
9 New perspectives on the evolution of south-east Asian pitvipers (genus *Trimeresurus*) from molecular studies

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Synopsis

The genus *Trimeresurus* contains about 36 species, several of which are remarkably similar in superficial appearance, and it is unclear whether this similarity relates to phylogenetic relationships or to ecological convergence. Whatever the cause, it has resulted in considerable confusion which hinders the understanding of the evolution of the genus by biologists as well the advancement of snakebite treatment by medical workers. Our ongoing study of this group (part of a larger study of the systematics of medically significant snakes) focuses on a core group of species (*T. albolabris*, *T. popeorum*, *T. stejnegeri*, *T. erythrurus*, *T. macrops*) known to be or potentially sympatric in Thailand and adjacent areas and involves the use of multivariate morphometry and mitochondrial DNA analysis to study both inter- and intra-specific relationships. We propose a preliminary phylogenetic hypothesis for a set of species based on PCR-RFLP of the cytochrome *b* gene of mitochondrial DNA and discuss this in relation to its ability to shed light on morphological evolution in the genus. We then illustrate geographic variation in morphology of *T. albolabris*, a species with a large range, and relate this to current subspecies and to the phylogenetic history of populations as revealed by cytochrome *b* sequencing. Mantel tests are used to evaluate various causal relationships.

Introduction

The genus *Trimeresurus* contains a large number of species, found in most of southern Asia from the Indian subcontinent to southern China and Japan, Indochina, the Philippines and the Indo-Malayan archipelago (excluding Sulawesi). The most recent checklist (Golay *et al.* 1993) lists 36 species under *Trimeresurus*; another four formerly considered part of this genus are now listed under the genus *Ovophis* (Burger 1971; H. M. Smith & Chiszar 1988), and the species *wagleri* in a monotypic genus, *Tropidolaemus* (Hoge & Romano-Hoge 1978/79).

The genus *Trimeresurus* (*sensu stricto*) still contains a diversity of forms which can be divided, on the basis of superficial resemblance, into several groups. There are ground-living, egg-laying forms (e.g. *T. mucrosquamatus*, *T. flavoviridis*, *T. jerdoni*).

The majority of species of *Trimeresurus*, however, are arboreal. Some of these are brown (e.g. *T. puniceus*) and some are patterned (e.g. *T. trigonocephalus*, *T. kanburiensis*, *T. sumatranus*). Quite a few species, however, can be described as 'green pitvipers' (e.g. *T. albolabris*, *T. popeorum*, *T. erythrurus*, *T. stejnegeri*, *T. tibetanus*, *T. macrops*, *T. gramineus*, *T. medoensis*, *T. flavomaculatus*, *T. hageni*). Many of these were originally included in a single species, *T. gramineus* (Shaw 1802). Later the work of Stejneger (1927) and Pope (Pope & Pope 1933) revealed that this contained several species, the status and relationships of which have since been the subject of much debate and rearrangement (e.g. M. A. Smith 1943; Hoge & Romano-Hoge 1978/79; Regenass & Kramer 1981). Identification of the green species remains fraught with problems, as illustrated by the number of herpetological books which contain pictures of misidentified snakes (e.g. Engelmann & Obst 1981), including some guides to snakes of particular countries or regions (e.g. Nootpand 1971).

Apart from its general biological interest, there are likely to be medical benefits arising from a thorough revision of the genus. While less life-threatening than some of the elapids and true vipers found in the same region, *Trimeresurus* species are frequently the most important cause of snake-bite as they are common and frequently come into contact with man (Romer 1963; Kuo & Wu 1972; Viravan *et al.* 1992). The problems associated with identification have led to a situation where most of the medical literature on aspects of snakebite and venom involving these species is virtually meaningless, as the actual species involved are unknown (Mitrakul 1973; Mitrakul & Impun 1973; Visudiphan *et al.* 1989; but see Hutton *et al.* 1990).

In this paper, we will briefly review some of the characters which have been used for identification purposes and some of the problems which contribute to the general confusion surrounding the 'green' species. We then give a progress report on our work on this genus to date, illustrating our approach to resolving some of these problems.

Morphological characters of use in identification of 'green' *Trimeresurus*

Various characters have been considered important in distinguishing between the species. Two major ones, which have been given considerable emphasis by previous workers in defining species affinities, are the type of hemipenis (Pope & Pope 1933; Maslin 1942) and the condition of the first supralabial scale (M. A. Smith 1943).

