

Maximizing information in systematic revisions: a combined molecular and morphological analysis of a cryptic green pitviper complex (*Trimeresurus stejnegeri*)

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Although the integration of DNA information in taxonomy has been invaluable, logistical problems relating to sampling can seriously limit its applicability. Here we describe the analysis of a morphologically cryptic species complex, in which we maximize the information present by using both a DNA phylogeny and a multivariate morphometric approach. The green pitviper *Trimeresurus stejnegeri s.l.* is widespread in Asia, with a number of described subspecies (some of which are considered full species by some workers) and two new species that have recently been described from Thailand. The phylogeny indicates three clades, which can also be discerned in the principal component analyses of morphological variation. Combining molecular and morphological information permits evaluation of the taxonomic position of populations not represented in the phylogeny – in particular, the subspecies *T. s. chenbihui* and *T. s. yunnanensis*. We discuss nomenclatural issues raised by this analysis, although these cannot be fully resolved until the holotypes of these subspecies can be examined. Finally, we apply a molecular clock calibrated in New World pitvipers, and discuss some of the palaeoclimatic changes that might have impacted upon diversification in this group. © 2004 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2004, 82, 219–235.

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INTRODUCTION

In some species there appear to be selective or developmental constraints that either prevent morphological divergence (e.g. Colborn *et al.*, 2001) or promote convergence (e.g. Wake, 1991), complicating detection of the actual evolutionary units. In such cases, molecular studies are invaluable. However, despite the advances in data gathering and analysis techniques, interpreting species limits from gene trees remains far from straightforward (Puerto *et al.*, 2001; Templeton, 2001). The applicability of gene trees to resolving complexes of cryptic species also depends on obtaining an adequate genetic sampling of the range. This is not a trivial matter, especially for those taxa where normal museum preservation techniques, such as fixing in formalin, have made existing specimens unavailable for

DNA analysis. A combined molecular and morphological approach may help to maximize the contribution of information available from decades of collecting effort.

Asian *Trimeresurus* Lacépède, 1804 represents one of the most diverse radiations of pitvipers (McDiarmid, Campbell & Toure, 1999). The *Trimeresurus* radiation is a monophyletic assemblage of over 30 species (Malhotra & Thorpe, 2000a; Malhotra & Thorpe, 2004), which vary considerably in ecology, life-history and behaviour, and thus is a promising group for comparative analysis. The best known members of the genus, the green pitvipers, are remarkable for their extreme morphological similarity. They share a common colour pattern consisting of a uniform green background and a red tail (facets which are often reflected in their vernacular and scientific names), often also with lateral lines and postocular stripes. Other pattern elements (e.g. spots, bands) may be present, but are usually not conspicuous. During almost a century

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of study, successive splitting has brought the current tally of green pitvipers to c. 11 species, yet some are not distinguishable on external characters alone. As they include the most common venomous snakes in Asia and are the commonest cause of snakebite in many Asian countries, this uncertainty creates fundamental problems with snakebite management and treatment (Hutton *et al.*, 1990; Chippaux, 1998). It has also seriously hampered study of their venom (Wüster & McCarthy, 1996), and molecular systematics (see, for example, Parkinson, Campbell & Chippindale, 2002; a specimen they included as *T. popeiorum* has also been studied by the present authors and is in fact referable to *T. vogeli*).

The potential presence of still unrecognized cryptic species exacerbates these practical problems and impinges upon our ability to understand the evolution of current patterns of diversity in this group. Previous genetic work has already highlighted examples of this in the white-lipped pitviper, *T. albolabris* Gray, 1842 (Malhotra & Thorpe, 2000a; Giannasi, Thorpe & Malhotra, 2001). In this case, the cryptic species were fairly easy to detect since they involved parapatry. However, some monophyletic clusters also contain considerable genetic diversity and substructure, and may also represent species complexes.

This study is part of a long-term project on Asian pitviper evolution, which aims to resolve this problem by adding new material and characters to the available data through collecting in the field wherever possible. This has also allowed further information on morphology (particularly on colour pattern), distribution, ecology and behaviour to be obtained, and for DNA analysis to be performed alongside morphological analysis.

In this paper, we focus on *T. stejnegeri* Schmidt, 1925. This is one of the most northerly distributed *Trimeresurus* species and its range has been considerably underestimated in the past (Regenass & Kramer, 1981). Several subspecies – *T. s. stejnegeri*, *T. s. yunnanensis* (Schmidt, 1925), sometimes considered a distinct species, and *T. s. chenbihui* Zhao, 1997 – have been proposed (Fig. 1). Recently, two populations from Thailand have been described as new species: *T. vogeli* (David, Vidal & Pauwels, 2001) from the southern edge of the Khorat Plateau and south-eastern Thailand (Nakhon Ratchasima, Prachin Buri, Chantaburi and Trat provinces) and *T. gumprechtii* (David *et al.*, 2002) from Loei, Phitsanulok, Petchabun and Chaiyaphum provinces, north-eastern Thailand.

Here, we evaluate the presence of cryptic species in *T. stejnegeri*, based on a phylogenetic tree inferred from mitochondrial cytochrome *b* sequence data, and on the basis of this information attempt to define morphological boundaries for the species detected. Once these have been defined, it becomes possible to relate

specimens for which genetic data are not available to these species. Thus we attempt a complete revision of the taxonomy of the species, rather than just a partial solution that would result from using just a molecular approach on its own, with large gaps in geographical coverage arising from the difficulty of obtaining genetic samples from some countries in the region.

MATERIAL AND METHODS

SAMPLING

Specimens were obtained by a number of methods. Fieldwork was carried out in various parts of Thailand, Taiwan, western Malaysia and Vietnam between 1994 and 1999. Blood (100–200 µL) was extracted from the caudal vein, placed in 1 mL 5% EDTA, and preserved by the addition of 2–4 mL SDS-Tris buffer (100 mM Tris, 3% SDS). In most cases, conditions attached to permission did not allow permanent collections to be made. A number of morphological characteristics were measured from anaesthetized specimens, and macro photographs were taken. Where permission conditions allowed, voucher specimens were deposited in local collections. Tissues were also obtained from museum collections (Table 1) and from private collections only when locality information was available and identity could be verified. In these cases, liver or muscle tissue, preserved in 80% ethanol, was used.

DNA ISOLATION, AMPLIFICATION AND SEQUENCING

Whole genomic DNA was extracted from 0.01–0.02 g of ethanol-preserved muscle or liver tissue, or 200–500 µL of blood/buffer, using standard protocols (Sambrook, Frisch & Maniatis, 1989). Cytochrome *b* (*cyt b*) sequences were obtained as described in Malhotra & Thorpe (2000a). Unincorporated nucleotides and primers were removed using a variety of commercially available kits [e.g. Prep-a-gene (Biorad), Wizard minicolumns (Promega), or QIAquick columns (QIAGEN)]. The double-stranded product was then either manually sequenced in both directions using a modification of the Sequenase v2.0 protocol (Perkin-Elmer), or sequenced using dye-labelled terminators (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit), and subsequently run on an ABI Prism 377 automated DNA sequencer.

SEQUENCE ANALYSIS

The phylogenetic analysis included 737 bp of *cyt b* from 24 specimens of the *T. stejnegeri* group (Table 1), with four outgroups representing the other species groups of *Trimeresurus s.s.* chosen on the basis of previous analyses (Malhotra & Thorpe, 2000a). Align-

