

Ecological diversification in a group of Indomalayan pitvipers (*Trimeresurus*): convergence in taxonomically important traits has implications for species identification

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Abstract

We analyse molecular and phenotypic evolution in a group of taxonomically problematic Indomalayan pitvipers, the *Trimeresurus sumatranus* group. Mitochondrial DNA sequencing provides a well-resolved phylogeny, with each species representing a distinct lineage. Multivariate morphological analysis reveals a high level of phenotypic differentiation, which is congruent between the sexes but does not reflect phylogenetic history. An adaptive explanation for the observed pattern of differentiation is supported by independent contrasts analysis, which shows significant correlations between current ecology and the characters that most account for the variation between taxa, including those that are presently used to identify the species. Reduced precipitation and altitude, and increased temperature, are correlated with higher numbers of scales on the head, body and tail. It is hypothesized that scale number plays an important role in heat and water exchange by influencing the area of exposed of interstitial skin, and that colour pattern variation reflects selection pressures involving camouflage and thermoregulation. Ecological convergence in traits used for classification is found to have important implications for species identification where taxa are distributed over varying environments.

Introduction

Despite the increased availability of molecular systematic techniques that allow phylogeny to be inferred independent of phenotype, most species are still described on the basis of morphological traits of unknown phylogenetic content. When shared ancestry is taken into account in comparative studies of character variation, the evolutionary forces involved in phenotypic differentiation can be assessed by the distribution of traits within a group of taxa in relation to current ecological factors (Felsenstein, 1985; Harvey & Purvis, 1991; Garland, 1992; Bauwens *et al.*, 1995; Martins & Hansen, 1997; Kohlsdorf *et al.*, 2001). Ecological convergence in traits used for classification can confuse species identification

in groups that are widely distributed over varying environments. Comparative studies can provide an indication of the utility of morphological characters used in classification, and can lead to new hypotheses concerning the adaptation of organisms to their environments (Brooks & McLennan, 1991).

Numerous studies of geographic variation in reptilian scalation and colour pattern have been reported in the literature. However, only a minority have addressed the role of natural selection in the observed pattern of variation, and most of these have been carried out at the intraspecific level on island lizard populations (e.g. Thorpe, 1989, 1991; Losos *et al.*, 1994; Malhotra & Thorpe, 1997). Such investigations have elucidated the relative roles of ecogenetic and phylogenetic factors in the variation of island populations, and have revealed correlations between morphological characters and various facets of the environment, including temperature, rainfall, altitude and vegetational type. Additional studies have shown that adaptive differences can arise very

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quickly at the population level. This has been observed in *Anolis* lizards, in which scalation and colour pattern characters were found to be prone to rapid adaptive change in response to change of ecotype (Malhotra & Thorpe, 1993).

The *Trimeresurus sumatranus* group of Indomalayan pitvipers provides an excellent opportunity to investigate the role of ecological divergence in differentiation at the interspecific level. The taxa are closely related, yet have isolated gene pools because of their allopatric distribution across an environmentally varying archipelago (Malhotra & Thorpe, 2004). In this study, we use mtDNA sequences to infer phylogenetic relationships in the *T. sumatranus* group, and elucidate the pattern of phenotypic differentiation between taxa using multivariate analysis on scalation and colour pattern characters. The characters that account for most of the variation in the morphological analysis, and those that are currently used to identify the species, are then subjected to the method of phylogenetically independent contrasts (Felsenstein, 1985). Our aims are to investigate the role of environmental factors in the differentiation of taxa, and to assess the implications of ecological adaptation in taxonomically important traits for species identification. Felsenstein's independent contrasts analysis (Felsenstein, 1985) is a standard phylogenetic method and has proved useful in detecting correlations in numerous comparative studies at the interspecific level (Garland *et al.*, 1993; Martins *et al.*, 2001).

Materials and methods

Model group

The *T. sumatranus* group comprises five nominal species, which are widely distributed over the Indomalayan archipelago (Fig. 1) and display considerable morphological and ecological diversity. They are: *T. sumatranus*, *T. hageni*, *T. malcolmi*, *T. schultzei* and *T. flavomaculatus*. These species are arboreal and oviparous, occurring almost exclusively in undisturbed rainforests. There is substantial variation in the forest types they occupy, reflecting the latitudinal range of the complex. *Trimeresurus schultzei*, *T. f. flavomaculatus* and *T. f. mcgregori* occupy seasonal rainforests in the Philippine Islands, which receive consistently lower, more periodic rainfall than those in equatorial Indonesia (2000–3000 mm year⁻¹ vs. 3000–4000 mm year⁻¹) (Whitmore, 1975; Anon., 1995). Moreover, whereas *T. sumatranus* from Borneo, *T. hageni*, *T. schultzei*, *T. f. flavomaculatus* and *T. f. mcgregori* are rarely found above 300 m (David & Vogel, 1996; Cox *et al.*, 1998; Stuebing & Inger, 1999), *T. malcolmi* is found only in montane rainforest over 1000 m (Stuebing & Inger, 1999), and the Sumatran population of *T. sumatranus* occurs between 650 and 800 m (unpublished data). The species also differ in prey utilization; *T. sumatranus*, *T. hageni*, *T. malcolmi* and *T. f. mcgregori* feed mainly on mammals, whereas *T. f. flavomaculatus* seems to feed exclusively on amphibians and the diet of *T. schultzei* includes lizards and frogs (unpublished data).