1. Hemipenis type. There are two markedly different types of hemipenes. One is short and covered by spines in the region of the fork, with the tips being calyculate (pictured in Mao, Yin & Guo 1984). This resembles that of other pitvipers and true vipers. The second type is long and slender and completely lacks spines; again it is calyculate for much of the forked region (pictured in Vogel 1991). These are not subtle differences, and can be easily detected without even everting the hemipenes, by the pronounced bulge in the top of the tail produced by the spiny type compared to a more gradually tapering tail in those with the slender type of hemipenis. In spite of the weight given to this character in determining species groups, descriptions of new species are often published with no mention of the hemipenis (e.g. *T. medoensis*, Zhao & Jiang 1977) and literature reports are often inconsistent.

2. Fusion of the first labial and the nasal scales. In some species (*T. albolabris*, *T. erythrurus*), the first supralabial scale is partly or completely fused to the nasal scale. In others (e.g. *T. stejnegeri*, *T. popeorum*), they are completely separated by a suture. Scoring this character can be problematic as fused scales are often creased at the point at which the suture normally occurs, therefore close examination may be necessary in order to distinguish the states.
3. Size of internasals and their contact or separation by intervening scales. *T. albolabris* normally has internasals in contact, while similar species (e.g. *T. erythrurus*) normally have one or more scales between the internasals.
4. Presence or absence of one or more scales between the nasal and the shield bordering the pit anteriorly.
5. Keeling of scales. Some species have smooth or feebly keeled scales while others have extremely keeled scales (e.g. *T. erythrurus*). Keeling of head scales may be of particular diagnostic utility.
6. Size of the eye. *T. macrops* (meaning 'big eye') was confused with *T. albolabris* until recently (Regenass & Kramer 1981) in spite of the coloration and head shape differences between the two. This confusion had particular medical significance since *T. macrops* is the second most common species in the heavily populated Bangkok area (the most common being *T. albolabris*) (Mahasandana & Juntakune 1990).
7. Number of scales around the body. In the green species, this varies between 25 (*T. erythrurus*) and 12 (*T. macrolepis*), but is usually 21 in the species most difficult to distinguish (e.g. *T. popeorum*, *T. stejnegeri*, *T. albolabris*, *T. macrops*).
8. The size and arrangement of gular scales. These may be arranged in regular pairs (e.g. *T. albolabris*) or may be more irregular (*T. stejnegeri*).

As already indicated, in spite of the fact that several of these characters can be used to separate species, misidentification of species (even by professional herpetologists) continues to be frequent.

Problems of identification of *Trimeresurus*

Within-species variability

A mixture of ontogenetic variation, sexual dimorphism and geographic variation is present in almost every character. An example of geographic variation especially relevant to identification in the field is colour pattern in a widespread species, *T. albolabris*. In a typical *albolabris* specimen from central Thailand, the upper lip is sharply demarcated from the upper part of the head, being the same yellow colour as the ventral scales. Sexual dimorphism is rather subtle in this species, males having a distinct white stripe down the side of the body which tends to be indistinct or absent in females. In the eastern Indonesian islands (e.g. Flores and Komodo), the stripe is absent in both sexes, and the lip is not sharply demarcated from the rest of the head. Since these islands are on the edge of the range of this species, it perhaps is not unexpected that they look different. However, only a few hundred kilometres north-

east of Bangkok, *albolabris* are also a much more uniform green all over and have an orange eye rather than a yellow eye. A few hundred kilometres in the other direction, in the region of Chiang Mai in north Thailand, specimens that resemble *albolabris* in scalation characters (e.g. internasals in contact and fused first labial) nevertheless look quite different, having a deep-red eye and rather prominently keeled temporal and body scales. These specimens will be referred to as *Trimeresurus* sp. in the rest of the paper.

Another example of geographic variation is given by *T. stejnegeri*. Males of this species from Taiwan have a prominent lateral white stripe which continues onto the head, edged with a narrow red stripe (below the white stripe on the body, above it on the head). Females lack the lateral red pigmentation, but the eyes are red in both sexes. They are small snakes, typically reaching 0.5 m. In *T. stejnegeri* from north-east Thailand (Loei Province) the red stripe in males is much more pronounced and the eye is also deep red. Females, however, have only the white stripe and have yellow eyes. In this area this species reaches a large size, with females commonly exceeding 1 m in length. Ontogenetic variation is also present, notably in the colour of the eye in juvenile males, which are yellow, although they have a broad red lateral stripe from birth.