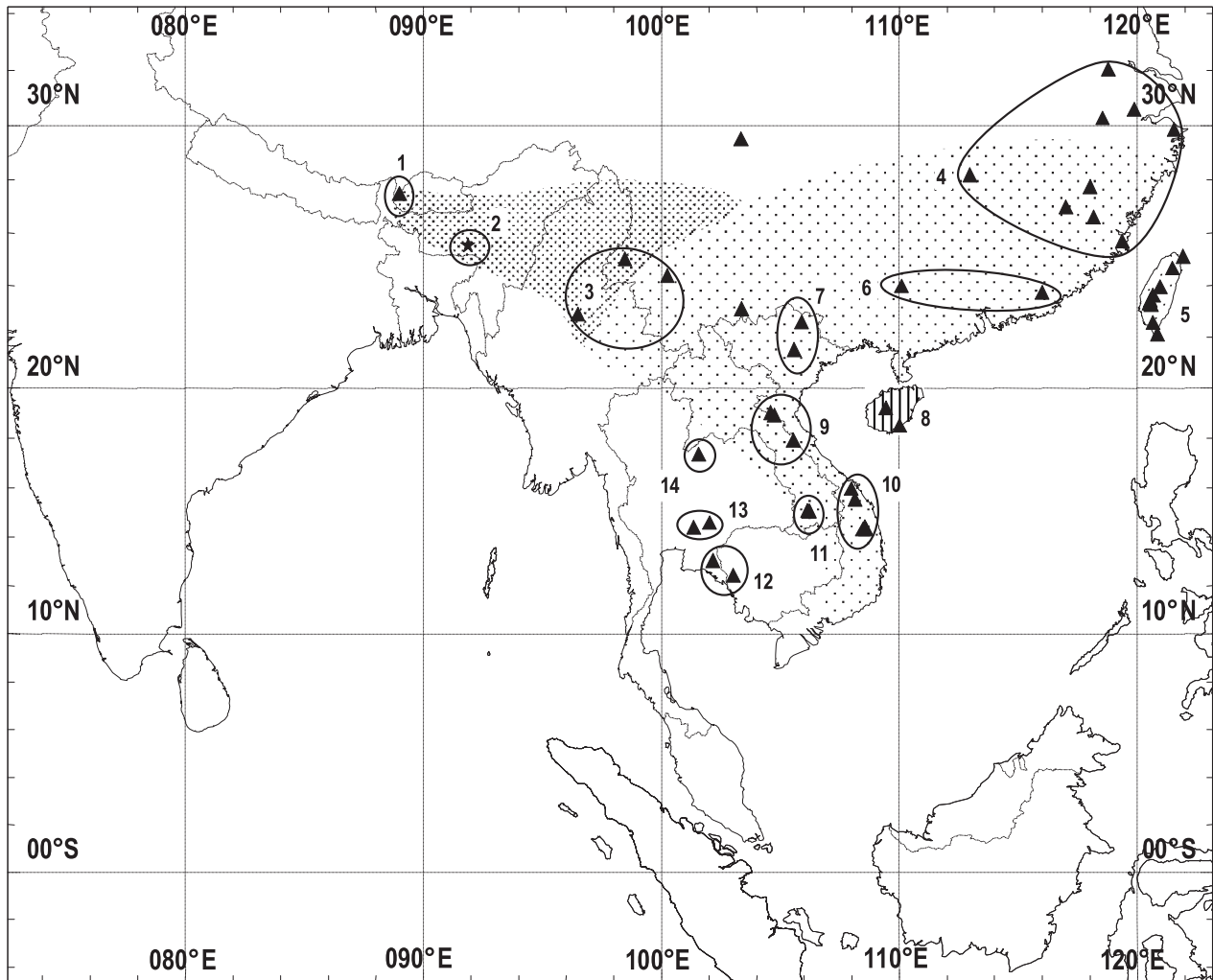


Figure 1. The range of *T. stejnegeri* (light stippling; *T. s. stejnegeri*; heavy stippling; *T. s. yunnanensis*) according to Regenass & Kramer (1981) in the last major revision of the green pitvipers. Vertical hatching indicates the range of *T. s. chenbihui* (Zhao, 1997). Composite localities used where a priori grouping was required for the preliminary morphological analysis of *T. stejnegeri* are circled and numbered as follows: 1. Himalayas. 2. North-eastern India. 3. North Myanmar and western Yunnan. 4. Northern China. 5. Taiwan. 6. South-eastern China. 7. northern Vietnam. 8. Hainan. 9. Annamite mountains. 10. Central Vietnam. 11. Bolovens Plateau. 12. Cardamom mountains and south-east Thailand. 13. Dongraek mountains (corresponding to the type locality of *T. vogeli*). 14. North-east Thailand (corresponding to the type locality of *T. gumprechtii*). Some individual specimens of uncertain affinity were not grouped.

ment of this coding region was trivial and was done by eye. Since pseudogenes of *cyt b* have been amplified in some studies (Zhang & Hewitt, 1996), the sequences were first translated into amino acid sequences using MEGA version 2.1 (Kumar *et al.*, 2001) to check for the unexpected occurrence of stop codons. The presence of phylogenetic signal in the data matrix was tested using the g_1 statistic (Hillis & Huelsenbeck, 1992) for the skewness of tree length distributions, estimated from 10^6 random trees. The possibility of non-neutral evolution was tested using a variety of tests implemented in the program DnaSP 3.53 (Rozas & Rozas, 1999), including McDonald & Kreitman's (1991) test,

Fu & Li's D^* and F^* , and their modifications for use with an outgroup sequence (Fu & Li, 1993), and Tajima's D (Tajima, 1989). We also checked the dataset for homogeneity of base composition among taxa, to detect problems with the assumption of a similar underlying substitutional model.

We used both parsimony and Bayesian Markov Chain Monte Carlo (MCMC) approaches to reconstruct phylogenies, using PAUP* 4.0b8 (Swofford, 2003) and MrBayes v.2.01 (Huelsenbeck & Ronquist, 2001) respectively. Parsimony trees were unweighted, as a now substantial literature documents the general ineffectiveness of weighting schemes in decreasing

Table 1. Details of the ingroup specimens used in the phylogenetic analysis of *cyt b* sequences of the *T. stejnegeri* group. '*T. khoratensis*' is the trade name of a specimen bought from the captive trade. *Abbreviations:* BMNH: The Natural History Museum, London; FMNH: Field Museum of Natural History, Chicago; NMNS: National Museum of Natural Science, Taiwan; ROM: Royal Ontario Museum, Toronto. CAT are the author's own reference numbers

Locality	Museum	CAT	GenBank accession codes
China, Fujian Province	NMNS 3651 : 12347	A222	AF277677
China, Yunnan Province	NMNS 3113	B15	AY321487
China, Yunnan Province	BMNH 2002.53, 2002.54	B497, B553	AY321489, AY321488
Taiwan, Taitung County	NMNS 2684	A161	AF171880
Taiwan, Nantou County	NMNS 3593	TST110	AF277678
Taiwan, Hualien County	NMNS 2768	TST66	AF277681
Taiwan, Taipei county	NMNS 2558	A160	AF171896
Taiwan, Pingtung County	NMNS 2692	TST43	AF277679
Northern Vietnam, Vin Phuc Province	–	B109, B110	AF277709, AF277710
Northern Vietnam, Cao Bang Province	ROM 35321	B181	AF278711
Northern Vietnam, Nghe An Province	FMNH 255577, 255579–80	B127, B170, B174	AY059575, AY059576, AY059573
Central Vietnam, Gia Lai Province	ROM 30790–91, 34565	A198, A199, B182	AY059577, AF171903, AY059578
Lao PDR, Khammouane Province	FMNH 256419	B128	AY059579
Lao PDR, Champasak Province	FMNH 258945–46	B124, B125	AY059580, AY059581
Thailand, Loei Province	–	A181	AF171898
Thailand, Nakhon Ratchasima Province	–	B97	AY059574
' <i>T. khoratensis</i> ', locality unknown	–	A65	same as AY059574

homoplasy without also reducing useful phylogenetic information (Allard & Carpenter, 1996; Philippe *et al.*, 1996; Milinkovitch & Lyons-Weiler, 1998; Vidal & Lecointre, 1998; Baker, Wilkinson & DeSalle, 2001). Searches were heuristic, with starting trees obtained by random addition with 100 replications, and tree-bisection-reconnection (TBR) branch swapping. Confidence in the inferred branches of the optimal trees was obtained by bootstrapping (1000 replications) with a modified search strategy, with the starting tree obtained from only ten replications.

Prior to the Bayesian analysis, ModelTest version 3.0 (Posada & Crandall, 1998) was used to infer the simplest best-fit model of evolution for the combined dataset based on hierarchical log-likelihood ratio tests comparing successively complex models (Huelsenbeck & Crandall, 1997; Posada & Crandall, 2001). All MCMC phylogenetic reconstructions were initiated with uniform priors, model parameters estimated as part of the analyses, and the best-fit model as indicated via ModelTest. Three heated chains and a single cold chain were used; runs were initiated with random trees (the default settings of the program), run for 1 020 000 generations, and sampled every 100 generations. Majority-rule consensus phylograms and posterior probabilities for nodes were assembled from all post burn-in sampled trees (i.e. after the likelihood scores approached stationarity, in this case, after

the first 1500 generations). Finally, PHYLTEST (Takezaki, Rzhetsky & Nei, 1995) was used to conduct a relative rate test between clades, and to calculate divergence times (with confidence intervals) of the clades.