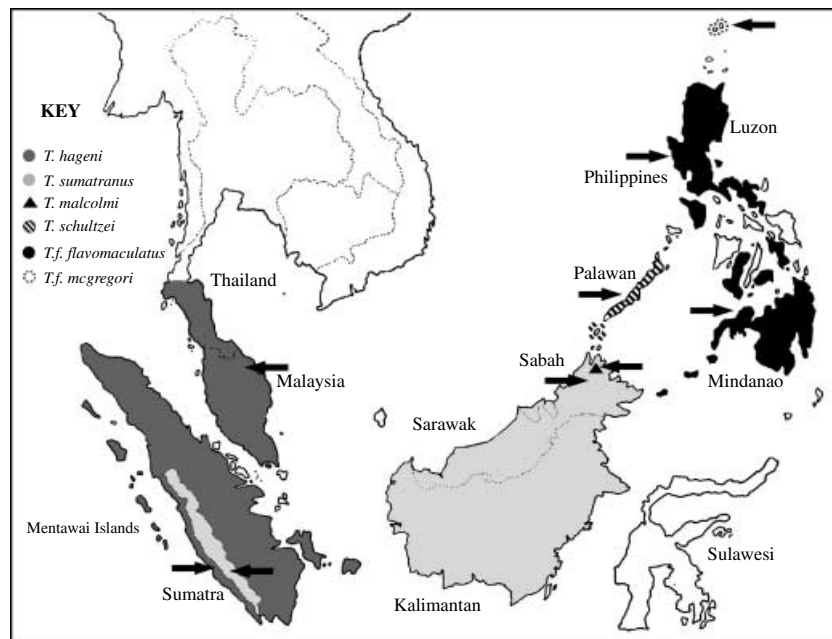


Fig. 1 Distribution of the *Trimeresurus sumatranus* group in the Indomalayan archipelago. Black arrows indicate geographic origin of specimens used in phylogenetic analysis.

The systematics of the group, and the precise distribution of certain species, is an area of long-standing confusion. The distinction between *T. sumatranus* and *T. hageni* is especially problematic (Sanders *et al.*, 2002), and most workers assign both species to *T. sumatranus* by default (Tweedie, 1983; Lim, 1991; Jintakune & Chanhome, 1995; David & Vogel, 1996). The status of *T. malcolmi* is also an area of contention; this taxon was recently elevated from a subspecies of *T. sumatranus* on the basis of scalation and colour pattern (Stuebing & Inger, 1998). *Trimeresurus flavomaculatus* occurs over much of the Philippine Islands and comprises three subspecies: *T. f. halieus*, *T. f. mcgregori* and *T. f. flavomaculatus* (Leviton, 1963). *Trimeresurus f. halieus* is not generally recognized as a valid subspecies. However, many workers treat *T. f. mcgregori* as a full species because of striking differences in colour pattern (individuals are frequently yellow or grey, always lacking green pigmentation) (Gumprecht, 2002) and extreme isolation on the Batanes islands, which lie 130 miles north of Luzon and have never been connected to the mainland (Leviton, 1963).

DNA preparation, amplification and sequencing

Blood or tissue samples were obtained from wild-caught and captive specimens (of known locality) of *T. sumatranus* from Sabah and west Sumatra, *T. hageni* from West Malaysia and west Sumatra, *T. malcolmi* from Mt Kinabalu (Sabah), *T. schultzei* from Palawan, *T. f. flavomaculatus* from Luzon and Mindanao and *T. f. mcgregori* from Batanes (Table 1 and Fig. 1). Blood samples were taken from the caudal vein, placed in 1 mL 0.1 M ethylenediaminetetraacetic acid (EDTA), and stored in sodium dodecyl sulphate (SDS)-Tris buffer (2% SDS, 100 mM Tris); liver and muscle tissue was preserved in 80%

ethanol. Whole genomic DNA was extracted using standard proteinase K protocols (Sambrook *et al.*, 1989).

Four mitochondrial genes were amplified via polymerase chain reaction (PCR): these were cytochrome *b* (*cytb*, 750 bp), NADH4 dehydrogenase subunit 4 (650 bp), 12S ribosomal RNA (400 bp) and 16S large subunit ribosomal RNA (500 bp). *Cytb* sequences were obtained as described in Malhotra & Thorpe (2000). NADH dehydrogenase subunit 4 (ND4) sequences were obtained as described in Parkinson *et al.* (2000), 12S rRNA as described in Knight & Mindell (1993), and 16S rRNA as in Parkinson *et al.* (1997).

Thermal cycling parameters for *cytb* and ND4 were denaturation at 94 °C for 3 min followed by 35 cycles of: denaturation at 93 °C for 1 min, annealing at 48 °C for 2 min and extension at 72 °C for 2 min. At the end of the reaction there was a final extension step at 72 °C for 3 min. Amplification conditions for 12S and 16S were denaturation at 94 °C for 2 min followed by 30 cycles of: denaturation at 94 °C for 2 min, annealing at 45 °C for 0.5 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 3 min.

The PCR products were concentrated and purified using Wizard minicolumns (Promega UK Ltd, Southampton, UK). Single-stranded sequencing was then carried out using dye-labelled terminators (ABI PRISM™, BigDye™, Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Warrington, UK) and run on an ABI 377 DNA sequencer.