This variation poses problems for identification, in some cases producing pronounced confusion. In Loei Province, *T. stejnegeri* are, according to current range maps (Regenass & Kramer 1981), sympatric with *T. popeorum*. These two species present perhaps the most difficult challenge in identification as their external morphology seems to be almost perfectly convergent. The males, however, have different hemipenes, with *stejnegeri* having the short spiny type and *popeorum* the long slender type. While this means that it should be possible to correctly identify males of the two species, females are virtually indistinguishable by any currently known criteria. From time to time, characters are proposed which purport to do this, a recent example being the continuity of the reddish tail colour (continuous vs. broken up into patches). However, a recent examination of a series of 40 *T. stejnegeri* from Loei showed that this character varies ontogenetically within the species, being broken up in young animals and solid in older ones.

Inaccurate range descriptions

Snakes are cryptic animals and, in much of the area in which this genus occurs, access is politically and physically difficult. These facts make it highly likely that current range descriptions are inaccurate, yet identifications sometimes seem to be based on the species known to occur in the area. For example, a classic paper on guild structure in reptiles (Inger & Colwell 1977), carried out in Nakhon Ratchasima Province (north-east of Bangkok), lists all *Trimeresurus* specimens caught as *T. popeorum*. However, only a cursory examination of the preserved specimens is necessary to confirm that the species is actually *T. stejnegeri*, the hemipenes having been carefully everted and clearly of the spiny type. The simplest explanation that can be proposed for such a basic error is that, since it was known that *stejnegeri* did not occur so far south in Thailand, the hemipenis had not been checked. In fact, it is clear that this species occurs considerably further south than Nakhon Ratchasima, a confirmed

specimen now being known from as far south in peninsular Thailand as Krabi (P. Juntakune, pers. comm.). Since this confusion came to light, we have examined almost every available male '*popeorum*' from north-east Thailand and in every case it has proved to be *T. stejnegeri*, which raises the possibility that *popeorum* does not occur in the Korat plateau at all.

Discovery of new species

Many currently accepted species will almost certainly eventually be found to be polytypic (e.g. *T. flavomaculatus* in the Philippines). Entirely new species are regularly being described (e.g. *T. medoensis*, *T. tibetanus*, *T. xiangchengensis*) although not all of them may be validated as new material surfaces (e.g. new specimens of *T. venustus* have recently been found which call into question the validity of its separation from *T. kanburiensis*). Since *Trimeresurus* are very popular with hobbyists, animals are sometimes imported into Europe that do not obviously fit into any of the currently described species. In many cases even the country of origin of these animals is unknown, making it difficult to judge whether the differences relate to intraspecific geographic variation or to interspecific differentiation.

A molecular phylogenetic approach: can new techniques help?

As we have already described, some of the 'green' species are so similar in external morphology that they may be impossible to tell apart except by hemipenis morphology (Pope & Pope 1933). This last fact suggests that they are not closely related sister species, implying a remarkable degree of evolutionary convergence. This also suggests that studying morphology alone may not be appropriate in determining the evolutionary relationships within the group. The use of molecular markers has been shown to be a powerful means of recovering phylogenetic histories for many organisms (see references in Avise 1994). While not being entirely free from selective effects, molecular markers will almost certainly show less homoplasy than morphology in this case. Nevertheless, considerable morphological variation may be present within currently recognized species, so character analysis is still appropriate to define the taxonomic units to be represented in the molecular phylogenetic tree.

Methods

Because of the problems of identification already alluded to, the samples used in this study were collected by a few people who could be relied on to make an accurate identification, and are supported by photographs or voucher specimens. The technique used to obtain the phylogeny presented here is enhanced restriction fragment length polymorphism (RFLP), also known as PCR-RFLP (Quinn 1992; Simon, McIntosh & Deniega 1993). The advantages of this technique over conventional RFLP of whole mitochondrial genomes is that it involves cutting of polymerase chain reaction (PCR)-amplified fragments, hence small amounts of tissue (blood or small section of tip of tail or even shed skin) provide sufficient DNA. Also, since the fragment is smaller (a 767 bp fragment of cytochrome *b* was used in this study), more sensitive four-cut enzymes can be used while retaining the ability to easily map the restriction

