

Combining molecular, morphological and ecological data to infer species boundaries in a cryptic tropical pitviper

KATE L. SANDERS*, ANITA MALHOTRA and ROGER S. THORPE

School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UW, UK

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Few operational methods exist for delimiting species boundaries, and these usually require sampling strategies that are unrealistic for widespread organisms that occur at low densities. Here we apply molecular, morphological and ecological species delimitation criteria to a wide-ranging, fragmented group of Asian green pitvipers, the *Popeia popeiorum* complex. A mitochondrial DNA phylogeny for the group indicates two well-differentiated clades, corresponding mainly to northern and southern parts of its range. Strong phylogeographical structure within each clade suggests isolation in forest refugia during the Pliocene and a southward colonization of the Sunda islands during the Pleistocene. Multivariate analysis of morphological characters reveals a generally conserved pattern of geographical variation, incongruent with the recovered phylogenetic history. We compare groups delineated by mtDNA variation to morphological and ecological divisions in the complex, and discuss the implications of these for the taxonomy of the group. Discordance between species boundaries inferred from different criteria suggests that combining independent sources of data provides the most reliable estimation of species boundaries in organisms that are difficult to sample in large numbers. © 2006 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2006, 87, 343–364.

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INTRODUCTION

A reliable estimate of species boundaries is of central importance to conservation management (Greene, 1997) and to the large body of research that concerns this taxonomic level (Goldstein & Brower, 2002). However, there is no universally applicable, operational definition of species (Harrison, 1998). The literature is instead saturated with ‘species concepts’ promoting a combination of species criteria that reflect the diversity of events associated with the speciation process and the differing research interests of authors (de Queiroz, 1998; Hey, 2000).

Approaches to species delimitation can be broadly divided into those based on knowledge of the evolutionary process (categorized as mechanistic species concepts) and those based on historical patterns of evolution (historical species concepts) (Luckow, 1995;

de Queiroz, 1998). Mechanistic species concepts emphasize species criteria that influence the future cohesion of populations (de Queiroz, 1998). Under the reproductive isolation criterion, gene exchange is reduced either at the whole genome level (Mayr, 1963; Avise & Wollenberg, 1997) or at loci of differential adaptation (Wu, 2001). The ‘nonrelational’ recognition criterion promotes common fertilization and shared mate recognition systems (Patterson, 1985). Cohesion species criteria include phenotypic and ecological exchangeability (Templeton, 1989).

Alternatively, historical approaches to species delimitation promote criteria that can be used to infer species status from patterns of variation (de Queiroz, 1998). The phenetic criterion (Sokal & Crovello, 1970) distinguishes species as separate clusters in multivariate morphometric analysis. Phylogenetic species criteria equate species with segments or branches of phylogenetic trees (Cracraft, 1983). Some phylogenetic concepts require strict monophyly (Donoghue, 1985; Mishler, 1985; Smith, 1994; Baum & Shaw,

*Corresponding author. E-mail: katelsanders@hotmail.com

1995) based on apomorphic characters (Hennig, 1966). Others emphasize diagnosability regardless of whether characters are derived (Eldredge & Cracraft, 1980) or require that diagnostic characters be fixed combinations of character states (Nixon & Wheeler, 1990; Davis & Nixon, 1992).

Although the theoretical framework underlying species criteria has been developed extensively, very few specific operational methods have been proposed for the practical delimitation of species (summarized by Mallet, 2001; Sites & Marshall, 2003). Moreover, the statistical power necessary for their application usually requires a level of population sampling (and knowledge of ecology and reproductive biology) that is unrealistic for many taxa. Organisms that occur at naturally low densities over a wide geographical area are particularly problematic. Political constraints and recent habitat fragmentation (MacKinnon, 1997) further preclude complete sampling in the tropics, although a reliable taxonomy is most important for vulnerable populations in areas of high conservation interest.

The *Popeia popeiorum* complex of South-east Asian pitvipers (*sensu* Malhotra & Thorpe, 2004a) provides an excellent example of the practical difficulties of delimiting species. The complex has a wide geographical distribution in South-east Asia, comprising both transcontinental and island populations (Fig. 1) that occur almost exclusively in undisturbed rainforests at naturally low densities. *Popeia popeiorum* is one of numerous, strikingly convergent, 'green pitviper' species formerly classified in *Trimeresurus*, whose taxonomic resolution and field identification have proved problematic even to professional herpetologists (see e.g. Orlov *et al.*, 2002, in which a specimen from Vietnam is incorrectly identified as *P. popeiorum*). However, the recent application of molecular methods has resolved the status and relationships of many of the green species and in several cases has revealed previously undetected, cryptic species (Malhotra & Thorpe, 1997, 2000, 2004b, 2004c; Giannasi, Thorpe & Malhotra, 2001). The most recent taxonomic revision of *P. popeiorum* (Regenass & Kramer, 1981) split the complex into three subspecies on the basis of ventral scale counts and the number of scale rows at mid-body, although the geographical range of these was significantly underestimated. *Trimeresurus p. popeiorum* was recognized on the mainland and its range was given as India, Myanmar, Laos, Thailand, Vietnam and Malaysia. However, on the basis of our identification of museum specimens, this species only occurs in north-east India, Myanmar, Laos, Thailand and Malaysia. Regenass and Kramer coined the name *T. p. barati* for the Barisan range population in west Sumatra, although specimens referable to *P. popeiorum* of which they were apparently unaware have also been col-

lected from north Sumatra. Finally, *T. p. sabahi* was coined for the population that is restricted to Borneo (Fig. 1).

In this study, we attempt to revise the taxonomy of the *P. popeiorum* complex by combining molecular, morphological and ecological pattern-based species criteria. Mitochondrial DNA (mtDNA) sequences were used to reconstruct phylogenies, estimate divergence times and compare between-operational taxonomic unit (OTU) frequencies of fixed nucleotide differences. Generalized morphological variation was investigated using multivariate ordination methods. Ecological data, including vegetational type and altitudinal range, was collected from field and museum records. We discuss concordant support for groups delineated by these data with respect to species boundaries in the complex. Finally, we use our data for *P. popeiorum* to assess the limitations of several pattern-based species criteria and their combined utility with respect to delimiting species boundaries in organisms that are difficult to sample in large numbers.

MATERIAL AND METHODS

SAMPLE COLLECTION

Fieldwork was carried out in Thailand, Malaysia and Indonesia. Wild-caught specimens provided blood samples and morphometric data (under anaesthesia), allowing correspondence between genetic, morphological and ecological data sets for at least one specimen in most OTUs. Blood and tissues were also obtained from museum and private collections when locality information was available and species identity could be verified. In total, 32 specimens of *P. popeiorum* were sampled from Myanmar, Thailand, Laos, peninsular Malaysia, Borneo and Sumatra (Fig. 1, Table 1). Blood samples were taken from the caudal vein with a hypodermic syringe, placed in 1 mL 0.1 M EDTA, and stored in SDS-Tris buffer (2% SDS, 100 mM Tris); liver and muscle tissue was preserved in 80% ethanol.

DNA PREPARATION, AMPLIFICATION AND SEQUENCING

Whole genomic DNA was extracted from blood and tissues using standard proteinase K protocols (Sambrook, Frisch & Maniatis, 1989). Four mitochondrial genes were amplified via polymerase chain reaction: these were cytochrome *b* (*cyt b*), NADH dehydrogenase subunit 4 (ND4), 12S small subunit ribosomal RNA (12S) and 16S large subunit ribosomal RNA (16S). *Cyt b* sequences were obtained as described in de Queiroz, Lawson & Lemos-Espinal (2002). ND4 sequences were obtained as described in Parkinson, Zamudio & Greene (2000), 12S as described in Knight & Mindell (1993), and 16S as in Parkinson, Moody & Ahlquist (1997). Unincorporated nucleotides and primers were removed from PCR products using