Phylogenetic analyses

Outgroups were selected to represent three *Trimeresurus* species groups (*sensu* Malhotra & Thorpe, 2000); these were *T. insularis*, *T. stejnegeri* and *T. vogeli*. Alignment of

Table 1 Localities and GenBank accession numbers for sequences used in this study.

| Sample | Author's catalogue numbers | Geographic origin | Accession numbers | | | |
|---------------------------------------|----------------------------|-----------------------|-------------------|----------|----------|----------|
| | | | Cytb | ND4 | 12S | 16S |
| <i>Trimeresurus insularis</i> | B7 | Java | AY059568 | AY059586 | AY059534 | AY059550 |
| <i>Trimeresurus vogeli</i> | B9 | Taiwan | AY059574 | AY059596 | AY059546 | AY059562 |
| <i>Trimeresurus stejnegeri</i> | A100 | Nepal | AF171909 | AY059592 | AY059543 | AY059559 |
| <i>Trimeresurus hageni</i> | B131 | West Malaysia | AY371826 | AY371868 | AY371761 | AY371787 |
| <i>Trimeresurus hageni</i> | B229 | West Malaysia | AY371827 | AY371867 | AY371755 | AY371794 |
| <i>Trimeresurus hageni</i> | B360 | Bengkulu, Sumatra | AY371829 | AY371862 | AY371764 | AY371789 |
| <i>Trimeresurus hageni</i> | B364 | Bengkulu, Sumatra | AY371825 | AY371863 | AY371763 | AY371790 |
| <i>Trimeresurus sumatranus</i> | B347 | Sabah, Borneo | AY371823 | AY371859 | AY371759 | – |
| <i>Trimeresurus sumatranus</i> | B348 | Sabah, Borneo | AY371828 | AY371866 | AY371760 | AY371788 |
| <i>Trimeresurus sumatranus</i> | B367 | Bengkulu, Sumatra | AY371864 | AY371824 | AY371765 | AY371791 |
| <i>Trimeresurus sumatranus</i> | B368 | Bengkulu, Sumatra | AY371865 | AY371830 | AY371762 | AY371792 |
| <i>Trimeresurus malcolmi</i> | B295 | Sabah, Borneo | AY371822 | AY371860 | AY371758 | AY371793 |
| <i>Trimeresurus malcolmi</i> | B349 | Sabah, Borneo | AY371832 | AY371861 | AY371757 | AY371786 |
| <i>Trimeresurus f. flavomaculatus</i> | B3 | Luzon, Philippines | AF171916 | AY059584 | AY059535 | AY059551 |
| <i>Trimeresurus f. flavomaculatus</i> | B4 | Mindanao, Philippines | AY352764 | AY352830 | AY352796 | A7352734 |
| <i>Trimeresurus f. mcgregori</i> | B290/4 | Batanes, Philippines | AY371831 | AY371858 | AY371756 | AY371795 |
| <i>Trimeresurus schultzei</i> | B210 | Palawan, Philippines | AY352756 | AY352819 | AY352785 | AY352725 |

cytb 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 region of 16S because of insertions found in some of the new sequences obtained. The coding genes were translated into amino acid sequences to check for the presence of stop codons that might indicate that pseudogenes had been amplified. The four mitochondrial genes were combined into a single data set; mitochondrial genes are inherited as a single linkage group and as such do not provide independent estimates of phylogeny (Moore, 1995; Page, 2000). In addition, an increased number of genes are likely to provide a higher number of potentially variable sites for phylogenetic analysis (Chippindale & Wiens, 1994; Cummings *et al.*, 1995). A model of molecular evolution was assigned to the combined data set using the log likelihood function of MODELTEST 3.0 (Posada & Crandall, 1998). MODELTEST compares 56 different nested substitutional models and uses log likelihood scores to infer the simplest best-fit model of evolution. Corrected pairwise sequence comparisons were then made in MEGA version 2.1 (Kumar *et al.*, 2001) using the model of evolution identified by MODELTEST.

PAUP* 4.0b8 (Swofford, 1998) was used for all further phylogenetic analysis. Maximum likelihood (ML) analysis, using a heuristic search with a starting tree obtained by 10 random additions of taxa and tree-bisection-reconnection (TBR) branch swapping, was conducted on the parameters identified by MODELTEST. The parameters were then re-estimated from the resulting tree and used in a new ML analysis. A total of 500 bootstrap replicates using the same options were performed to assess node support. A maximum parsimony (MP) tree was also produced using the same search options as in the ML analysis. All sites were equally weighted, and 1000 bootstrap replicates were performed.

Multivariate morphometrics

We examined 143 specimens from museum collections in the United States, Europe, the Philippine Islands, Indonesia and Malaysia. Specimens were grouped into operational taxonomic units (OTUs) on the basis of allopatric populations. *Trimeresurus hageni* was represented by two OTUs, one comprising specimens from Thailand, West Malaysia and Singapore and another comprising specimens from south Sumatra. *Trimeresurus sumatranus* was represented by OTUs from Borneo (Sabah and Sarawak) and west Sumatra. *Trimeresurus schultzei* was represented by specimens from Palawan and Balabac island. *Trimeresurus f. flavomaculatus* was represented by separate OTUs of specimens from Luzon and Mindanao. *Trimeresurus f. mcgregori* was represented by specimens from the Batanes islands. *Trimeresurus malcolmi* corresponds to specimens from Mt Kinabalu (Sabah). The OTUs

used and their sample size for each sex are listed in Table 2.