sites. Its advantage over sequencing is that it is a faster, easier and cheaper method of screening large numbers of samples. However, loss of restriction sites can result from mutations occurring at any one of four positions (in the case of a four-base cutter) and involving three possible changes at each position. The chance of non-homologous site loss is therefore considerably higher than that of non-homologous site gain and this must be taken into account in the analysis. Also, as only a subset of the polymorphisms present will be detected, the technique is less sensitive than sequencing. Although it is likely to provide enough information at the interspecific level, and in some cases sufficient intraspecific variation for a phylogenetic analysis may be detectable by PCR-RFLP alone (e.g. Daltry, Wüster & Thorpe, this volume pp. 155–171), in general levels of differentiation are likely to be much lower at the within-species level. We therefore chose to sequence the fragment to provide more detailed information.

Whole genomic DNA extracts from muscle or liver tissue were PCR-amplified using primers L14841 (Kocher *et al.* 1989) and MVZ16 (Moritz, Schneider & Wake 1992). Thermal cycle parameters were 1 min at 94 °C, 1 min at 45 °C and 1 min at 72 °C for the first five cycles, followed by 94 °C for 1 min, 50 °C for 1.5 min and 72 °C for 1.5 min for an additional 30 cycles and a final 5 min extension step at 72 °C. A negative control was always included in order to exclude the possibility of contamination. Purified product was either sequenced (see Salomão *et al.*, this volume pp. 89–98, for protocol details) or digested with eight restriction enzymes (*Aci*1, *Cfo*1, *Dde*1, *Hae*3, *Nla*3, *Mse*1, *Rsa*1, *Taq*1). Digestion products were separated on a 6% acrylamide gel alongside a size marker, and visualized by staining with ethidium bromide.

Restriction site analysis was carried out using the programme REAP (McElroy *et al.* 1992) and genetic distances from this were input into PHYLIP 3.5 (Felsenstein 1993) for phylogenetic analysis. Sequence data (confirmed by sequencing from both primers) could be aligned easily by eye (since the cytochrome *b* gene is a protein coding region), and subjected to maximum parsimony analysis using PAUP 3.3 (Swofford 1993) with default settings. All sites were weighted equally.

Results

Phylogenetic tree for *Trimeresurus*: a preliminary hypothesis

At present 10 species are represented in the phylogeny. A Fitch–Margoliash tree (Fig. 1) was constructed from information on 42 restriction sites (from seven four-cut enzymes and one five-cut), using *Bothrops moojeni* and two specimens of *Tropidolaemus wagleri* as outgroups. The branch lengths are not shown, as it is the topology that is of primary interest at this stage. The tree is quite pectinate, but some interesting patterns emerge nevertheless. *T. mucrosquamatus* is close to the base of the tree, as might be expected from its ground-living, egg-laying habits. *T. albolabris* forms a monophyletic group, with a second cohesive group being formed by *T. purpureomaculatus*, *T. erythrurus*, *T. cantori* and the red-eyed *Trimeresurus* sp. from Chiang Mai. The species *purpureomaculatus* as currently recognized is clearly polyphyletic, with *erythrurus* intervening between the mainland subspecies (*T. p. purpureomaculatus*) and the Andaman island subspecies (*T. p. andersoni*).