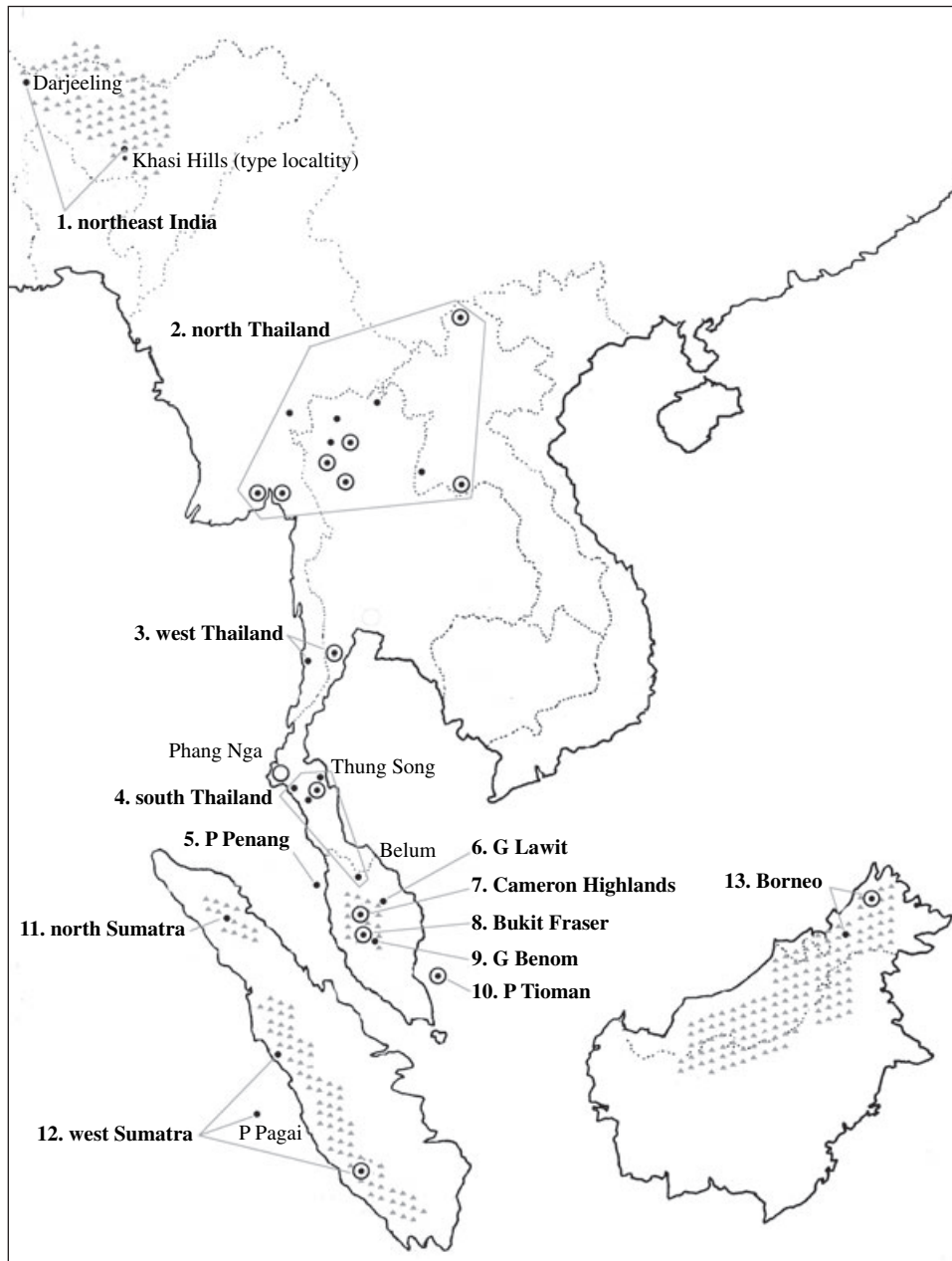


Figure 1. Distribution of samples included in morphological (bold dots) and molecular (open circles) analysis. Stippled triangles indicate montane habitats exceeding 1000 m. Numbered OTUs represent localities that were grouped in multivariate morphometric analysis.

QIAquick columns (QIAGEN). Single stranded product was then sequenced using dye-labelled terminators (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit) and run on an ABI Prism 377 DNA automated sequencer.

SEQUENCE ANALYSES

Outgroups were selected to represent four genera formerly part of *Trimeresurus* (Malhotra & Thorpe,

2004a). These were *Himalayophis tibetanus*, *T. malabaricus*, *Viridovipera vogeli* and *Cryptelytrops septentrionalis*. Alignment of *cyt b* and ND4 was trivial as there were no indels. The 12S and 16S sequences were aligned by eye following Parkinson (1999) with the exception of minor changes, which were required in one region of 12S and one of 16S due to insertions found in some of the new sequences obtained. Coding genes were translated into amino acid sequences to check for the presence of stop codons that might indi-

Table 1. OTU sample sizes for morphological (MOR) and molecular analyses (MOL)

OTU	Locality	MOR		MOL
		M	F	M & F
1	North-east India (Darjeeling)	1	0	0
	North-east India (Khasi Hills)	1	0	0
2	North Thailand	7	4	3
	Myanmar (central)	4	1	3
	Laos	0	2	2
3	West Thailand	1	2	2
	Myanmar (south)	1	0	0
	South Thailand (Phang Nga)	0	0	1
4	South Thailand (Thung Song area)	8	4	4
	Thailand/Malaysia border (Belum)	1	0	0
5	West Malaysia (Pulau Penang)	1	0	0
6	West Malaysia (Gunung Lawit)	2	2	0
7	West Malaysia (Cameron Highlands)	1	7	8
8	West Malaysia (Bukit Fraser)	3	2	3
9	West Malaysia (Gunung Benom)	0	1	0
10	Pulau Tioman	2	1	1
11	North Sumatra	2	0	0
12	West Sumatra	9	8	1
13	Borneo (East Malaysia)	4	6	4

cate that pseudogenes had been amplified. The four mitochondrial genes were combined into a single data set under the rationale that they belong to a single linkage group and an increased number of genes are likely to provide a higher number of potentially informative sites for phylogenetic analysis (Chippindale & Wiens, 1994; Cummings, Otto & Wakeley, 1995). PAUP* 4.0b10 (Swofford, 2003) was used to calculate skewness (g_1) statistics from 10^6 randomly generated trees to evaluate the adequacy of phylogenetic signal in the data (Hillis & Huelsenbeck, 1992).

PAUP* 4.0b10 was used for maximum parsimony (MP) and maximum likelihood (ML) analyses. A model of molecular evolution was first assigned to the data using the log-likelihood function of MODELTEST 3.0 (Posada & Crandall, 2001). MODELTEST compares 56 different nested substitutional models and uses log-likelihood scores to determine which model best fits the data. ML analysis, using a heuristic search with a starting tree obtained by ten random additions of taxa and tree-bisection-reconnection (TBR) branch swapping, was conducted using the parameters identified by MODELTEST. The parameters were then re-estimated from the resulting tree and used in a new ML analysis. The final tree was not bootstrapped to save computational time. An MP tree was produced using the same search options as in the ML analysis, but with a starting tree obtained by 100 random additions of taxa. All sites were

equally weighted and 1000 bootstrap replicates were performed.

MrBayes v.2.01 (Huelsenbeck & Ronquist, 2001) was used to conduct Bayesian Markov Chain Monte Carlo (MCMC) phylogenetic inference, using the best-fit model indicated by MODELTEST. Substitution model parameters were estimated as part of the analysis. Three heated chains and one cold chain were initiated with a random starting tree, run for 10^6 generations, and sampled every 100 generations. The log-likelihood scores of sample points were plotted against generation time to determine when sample points reached stationarity, and samples prior to this point were discarded as 'burn-in' samples. The topologies of all remaining samples were used to generate a majority rule consensus tree. Three additional MCMC phylogenetic reconstructions were performed to confirm convergence of resulting tree topologies, and post burn-in trees were combined in a final majority rule consensus tree, with the percentage of samples that recovered each clade representing posterior clade probabilities (Huelsenbeck & Ronquist, 2001).

SEQUENCE DIVERGENCE AND FIXED NUCLEOTIDE DIFFERENCES

MEGA version 2.1 (Kumar *et al.*, 2001) was used to make between-OTU pairwise sequence comparisons using the model of best-fit indicated by MODELTEST.

Only ND4 and *cyt b* were used to allow comparison with the published literature and application of a molecular clock calibrated in New World pitvipers (Wüster *et al.*, 2002). Equality of substitution rates has been tested between the taxa used in the calibration of this molecular clock and the *P. popeiurum* group, and confirmed not to be significantly different (Malhotra & Thorpe, 2004a).

We systematically compared fixed nucleotide differences between mtDNA lineages using the diagnostic framework of population aggregation analysis (PAA) (Davis & Nixon, 1992). Only the highly conserved rRNA genes were used (12S and 16S), as character-based delimitation requires that potentially diagnostic characters evolve relatively slowly. Under PAA, sets of populations that have fixed differences at one or more sites are considered separate species (Davis & Nixon, 1992). However, if too few individuals are sampled the number of species may be overestimated, as polymorphic traits may appear to be fixed (Walsh, 2000). This is evidently the case in our data, in which fixed differences separate all *T. popeiurum* OTUs. For this reason, we interpret our results in the context of frequencies of fixed nucleotide differences between OTUs.