A total of 93 characters relating to scalation and colour pattern were recorded for each specimen. Ventral scales were counted from head to vent, with the first ventral identified according to the method of Dowling (1951). The positions of scale reductions along the body (recorded as the number of the ventral or subcaudal scale opposite which it was situated) were transformed to percentage ventral scale (%VS) or subcaudal scale (%SC) position, in order to compensate for variation in VS and SC number. Male and female specimens were treated separately in all analyses to avoid bias caused by sexual dimorphism.

Variation between OTUs was tested for individual characters by means of one-way ANOVA. Only characters showing significant between OTU variation were used in subsequent analyses. These are highlighted in Appendix I. Principal components analysis (PCA) was used to check for the possible inclusion of sympatric species within OTUs. Canonical variate analysis (CVA) was then used to investigate multivariate patterns of geographic variation between OTUs (Thorpe, 1976, 1983). Characters that are invariable in some OTUs and variable in others violate the assumptions of CVA, and were excluded from further analyses at this stage. CVA maximizes the separation between groups relative to variation within-groups, and has been applied successfully to numerous models of geographic variation.

Independent contrasts

We analysed our morphological data in a phylogenetic context, using Felsenstein's (1985) method of independent contrasts in the PDTREE program of Garland *et al.* (1999). Because of the hierarchical nature of phylogenetic relationships, closely related taxa are more likely to share characteristics through descent from common ancestors than distantly related species (Garland, 1992).

Table 2 List of operational taxonomic units (OTUs) and sample size for each sex.

| Species | Sample size | |
|---|-------------|---------|
| | Males | Females |
| <i>Trimeresurus hageni</i> (Thailand, West Malaysia, Singapore) | 16 | 15 |
| <i>Trimeresurus hageni</i> (Sumatra) | 5 | 3 |
| <i>Trimeresurus sumatranus</i> (Borneo) | 7 | 23 |
| <i>Trimeresurus sumatranus</i> (Sumatra) | 5 | 10 |
| <i>Trimeresurus schultzei</i> | 8 | 4 |
| <i>Trimeresurus malcolmi</i> | 3 | 3 |
| <i>Trimeresurus flavomaculatus</i> (Mindanao) | 3 | 4 |
| <i>Trimeresurus flavomaculatus</i> (Luzon) | 9 | 9 |
| <i>Trimeresurus f. mcgregori</i> (Batanes Is) | 4 | 11 |
| Total | 61 | 82 |

The method of independent contrasts attempt to account for this lack of independence in cross-species data sets; correlations between ecology and phenotype are estimated using information on the amount of character change between pairs of closely related species since their most recent shared ancestor. The values of N species are transformed into $N - 1$ statistically independent and identically distributed contrasts (Garland, 1992).

Overall phenotype (derived from all characters generalized on CV1), maximum SVL, the characters most important in the canonical variate analyses and the characters presently used to distinguish the species were subjected to independent contrasts analysis. These data were analysed for nine allopatric populations (*T. hageni* from West Malaysia; *T. hageni* and *T. sumatranus* from Sumatra; *T. sumatranus* from Borneo; *T. malcolmi*; *T. schultzei* and *T. f. flavomaculatus* from Luzon; *T. f. flavomaculatus* from Mindanao; and *T. f. mcgregori*), using the ML tree. Mean values were used for scalation characters; maximum snout-to-vent length (SVL) was estimated from our data and the published literature (Taylor, 1919; Loveridge, 1938). Mean annual precipitation and temperature data were obtained from on-line world climate databases (Buttle & Tuttle Ltd, 2001; Qwikcast, 2002). The upper limit of the range of each population was used for analyses involving altitude; this was taken from data collected in the field and the published literature (Taylor, 1919; Loveridge, 1938; Stuebing & Inger, 1999).

Reported correlation coefficients and significance values refer to those obtained from regression of standardized contrasts based on the ordinary least-squares method, using phylogenetically independent contrasts. Statistical tests are two-tailed. Results are Bonferroni corrected nonsequentially by row. Diagnostic plots (Garland, 1992) of the absolute values of standardized contrasts vs. their SD (square root of sums of branch lengths) were checked to assess the adequacy of branch lengths for standardization of independent contrasts. The adequacy of the branch lengths used is confirmed in all analyses other than those involving precipitation, in this case branch lengths are \log_{10} transformed to properly standardize independent contrasts (Garland, 1992; Garland *et al.*, 1999).

Results

Preliminary sequence analysis

A total of 2424 bp was used to represent 13 ingroup accessions and four outgroup taxa in the final phylogenetic analysis (GenBank accession numbers are given in Table 1). These contained 638 variable sites, of which 401 (16.5% of all sites) were parsimoniously informative. There was no indication that pseudogenes had been amplified as no stop codons or indels were found in either of the protein coding genes. The substitutional model of evolution assigned by MODELTEST was TrN

(Tamura & Nei, 1993) with a γ -distance shape parameter of 0.8838.