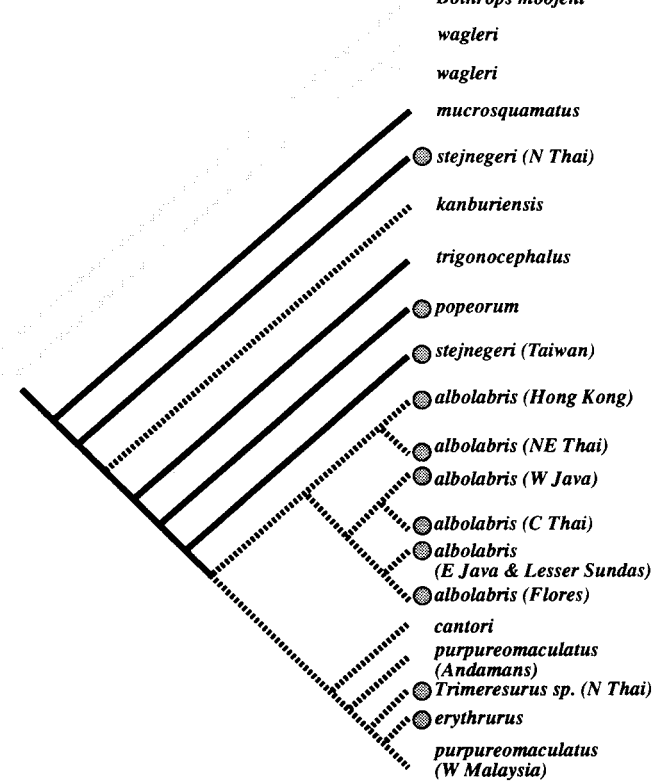


Fig. 1. Phylogenetic tree for some species of *Trimeresurus*. Outgroups are *Bothrops moojeni* and *Tropidolaemus wagleri*. The dashed lines represent the presence of the long slender hemipenis type and the fusion of the first labial and nasal scale. The grey circles indicate the green species. See text for details.

Another interesting feature is that the two *stejnegeri* specimens (from Taiwan and north-east Thailand) are quite separated in the tree, which indicates that they may be different species. It would be useful to compare them with *T. stejnegeri* from Fujian (the type locality) to determine their affinities.

Phylogenetic utility of morphological characters

How do the characters described above, that have been used to determine species affinities in the past, map onto the tree?

1. Fusion of first labial and nasal. Species within the *albolabris/purpureomaculatus* clade all possess this character (Fig. 1) but it is not restricted to this clade, also being found in *T. kanburiensis* (although it appears to be variable in this species). This could be the result of an independent evolution or loss of the character, or it might result from the hypothesis being wrong regarding the relationships of this species. It is interesting to speculate that if *T. kanburiensis* actually belongs to the *albolabris/purpureomaculatus* lineage, only a single evolutionary event would be necessary to explain the distribution of this character.

2. Hemipenis. The *albolabris*/*purpureomaculatus* lineage is also characterized by the possession of the long, slender hemipenis type. The speculation that *kanburiensis* may be part of this lineage is reinforced by the observation that it shares this hemipenis type. Only one other species represented on the tree has this hemipenis type, *T. popeorum*. It is possible that the *popeorum* hemipenis has evolved independently as it differs from all the rest in lacking finger-like papillae in the region of the fork, below the calyculate region.

3. Green coloration. To emphasize the point about morphological convergence, it can be seen from Fig. 1 that the green species (indicated by grey circles at the tips of the tree) have evolved independently.

Within-species evolution in *Trimeresurus albolabris*

Since significant substructure was observed within the *albolabris* clade (Fig. 1), this was subsequently examined in more detail, with particular attention to the correspondence between morphological variation and phylogenetic relationships revealed by mitochondrial DNA analysis.

Figure 2 illustrates the ranges of the three currently recognized subspecies (Regenass & Kramer 1981). The presence of *albolabris* on Sumatra and Borneo is in some doubt, depending on the existence of a few specimens with imprecise locality data (which may possibly be in error). Tissue samples have been obtained from most parts of the species range, with the exception of the subspecies *septentrionalis* from Nepal. For this within-species study, sequence was obtained for a 660 bp region of the cytochrome *b* gene (mitochondrial DNA).

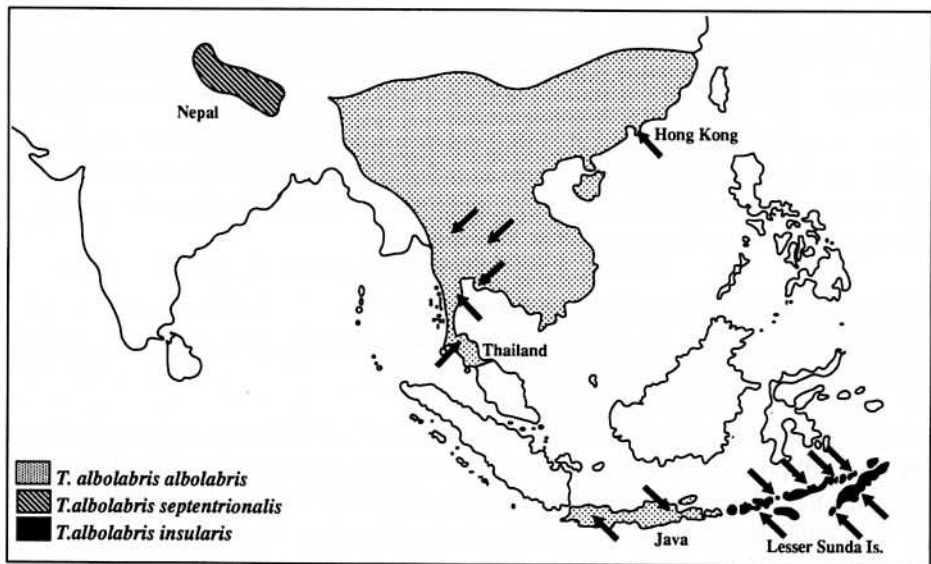


Fig. 2. Distribution of the currently recognized subspecies of *T. albolabris*. Black arrows indicate populations sampled for DNA analysis. Most parts of the illustrated range were represented in the morphological analysis.