MULTIVARIATE MORPHOMETRICS

We examined 74 morphological characters relating to scalation, colour pattern and body proportions (Appendix 1) from 88 museum and wild-caught specimens spanning the geographical range of the complex (Appendix 2). Male and female specimens were treated separately in all analyses to avoid bias caused by sexual dimorphism. Specimens were grouped geographically into operational taxonomic units (OTUs) on the basis of a preliminary ordination using principal components analysis (PCA). PCA was chosen in preference to canonical variate analysis (CVA) as it summarizes multivariate patterns of variation without requiring that specimens be grouped into specific taxonomic units prior to analysis, and does not require homoscedastic data (Thorpe, 1976). In all further analyses, specimens from north Thailand and proximate localities in Myanmar and Laos were grouped separately from localities in north-east India; west Thailand and an adjacent locality in Myanmar; southern Thailand and Belum (Malaysia). All remaining west Malaysian localities were treated as separate OTUs due to high levels of between-locality variation; north and west Sumatra were also treated separately and localities within Borneo were grouped. The OTUs used are illustrated in Figure 1 and sample sizes for each sex are listed in Table 1.

One-way analysis of variance and covariance (ANOVA/ANCOVA) was used to identify characters

showing significant between-OTU variation. Non-significant characters were excluded in subsequent analyses. Size-correlated characters were adjusted using a pooled within-group regression coefficient against either snout-vent length or head length. PCA analyses were performed on all OTUs for each sex to investigate multivariate patterns of variation in the group.

In order to distinguish between clinal and categorical patterns of variation between parapatric mainland OTUs, these were subjected to separate PCAs. This involved male specimens from Thailand and north-east India, and female specimens from mainland peninsular Malaysia (the sex used was decided on the basis of highest available sample size). First principal component (PC1) scores were then plotted against the latitudinal position of OTUs.

RESULTS

PRELIMINARY SEQUENCE ANALYSIS

A total of 2419 bp (*cyt b*: 809 bp, ND4: 668 bp, 12S: 426 bp, 16S: 516 bp) was used to represent 32 ingroup taxa and four outgroup taxa in the final phylogenetic analysis (GenBank accession numbers are given in Appendix 3). These contained 638 variable sites, of which 367 (15.2% of all sites) were parsimoniously informative. There was no indication that pseudogenes had been amplified as no stop codons or indels were found in coding genes. Tree length distribution was significantly skewed to the left, indicating that there was significant structure in the data ($g_1 = -0.57$, $P < 0.01$). The substitutional model of evolution assigned by MODELTEST was Tamura-Nei (TrN) distance (Tamura & Nei, 1993) with gamma correction shape parameter = 0.8448.

PHYLOGENETIC RELATIONSHIPS

Bayesian, ML and MP analyses of the combined data set resulted in identical tree topologies, with generally high support (Fig. 2). The Bayesian tree had a mean log-likelihood score of -7562.72 . The final ML tree was obtained using the TrN model with gamma distributed rates and had a log-likelihood score of $-\ln 8044.23$. MP analysis revealed a single most parsimonious tree with a length of 1186, a consistency index (CI) of 0.63, a retention index (RI) of 0.73 and a rescaled consistency index (RC) of 0.46.

Two strongly supported, well-differentiated clades correspond to overlapping lineages in the north and south. The northern clade contains three well-supported clusters. The first comprises north Thailand/Laos and Myanmar, the second the Cameron Highlands, and the third comprises specimens from west Thailand. A specimen from Phang Nga (south Thailand) is also supported as being in the northern

clade. Relationships among these clusters are less well resolved. The southern clade also contains several well-supported clusters, including Bukit Fraser, Borneo and south Thailand (Thung Song area). Relationships among these clusters, and two sequences from west Sumatra and Pulau Tioman, are less well resolved.

SEQUENCE DIVERGENCE AND FIXED NUCLEOTIDE DIFFERENCES

Levels of average sequence divergence (Tamura-Nei with gamma correction) in ND4 and *cyt b* range from $7.2\% \pm 1.6$ – $14.8\% \pm 2.4$ between ingroup and outgroup taxa; $3.0\% \pm 1.0$ and $5.7\% \pm 1.4$ (mean $4.2\% \pm 1.1$) between northern clade OTUs, and $1.7\% \pm 0.8$ and $2.7\% \pm 0.7$ (mean $2.1\% \pm 0.8$) between southern clade OTUs. Average corrected sequence difference between the northern and southern clades is $5.4\% \pm 1.4$ (Figure 5).

Frequencies of between-OTU fixed nucleotide differences were higher within the northern clade (mean 9.66 fixed differences) than within the southern clade (mean 3.33), and more fixed differences were found between these clades (mean 12.88) than within either clade (Table 2). The frequency of fixed differences between parapatric lineages is comparable to that between allopatric lineages. For example, the Cameron Highlands population has a comparable number of fixed differences to the parapatric Bukit Fraser lineage as to geographically isolated populations in Thailand (Table 2).

MORPHOMETRIC VARIATION

PCA plots (Fig. 3A, B) show a clearer pattern of geographical variation in males than in females, although OTUs do not form distinct clusters in either sex. In both sexes, the specimens from the Cameron Highlands, Sumatra and Borneo are separated from all

remaining OTUs on PC1 by the more anterior occurrence of several scale reductions, lower ventral, supralabial and sublabial scale counts, fewer scales between the edge of the mouth and the first ventral scale, and fewer scales between the supraoculars.

Female specimens from Bukit Fraser and Gunung Lawit (Malaysia), north Thailand, Myanmar, Laos, west Thailand, south Thailand and Pulau Tioman are undifferentiated in our multivariate analysis. However, females from north Thailand, Myanmar, Laos and west Thailand can be distinguished from south Thailand, Malaysia and Tioman females by a more distinct lateral stripe that covers at least 100% (vs. < 50%) of the first dorsal scale row. Females can be further separated by eye colour, which is orange in the Sumatra and Borneo populations, and yellow or green in all other OTUs.

Male specimens from Thailand, Tioman, Bukit Fraser and Gunung Lawit, and north-east India are separated on PC2 primarily by colour pattern characters (they can also be distinguished by eye colour, although this was not included in our analyses), and also by scalation (scale reduction characters and subcaudal scale counts). The presence of dorsal crossbands along the body and tail and yellow eyes distinguish males from Thailand south of Phang Nga, Belum and Pulau Tioman. Males from west and north Thailand form a separate group on the basis of red eyes and a red lateral stripe that runs above a white stripe from eye to neck, and below a white stripe from neck to vent. A photograph of the Phang Nga specimen includes it in this group due to the presence of red eyes and a red postocular stripe. Males from Bukit Fraser and Gunung Lawit have a red (below) and white (above) lateral stripe from neck to vent, but there is no postocular stripe and the eyes are green (A. Gumprecht, pers. comm.). Cameron Highlands males are distinguished from these groups by a complete lack of red pigmentation on the body, a reduced white lateral

Table 2. Between-OTU frequencies of fixed nucleotide differences for 12S and 16S sequences. The mean between-OTU frequency of fixed differences is 3.33 in the southern clade (above diagonal), and 9.66 in the northern clade (below diagonal). The mean between-clade frequency of fixed differences is 12.88

	N	N. Thailand & Laos	Myanmar	W. Thailand	Cam h'lands	S. Thailand	B. Fraser	Borneo
N. Thailand & Laos	5	0	7	11	8	11	9	10
Myanmar	2	7	0	11	14	15	15	17
W.Thailand	2	11	11	0	11	13	12	14
Cam h'lands	8	8	14	11	0	17	11	15
S. Thailand	4	11	15	13	17	0	3	5
B. Fraser	3	9	15	12	11	3	0	2
Borneo	4	10	17	14	15	5	2	0

