). While no strong geographic structure was inferred from the phylogenetic analysis, the well-supported (posterior probability = 0.99; Fig. 3) basal placement in the *C. sphinx* clade of haplotypes from the northern end of the sampling transect suggested a pattern of north–south colonization of the peninsula.

Discussion

Concordance between mitochondrial and nuclear markers strongly supports the recognition of *Cynopterus sphinx*, *Cynopterus horsfieldi*, *Cynopterus brachyotis* Sunda and *Cynopterus brachyotis* Forest as four distinct species. Likewise, overall agreement between genetic data and species assignments based on morphology and habitat associations indicates that genetic differentiation corresponds to biologically meaningful differences in ecology and habitat use. We found no indication of mitochondrial introgression and evidence for nuclear introgression was minimal and not restricted to any species pair. Rare introgression of nuclear but not mtDNA among all four species seems biologically improbable. Although homoplasy and/or introgression cannot be ruled out as an explanation for the relatively high proportion of alleles that were observed in all taxa, this pattern is also consistent with incomplete lineage sorting in the nuclear genomes of closely related species.

While the deep level of mitochondrial structure in *C. brachyotis* Forest contrasted with shallow divergence in

C. horsfieldi, from a phylogenetic perspective, the *C. horsfieldi* clade is one of seven well-supported subclades within *C. brachyotis* Forest (Fig. 3). Given this observation, it is logical to ask whether other divergent mitochondrial lineages within *C. brachyotis* Forest may correspond to additional unrecognized diversity in a taxonomically cryptic genus. However, we have no evidence for consistent patterns of ecological or morphological differentiation within *C. brachyotis* Forest. Likewise, both the absence of spatial cohesion within haplotype subclades and the lack of concordance between haplotype and nuclear genotype groups make it unlikely that intraspecific genetic differentiation reflects biologically meaningful differences within populations. A more reasonable explanation is that high divergence among haplotypes within localities reflects the stochastic process of lineage extinction in a species with a long demographic history.

Given that multiple criteria support the species designations used in this study, we asked whether historical environmental change had a differential or overriding impact on the geographic and demographic structure of the four species, and whether interspecific differences in population structure correspond to differences in ecology. Contrary to our expectations based on contemporary habitat associations, we did not find concordance between the ecologically paired species. While demographic analyses suggested a unique population history for each species, there were greater interspecific similarities between than within forest and open-habitat types. Genetic structuring in *C. brachyotis* Forest and *C. sphinx* was characterized by equilibrium population dynamics and evidence for long-term demographic stability. Evolutionarily recent but noncoincident demographic fluctuations were inferred for both *C. brachyotis* Sunda and *C. horsfieldi*. These results suggest that multiple factors, including historical and contemporary environmental change and interspecific interactions may have played differential roles in shaping the recent evolutionary history of *Cynopterus* in Southeast Asia.

Impact of historical environmental change on species distributions and demography

In keeping with expectations for a species characterized by long-term association with a historically unfragmented habitat type, the genetic structure of *C. brachyotis* Forest implicates a long and relatively stable demographic history. Lack of evidence for demographic expansion suggests that this species' occurrence on the island of Borneo is a consequence of dispersal at sea-level minima, rather than widespread occupation of the exposed area. During periods of lowered sea levels, the Malay peninsula, Borneo, and Java were connected by several major river systems that are likely to have supported gallery forests, providing dispersal routes for forest taxa (Vorris 2000). Pleistocene gene flow between Borneo and peninsular Malaysia is supported by the fact that Bornean and peninsular Malaysian *C. brachyotis* Forest mitochondrial lineages have not achieved reciprocal monophyly (Campbell *et al*. 2004).

In contrast, phylogeographic analysis of three rainforest rodent species, with distributions similar to *C. brachyotis* Forest, identified well-defined haplotype clades from Sumatra, Java, Borneo, and peninsular Malaysia, suggesting that Pleistocene land connections did not facilitate gene flow among island and mainland populations (Gorog *et al*. 2004). While the high dispersal potential of bats relative to rodents seems sufficient to account for these contrasting results (e.g. Ditchfield 2000; but see Heaney *et al*. 2005), greater ecological flexibility in *C. brachyotis* Forest may provide an additional explanation. Present-day populations of this species use a range of forest types, from evergreen rainforest to seasonal monsoon forest, suggesting that this bat may have been ecologically well-suited to dispersal along corridors provided by Pleistocene riverine gallery forests.