Molecular phylogeny of *T. albolabris*

Figure 3 illustrates the majority-rule consensus tree (parsimony) with the bootstrap values (out of 100 bootstraps) given above the nodes. Three main lineages are discernible, the first comprising all specimens from the Lesser Sunda islands, together with the east Javan specimens. This clade is very well supported by the bootstrap, appearing in all 100 bootstrapped trees. The second lineage consists of all specimens from southern Thailand (Trang and Krabi) and the third consists of all other samples (north-east Thailand, central Thailand and Hong Kong and west Java). The relationships between these three clades are unresolved in the consensus tree. Although the affinities of the west Javan population appear to make no biogeographic sense on the basis of current geography, in the Pleistocene when sea levels were lower these islands formed part of the Sunda shelf (Heaney 1991). It is possible that Java was colonized from Indochina, bypassing south Thailand (Fig. 4). Many species do show boundaries between southern Thailand and northern west Malaysia, e.g. *Calloselasma rhodostoma* (Daltry *et al.*, this volume pp. 155–171), so there may have been some kind of a biogeographic barrier here. However, why east Java should be so divergent from west Java is inexplicable, unless it represents a westward recolonization of Java from the Lesser Sundas.

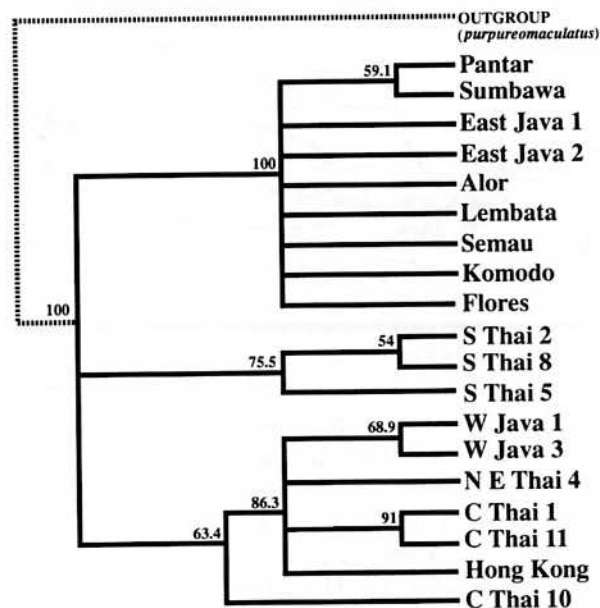


Fig. 3. Haplotype tree (50% majority-rule consensus tree from parsimony analysis) for *T. albolabris* from a 660 bp sequence of cytochrome *b*. Bootstrap values (out of 100 bootstraps) are given adjacent to the nodes. Haplotype numbers are the author's reference numbers.

Geographic variation in morphology

The phylogenetic lineages described above do not relate to the subspecific designations, since the subspecies *insularis* does not include any Javan populations. However, subspecies described by conventional taxonomic methods frequently fail

to portray the real patterns of variation (Thorpe 1987). Consequently, we reanalysed morphological variation in this species.