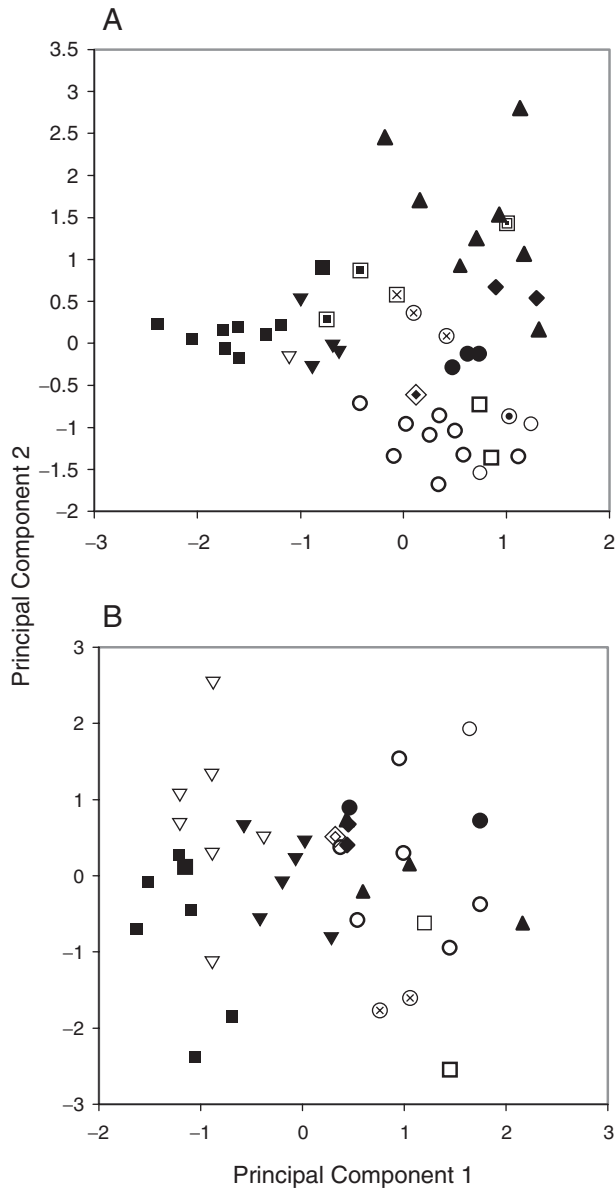


Figure 3. Principal Components Analysis of the *P. popeiorum* complex.: A, males. B, females. Northern clade OTUs are shown as open symbols, southern clade OTUs are shown as bold symbols and specimens of unknown clade affinity are shown as patterned symbols. ○, North Thailand (+ Myanmar/Laos); □, West Thailand (+ Myanmar); ▽, Cameron H'Lands; ▲, South Thailand; ◆, Pulau Tioman; ●, Bukit Fraser; ■, West Sumatra; ▼, Borneo; ▣, Belu; ⊗, Gunung Lawit; ⊠, Pulau Penang; ⊞, Gunung Benom; ▣, North Sumatra; ⊙, Khasi Hills (NE India); ⊠, Darjeeling, (NE India).

stripe, and green eyes (A. Gumprecht, pers. comm.). Males from Sumatra and Borneo have a single pale orange lateral stripe from neck to vent, and orange eyes.

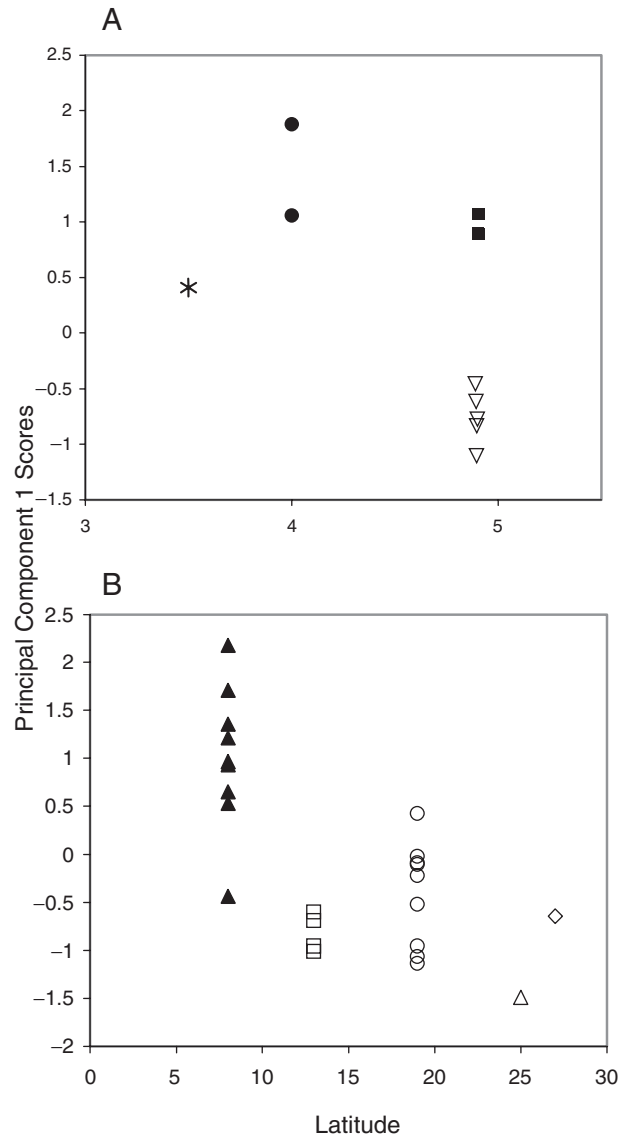


Figure 4. Plot of first principal component scores of individual specimens against latitude. A, female specimens from parapatric OTUs in Malaysia. B, male specimens from parapatric OTUs in Thailand and India. *, Gunung Benom; ●, Bukit Fraser; ▽, Cameron Highlands; ■, Gunung Lawit; ▲, South Thailand/Belum; □, West Thailand; ○, North Thailand; △, Khasi Hills (NE India); ◇, Darjeeling (NE India).

Plots of PC1 scores against latitudinal position for parapatric OTUs (Fig. 4A, B) indicate categorical patterns of variation between northern and southern clade populations. Only one south Thailand specimen overlaps with the specimens from west and north Thailand and north-east India, which are weakly differentiated on PC1 despite high latitudinal separation (Fig. 4A). The morphological discontinuity

between the Cameron Highlands and parapatric Malaysian OTUs also indicates categorical variation, with no intermediates present in our analysis (Fig. 4B).

The clade membership of specimens from localities not represented in the genetic analysis can be deduced by comparison with specimens included in both morphometric and phylogenetic analyses. This includes Malaysian OTUs Gunung Lawit, Pulau Penang and Gunung Benom. Their phenotypic similarity to Bukit Fraser specimens (Figs 3A, 4B) indicates that these are likely to belong in the southern clade. The Belum specimen clearly groups with southern clade Thailand specimens (Fig. 3A). North Sumatra specimens show phenotypic similarity to specimens from southern clade OTUs in west Sumatra and Borneo (Fig. 3A). Specimens from north-east India include the lectotype for *P. popeiorum*, and group closely with northern clade specimens from west and north Thailand (Fig. 3A).

The morphological divisions described here are not concordant with the subspecies diagnosed by Regeness & Kramer (1981). In their scheme, the mainland subspecies (*T. p. popeiorum*) is diagnosed by ventral scale counts higher than 155. However, in our study, female specimens from the Cameron Highlands have an average of 151 ventral scales. The west Sumatran subspecies (*T. p. barati*) is diagnosed by fewer dorsal scale rows at mid-body (19 vs. 21). However, the presence of 21 scale rows at mid-body in a specimen from west Sumatra indicates that this is not a valid character for diagnosing the population.

ECOLOGICAL DIVERGENCE

Between-OTU differences in habitat utilization reflect the latitudinal range of the complex. Semi-evergreen rainforest in north-east India, Myanmar, Laos and Thailand is characterized by lower rainfall (2000–3000 mm/year vs. 3000–4000 mm/year) and more pronounced seasons than the equatorial wet-evergreen rainforest in Malaysia, Sumatra and Borneo (Whitmore, 1975; Anon, 1995). *Popeia popeiorum* populations also vary in altitudinal range. Thailand (both northern and southern lineages), Myanmar and Laos populations occur at moderate altitudes, mostly between 300 m and 1000 m. The collection of a specimen from Darjeeling (1500 m) indicates that *P. popeiorum* may occupy higher altitudes in north-east India. The Cameron Highlands (Malaysia) population occurs between 1500 m and 2000 m. In Bukit Fraser, Penang and Gunung Lawit (Malaysia), Sumatra and Borneo, *T. popeiorum* occurs between 800 m and 2000 m, but is most commonly found over 1000 m. Specimens from Tioman Island have been collected between 400 m and 1050 m.