Despite high overlap with *C. brachyotis* Forest in current habitat associations and geographic distribution, results for *C. horsfieldi* suggest a contrastingly unstable demographic history characterized by a period of low effective population size, and an evolutionarily recent demographic expansion. Determining what, if any, environmental factors promoted the expansion is largely a matter for conjecture. Given that recent shared ancestry with a forest-restricted species suggests a forest origin for *C. horsfieldi*, the genetic signature of expansion may reflect a Pleistocene shift into novel open habitat. However, the low abundance of *C. horsfieldi* in present-day open habitats suggests that ecological expansion out of the forest may be a more recent response to anthropogenic deforestation, whose genetic signature will not have had time to accumulate. It is possible then that the inferred expansion in *C. horsfieldi* was promoted by initial divergence from *C. brachyotis* Forest. A potential relationship between speciation and demographic and geographic expansion is outlined in more detail below.

The only inference of fragmentation in the peninsular distribution of *C. horsfieldi* comes from highly significant nuclear differentiation between Endau Rompin and all other populations, independent of geographic distance. While we have no alternative explanation for this result, a historical or recent disjunction in the distribution of forest is not well supported since corresponding differentiation is not observed in the mitochondrial data for this species, or in either the mitochondrial or nuclear data set for codistributed *C. brachyotis* Forest populations.

Patterns of differentiation in both mitochondrial and nuclear DNA support a previously proposed expansion in *C. brachyotis* Sunda (Campbell *et al*. 2004). The strength of

the genetic signature of expansion, combined with occurrence of undifferentiated populations across more than 18° of latitude throughout the Sunda region (Campbell *et al*. 2004) suggests that the periodic exposure of the Sunda shelf not only facilitated dispersal, but greatly increased the contiguous range of this bat. Whether the expansion was coincident with a shift from forest into open habitat or whether *C. brachyotis* Sunda originated in open habitat is impossible to determine since major demographic expansion effectively erases prior population history and a large part of the species' presumptive Pleistocene range is covered today by the South China Sea. In either case, *C. brachyotis* Sunda populations that were successful in colonizing the Sunda shelf are likely to have experienced an ecological release in response to decreased competition in a novel environment. Allozyme-based population genetic analyses of the ecologically similar Philippine *C. brachyotis* lineage found comparable evidence for high levels of gene flow both within island groups that formed single land masses during the Pleistocene, and between neighbouring Pleistocene islands (Peterson & Heaney 1993; Heaney *et al*. 2005).

Based on the current predominance of *C. sphinx* in open habitat, we expected to find evidence for a significant demographic expansion concordant with that inferred for *C. brachyotis* Sunda. Instead, the genetic structure of this species suggests a large historical effective population size and demographic perturbations whose magnitude was not sufficient to eliminate evidence of a long demographic history. One feasible explanation for this species' apparent lack of response to historical increase in open habitat is that the Pleistocene range of *C. sphinx* did not extend across the Sunda shelf. The presumed occurrence of *C. sphinx* on Borneo (Payne *et al*. 1985; Corbet & Hill 1992) is called into question by studies, which have recorded only *C. brachyotis* Forest, *C. brachyotis* Sunda and *C. horsfieldi* from that island (Francis 1990, 1994; Campbell *et al*. 2004; Hall *et al*. 2004).

Interestingly, *C. sphinx* and *C. brachyotis* Sunda exhibit similarly weak isolation-by-distance effects for mitochondrial but not nuclear DNA, suggesting that both species are approaching, but have yet to reach, demographic equilibrium. However, differences in both the inferred demographic histories and geographic distributions of the two species suggest that the similarity may be an artefact of the spatial scale of sampling. While the area sampled in this study covers a substantial portion of the mainland distribution of *C. brachyotis* Sunda, for *C. sphinx*, the Malay peninsula lies at the eastern periphery of an uninterrupted range that extends northwest through Myanmar and Bangladesh and south through the Indian subcontinent (Corbet & Hill 1992; Bates & Harrison 1997). Minimal, but significant, differentiation between *C. sphinx* haplotypes from southwestern India and the Sunda region indicates a lack of historical disjunctions in this distribution (Campbell *et al*. 2004). A latitudinal study of *C. sphinx* on the Indian subcontinent conducted at a geographic scale similar to that of the present study found evidence of high gene flow and equilibrium population dynamics (Storz 2002). It is likely then, that systematic sampling across the full range of the species would reveal a much stronger pattern of isolation by distance for both mitochondrial and nuclear DNA than that inferred in the present study.