Phylogenetic relationships

Maximum likelihood and MP analyses of the combined data set resulted in identical tree topologies, with generally high bootstrap support that is similar in the two analyses (Fig. 2). The log likelihood score for the final ML tree was $-\ln 8200.22$, and was obtained using the Tamura-Nei model with γ -distributed rates. MP analysis revealed a single most parsimonious tree with a length of 1071, a consistency index (CI) of 0.65, a retention index (RI) of 0.71 and a rescaled consistency index (RC) of 0.47.

Our analyses reveal five clearly distinct lineages, corresponding to currently recognized species. *Trimeresurus hageni* diverges earliest in the group, followed by *T. flavomaculatus*, and then *T. malcolmi*; *T. schultzei* and *T. sumatranus* are sister species. *Trimeresurus f. flavomaculatus* from Mindanao diverges earlier than *T. f. flavomaculatus* from Luzon and *T. f. mcgregori*, which are weakly supported as sister taxa.

Pairwise sequence differences were estimated using the Tamura Nei model with Gamma correction (Tamura & Nei, 1993). Mean levels of sequence divergence between ingroup species ranged from 7.9 ± 0.9 (between *T. schultzei* and Bornean *T. sumatranus*) to $12.8 \pm 1.4\%$ (between *T. hageni* and *T. sumatranus*), and from 13.0 ± 1.0 to $15.4\% \pm 1.2\%$ between ingroup and outgroup taxa. Within-locality sequence divergence was low: $0.3 \pm 0.2\%$ between *T. hageni* from West Malaysia,

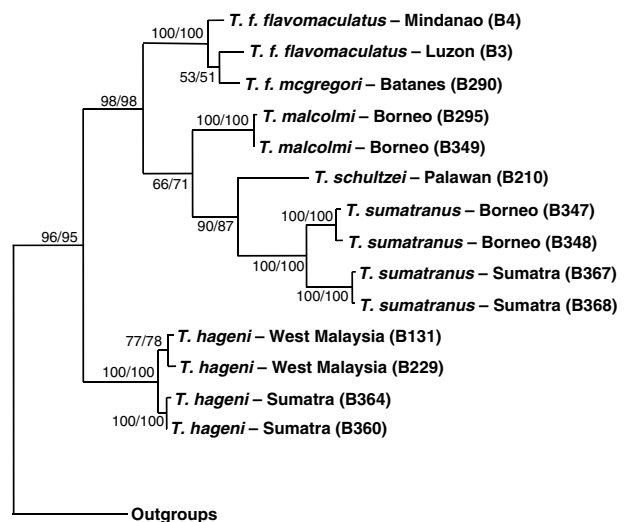


Fig. 2 Mitochondrial gene tree derived from maximum likelihood (ML) analysis of combined data (2424 bp), with bootstrap support values for ML and maximum parsimony trees, respectively, reading left to right.

0.2 ± 0.1% between *T. hageni* from Sumatra, 0.1 ± 0.1% between *T. sumatranus* from Sumatra, 0.6 ± 0.2% between *T. sumatranus* from Borneo, and 0.1 ± 0.01% between *T. malcolmi*. Sequence differences between allopatric populations were 1.0 ± 1.3% between *T. hageni* from West Malaysia and Sumatra; 3.3 ± 1.5% between *T. sumatranus* from Borneo and Sumatra; 1.9 ± 1.4% between *T. f. flavomaculatus* from Luzon and Mindanao, and 1.6 ± 1.4% between *T. f. flavomaculatus* from Luzon and *T. f. mcgregori*.

Morphological variation

The CVA analysis revealed a pattern of morphological variation that is generally congruent between males and females (Fig. 3). Most taxa are clearly distinct, with strong separation between *T. hageni* and *T. sumatranus*, and between these two species and *T. flavomaculatus*. *Trimeresurus malcolmi* is most similar to *T. sumatranus*, and *T. schultzei* is closest to *T. f. flavomaculatus*.

Conspecific OTUs of *T. hageni*, *T. sumatranus* and *T. f. flavomaculatus* show weak differentiation in this analysis, although the separation between these group is clearer in males than in females. *Trimeresurus f. mcgregori* is strongly differentiated from *T. f. flavomaculatus* in males, but is weakly differentiated in the analysis of females.

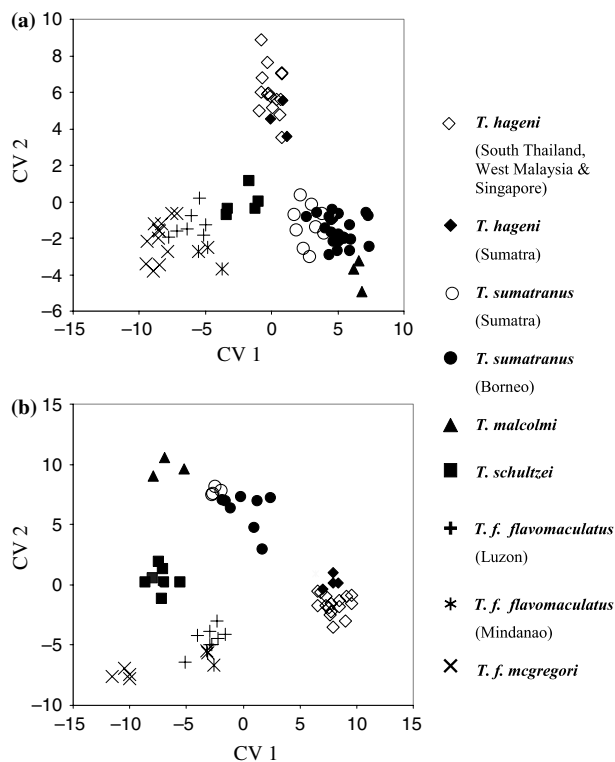


Fig. 3 Canonical variate analysis of the *Trimeresurus sumatranus* group (a = males; b = females).