DISCUSSION

EVOLUTIONARY HISTORY AND BIOGEOGRAPHY

Popeia popeiorum mtDNA haplotypes exhibit a strong geographical structure, comprising two well-supported northern and southern clades. The northern clade includes parapatric OTUs in the north that occur at moderate altitudes, and a high altitude allopatric population within the range of the southern clade. The southern clade comprises more closely related, mostly high altitude, allopatric OTUs, including the Sunda island populations. Morphological variation is generally more conserved in the northern clade than in the southern clade island populations, which is unsurprising given that both adaptive and nonadaptive differences are expected to accumulate most quickly in isolated founder populations (Berry, 1998; Whittaker, 1998). Ecological convergence is indicated in high altitude populations, whose phenotypic similarity is based on lowered scale counts for characters that were found to be negatively correlated with cool, wet habitats in the related *Trimeresurus sumatranus* Indomalayan species group (Sanders, Malhotra & Thorpe, 2004a). Parapatric northern and southern clade populations in Thailand and Malaysia display categorical patterns of variation despite shared habitat preferences. Figure 5 illustrates the geographical distribution of the main phylogenetic and morphological divisions in the complex.

Divergence times can be estimated given a calibrated estimate of the rate of sequence evolution in a comparable taxon. Wüster *et al.* (2002) proposed a rate of sequence evolution for cyt *b* and ND4 of between 1.09 and 1.77% Myr⁻¹ in New World pitvipers. Applying these rates would date the split between the northern and southern *P. popeiorum* clades (using the mean sequence difference of 5.4% ± 1.4) at between 2.29 and 6.25 Mya, i.e. during the late Miocene and Pliocene. Most divergence events within the northern clade (mean sequence difference of 4.2% ± 1.1) are also likely to have taken place in the Pliocene, 1.75–4.94 Mya. Divergence events in the southern clade (mean sequence difference of 2.1% ± 0.8) probably occurred more recently, between 0.75 and 2.63 Mya. This indicates a southward colonization of the Indomalayan archipelago during the end of the Pliocene and the Pleistocene.

Climatic fluctuations occurred throughout the Miocene, Pliocene and Pleistocene epochs (Morley, 1998), and have been linked to the diversification of numerous species complexes (e.g. Wüster & Thorpe, 1990; Schneider, Cunningham & Moritz, 1998). Glacial periods were accompanied by lowered sea levels and increasing aridity and seasonality. In South-east Asia, this resulted in the fragmentation of forest hab-

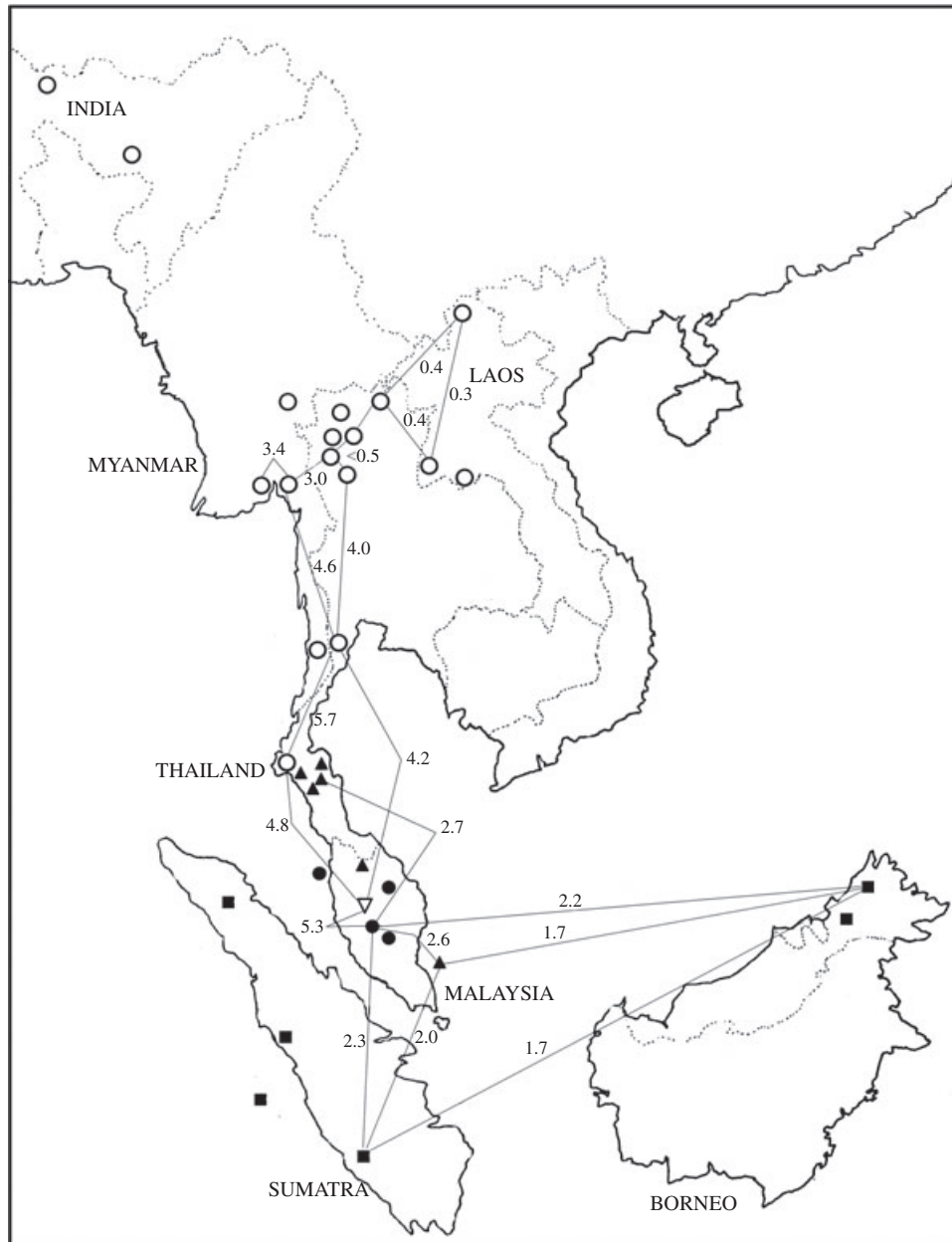


Figure 5. Geographic distribution of phylogenetic and morphological divisions in the *P. popeiorum* complex. Corrected pairwise sequence divergence (ND4 and *cyt b*) is shown between OTUs, represented by symbols indicating the main groupings in morphological analyses. Northern clade OTUs are shown as open symbols, southern clade OTUs are shown as bold symbols.

itats on the mainland and the formation of land bridges in the Sunda shelf (Hall & Holloway, 1998). The geographical structuring of *P. popeiorum* haplotypes is suggestive of isolation in mainland forest refugia and subsequent habitat expansion leading to secondary contact. Dispersal to the Sunda shelf is likely to have occurred via land bridges; rising sea levels would have subsequently led to the isolation of

island populations, which may have followed their retreating habitats to higher elevations.

DELIMITING SPECIES BOUNDARIES

Lineage-based species concepts recognize species on the basis of reciprocal monophyly of gene genealogies (Cracraft, 1983, 1987; Donoghue, 1985; Mishler, 1985;

Smith, 1994; Baum & Donoghue, 1995; Baum & Shaw, 1995). However, there are a number of problems associated with tree-based species diagnosis. Gene trees may not always be congruent with species trees due to lineage sorting of ancestral polymorphisms (Moore, 1995). Furthermore, the matrilineal nonrecombining mode of mtDNA inheritance results in a phylogenetic pattern of descent even among interbreeding organisms (Davis, 1996); thus, even a gene tree that accurately represents phylogenetic history is of limited value in predicting hybridization between closely related taxa (Mallet, 2001; Schluter, 2001). Paraphyly is particularly common in groups containing peripheral isolates, such as divergent island populations, and is a result of the fundamental process of speciation in these cases (Harrison, 1998; Funk & Omland, 2003).

Monophyly exists at all levels in a phylogeny, and the actual level at which species should be recognized is unclear. We could delimit two monophyletic species in the *P. popeiorum* complex, corresponding to the strongly supported northern and southern clades. Alternatively, we could delimit four species, corresponding to the two monophyletic groups in each primary clade. However, the monophyly criterion is unlikely to lead to reliable species delimitation in the *P. popeiorum* complex. Groupings based on monophyly do not correspond either to the geographical distribution of OTUs or to morphological and ecological divisions in the complex.