MORPHOMETRIC ANALYSIS

Specimens used are listed in Appendix 1, and characters measured (with their abbreviations), are given in Appendix 2. Where localities were represented by single specimens and needed to be grouped into composite localities (Fig. 1), every attempt was made to keep these as small as possible, and to define them with attention to potential biogeographical boundaries (Zhao & Adler, 1993; MacKinnon, 1997). The species identification was verified by examination of the hemipenis of male specimens. The presence of stout spines was a clear indicator of *T. stejnegeri*, and could be detected with careful dissection even in the smallest specimens. Although females are potentially confused with *T. popeiorum*, this study was performed as part of a larger study of *Trimeresurus* and the range of the species could be shown (from the males) to be non-overlapping in all but one locality (A. Malhotra & R. S. Thorpe, unpubl. data). This simplified the assignment of females to species.

All characters were first screened for significant between-locality differences using one-way analysis of variance and covariance (ANOVA/ANCOVA). Levene's test was used to test for violations of the assumption of homogeneity of variance, and where this was significant, the Brown–Forsythe variant which relaxes this assumption was used instead (Brown & Forsythe, 1974). Non-significant characters were not used in further analyses. Males and females were analysed separately to eliminate variation due to sexual dimorphism, and all size-correlated characters were adjusted using the pooled within-group regression coefficient against either snout–vent length (SVL) or head length (LHEAD) to remove size-related bias. Included characters showed no sign of recording drift or preservation effects.

Principal component analysis (PCA) was performed in preference to the more powerful canonical variate analysis (CVA) as the latter has been shown to be sensitive to heteroscedasticity in the data. Also, as PCA does not require a priori grouping of the specimens, it is more robust to any possible misclassification arising from the grouping procedure described above. A few characters were highly correlated with each other ($r > 0.7$), indicating that they may not provide independent information. In CVA, these correlations are taken into account, but in PCA they may result in over-emphasis of the correlated variables (Thorpe, 1976). Thus, only one of the characters from the correlated character sets was used in PCA. Two different PCA analyses were performed for all datasets. The first included external characters such as scalation, colour pattern and shape. A second analysis included internal characters as well, such as tooth number and position of major internal organs. The latter had a considerably reduced sample size, as internal data were missing for museum specimens where permission had not been granted for the necessary dissections, or where specimens had been gutted, and for all live animals examined. Lists of characters included in these analyses are given in Appendix 3.

The inferred clades from the phylogenetic analysis were used to make an informed interpretation of the PCA ordination. Using the specimens from localities that are represented in the genetic analysis as a guide, lines defining the morphological limits of the clades can be drawn on the plot. This allows predictions to be made regarding membership of the clades by specimens from populations not represented in the molecular analysis.

EVALUATING ENVIRONMENTAL EFFECTS

Morphology may be significantly affected by adaptations to the environment either through natural selection (Thorpe *et al.*, 1994; Malhotra & Thorpe, 2000b)

or plasticity (Elphick & Shine, 1998; Qualls & Shine, 1998; Queral Regil & King, 1998). This can interfere with its ability to reflect population history and genetic discontinuity, make distinct genetic entities morphologically similar, and obscure any taxonomic differences between them. To evaluate this, we used matrix correlation tests (MCTs) (Manly, 1991; Thorpe & Malhotra, 1996; Thorpe, 1996, and references therein; Ritchie, Kidd & Gleason, 2001), in which each character was tested individually against separate environmental factors. The advantage of using MCTs is that one can attempt to remove spatial effects by including a matrix of geographical proximity (calculated from latitude and longitude co-ordinates), as one of the independent matrices. These spatial effects may arise due to the fact that localities that are closer together are also likely to be more environmentally similar. Elevation was obtained from field notes accompanying specimens or from <http://www.calle.com/world/>. Average annual temperature and average annual rainfall obtained from online databases (<http://www.worldclimate.com>, <http://www.weatherbase.com>) or the *Climatic Atlas of Asia* (Anonymous, 1981).

Vegetation type was inferred from maps of actual (MacKinnon, 1997) or potential (Adams & Faure, 1997) vegetation cover. As these two sources did not use the same classification scheme, this variable was recoded from 1 to 8 according to seasonality and judged from the descriptions of the vegetation types contained in each source. If morphological characters that are highly correlated with one or more environmental factors (after Bonferroni correction for multiple comparisons) in the MCTs have also contributed significantly to the multivariate ordination of clades, it may indicate that our allocation of specimens to clades is biased.

RESULTS

PHYLOGENETIC ANALYSIS OF THE *T. STEJNEGERI* GROUP

No stop codons were detected, indicating that amplification of pseudogenes was unlikely. The data differed significantly from random ($g1 = -0.57$, $P < 0.01$). Base composition was not significantly different between ingroup taxa ($\chi^2 = 4.6$ $P = 1.00$). None of the neutrality tests showed a significant departure from neutrality. The time reversible model with gamma distributed rates was used for MCMC analyses.

The parsimony and Bayesian trees were identical in topology, showing the presence of three major clades. The model parameters were estimated by MrBayes, with mean and 95% credible intervals. Base frequencies were as follows. A: 0.3164 (0.2865–0.3465), C: 0.3209 (0.2933–0.3489), G: 0.0999 (0.0800–0.1202), T:

0.2640 (0.2378–0.2917); $\alpha = 0.29419$ (0.23388–0.36612). The resulting tree had a mean likelihood score of -3639.3596 (-3652.14 to -3628.0). The presence of three well-differentiated clades within *T. stejnegeri* is indicated (Fig. 2).

The basal clade, Clade A, is strongly supported (posterior probability = 100%, parsimony bootstrap = 100%), and contains the most southern populations from central and south-eastern Thailand (including the population recently described as *T. vogeli*; David *et al.*, 2001), Cambodia, southern Laos, and central Vietnam. It also includes a specimen bought from the trade under the name '*T. sumatranus*' and, when it was recognized that it was misnamed, later also known as *T. khoratensis*.

Clade B is supported by a posterior probability of 83% and a parsimony bootstrap of 68%. However, if the specimen from south-eastern Yunnan is excluded, support increases substantially (posterior probability of 100% and parsimony bootstrap of 95%). Clade B contains a cluster of populations ranging from north-eastern Thailand to the Annamite mountain chain along the border of Vietnam and Laos, and the southern part of Yunnan province, China. It includes the population from Loei Province, Thailand, that has recently been described as *T. gumprechtii* (David *et al.*, 2002).

The remaining clade, Clade C, is strongly supported by a posterior probability of 98%, although the parsimony bootstrap value is lower (58%). It consists of more northern populations from northern Vietnam, China, and Taiwan, including those from the type locality of *T. stejnegeri* s.s. in Fujian province. However, the internal substructure of this clade is not well resolved. This may partially be due to a lack of samples from mainland China, leaving a rather large sampling gap between the northern Vietnamese populations and the remaining samples from Fujian and Taiwan.

PHYLTEST revealed no rate differences between any of the clades (even when treating the northern Vietnam subclade separately). The estimated average divergence (Kimura 2 parameter with gamma correction, using the estimate of α produced by MrBayes) between the *T. stejnegeri* group and the outgroups was 0.2360 ± 0.0286 , between Clade A and Clades B and C was 0.1494 ± 0.0202 , and between Clades B and C was 0.0853 ± 0.0111 .

MORPHOMETRIC ANALYSIS OF *T. STEJNEGERI*

PCA plots using external characters alone show some separation of the three clades in two dimensions, although this is more obvious in females. In Figure 3, the specimens of unknown affinity are represented by solid or grey symbols. The analysis includes paratypes from the type locality for *T. s. yunnanensis*, Tongchong

(formerly known as Tengyueh; Zhao & Adler, 1993), Yunnan Province, China.

The external analysis (Fig. 3A) is dominated by the separation of an outlying specimen (which is referable to *T. s. yunnanensis* on the basis of its having 19 scale rows at mid-body) primarily on PC1. This is due to some scale reductions (SC10to8, SC6to4, SC12to10, VS27to25, VS19to17, VS29to27, VS31to29) occurring more anteriorly, as well as a more pronounced lateral stripe, in the outlying specimen. On PC2, it is separated from the other specimens by the presence of a broad postocular stripe (OCTSRIPPE), fewer ventral scales (VSC) and sublabials (SUBLAB) and scales between the rear edges of the supraoculars (BTWSUPOC2).