The CVA can be used to identify the characters that account for most variation between groups (Table 3). The characters with highest eigenvector coefficients for the first two canonical variables are the minimum and maximum number of scales separating the supraocular scales, the number of scales between the nasal scale and the shield bordering the pit anteriorly, the number of internasal scales, the position of scale reductions (on the body) from 17 to 15 scale rows, and (on the tail) from 10 to 8 rows, the number of scales bordering supraoculars, the number of scales separating the fourth supra-labial from the subocular scale, and the presence of dark edges on head and body scales.

Independent contrasts

Phylogenetically independent contrasts reveal significant correlations between all three ecological factors and most of the characters tested in males and females (Table 4). In addition, generalized phenotype is significantly correlated with precipitation in males (Table 4). Mean annual precipitation has a negative influence on head scale number, tail scale reduction formula and the presence of a stripe, but is positively correlated with dark edges on head and body scales. Altitude is negatively correlated with scale counts and the presence of a stripe, and is positively correlated with maximum SVL in males, although not in females. Altitude is also positively correlated with dark edges on head and body scales. Mean annual temperature is positively correlated with scale number for several head scale characters, scale reduction on the body and the presence of a stripe, but is negatively correlated with dark edges on head and body scales. In general, reduced precipitation and altitude, and increased temperature, are correlated with higher number of scales on the head and cause scale reductions to occur closer to the head and vent, resulting in fewer scales for more of the body and tail length. Dark edges on head and body scales increase with precipitation and altitude, and decrease with temperature; the opposite trend is found for the presence of a stripe on dorsal scale row 1.

Discussion

Phylogeny and systematics

This study reveals a well-resolved phylogeny for the *T. sumatranus* group. Distinct interspecific divisions correspond to five well-separated lineages: *T. hageni* diverges earliest in the phylogenetic history of the group, followed by *T. flavomaculatus*, and then *T. malcolmi*; *T. schultzei* and *T. sumatranus* are sister species. Our data are also consistent with the species status of *T. malcolmi*, which was recently elevated from a subspecies of *T. sumatranus* on the basis of having fewer scale rows at mid-body and increased dark edges on scales (Stuebing & Inger, 1998).

Table 3 Standardized canonical variate coefficients for CV1 and CV2.

| Character | Males | | Females | |
|--|--------------|--------------|--------------|--------------|
| | CV1 | CV2 | CV1 | CV2 |
| Proportion of total variation explained (%) | 50 | 28 | 56 | 22 |
| Maximum number of scales separating the supraocular scales | 0.32 | -0.62 | 0.38 | -0.01 |
| Minimum number of scales separating the supraocular scales | 0.38 | 0.65 | -0.34 | -0.29 |
| Number of internasal scales | -0.51 | 0.21 | -0.07 | -0.29 |
| %SC position of reduction from 10 to 8 tail scale rows | 0.48 | -0.36 | 0.40 | 0.18 |
| %SC position of reduction from 6 to 4 tail scale rows | -0.36 | 0.29 | 0.06 | 0.40 |
| %VS position of reduction from 17 to 15 body scale rows | 0.11 | 0.50 | -0.49 | -0.12 |
| Number of sublabial scales | 0.12 | 0.12 | -0.49 | -0.26 |
| Number of supralabial scales | -0.37 | -0.37 | -0.23 | 0.10 |
| Number of scales separating the fifth supralabial scale from the subocular scale | 0.29 | 0.29 | 0.13 | -0.04 |
| Number of scales separating the fourth supralabial scale from the subocular scale | 0.83 | -0.46 | 0.42 | -0.01 |
| Number of scales bordering the supraocular scales | -0.62 | 0.04 | 0.37 | 0.07 |
| Number of scales between the nasal scale and the shield bordering the pit anteriorly | -0.65 | 0.69 | -0.04 | -0.12 |
| Number of spots on dorsal scale row one | 0.03 | -0.32 | 0.21 | -0.68 |
| Presence of dark edges on head scales | 0.67 | -0.13 | 0.02 | -0.40 |
| Presence of dark edges on body scales | -0.21 | -0.13 | -0.21 | 0.43 |

Characters with highest eigenvector coefficients on each CV are highlighted in bold.

% SC, percentage subcaudal scale; %VS, percentage ventral scale.

A varied level of divergence is found at the intraspecific level. Mainland and Sumatran populations of *T. hageni* represent recently diverged populations that are weakly differentiated by phenotype. A higher level of sequence divergence is found between *T. flavomaculatus* populations. *Trimeresurus f. mcgregori* and *T. f. flavomaculatus* are phenotypically distinct in males, but weakly differentiated in our analysis of females. However, when *T. flavomaculatus* is analysed separately from the remainder of the group, *T. f. mcgregori* forms the most distinct grouping in both sexes (not shown). Mean sequence divergence between *T. sumatranus* from Borneo and Sumatra is comparatively high ($3.3 \pm 1.5\%$). These groups also show phenotypic and ecological differences (the Bornean population occupies low altitudes, whereas the Sumatran population is not found below 650 m), and with further study they may qualify as separate species under the phylogenetic species concept (Cracraft, 1983) and the cohesion species concept (Templeton, 1989, 2001).