For example, the Thailand OTU of the southern clade is closer to northern clade lineages, in terms of geographical and altitudinal range, than to southern clade lineages with which it shares a more recent common ancestor. Furthermore, Thailand, Myanmar, Laos and Cameron Highlands populations of the northern clade are monophyletic with respect to the west Thailand population. However, the west Thailand population is phenotypically and ecologically undifferentiated from parapatric north Thailand, Myanmar and Laos populations. Therefore, these OTUs are more likely to represent an independent group with respect to the phenotypically divergent, high altitude allopatric Cameron Highlands population.

SEQUENCE DIVERGENCE

Mitochondrial pairwise sequence differences, ranging between 1.6% and 6.2%, are often used to delimit snake species (Kraus, Mink & Brown, 1996; Zamudio & Greene, 1997; Rodriguez-Robles & de Jesus-Escobar, 2000; Ashton & de Queiroz, 2001; Keogh, Barker & Shine, 2001; Rawlings & Donnellan, 2003). Comparable levels of divergence are found between *P. popeiorum* lineages (1.7 ± 0.8 – $5.7\% \pm 1.6$). However, relatively ancient haplotypes can coexist in a single

interbreeding population (Thomaz, Guiller & Clarke, 1996; Ogden & Thorpe, 2002) and can mask high levels of gene flow mediated by dispersing males (Palumbi & Baker, 1994; Thorpe, Black & Malhotra, 1996; Stenson, Malhotra & Thorpe, 2002). Consequently, mtDNA haplotype distribution alone is an insufficient criterion for recognizing species, and should be combined with concordant support from at least one independent source of data. Parapatric northern clade OTUs in Thailand and Myanmar are strongly differentiated by mtDNA haplotype (average divergence = $4.14\% \pm 1.0$), but are phenotypically indistinguishable in both sexes. Gene exchange between these populations is especially likely given that male pitvipers are known to disperse more widely than females (Shine, 1993).

FIXED NUCLEOTIDE DIFFERENCES

This diagnostic criterion predicts that differences in inherited character states will most often reach fixation in the context of reproductive isolation, and uses these as evidence for barriers to gene exchange in sympatric and parapatric populations (Davis & Nixon, 1992). In the present study, higher frequencies of fixed differences are found between northern clade lineages than between the more recently diverged southern clade lineages. There are no fewer fixed nucleotide differences between parapatric lineages than between allopatric lineages. This could be viewed as evidence for reduced gene flow between parapatric populations. However, the extent of introgression can vary across the genome (Harrison, 1986) and gene flow is possible despite fixed differences at some loci (Harrison, 1998). Furthermore, spurious determination of fixed nucleotide differences may result from insufficient sampling within OTUs (Walsh, 2000), or the extinction of haplotypes (Templeton, 1989). Unfortunately, the sample sizes currently available do not allow us to distinguish between alternative hypotheses of character fixation.

PHENETIC SPECIES CRITERIA

The most widely applied morphological method of delimiting species is to base species status on the presence of fixed or nonoverlapping character differences between geographical samples (Wiens & Servedio, 2000). Alternatively, multivariate analysis of generalized phenotypes can be used to identify groupings (phenetic clusters), which are considered species in the absence of intermediates (Sokal & Crovello, 1970; Mallet, 1995).

The lack of discrete clusters in our multivariate analyses of *P. popeiorum* morphology is incompatible with recognizing multiple species according to this cri-

terion. However, extreme morphological conservatism is typical even between distantly related green species of the *Trimeresurus* radiation (Malhotra & Thorpe, 2004a). *Popeia popeiorum* OTUs can be grouped tentatively on the basis of scalation and colour pattern characters. These groupings correspond to: (1) north-east India and northern clade Thailand, Myanmar and Laos; (2) southern clade Thailand, Belum and Pulau Tioman; (3) Bukit Fraser, Gunung Lawit and Pulau Penang (Malaysia); (4) Borneo, west Sumatra and north Sumatra; (5) the Cameron Highlands population, although this OTU partially overlaps with Sumatran and Bornean specimens belonging to the southern clade in both sexes.

Potentially informative morphological differences also exist between parapatric lineages in Malaysia and Thailand. There are no known colour pattern intermediates between northern and southern clade males in southern Thailand, and only one intermediate is present in our analysis of generalized phenotype. Morphological discontinuity between the Cameron Highlands and parapatric Malaysian lineages also indicates a categorical pattern of variation. This concordance between morphological and molecular variation in parapatric populations suggests that in Malaysia and Thailand northern clade OTUs represent independent populations with respect to southern clade OTUs.

ECOLOGICAL SPECIES CRITERIA

Although relatively few species concepts promote ecological criteria (Van Valen, 1976; Templeton, 1989), ecology is an important component of current speciation research. Some authors have argued that species delimitation should be treated independently from investigations of the speciation process due to a risk of circularity and compromised generalizability (Rieppel, 1986; Luckow, 1995; Goldstein & DeSalle, 2000). However, given that both sympatric and allopatric populations are more likely to speciate in the context of adaptive divergence (Endler, 1992; Marchetti, 1993; Schluter, 2001), ecological compatibility may provide a useful indication of whether two closely related populations will hybridize (Schluter, 2001; Templeton, 2001).

Overlapping altitudinal range can be used as a basis for ecological compatibility in the *P. popeiorum* group. Southern clade *P. popeiorum* OTUs share moderate and high altitudes, and overlap in altitudinal range with moderate altitude northern clade OTUs in Thailand, Myanmar and Laos. However, the exclusively high altitude Cameron Highlands population is ecologically incompatible with the remaining northern clade populations in Thailand, Myanmar and Laos, from which it is also strongly differentiated by pheno-

type. Shared habitat may facilitate introgression between the Cameron Highlands and parapatric Malaysian lineages. However, the morphological discontinuity between these OTUs indicates that this is improbable, and introgression is more likely between northern clade OTUs in Thailand, given their lack of phenotypic differentiation.

TAXONOMIC RECOMMENDATIONS FOR THE *P. POPEIORUM* COMPLEX

The current subspecific taxonomy of the *P. popeiorum* complex is clearly inconsistent with the molecular, morphological and ecological divisions revealed in this study. We propose a taxonomic reorganization of the complex into three species that conservatively represent the evolutionary units delineated by our data. Northern clade OTUs in north-east India, Myanmar, Laos and Thailand are morphologically undifferentiated in females, and group closely in our analysis of males. Furthermore, there is no known biogeographical barrier to introgression of these, mostly parapatric, lineages. Therefore, despite relatively high levels of mtDNA haplotype divergence, we recommend that these populations be considered as a single species. Since it includes the lectotype of *P. popeiorum*, this species corresponds to *P. popeiorum* *s.s.* The Cameron Highlands population represents a morphologically and ecologically divergent allopatric lineage with respect to the remaining northern clade OTUs. In addition, we find no evidence of intergradation with ecologically compatible, parapatric southern clade OTUs, either in terms of mtDNA haplotype distribution or the presence of morphological intermediates. On this basis we propose full species status for the Cameron Highlands population (Sanders *et al.*, 2004b). The southern clade OTUs (south Thailand, Malaysia, Sumatra, Borneo and Pulau Tioman) represent recently diverged populations with compatible habitat preferences. Morphological differentiation is observed within this group, but cannot be interpreted in the context of intrinsic barriers to gene flow between these populations due to their allopatric distribution. Furthermore, intergradation with parapatric, ecologically compatible northern clade populations is unlikely in light of the categorical patterns of variation between these OTUs. Therefore, we suggest a conservative arrangement of the southern clade populations as a single, polytypic, equatorial species. This newly defined species includes two subspecies (*T. p. barati* and *T. p. sabahi*) of equal priority since they were published in the same paper. We propose to use the name *P. sabahi* (comb. nov.). The geographical distributions of the species of *Popeia* delimited by this study are illustrated in Figure 6.



Figure 6. Geographic distribution of *Popeia* species delimited by this study. Circles represent *P. popeiorum* s.s.; squares represent *P. sabahi*; a triangle represents *P. nebularis*.

COMPARISON WITH ALTERNATIVE SYSTEMATIC ARRANGEMENTS

An alternative interpretation of the systematic relationships within the *Popeia popeiorum* complex was published after this paper was accepted (Vogel *et al.*, 2004), in which the subspecies *T. p. sabahi* and *T. p. barati* are raised to species status, and two new species, *T. nebularis* (Cameron Highlands) and *T. fucatus* (southern Thailand and West Malaysia excluding the

Cameron Highlands) are described. *T. fucatus* is referred to *P. sabahi* in our arrangement along with populations from Sumatra and Borneo, while *P. nebularis* (comb. nov.) takes precedence over *P. inornata* (Sanders *et al.*, 2004b).