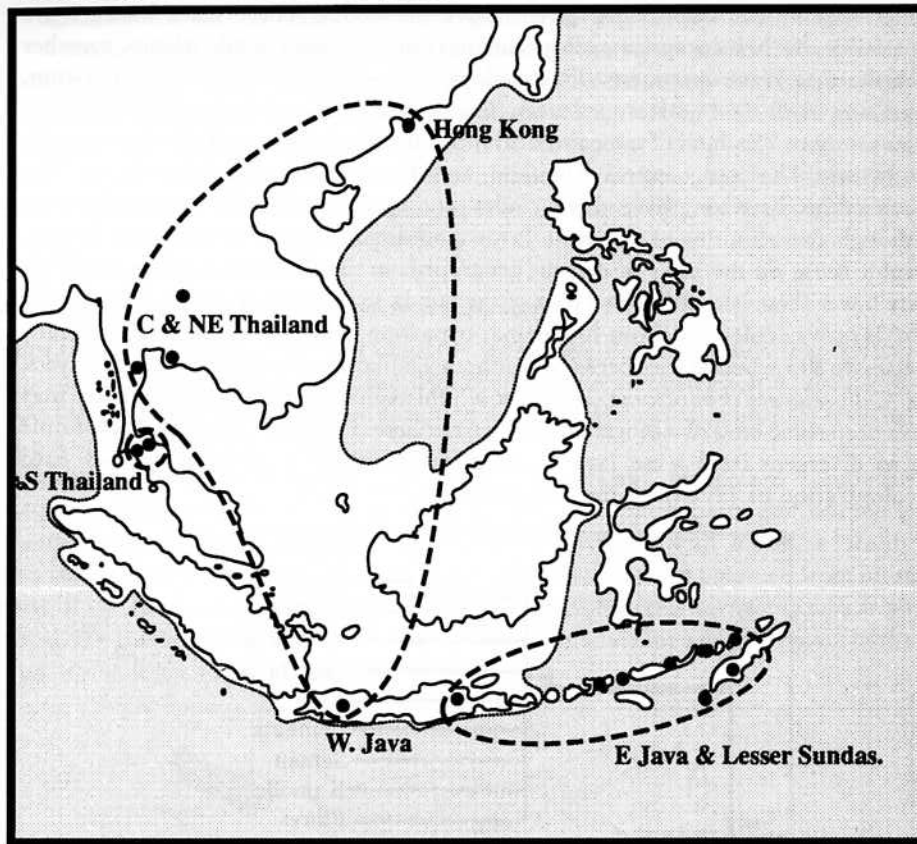


Fig. 4. Map indicating sea levels in the Pleistocene (stippled area) in relation to current sea levels (thin black line) and the three lineages represented on the phylogenetic tree (thick dashed line).

A multivariate (canonical variate) analysis on scalation characters showed some evidence of geographic variation. If the canonical variate scores are contoured on a map of Asia (Fig. 5), the closeness of the contours represents the gradient of morphological change between populations. The first canonical variate (CV1) distinguishes the Lesser Sundas from the rest, while the second canonical variate separates the north-eastern populations (Hong Kong, North Vietnam, China) from all other populations. Note that the east and west Javan populations are virtually indistinguishable and the Nepalese population is also not distinguishable from those further east in Thailand. A similar pattern of geographic variation is obtained for both sexes (but is not illustrated for males). A single female specimen from Borneo was fitted onto the axes, and appears different to any other population (Fig. 5), indicating that the locality may not be in error as suggested earlier.

The morphological analysis has shown, yet again, that the conventional subspecies are probably invalid. The groups of morphologically similar populations, however,