Adaptive evolution

The results of our multivariate analyses reveal a high level of morphological differentiation between most of the nominal species in the group. The pattern of phenotypic variation is largely congruent between the sexes, but clearly does not reflect the phylogenetic history revealed by the mitochondrial gene tree. *Trimeresurus malcolmi* and *T. sumatranus* are phenotypically very similar, despite their strong phylogenetic separation. *Trimeresurus schultzei* is similar in phenotype to *T. flavomaculatus*, but is most closely related to *T. sumatranus*, from which it is well-differentiated by phenotype.

An adaptive explanation for the observed pattern of differentiation is supported by independent contrasts analysis, which reveals significant correlations between current ecological conditions and most characters. A combination of selective pressures is likely to be involved in the diversification of the group. The skin plays an important role in both heat and water exchange in reptiles (Pough *et al.*, 2001), and our results are consistent with numerous studies that report trends of increasing scale number in drier, warmer environments (Klauber, 1941, 1972; Soulé & Kerfoot, 1972; Thorpe & Baez, 1987; Brown *et al.*, 1991; Malhotra & Thorpe, 1997). The reverse relationship has also been observed, with lizard populations occupying hot, dry habitats having larger (and therefore fewer) scales (Hellmich, 1951; Horton, 1972; Lister, 1976). However, these studies highlighted the surface area of scales in relation to radiation of heat; larger scales tend to be more sculptured and have higher surface areas. Keeling of the head and body scales was not found to vary significantly in the *T. sumatranus* group, and given that these species are nocturnal and occupy closed habitats, protection from excess heat and ultraviolet light is unlikely to be an important factor.

Cutaneous evaporation has been reported to be the primary avenue of water loss in reptiles (Bentley & Schmidt-Nielsen, 1966). In the *T. sumatranus* group, it may be that an increase in scale number results in a tighter fit between scales, reducing the surface area of exposed interstitial skin, and hence facilitating more efficient water retention in hotter, drier climates. This may be of particular relevance for animals that specialize in consuming large prey by distending the body, consequently increasing the surface area of exposed interstitial skin.

Table 4 *r*- and *P*-values from independent contrasts regression of morphological characters and generalized phenotype against ecological variables.

| Trait | Precipitation | | | | Altitude | | | | Temperature | | | |
|---|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| | Males | | Females | | Males | | Females | | Males | | Females | |
| | <i>r</i> -Value | <i>P</i> -value | <i>r</i> -Value | <i>P</i> -value | <i>r</i> -Value | <i>P</i> -value | <i>r</i> -Value | <i>P</i> -value | <i>r</i> -Value | <i>P</i> -value | <i>r</i> -Value | <i>P</i> -value |
| Minimum number of scales separating the supraocular scales | - | - | - | - | - | - | - | - | - | - | - | - |
| Maximum number of scales separating the supraocular scales | -0.8012 | <0.05 | - | - | - | - | - | - | - | - | - | - |
| Number of internasal scales | - | - | - | - | - | - | - | - | - | - | - | - |
| %SC position of reduction from 10 to 8 tail scale rows | - | - | - | - | - | - | - | - | - | - | - | - |
| %SC position of reduction from 6 to 4 tail scale rows | -0.6929 | <0.05 | - | - | - | - | - | - | - | - | - | - |
| %VS position of reduction from 17 to 15 body scale rows | - | - | - | - | - | - | - | - | - | - | 0.6469 | <0.05* |
| Number of sublabial scales | - | - | -0.6739 | <0.05* | -0.7546 | <0.05* | - | - | 0.8020 | <0.01 | 0.7511 | <0.05* |
| Number of supralabial scales | - | - | - | - | - | - | - | - | - | - | - | - |
| Number of scales separating the fifth supralabial scale from the subocular scale | -0.6634 | <0.05* | - | - | -0.7738 | <0.05* | 0.6654 | <0.05* | 0.7743 | <0.05* | - | - |
| Number of scales separating the fourth supralabial scale from the subocular scale | - | - | - | - | - | - | - | - | - | - | - | - |
| Number of scales bordering the supraocular scales | - | - | - | - | - | - | - | - | 0.6929 | <0.05* | - | - |
| Number of scales between the nasal scale and shield bordering the pit | - | - | - | - | - | - | - | - | - | - | - | - |
| Presence of dark edges on head scales | 0.7843 | <0.05* | 0.6406 | <0.05* | 0.8663 | <0.01 | 0.8032 | <0.01 | -0.8691 | <0.01 | -0.7936 | <0.05* |
| Presence of dark edges on body scales | 0.8115 | <0.01 | - | - | 0.8296 | <0.01 | 0.9089 | <0.01 | -0.8368 | <0.01 | -0.8877 | <0.01 |
| Presence of stripe on dorsal scale row 1 | -0.7466 | <0.05* | -0.6401 | <0.05* | -0.6904 | <0.05* | -0.7277 | <0.05* | 0.7752 | <0.05* | 0.7789 | <0.05* |
| Number of dorsal body spots | - | - | - | - | - | - | - | - | - | - | - | - |
| Number of scales above supralabials covered by ventral colour | - | - | - | - | - | - | - | - | - | - | - | - |
| Maximum snout-to-vent length | - | - | - | - | 0.7662 | <0.05* | - | - | -0.7898 | <0.05* | - | - |
| Generalized phenotype | 0.7334 | <0.05* | - | - | - | - | - | - | - | - | - | - |

Significant values are Bonferroni-corrected by row (* $P < 0.05/0.06$).