If this specimen does in fact represent *T. s. yunnanensis*, it is surprising that the diagnostic character (the lower number of scale rows at mid-body) does not seem to play a major role in the differentiation of this taxon. Note that the paratype of *T. s. yunnanensis*, which also has 19 scale rows at mid-body, falls right in the middle of the ordination, in the zone of overlap between Clades B and C (i.e. *T. gumprechtii* and *T. stejnegeri* s.s., respectively). Because the lateral and postocular stripes are unusually broad in this specimen, it could simply be an aberrant specimen, or represent an unknown taxon. We therefore re-ran the PCA without it (Fig. 3B). The separation of the clades was greatly improved, with A being mainly separated from B and C on PC1 by the more anterior occurrence of some scale reductions (SC10to8, SC6to4, SC12to10, VS19to17 and VS27to25). Clade C (*T. stejnegeri* s.s.) is primarily distinguished from Clade B (*T. gumprechtii*) on PC2 by the greater number of subcaudals (SCS), a broader lateral stripe (SCR1), a more posterior occurrence of the reduction from 21 to 19 scale rows (VS21to19) and from 19 to 17 rows (VS19to17), more strongly keeled temporal (KTEMP) and rear head scales (KHEADSC), the presence of a postocular stripe (OCSTRIPPE) and a more pronounced lateral stripe (STRIPPE).

Here, the female paratype of *T. s. yunnanensis* falls on the boundary of Clade B (Fig. 3B); it does not appear to be sufficiently morphologically distinct from specimens that clearly fall within this clade in the molecular analysis to justify its status as a member of a distinct taxon. Specimens from south-eastern China are similarly unambiguously classified as members of Clade C (*T. stejnegeri* s.s.), consistent with their geographical location. While one of the specimens from south-eastern Thailand falls within the exclusive region of Clade A (*T. vogeli*), the other falls outside the clade boundaries as defined by specimens of known affinity, although not by very much.

However, a colour pattern character (the lack of red pigment on the tail) that is diagnostic of Clade A

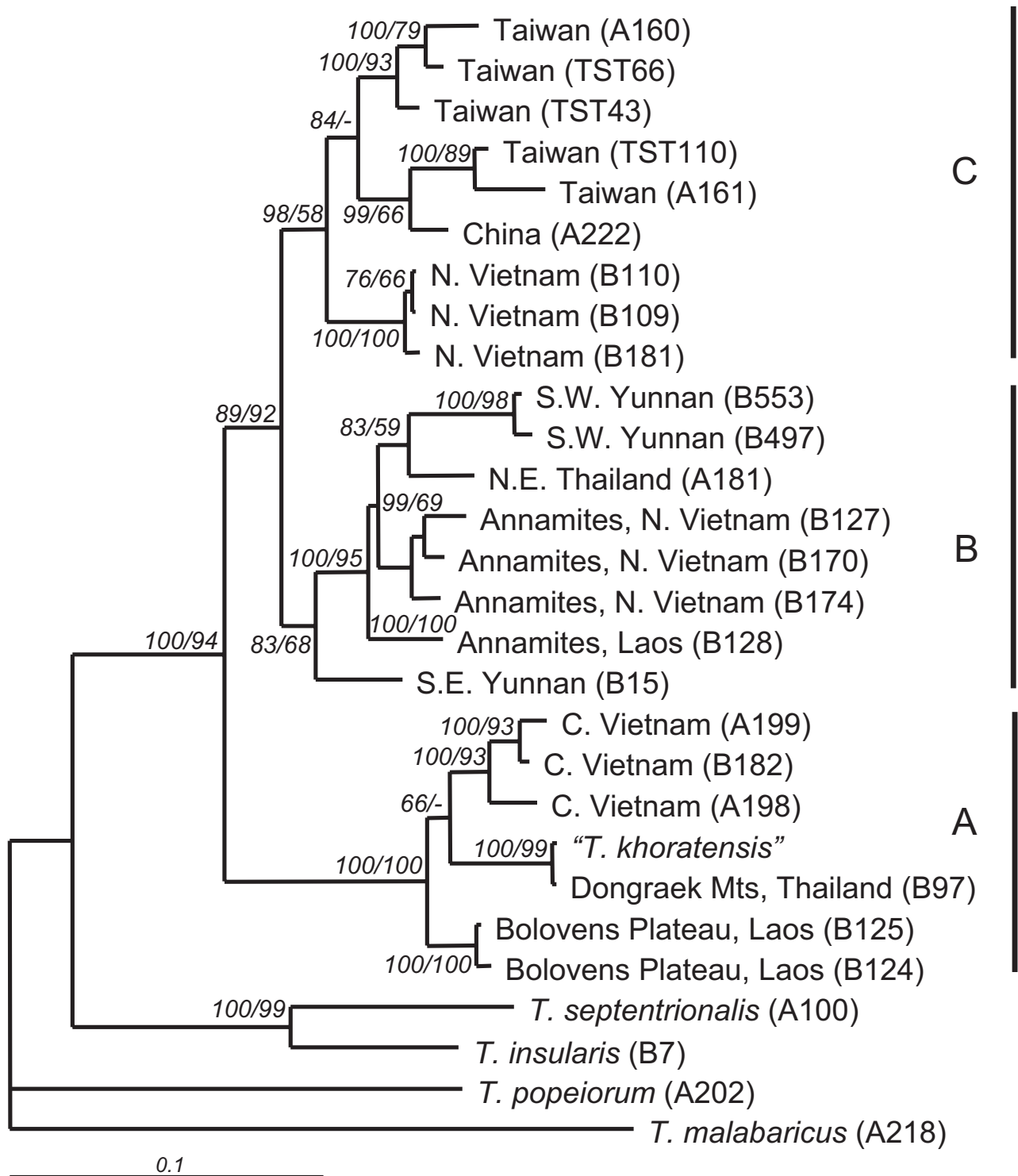


Figure 2. Bayesian tree for *Trimeresurus stejnegeri sensu lato*, showing the presence of three clades (A–C). Posterior probabilities for the clades are shown below the node to which they refer, with corresponding bootstrap values from the parsimony analysis given after the diagonal slash. Branch lengths shown are average branch lengths of all sampled trees.