Although the species definitions in Vogel *et al.* (2004) are also based on the identification of morphologically diagnosable clusters in multivariate analyses, an examination of their figures 1 and 2, which show plots of the first two principle components of an

analysis based on 18 scalation characters for males and females respectively, clearly shows that the actual distribution of points representing individual specimens bears little relationship to the definition of the clusters which have been superimposed on them (e.g. there is almost complete overlap between Clusters I and II for both sexes, and Clusters III to V in males). Hence, we believe that the authors' statement that 'the results of the PCA . . . show in both cases the occurrence of five clusters of plots' (Vogel *et al.*, 2004) is unfounded. All subsequent analyses (including definition of species boundaries) are based on these clusters, and hence have limited utility [see Thorpe (1983) for further discussion of the ability of canonical variate analysis, which is identical to Discriminant Canonical Analysis as used by Vogel *et al.* (2004), to find differences between predefined categories even if the predefined categories do not match the natural categories]. In fact, the main result discernible from Vogel *et al.*'s morphological analysis is very similar to ours, i.e. that the Sumatran, Bornean and Cameron Highlands populations are morphologically distinguishable from the remaining populations. For these reasons, we do not believe that the delimitation of species in Vogel *et al.* (2004) reflects biologically meaningful units.

IMPLICATIONS FOR THE SPECIES DEBATE

Our study illustrates the discordance between species boundaries inferred from different pattern-based criteria. This underscores the dichotomy between evolutionary groups and the categories that we use to define them (Hey, 2000) and indicates that taxonomic revisions based on a single species criterion, despite advantages of comparability, are unlikely to lead to a realistic delimitation of species. Therefore, following Mishler & Donoghue (1982), Baum & Shaw (1995), Mallet (2001), Puerto *et al.* (2001) and Wiens & Penkrot (2002), we recommend an approach to species delimitation that combines as many independent sources of data as are available. We further emphasize the importance of critical analysis of species criteria, particularly with respect to delimiting species on the basis of data sets compromised by incomplete sampling.

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APPENDIX 1

Characters used in morphological analyses. *indicates significant between-OTU variation ($P < 0.05$, ANOVA)

(A) Scalation

Character	Males	Females
No. of ventral scales – first ventral identified according to the method of Dowling (1951)	*	*
No. of pairs of subcaudal scales	*	–
No. of unpaired subcaudal scales	–	–
Division of anal scale (recorded as 0 = undivided; 1 = divided)	–	–
Keeling of body scales at mid-body (recorded as 0 = none; 0.5 = weak; 1 = strong)	*	–
No. of scale rows anterior to the vent	–	–
No. of supralabial scales (average on left and right hand sides)	*	*
No. of sublabial scales (average on left and right hand sides)	*	*
No. of postocular scales	*	–
No. of preocular scales	–	–
No. of scales bordering the supraocular scales, excluding the preoculars and postoculars	*	*
Minimum no. of scales separating the supraocular scales	–	*
Maximum no. of scales separating the supraocular scales	*	*
No. of sutures dividing supraoculars	–	–
No. of scales between nasal scale and shield bordering the pit anteriorly	–	*
No. of internasal scales	*	*
Minimum no. of scales separating third supralabial and subocular scale	*	*
Minimum no. of scales separating fourth supralabial and subocular scale	*	–
Minimum no. of scales separating fifth supralabial and subocular scale	–	*
Minimum no. of scales bordering suboculars, excluding the preoculars and postoculars	*	*
Keeling of temporal scales	*	–
Keeling of scales on the back of the head	*	–
No. of scales between first ventral and anterior chin shields	*	*
No. of scales between the edge of the mouth and first ventral scale, including last sublabial	*	*

(B) Scale reduction formula. Scale reductions along the body were recorded as the number of the ventral scale (VS) or subcaudal scale (SC) opposite which they were situated and the dorso-ventral position (DV) of the merging scale rows. These were transformed to percentage ventral scale (%VS) or subcaudal scale (%SC) position prior to analysis, to compensate for variation in ventral and subcaudal scale number.

Character	Males	Females
%VS position of reduction from 31 to 29 body scale rows	–	–
%DV position of reduction from 31 to 29 body scale rows	–	–

APPENDIX 1: *Continued*

Character	Males	Females
%VS position of reduction from 29 to 27 body scale rows	—	—
%DV position of reduction from 29 to 27 body scale rows	—	—
%VS position of reduction from 27 to 25 body scale rows	—	—
%DV position of reduction from 27 to 25 body scale rows	—	—
%VS position of reduction from 25 to 23 body scale rows	—	—
%DV position of reduction from 25 to 23 body scale rows	—	—
%VS position of reduction from 23 to 21 body scale rows	*	—
%DV position of reduction from 23 to 21 body scale rows	*	—
%VS position of reduction from 21 to 19 body scale rows	*	*
%DV position of reduction from 21 to 19 body scale rows	—	—
%VS position of reduction from 19 to 17 body scale rows	*	*
%DV position of reduction from 19 to 17 body scale rows	*	—
%VS position of reduction from 17 to 15 body scale rows	*	*
%DV position of reduction from 17 to 15 body scale rows	—	—
%SC position of reduction from 12 to 10 tail scale rows	—	*
%DV position of reduction from 12 to 10 tail scale rows	—	—
%SC position of reduction from 10 to 8 tail scale rows	—	*
%DV position of reduction from 10 to 8 tail scale rows	—	—
%SC position of reduction from 8 to 6 tail scale rows	*	*
%DV position of reduction from 8 to 6 tail scale rows	—	—
%SC position of reduction from 6 to 4 tail scale rows	—	—
%DV position of reduction from 6 to 4 tail scale rows	—	—

(C) Colour pattern

Character	Males	Females
Presence of stripe on dorsal scale row one (recorded as 0 = absent; 1 = indistinct; 2 = distinct)	*	*
No. of scale rows involved in stripe	*	*
Presence of postocular stripe (recorded as 0 = absent; 1 = indistinct; 2 = distinct)	*	*
No. of scale rows involved in postocular stripe	*	*
No. of scales above lip covered by ventral colour	*	*
Presence of dark edges body scales (recorded as 0 = none; 1 = narrow; 2 = broad)	—	—
No. of spots on the dorsal surface	*	*
Mean no. of scales covered by the three largest dorsal spots	*	*
Proportion of the first scale row covered by the light area	*	*
No. of bands on body	*	—
Mean width (in no. of scales) of three half bands at 50% VS length	*	—
Mean width (in no. of scales) of three intrahalfband gaps at 50% VS length	*	—
% of ventral scales with darker pigmentation	—	—

(D) Body dimensions. These were measured on the right side of the head unless damaged, in which case measurements made on the left side.

Character	Males	Females
Snout to vent length, between tip of snout and cloaca	—	—
Tail length, measured between first subcaudal and tip of tail	—	—
Width of head between the outer edges of the supraoculars	*	—
Width of head at the widest point between the jaw bones	—	—

APPENDIX 1: *Continued*

Character	Males	Females
Length of head between tip of snout and posterior edge of the lower jaw bone	–	–
Diameter of eye measured between outer edges of surrounding scales	–	*
Distance between eye and nostril, from anterior edge of preoculars to inner edge of nostril	–	–
Distance between eye and pit, from anterior edge of preoculars to inner edge of pit	–	*
Distance between eye and nostril, between outer edges	–	–
Width of internasals at widest part	*	–
Width of supraoculars at widest part	–	*
Length of supraoculars	–	–
Ratio of anterior margin of the rostral scale to the posterior margin	–	–