Although palaeoclimatic data suggest two major cycles of forest contraction and open habitat expansion on the Indian subcontinent during the late Quaternary (Sukumar *et al*. 1995; Ravindranath & Sukumar 1998), a coalescent-based Bayesian analysis of a large sample of *C. sphinx* genotypes from one of the eight localities included in the Indian latitudinal study (Storz 2002), found evidence for a significant demographic contraction (Storz & Beaumont 2002). This result is surprising, given that Indian *C. sphinx* are strongly associated with open habitat (Bates & Harrison 1997; Storz *et al*. 2001). Confirmation that this local contraction reflects a larger geographic pattern of recent demographic reduction in Indian *C. sphinx* populations awaits broader sampling.

Impact of contemporary environmental change on gene flow and distribution

As global temperatures stabilized following the last glacial maximum and major land connections across the Sunda shelf were submerged, rainforest returned as the dominant habitat type in the Sunda region (Adams & Faure 1997). At the beginning of the 20th century an estimated 90% of peninsular Malaysia was covered by evergreen rainforest (Mittermeier & Mittermeier 1997). Inferences from palaeoclimatic data suggest that present-day rainforest cover on the Malay Peninsula is at a minimum since the geological origin of the region (Heaney 1991). Twentieth-century timber harvest and land conversion have reduced forest cover in peninsular Malaysia to approximately 45% of the total land area (Malaysian Timber Council; www.mtc.com.my/publication/library/F-Sheets), an estimate that includes fragmentary secondary forest and monocrop plantations.

In the course of extensive field surveys in peninsular Malaysia, *C. brachyotis* Forest was never captured > 2 km from the nearest forest edge (P. Campbell, unpublished), suggesting that current gene flow across wide areas of anthropogenic habitat is unlikely. However, we found no evidence for a contemporary reduction in gene flow among recently disjunct populations. The simplest explanation for this result is that insufficient time has elapsed for the fixation of new mutations via genetic drift. The time required for population fragmentation or reduction to manifest in genetic differentiation is greatly dependent on prior effective population size and demographic history (Berry & Gleeson 2005). For example, the mitochondrial genome

of a species, whose decline over the past 1000 years is well documented, retained the signature of a Pleistocene expansion (Lavery *et al*. 1996). In the case of *C. brachyotis* Forest, extremely high allelic diversity, indicative of large and historically stable effective population sizes across an unfragmented range, may further increase the lag time between cessation of gene flow and population differentiation.

The lack of differentiation among *C. brachyotis* Sunda mainland and Bornean and Javan populations (Campbell *et al*. 2004), and evidence for approach to equilibrium in mitochondrial lineages, indicates that the expansion inferred for this species pre-dates 20th century deforestation. However, the abundance of this bat in highly disturbed habitat (Tan *et al*. 1997) does suggest that lack of observed geographic structure over > 1000 km may be maintained by presently high levels of gene flow among mainland and peninsular populations.

It is not clear whether recent deforestation has had a similar or opposite effect on gene flow in *C. sphinx*. The apparent absence of this species from the southern half of the Malay peninsula, despite current abundance of seemingly suitable habitat, might be interpreted either as a recent extirpation or a recent range expansion from the north. Both scenarios could have been effected by anthropogenic habitat modification. However, the preference of *C. sphinx* for relatively open, anthropogenic landscapes throughout the rest of its distribution renders the possibility of a recent extirpation in the context of accelerating deforestation on the Malay peninsula highly unlikely. Likewise, the distribution of northern mitochondrial haplotypes throughout the *C. sphinx* clade, combined with the basal position of an exclusively northern subclade, suggests that colonization of the Malay peninsula by this species is not an ecologically recent event.

Speciation, competition and other ecological interactions

While the histories of genes and the species carrying them are not necessarily coincident (Pamilo & Nei 1988; Nichols 2001), both mitochondrial phylogeny and observed patterns of nuclear allele sharing strongly suggest that divergence of *C. horsfieldi* from *C. brachyotis* Forest has taken place on an evolutionarily brief timescale. A post-speciation range expansion in *C. horsfieldi* is supported geographically by the high overlap between the current ranges of the two species, and genetically by concordant nuclear and mitochondrial evidence of a short and unstable demographic history. Mayr (1942, 1963) and others (Wilson 1961; Ricklefs & Cox 1972) proposed a relationship between species age and geographic range size, whereby 'young' species undergo post-speciation dispersal and attain maximum range size early in their life cycle. While it is evident that numerous extrinsic and intrinsic factors can delay or interrupt early range expansion (Lawton 1993; Kirkpatrick & Barton 1997), comparative studies of fossil (Jablonski 1987; Vrba & DeGusta 2004) and extant taxa (Webb & Gaston 2000) have provided some support for a positive relationship between geographic range size and age of young species.