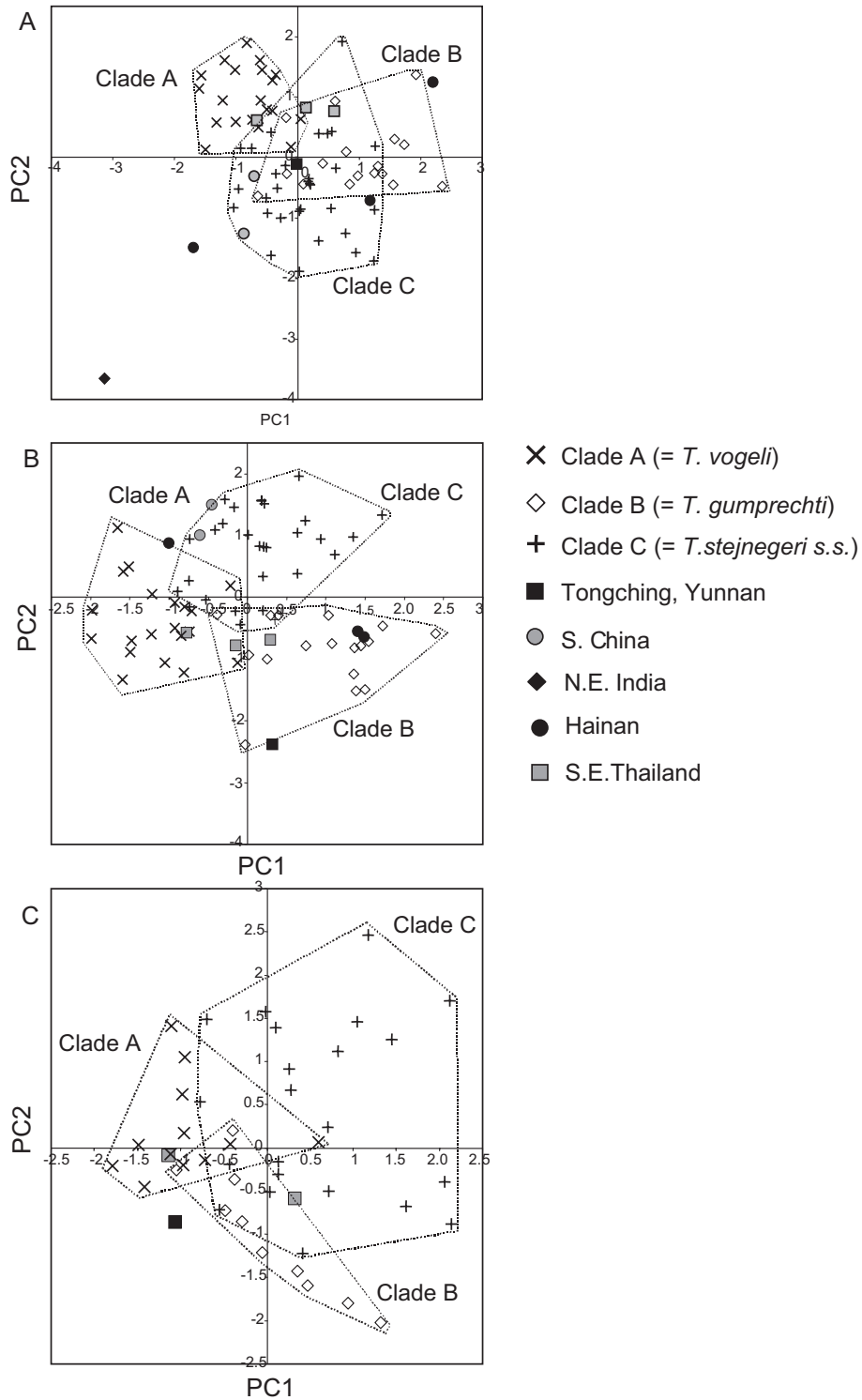


Figure 3. PCA on morphology of female *T. stejneri* s.l. A, external characters, including scalation and colour pattern but excluding shape characters to retain as many specimens in the analysis as possible. B, as in A, but excluding the specimen from north-eastern India. C, all characters, including the position of major internal organs, and tooth counts (excluding the specimen from north-eastern India). The dashed lines surround all individuals from localities allocated to one of these clades by the molecular analysis and represent their known morphological limits. Specimens which are not represented in the molecular analysis, and whose clade relationships are therefore not clear, are shown with solid black or grey symbols as indicated in the legend.

(*T. vogeli*), but which could not be included in the present analysis because it is not always clear in preserved specimens, allows the allocation of all these specimens to Clade A. Finally, specimens from Hainan provide an intriguing suggestion that there may be more than one taxon present on this island. One specimen falls near the boundary of *T. stejnegeri* s.s. (its similarity to Clade A can be disregarded due to the presence of red pigment on the tail) but the other two specimens fall clearly within the region exclusive to Clade B (*T. gumprechtii*).

In the analysis that included internal characters, the distinctness of the specimen from north-eastern India is maintained, although the addition of internal characters has the effect of reducing its separation

from Clade C on PC2 (not shown). When this specimen is not included in the ordination (Fig. 3C), the characters influencing separation on PC1 stay largely the same as in the external analysis, although with the addition of OCSTRIPE and KHEADSC. However, PC2 now primarily distinguishes Clade A from Clade B by a greater number of dentary teeth (DENT) and a more posterior position of the anterior tip of the right kidney (RKANT), together with some scale reduction characters occurring more anteriorly (VS31to29, VS29to27, SC12to10), a lower number of ventral scales (VSC) and a higher number of scales between the rear edges of the supraoculars (BTWSUPOC2). In this analysis, again, the paratype of *T. s. yunnanensis* appears most similar to Clade B.

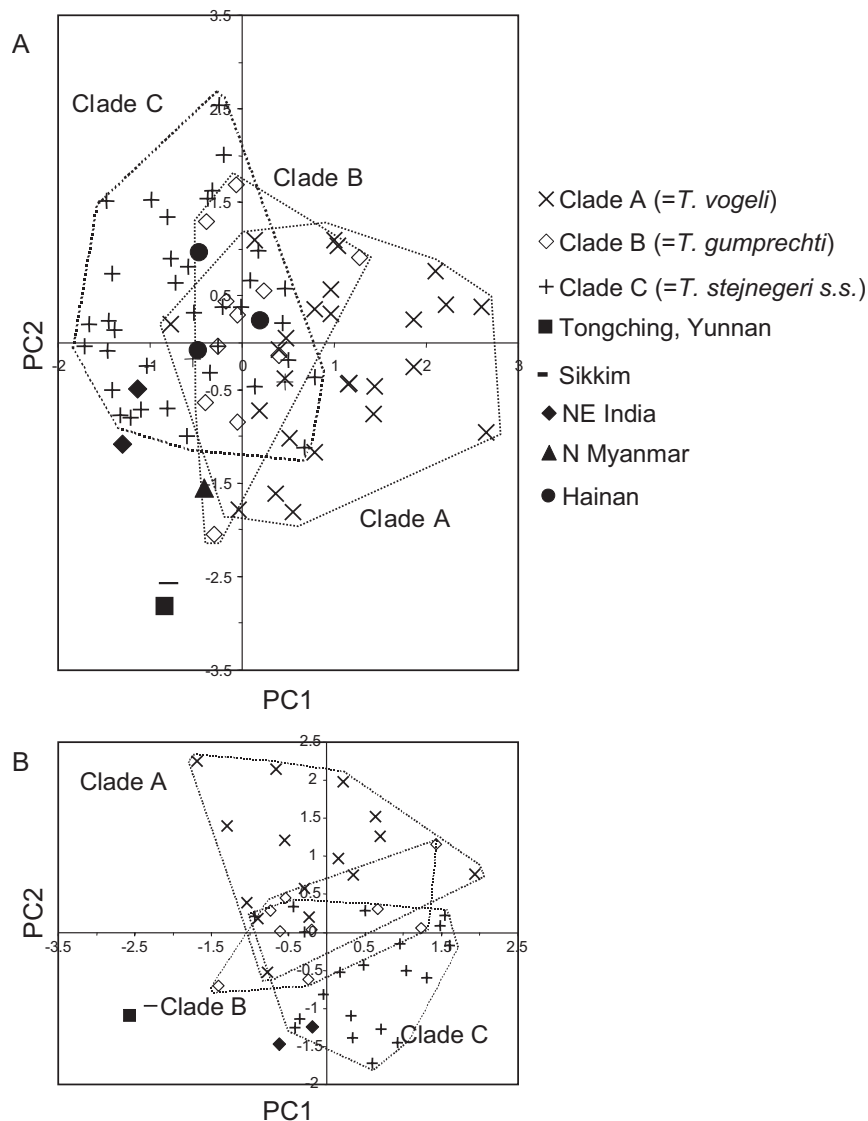


Figure 4. PCA on morphology of male *T. stejnegeri* s.l. A, external characters, including scalation, shape, and colour pattern. B, all characters, including the position of major internal organs, and tooth counts.

In males, the separation of clades in the analysis including external characters only (Fig. 4A) is mainly between Clades A and C along PC1, with Clade B occupying an intermediate position. This is due to some scale reductions (VS17to15) occurring more anteriorly on the body in Clade A. Clade A also has a lower incidence of postocular stripes (OCSTRIPE), a larger head (LHEAD, WHEAD), a larger distance between the nostril and the pit (NOS2PIT), and a higher number of dorsal spots (DORSPOT). PC2 does not effectively separate the clades but does separate two specimens from the range of *T. s. yunnanensis* (including the paratype and a specimen from the Himalayan foothills in Sikkim). These specimens are primarily distinguished from the remainder by scale reductions (VS23to21, VS21to19, VS17to15, SC6to4) occurring closer to the head, a lower number of sublabials and supralabials (SUBLAB, SUPLAB), a lower number of scales between supraoculars (BTWSUPOC1, BTWSUPOC2), a narrower lateral stripe (SCR1), fewer scales between the rear edge of the mouth and the chin shields (VENTEDGE) and wider supraoculars (WSUPOC).

Thus, while not greatly different, there is some support in this analysis for the distinctness of *T. s. yunnanensis*. However, other specimens that have been referred to this species, including those from north-eastern India and northern Myanmar, do not appear to be distinct from the clades represented in the phylogenetic analysis. Again, the possession of 19 scale rows at mid-body is not a useful diagnostic character, as it is found in several specimens that belong to Clade B by molecular analysis. The two north-eastern Indian specimens fall clearly within Clade C (*T. stejnegeri* s.s.) while the northern Myanmar specimen appears to be more similar to Clade B (*T. gumprechtii*) but is fairly close to the boundary of Clade C as well. In this analysis, the Hainan specimens fall within the region of overlap of all three clades, or of Clade B and C. However, all three specimens are similar to each other and there is no supporting evidence of the presence of two distinct taxa on this island from this analysis.