% SC, percentage subcaudal scale; %VS, percentage ventral scale.

Maximum SVL is increased in males at higher altitudes and lower temperatures, reducing sexual size dimorphism in the two montane taxa (the female is larger in all species). Endotherms occupying cooler environments tend to attain larger body sizes, which facilitate heat retention due to a reduction of the surface area to body volume ratio. However, a converse trend is observed in ectotherms, in which smaller body sizes are generally associated with cooler environments (Mousseau, 1997). Exceptions are known, including the western rattlesnake, *Crotalus viridis*, which attains larger sizes in cooler, more seasonal environments (Ashton, 2001).

Colour pattern characters also reflect adaptation to the local environment. An increase in the dark edges on head and body scales is most strongly associated with high altitude, cooler habitats in which darker pigmentation may provide a thermoregulatory advantage. Vegetational type is dependent on local climate, and habitat-driven selection for camouflage is also likely to be important given that these species are ambush predators that rely on cryptic colour and pattern.

Adaptation to differences in local climate may account for much of the taxonomic confusion among the taxa. Several of the characters presently used to distinguish the species are correlated with current ecology. These include the traits used to distinguish *T. hageni*, *T. sumatranus* and *T. malcolmi*: the number of scales separating the fifth supralabial from the subocular scale and the presence of dark edges on head and body scales (Lidth de Jeude, 1886; Brongersma, 1933; Stuebing & Inger, 1998); the characters used to distinguish between *T. hageni* and *T. sumatranus* and between *T. schultzei* and *T. flavomaculatus*, including the number of sublabial scales and the number of scales bordering supraoculars (Griffin, 1909; Taylor, 1919; Brongersma, 1933; Leviton, 1963); and the positions of scale reductions on the body, used to distinguish *T. malcolmi* from *T. sumatranus* (Stuebing & Inger, 1998).

Ecological adaptation has led to convergence between *T. hageni* and *T. sumatranus* where they overlap in range. In Sumatra, *T. hageni* share traits thought to be characteristic of *T. sumatranus*, including lowered scale counts on the head and dark edges on the head and body scales. Phenotypic convergence is especially pronounced in some of the Mentawai Island populations, and has led to confusion in Siberut where *T. hageni* has dorsal crossbands, and in Nias where the species has head and body scales that are edged in black to the same extent as found in *T. sumatranus* (Sanders *et al.*, 2002).

This example demonstrates that ecological convergence in traits used for classification can confuse species identification. However, by using multivariate methods on a broad range of morphological characters we were able to effectively delimit the species' boundaries. Therefore, although we do not suggest that morphological distinctiveness is an inappropriate criterion for species status, we emphasize the potential for error in basing

taxonomic decisions on few morphological characters that are of unknown phylogenetic utility.

In this paper, we have shown that ecological adaptation plays an important role in the phenotypic diversification of the *T. sumatranus* group. Most of the scalation and colour pattern characters that best account for the variation between taxa reflect some facet of the current environment, and may involve selection pressures relating to heat and water exchange, camouflage requirements and thermoregulation. Our study also shows that convergence in taxonomically important traits can lead to confusion in species identification, and is of particular relevance to taxonomically problematic groups that are widely distributed over varying environments.

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Note added in proof

Since this article was accepted, the taxonomy of *Trimeresurus* has been revised (Malhotra & Thorpe, 2004) and the *T. sumatranus* species group has been referred to the genus *Parias* Gray, 1849.

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Appendix I Characters used for multivariate analysis of *Trimeresurus sumatranus* group.

| Characters | Males | Females |
|--|-------|---------|
| Number of subcaudal scales | * | * |
| %VS position of reduction from 17 to 15 body scale rows | * | * |
| %SC position of reduction from 10 to 8 tail scale rows | * | * |
| %SC position of reduction from 8 to 6 tail scale rows | * | * |
| %SC position of reduction from 6 to 4 tail scale rows | * | * |
| Number of supralabial scales | * | * |
| Number of sublabial scales | * | * |
| Number of scales bordering the supraocular scales | * | * |
| Minimum number of scales separating the supraocular scales | * | * |
| Maximum number of scales separating the supraocular scales | * | * |
| Number of internasal scales | * | * |
| Number of scales separating the fourth supralabial scale from the subocular scale | * | * |
| Number of scales separating the fifth supralabial scale from the subocular scale | * | * |
| Number of scales contacting the suboculars, excluding the preoculars and postoculars | * | * |
| Presence of stripe on dorsal scale row 1 | * | * |
| Number of scale rows involved in stripe | * | * |
| Presence of postocular stripe | * | * |
| Number of scale rows involved in postocular stripe | * | * |
| Presence of dark edges on body scales | * | * |
| Number of bands on body | * | * |
| Mean number of scales of three half bands on body | – | * |
| Mean number of scales between three half bands on body | – | * |
| Presence of dark edges on head scales | * | * |

*Indicates significance value $P = <0.05$ (ANOVA).

% SC, percentage subcaudal scale; %VS, percentage ventral scale.