Although an early range expansion is sufficient to explain low allelic and haplotypic diversity in *C. horsfieldi* relative to *C. brachyotis* Forest, the low proportion of private alleles in *C. horsfieldi* relative to *C. brachyotis* Forest suggests that the signature of recent speciation may be retained in the nuclear genome of *C. horsfieldi*. Consideration of speciation as a genetic bottleneck has been mainly associated with various formulations of Mayr's controversial founder-effect model (Mayr 1954; Templeton 1980; Carson 1982), whereby reduced diversity in a small geographic isolate in itself promotes the evolution of reproductive isolation from the source population. Here, we do not attempt to reconstruct the geographic context, or the mechanism responsible for the evolution of reproductive barriers between *C. brachyotis* Forest and *C. horsfieldi*. We simply suggest that the demographic history of *C. horsfieldi* is sufficiently brief that the genetic consequences of speciation may be retained, whereby initially low genetic diversity due to small effective population size in a restricted geographic range is maintained by rapid geographic expansion and demographic growth.

Finally, it is probable that ecological factors other than habitat associations have impacted the demographic histories of *Cynopterus* fruit bats on the Malay peninsula. For example, the potential for competition among the four species may have played a role in shaping local and regional distributions, and by extension genetic structure. Rapid morphological divergence between *C. brachyotis* Forest and *C. horsfieldi* suggests selection to reduce niche overlap. The evolution of large body size in *C. horsfieldi* may have facilitated coexistence with *C. brachyotis* Forest and permitted expansion throughout the range of the smaller species. Likewise, the absence of *C. brachyotis* Forest from open habitat and of *C. sphinx* from the southern part of the peninsula may be due in part to their inability to compete with *C. brachyotis* Sunda, which is intermediate in size to the other two species and tolerant of high levels of human disturbance.

Conclusions

The relationship between ecology and biogeographic history in Southeast Asian *Cynopterus* is not a simple one. If a pattern can be inferred, it is one of geographically and historically uninterrupted gene flow in all four species, with major interspecific differences in the geographic scale of genetic structure and the time period for which population history can be recovered. Most notably, despite similarly high dispersal capabilities and shared geographic

distributions in a region characterized by major environmental change, genetic structure is not concordant across species. Rather, population history is species-specific and, in the case of *Cynopterus brachyotis* Sunda and Forest, readily interpretable in the light of present-day habitat associations. The results of this study highlight the value of comparative analysis of closely related codistributed species, and the importance of considering multiple aspects of species ecology when developing and testing phylogeographic and demographic hypotheses for tropical taxa.

While the phylogeographic effects of Pleistocene sea level changes have been extensively studied in Philippine bats and rodents (Peterson & Heaney 1993; Steppan *et al*. 2003; Heaney *et al*. 2005; Roberts 2005), and bats in the Nusa Tenggara region of the Indonesian archipelago (Hisheh *et al*. 1998; Schmitt *et al*. 1995; Maharadatunkamsi *et al*. 2000, 2003), surprisingly few phylogeographic studies have included taxa from the Malay Peninsula and mainland Southeast Asia (Ruedi & Fumagalli 1996; Gorog *et al*. 2004). Given a proposed Southeast Asian–Melanesian origin for pteropodid bats (Giannini & Simmons 2003) future studies of Old World fruit bats from these areas will be of great biogeographic and evolutionary interest.

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Supplementary material

The supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2769/MEC2769sm.htm

Table S1 Expected (H_E) and observed (H_O) heterozygosities, and departures from Hardy-Weinberg proportions (F_{IS}) for six microsatellite loci in four species of *Cynopterus* from Singapore, peninsular Malaysia and southern Thailand.

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Campbell's research seeks to understand the roles of ecological and historical processes in the diversification and coexistence of social mammals. This work was part of her dissertation at Boston University under the co-supervision of Kunz, whose research on bats addresses a broad range of questions in physiological ecology, evolution and conservation biology, and Schneider, who uses population genetic and phylogenetic approaches to study processes of diversification in tropical vertebrates. Adnan and Zubaid are Malaysian researchers. Adnan uses molecular genetics to study the effects of ecological and environmental processes on genetic variability in natural populations. Zubaid studies the ecology and conservation biology of small mammals in Southeast Asia, with special emphasis on the feeding and roosting ecology of bats.