In the analysis that includes internal characters (Fig. 4B), PC1 is dominated by internal characters, and largely discriminates the two '*T. s. yunnanensis*' specimens from the remainder. The former have the anterior tip of the right testis (RTANT), liver (LVANT) and right kidney (RKANT), and the posterior tip of the left kidney (LKPOST) occurring closer to the head than in Clades A–C, and a wider head (WHEAD). The three clades are primarily separated on PC2, with Clade B broadly overlapping the boundary between Clades A and C. Thus the contrast is mostly between Clades A and C, with Clade A having a longer head (LHEAD), longer nostril to pit distance (NOS2PIT), a

lower incidence of a postocular stripe (OCSTRIPE), higher number of supra- and sublabials (SUPLAB, SUBLAB) and dorsal spots (DORSPOT), wider internasals (WINTNAS), a more posterior transition between 31 and 29 scale rows (VS31to29), and a larger number of scales between the supraoculars (BTWSUPOC1). Again, the two specimens from north-eastern India are clearly similar to other specimens in Clade C and are referable to *T. stejnegeri* s.s.

EVALUATING ENVIRONMENTAL EFFECTS

MCTs showed that many characters are highly correlated with one or more environmental variables. In females, characters that are significantly correlated with environmental factors and also contribute to the ordination of specimens in Figure 3 are VSC, KTEMP, OCSTRIPE, SCR1, SUBLAB and DENT. In males, characters that are significantly correlated with environmental factors and also contribute to the ordination of specimens in Figure 4 are LHEAD, WHEAD, NOS2PIT, DORSPOT, LKPOST, RTANT, SUPLAB, SUBLAB, VS31to29, VS17to15, SC6to4, WHEAD, VSC and OCSTRIPE. However, when these characters are dropped and the PCA redone, there is less discrimination between clades but essentially no change in the conclusions (not shown). Thus characters that may be particularly prone to environmental effects do not seem to overly affect the conclusions of the morphometric analysis.

DISCUSSION

TAXONOMIC CONCLUSIONS FOR THE *T. STEJNEGERI* GROUP

A number of limitations of gene trees makes translating these into taxonomic recommendations problematic. First, there may be a difference between species trees and gene trees (Avice & Ball, 1990). There are theoretical reasons why mitochondrial DNA may be less prone to deviations from the species tree (Moore, 1995). Giannasi *et al.* (2001) showed that a nuclear tree was in broad agreement with a tree based on *cyt b* for *T. albolabris*, while a recent analysis by Creer, Malhotra & Thorpe (2003) indicated that of four mitochondrial gene regions, *cyt b* was the best single gene for recovering a highly resolved tree whose topology most closely matched that of a combined analysis that included nuclear genes.

Second, in any group with a range including areas that are politically and logistically difficult to sample, and especially in organisms which are difficult to sample in large numbers, there is likely to be a very patchy representation of the total variation in any molecular analysis. There are a multiplicity of species definitions

in existence (see Mayden, 1997 and DeQueroz, 1998 for a comprehensive review). However, the absence of information on ongoing processes of genetic change confines us to species definitions that focus on the nature of species as historical lineages, such as the diagnosability criterion (the phylogenetic species concept *sensu* Cracraft, 1983). However, where species names have been based on genetically unsampled populations (e.g. *T. s. chenbihui* and *T. s. yunnanensis* in this study), any reassessment of species boundaries within a group can only be partial. Moreover, the proper taxonomic treatment of putative species identified from the partial sampling will often be unclear.

The concurrent use of multivariate morphometrics goes some way towards resolving this problem. For example, it suggests that more than one taxon is present within the putative range of *T. s. yunnanensis*, with some specimens being referable to *T. stejnegeri s.s.*, some to *T. gumprechtii*, and some appearing distinct from either. This will complicate the interpretation of analyses in which allocation of specimens to *T. s. yunnanensis* has primarily been done on the basis of locality, or of the presence of 19 scale rows at mid-body. As we have shown, this character is not important in distinguishing clade boundaries, and has been found in specimens that are clearly referable to *T. gumprechtii* by molecular analysis. Moreover, morphological differences may be difficult to interpret where the population in question occupies a different environment, which may affect the expression of certain morphological traits. This may be pertinent to the present issue, as these specimens occur in the westernmost part of the range of the complex, in the foothills of the Himalayan massif, and are generally found at much higher altitudes than the populations further to the east. Explicit testing of the extent to which morphological characters are correlated with the environment has, however, allowed an at least partial assessment of the extent to which environmental factors may be affecting our interpretation of the morphological similarity or dissimilarity of populations, and it does not seem to be a significant problem.

The type locality of *T. stejnegeri* is Fujian province (formerly known as Fukien), China; this name clearly corresponds to Clade C. The genetic distinctness of the southernmost clade (Clade A) is clear, and is supported by the morphological differences revealed by multivariate analysis. This clade includes the populations from Thailand from which a new species, *T. vogeli*, has recently been described (David *et al.*, 2001). However, the range of the species clearly extends beyond Thailand, and a re-description of this new species, based on our more extensive data, will be published elsewhere (Malhotra, Thorpe & Stuart, in press).

The status of Clade B as a distinct taxon is clear; it contains specimens from the type locality of the

recently described *T. gumprechtii*, indicating that its range is greater than that stated in the species description (David *et al.*, 2002). However, there are several unresolved issues surrounding its nomenclature, as specimens referred to both *T. s. yunnanensis* and *T. s. chenbihui* show morphological affinities with this clade. While the male paratype of *T. s. yunnanensis* does appear to be rather distinct from the other clades, the female paratype has been shown to be clearly referable to Clade B. The name *T. yunnanensis* may therefore be the first available name for this clade, taking precedence over *T. gumprechtii*. However, this depends on the affinity of the holotype of *T. yunnanensis*, which was not available for examination. An added complication facing the nomenclature of the group is the possible presence of *T. gumprechtii* in Hainan. Ling Shui county, the type locality of *T. s. chenbihui* (Zhao, 1997), apparently has specimens referable to both *T. gumprechtii* and *T. stejnegeri s.s.* This poses a further problem for the nomenclature of Clade B; even if *T. yunnanensis* proves to be a distinct taxon, the name *T. chenbihui* would also take precedence over *T. gumprechtii*. Again, the holotype of this subspecies requires further examination before the matter can be fully resolved. For the moment, we suggest that the name *T. gumprechtii* is retained, the name *T. s. chenbihui* is synonymized with *T. stejnegeri s.s.*, and *T. yunnanensis* is given species status. The distribution of these putative taxa is shown in Figure 5.

A final point relevant to the nomenclature of these species arises from the study by Malhotra & Thorpe (2004) of the group in relation to other species of *Trimeresurus*, in which it has been proposed that the species discussed in this paper, together with *T. medoensis*, be placed in a distinct genus.

BIOGEOGRAPHY AND EVOLUTION OF *T. STEJNEGERI* S.L.