APPENDIX 2: SPECIMENS USED IN MORPHOMETRIC ANALYSIS

Museum/field ref:	Locality:	Sex
AFS96.3	Lampang, N. Thailand	M
AFS96.17	Chiang Rai, N. Thailand	F
AFS97B.13	Thung Song, S. Thailand	M
AFS98.1	Thung Song, S. Thailand	M
AFS98.16	Phetburi, W. Thailand	F
AFS98.34	Phetburi, W. Thailand	M
AFS00.12	Cameron Highlands, W. Malaysia	F
AFS00.13	Cameron Highlands, W. Malaysia	M
AFS00.14	Cameron Highlands, W. Malaysia	F
AFS00.15	Cameron Highlands, W. Malaysia	F
BMNH 62.7.28.1	Lao Mts, N. Thailand	F
BMNH 62.7.28.4	Lao Mts, N. Thailand	M
BMNH 72.4.17.137	Khasi Hill, N.E. India	M
BMNH 72.4.17.377	Darjeeling, N.E. India	M
BMNH 1937.2.1.24	Chiang Mai, N. Thailand	F
BMNH 1937.2.1.25	Chiang Mai, N. Thailand	M
BMNH 1940.39.43	Mergui, Myanmar	M
BMNH 1967.2289	Gunung Benom, W. Malaysia	F
BMNH 1974.4995	Gunung Lawit, W. Malaysia	M
BMNH 1974.4997	Gunung Lawit, W. Malaysia	F
BMNH 1974.4999	Gunung Lawit, W. Malaysia	F
BMNH 1974.5000	Gunung Lawit, W. Malaysia	M
BMNH (MLD 2007)	Pulau Tioman, W. Malaysia	F
CAS 205847	Bago Yoma, Myanmar	F
CAS 216609	Mon State, Myanmar	M
CAS 222195	Mon State, Myanmar	M
CAS-SU 8863	Cameron Highlands, W. Malaysia	F
DWNP (no number)	Bukit Fraser, W. Malaysia	M
FMNH 178655	Chiang Mai, N. Thailand	F
FMNH 233155	Sipitang, E. Malaysia	F
FMNH 243942	Sipitang, E. Malaysia	F
FMNH 178656	Chiang Mai, N. Thailand	M
NMBA 21026	Kinabalu, E. Malaysia	M
FMNH 178658	Chiang Mai, N. Thailand	M
FMNH 178659	Chiang Mai, N. Thailand	M

APPENDIX 2: *Continued*

Museum/field ref:	Locality:	Sex
FMNH 258950	Phongsaly, Laos	F
FMNH 258949	Vientiane, Laos	F
KLS00.001	Bukit Fraser, W. Malaysia	F
KLS01.103	Pulau Penang, W. Malaysia	M
KLS01.104	Kinabalu, E. Malaysia	F
KLS01.114	Kinabalu, E. Malaysia	M
KLS01.116	Kinabalu, E. Malaysia	F
KLS01.117	Kinabalu, E. Malaysia	M
KLS01.122	Kinabalu, E. Malaysia	F
KLS01.121	Kinabalu, E. Malaysia	F
LSUHC 4809	Pulau Tioman, W. Malaysia	M
MCZ 43612	Kinabalu, E. Malaysia	M
MCZ 43614	Kinabalu, E. Malaysia	F
NMBE 210b/197	Batak Mts, N. Sumatra	M
NMBE 210a/198	Batak Mts, N. Sumatra	M
NMW 23910 : 1	Pulau Pagai, Mentawai Islands	F
NMW 23910 : 2	Padang, W. Sumatra	M
NMW 23910 : 3	Padang, W. Sumatra	F
NMW 23910 : 4	Padang, W. Sumatra	M
NMW 23910 : 5	Padang, W. Sumatra	F
NMW 23917 : 1	Padang, W. Sumatra	M
NMW 23917 : 2	Padang, W. Sumatra	M
NMW 23917 : 3	Padang, W. Sumatra	F
NMW 23917 : 4	Padang, W. Sumatra	M
NMW 23917 : 5	Padang, W. Sumatra	M
NMW 23917 : 6	Padang, W. Sumatra	M
NMW 23917 : 7	Padang, W. Sumatra	F
NMW 23917 : 8	Padang, W. Sumatra	F
NMW 23917 : 9	Padang, W. Sumatra	M
NMW 23917 : 10	Padang, W. Sumatra	F
NMW 23923 : 1	Karen Mts, Myanmar	M
NMW 23923 : 2	Karen Mts, Myanmar	M
NMW 27947 : 1	Chiang Mai, N. Thailand	M
QSMI (no number)	Krabi, S. Thailand	F
QSMI (no number)	Krabi, S. Thailand	M
QSMI 13.1	Krabi, S. Thailand	M
QSMI13.2	Krabi, S. Thailand	M
QSMI 17	Krabi, S. Thailand	M
QSMI 7	Krabi, S. Thailand	M
QSMI 50.1	Krabi, S. Thailand	F
QSMI 50.2	Krabi, S. Thailand	F
QSMI 50.3	Krabi, S. Thailand	F
PCGV 34	Thung Song, S. Thailand	M
PCGV 223	Thung Song, S. Thailand	M
RMNH 16715	Uthai Thani, W. Thailand	F
SMF 21226	West Sumatra	M
ZRC 2.2884	Cameron Highlands, W. Malaysia	F
ZRC 2.2886	Cameron Highlands, W. Malaysia	F
ZRC 2.2889	Bukit Fraser, W. Malaysia	M
ZRC 2.2891	Bukit Fraser, W. Malaysia	M
ZRC 2.2892	Bukit Fraser, W. Malaysia	F

APPENDIX 2: *Continued*

Museum/field ref:	Locality:	Sex
ZRC 2.3493	Pulau Tioman, W. Malaysia	M
ZRC 2.5164	Cameron Highlands, W. Malaysia	F
ZRC 2.5361	Belum, W. Malaysia	M

Museum acronyms

BMNH	The Natural History Museum, London
CAS	California Academy of Sciences, San Francisco
DWNP	Department of Wildlife and National Parks, Malaysia
FMNH	Field Museum of Natural History, Chicago
LSUHC	La Sierra University Herpetology Collection, California
MCZ	Museum of Comparative Zoology, Harvard
NMBE	Naturhistorisches Museum Basel, Switzerland
NMW	Naturhistorisches Museum Wien, Austria
QSMI	Queen Savoabha Memorial Institute, Thailand
PCGV	Personal collection of Gernot Vogel
RMNH	Rijksmuseum van Natuurlijke Histoire, The Netherlands
SMF	Naturmuseum und Forschungsinstitut Senckenberg, Germany
ZRC	Raffles Museum of Biodiversity Research, National University of Singapore
AFS/KLS	indicates wild caught specimens examined under anaesthesia.

APPENDIX 3: GENBANK ACCESSION NUMBERS FOR SEQUENCES USED IN THIS STUDY

Locality	Catalogue No.	GenBank Accession Codes			
		Cyt <i>b</i>	ND4	12S	16S
Myanmar	B417 (CAS 216609)	AY371805	AY371845	AY371743	AY371776
	B419 (CAS 222195)	AY371806	AY371841	AY371738	AY371777
	B520 (CAS 205847)	AY371816	AY371855	AY371751	AY371783
North Thailand	B476	AY371809	AY371852	AY371745	AY371782
	A204	AF171902	AY371843	AY371742	AY371784
	A205	aF171906	AY371854	AY371741	AY371767
Laos	B195	AY371799	–	–	–
	B196	AY059571	AY059590	AY059538	AY059554
West Thailand	B34	AY059572	AY059591	AY059542	AY059558
	B52	AY371800	AY371836	AY371754	AY371768
South Thailand (Phang Nga)	B467	AY371807	AY371851	AY371744	AY371781
South Thailand (Thung Song)	A202	AF171904	AY371840	AY371739	AY371770
	A203	AY371796	AY059588	AY059537	AY059553
	A246	AY371820	AY371856	AY371749	–
	B19	AY371804	AY371844	–	AY371779
Cameron Highlands (Malaysia)	A196	AF171888	–	–	–
	A197	AY371808	AY371846	AY371746	AY371773
	B235	AY371812	AY371838	AY371740	–
	B236	AY371819	AY371847	AY371747	–
	B237	AY371813	AY371848	AY371748	–
	B238	AY371814	AY371839	AY371737	AY371774
	B345	AY371811	AY371849	–	AY371775
	B346	AY371810	AY371850	–	–
Bukit Fraser (Malaysia)	B246	AY059570	AY059589	AY059540	AY059556
	B278	AY371821	AY371857	AY371750	AY371780
	B469	AY371817	–	–	–

APPENDIX 3: *Continued*

Locality	Catalogue No.	GenBank Accession Codes			
		Cyt <i>b</i>	ND4	12S	16S
Tioman	B519	AY371818	AY371853	AY371752	AY371778
West Sumatra	B361	AY371801	AY371837	AY371753	AY371769
Borneo (East Malaysia)	B338	AY371798	AY371835	AY371733	AY371785
	B339	AY371802	–	AY371735	–
	B341	AY371803	AY371834	AY371734	AY371772
	B344	AY371815	AY371842	AY371736	AY371771