Estimating time of divergence of clades requires knowledge of the rate of sequence evolution, which in turn requires calibration from the fossil record or geological evidence. The fossil record of pitvipers, and snakes in general, is poor and at best indicates that pitvipers had evolved by the Miocene, *c.* 18–22 million years ago (Mya) (Greene, 1992). Wüster *et al.* (2002) calculated a rate of *cyt b* evolution of 1.09–1.77% per Myr, based on the divergence of the New World crocodyline genus *Porthidium*. Applying these rates, and taking the standard errors of the sequence divergence estimates into account, we estimate that the *T. stejnegeri* group is likely to have diverged from the rest of *Trimeresurus s.s.* between the late Oligocene and mid-Miocene (24.3–11.7 Mya). *Trimeresurus vogeli* is likely to have diverged from the rest of

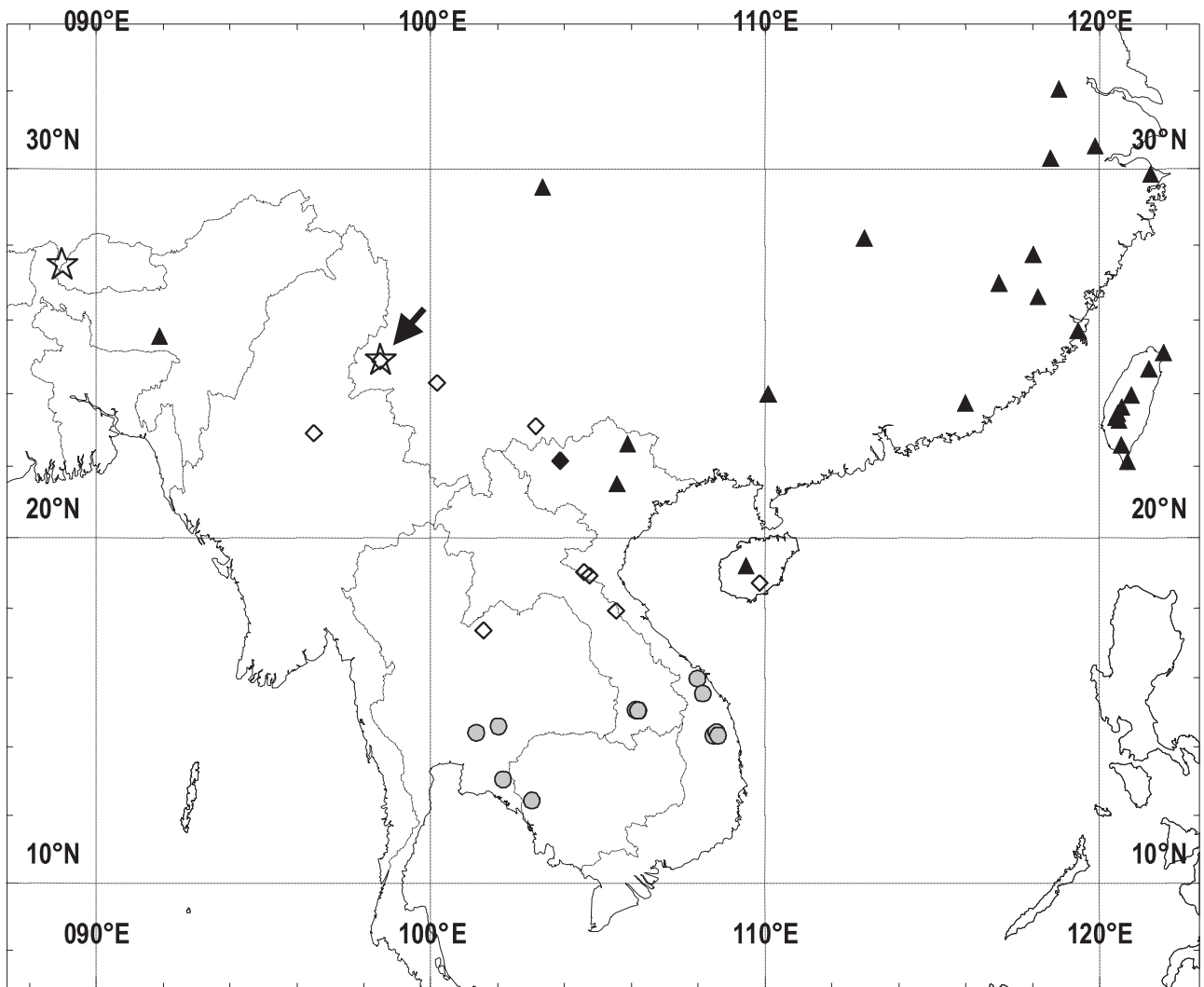


Figure 5. The revised distribution of species of *T. stejnegeri* complex, showing the distribution of the three species confirmed by DNA analysis (*T. vogeli*: ○, *T. gumprechtii*: ◇, *T. stejnegeri* s.s. ▲), and the putative species *T. yunnanensis* (☆). Note that two different taxa are present at the type locality of *T. yunnanensis* (indicated by an arrow). A specimen from Sapa, northern Vietnam, received after the analysis was completed and therefore not included, but which is known to group with *T. gumprechtii*, is indicated on the map by a solid diamond.

T. stejnegeri s.l. in the mid-late Miocene (15.6–7.3 Mya), and *T. stejnegeri* s.s. and *T. yunnanensis* diverged in the late Miocene to early Pliocene (8.9–4.2 Mya).

Since *Trimeresurus* s.s. is by no means among the most primitive pitvipers (Malhotra & Thorpe, 2000a), this suggests that the history of the crotalines extends further into the past than the fossil record indicates (see also Cadle, 1987; Vidal & Lecointre, 1998). During the period in which the divergence of the clades of the *T. stejnegeri* group was taking place, South-east Asia was already in the position it occupies today (Hall, 1998). A persistent, major montane connection from the tropics to northern latitudes is thought to have

existed throughout the Tertiary (Morley, 1998), although with a much reduced extent after about 20 Mya (early Miocene). The increasing fragmentation of the montane zone may have been the initial trigger for the divergence of the *T. stejnegeri* group, which presently occupies montane regions in the tropics and subtropics (between 10 and 32°N).

Other divergence events may have been linked with sea level changes that occurred throughout the Miocene and Pliocene, with low sea levels accompanied by increasing aridity and seasonality of the climate and high sea levels by moist, warm conditions with widespread tropical rain forests (Morley, 1998). In the Oligocene and earliest Miocene, dry conditions

prevailed, but pollen records show that rainforest vegetation extended as far north as Japan by the early to mid-Miocene (Morley, 1998), with repeated contractions and expansions throughout the mid- to late Miocene. Such fluctuations alter the availability of habitat, as well as its connectivity, and have frequently been implicated in the evolution of species complexes (Van Devender & Conant, 1990; Schneider, Cunningham & Moritz, 1998).

The sea level and climatic fluctuations that occurred during the Pleistocene (Heaney, 1991; Voris, 2000), on the other hand, have apparently not led to major differentiation in the group, although they may have affected their present distribution. The species in the group appear to be relatively adaptable with respect to habitat, but they undoubtedly require forested habitats. Although glaciation was never extensive in continental Asia, the cooler, drier climate would have caused extensive forest regression, with grassland in the lowlands and open woodland in the upland regions between 20 and 31°N (Adams & Faure, 1997). At lower latitudes, increase in connectivity of montane vegetation, which extended to lower elevations with apparently little decrease in rainfall, would have allowed extensive migration. The *T. stejnegeri* group may, however, have been prevented from migrating further south due to the presence of ecologically similar species already occupying that region (e.g. *T. popeiorum*), or by the presence of a drier equatorial belt (Adams & Faure, 1997). It remains the only major species group of *Trimeresurus* with no known representatives in the equatorial region. The expansion into China must have been a relatively recent event. However, the relatively large divergence between the haplotypes of northern Vietnam and China/Taiwan (between 6.7 and 2.9 Mya) argues for more than one Pleistocene refugium for Clade C (for example, the south-western Yunnan area seems to have retained a warm, moist climate through the last glacial maximum).

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APPENDIX 1

MUSEUM SPECIMENS EXAMINED FOR MORPHOLOGICAL ANALYSIS

Abbreviations

AFS: author's personal collection, field numbers; AMNH: American Museum of Natural History, New York; BMNH: The Natural History Museum, London; CAS: California Academy of Sciences, San Francisco; FMNH: Field Museum of Natural History, Chicago; MCZ: Museum of Comparative Zoology, Harvard; MHNG: Museum d'Histoire Naturelle Geneva; NMBA: Naturhistorisches Museum Basel; NMNS: National Museum of Natural Science, Taiwan; NMW: Naturhistorisches Museum Wien; QSMI: Queen Savabha Memorial Institute, Thailand; PLWRS: Phu Luang Wildlife Research Station, Thailand; PSGV: Gernot Vogel's private collection; RNHM: Rijksmuseum Van Natuurlijke Historie, Leiden; ROM: Royal Ontario Museum, Toronto; RTV: author's live collection; SNHM: Shanghai Natural History Museum; USNM: United States National Museum of Natural History, Smithsonian Institute, Washington.

T. stejnegeri group (arranged by composite locality, see Fig. 1)

1. Himalayas: BMNH 53.8.12.14;
2. NE India: BMNH 60.3.19.1121, BMNH 107.12.16.27, NMW 23805;
3. N Myanmar and Yunnan: BMNH 1901.4.26.7, FMNH 7064–5, BMNH 2002.53, BMNH 2002.54, NMNS 3113, NMNS 3653.
4. N China: SNHM 729159, MCZ 163259, AMNH 33222–9, BMNH 99.4.24.61, BMNH 54.2.10.18,

FMNH 25196–204, FMNH 170642, USNM 73140, NMNS 3651: 12347, 12349, 12351, 12354, NMW 23913: 1, CAS 71957, USNM 64022–3;
 5. Taiwan: FMNH 96807–11, FMNH 96816, FMNH 120772–5, NMNS 01882–7, NMNS 01889, NMNS 01334: 1–3, NMNS 01549, NMNS 01584, NMNS 01434, NMNS 01479, NMNS 01722, NMNS 01841, NMNS 01845;
 6. S China: SNHM 720068, SNHM 112–3;
 7. N Vietnam: NMW 23913: 3, ROM 35312–15, ROM 35318, ROM 35320–22, ROM 31066, ROM 31068, ROM 31072, AM99.15, AM99.17–18;
 8. Hainan: SNHM 500128–9, SNHM 720065, SNHM 720069–72;
 9. Annamite mountains: FMNH 255579–80, FMNH 256419;
 10. Central Vietnam: NMW 23913: 2, FMNH 11538, FMNH 252076, FMNH 252097, FMNH 252099, ROM 25403, ROM 30781–2, ROM 30785–6, ROM 30788, ROM 34559–61, ROM 35465, USNM 163967, ROM 30791;
 11. Bolovens Plateau: FMNH 258940–6;
 12. Cardamom mountains and SE Thailand: BMNH 2000.71, RNHM 16716: 1–2;
 13. Dongraek mountains: RTV9–10, AM99.5, FMNH 180242–44, FMNH 180247, FMNH 180256–61, FMNH 180263–5, FMNH 180269, FMNH 180272–4, FMNH 180277;
 14. NE Thailand: PLWRS 3–5, PLWRS 920503, AFS94.1, AFS94.3–18, AFS94.20;

APPENDIX 2

MORPHOLOGICAL CHARACTERS USED AND THEIR ABBREVIATIONS

(a) Scalation

VSC	number of ventral scales (VS), not including the anal scale, recorded by the Dowling (1951) method (i.e. the first VS is the one which contacts the first dorsal scale row on both sides).
SCS	number of pairs of subcaudal scales; any unpaired scales are treated as a pair.
USC	number of unpaired subcaudal scales.
BSCK	keeling of body scales (mid body); recorded as 0: none; 0.5: weak; 1: strong.
VENT	number of scale rows immediately anterior to the vent.
SUPLAB	average number of supralabials on the left and right hand side.
SUBLAB	average number of sublabials on the left and right hand side.
POSTOC	number of postocular scales.

PREOC	number of preocular scales.
BORSUPOC	number of scales bordering the supraocular scales (average of right and left), not counting pre- or postoculars.
BTWSUPOC1	minimum number of scales between the supraoculars.
BTWSUPOC2	number of scales between the posterior edge of the supraoculars.
DIVSUPOC	number of sutures dividing up the supraoculars.
NASPIT	number of scales between the nasal scale and the shield bordering the pit anteriorly.
INTNAS	number of scales separating the internasal scales.
WINTNASW	width of the internasals (in mm).
LAB3	minimum number of scales separating 3rd supralabial and subocular.
LAB4	minimum number of scales separating 4th supralabial and subocular.
LAB5	minimum number of scales separating 5th supralabial and subocular.
SOCBORD	number of scales contacting the subocular, not counting the scales immediately before and after it.
ROST	ratio of the anterior margin of the rostral scale to the posterior margin.
KTEMP	keeling of the temporal scales.
KHEADSC	keeling of the scales on the back of the head.
VENTEDGE	number of scales between the edge of the mouth and the ventral scales, starting at and including the last sublabial.
VENT	number of scales around the body immediately anterior to the vent.

(b) Scale reduction formula

This is recorded as a series of characters, each referring to a specific reduction. Each position of the reduction has two characters, the dorsoventral (DV) position of the reduction (the lowest of the two merging scale rows), and the ventral scale (VS) position (counted from the head), which is the ventral scale to which the scale reduction traces diagonally. Before analysis, the VS position was transformed into the percentage of the total number of ventral scales (%VS), to control for variation. (Subcaudal scale position: SC.)

VS31to29: ventral scale position of the reduction from 31 to 29 scale rows. DV31to29: dorsoventral position of the reduction from 31 to 29 scale rows. Then VS29to27, DV29to27, VS27to25, DV27to25, VS25to23, DV25to23,

VS23to21, DV23to21, VS21to19, DV21to19, VS19to17, DV19to17, VS17to15, DV17to15, SC12to10, DV12to10, SC10to8, DV10to8, SC8to6, DV8to6, SC6to4, DV6to4.

(c) *Body dimensions*

All measurements were made on the right side of the head only, unless this was damaged, in which case they were made on the left.

SVL	distance between the tip of the snout and the cloaca.
TAIL	distance between the anterior edge of the first subcaudal scale and the tip of the tail.
WHEAD	width of the head measured between the outer edges of the supraoculars.
LHEAD	length of the head, between the tip of the snout to the posterior edge of the lower jawbone.
DEYE	diameter of the eye measured between the edges of the scales surrounding it.
EYE2NOS	distance between the eye and the nostril, measured between the suture between the second and third preocular (from the bottom) and the inner edge of the nostril.
PIT2EYE	distance between the eye and the pit, measured between the inner edges, along the suture between the first and second preocular scales.
NOS2PIT	distance between the pit and the nostril, measured between the outer edges.
WSUPOC	the width of the supraoculars measured in mm, at the widest part.
LSUPOC	the length of the supraoculars measured in mm.

(d) *Internal characters*

Ventral and subcaudal scale positions (VSP, SCP) transformed to percentage before analysis (see scale reductions).

PAL	number of palatine teeth.
PTERY	number of pterygoid teeth.
DENT	number of dentary teeth.
HTANT	VSP thyroid gland.
HTPOST	VSP posterior tip of the heart.
LVANT	VSP anterior tip of the liver.
LVPOST	VSP posterior tip of the liver.
RKANT	VSP anterior tip of the right kidney.
RKPOST	VSP posterior tip of the right kidney.

LKANT	VSP anterior tip of the left kidney.
LKPOST	VSP posterior tip of the left kidney.
RTANT	VSP anterior tip of the right testis (males only).
RTPOST	VSP posterior tip of the right testis.
LTANT	VSP anterior tip of the left testis.
LTPOST	VSP posterior tip of the left testis.
CLOPOST	SCP posterior tip of the cloacal glands (females only).
RETRACT	SCP insertion of the hemipenis retractor muscle (males only).

(e) *Colour pattern*

STRIPE	presence of stripe covering dorsal scale row one (0: absent; 1: indistinct; 2: distinct).
SCRSTR	number of scale rows involved in stripe.
OCSTRIPE	presence of postocular stripe (0: absent; 1: indistinct; 2: distinct).
SCROC	number of scale rows involved in postocular stripe.
LIPCOL	number of scales above lip covered by ventral colour.
DORSPOT	number of spots on the dorsal surface.
SPOTSIZE	mean number of scales covered by the three largest dorsal spots.
SCR1	proportion of the first scale row covered by the light area.

APPENDIX 3

LISTS OF CHARACTERS INCLUDED IN MORPHOMETRIC ANALYSES

Females: VSC, SCS, VS31to29, VS29to27, VS27to25, VS25to23, DV25to23, VS21to19, VS19to17, DV19to17, DV17to15, DV12to10, SC12to10, SC10to8, SC6to4, SUBLAB, BORSUPOC, BTWSUPOC2, INTNAS, ROST, KHEADSC, VENTEDGE, WINTNAS, DEYE, STRIPE, SCRSTR, OCSTRIPE, LIPCOL, SCR1, PTERY, DENT, LVANT, RKANT.

Males: VSC, SCS, BSCK, VS31to29, VS27to25, DV27to25, VS23to21, VS21to19, DV19to17, VS17to15, DV17to15, SC6to4, SUPLAB, SUBLAB, BTWSUPOC1, BTWSUPOC2, INTNAS, SOCBORD, KHEADSC, ROST, VENTEDGE, WSUPOC, WINTNAS, DEYE, LHEAD, WHEAD, NOS2PIT, TAIL, OCSTRIPE, DORSPOT, SCR1, PTERY, DENT, LVANT, RKANT, LKPOST, RTANT, RETRACT.