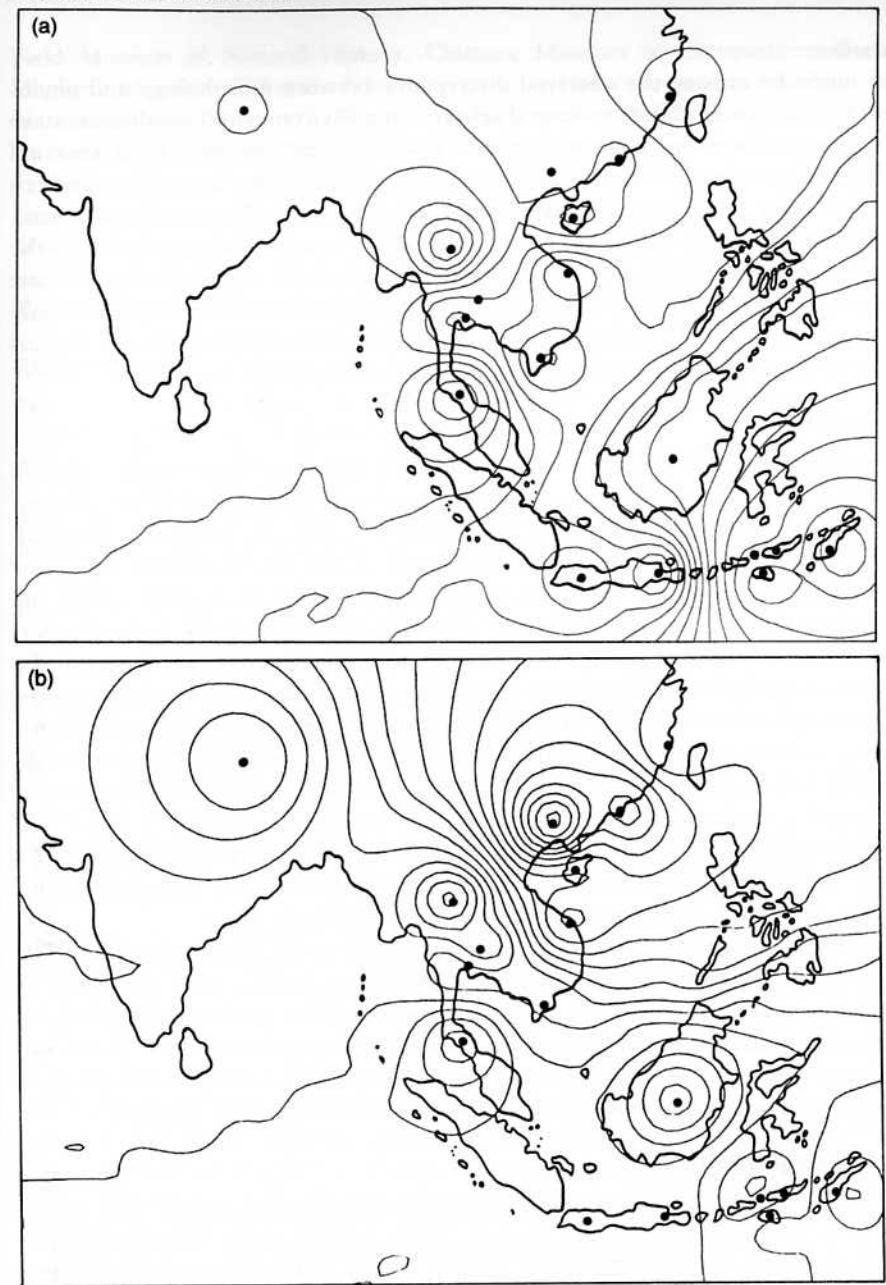


Fig. 5. Canonical variate scores contoured over a map of Asia illustrating geographic variation: scalation characters in females. (a) CV1; (b) CV2. See text for explanation.

do not correspond to lineages on the phylogenetic tree. The Hong Kong specimen, example, is not in a separate lineage to the Thai samples as implied by the ordination based on scalation characters.

Causation

What might be causing the observed discrepancy between morphology and phylogeny? An obvious candidate is natural selection for environmental conditions, since the species is found in a wide range of environmental regimes, from seasonal subtropical areas (e.g. southern China), relatively aseasonal wet areas (e.g. southern Thailand, west Java), and arid areas (the Lesser Sundas). To test this, a matrix correlation test or Mantel test (Thorpe *et al.* 1994) was run using scalation as the dependent matrix and climate (derived from four variables obtained from climatic maps of Asia: Anon. 1981, 1992) as the independent matrix. The result was highly significant ($P < 0.001$), indicating that taxonomic units based on morphological similarity may have little to do with historical evolutionary units.

Discussion

The results presented in this paper represent a progress report rather than a completed piece of work, and are only the first step towards resolving some of the challenges posed by this genus. It seems likely that the molecular approach adopted here will prove very rewarding, but there is a long way to go before all 36 (or more) species are represented on the tree. Many of the problems of resolving the systematics of this genus result from the difficulty of finding stable morphological characters. As far as it is possible to evaluate morphological characters against the preliminary molecular phylogeny presented here, it would seem that both the type of hemipenis and the fusion of the first labial scale with the nasal may be valuable for defining species affinities, although this requires confirmation by further work. However, at the intraspecific level, the molecular phylogeny of *T. albolabris* does not correspond with morphological differentiation, making it difficult to name subspecies on any consistent criterion. The inclusion of other morphological character systems (e.g. colour pattern and body shape) in the analysis is likely to exacerbate this problem, since they are unlikely to be congruent with each other.

The results presented here also suggest the existence of several new taxa. The *Trimeresurus* sp. from Chiang Mai is probably an entirely new species. The Taiwanese population of *T. stejnegeri* also appears to be distinct from mainland populations, as are the mainland and Andaman Islands populations of *T. purpureo-maculatus*. However, we feel it would be inappropriate to confuse the situation further by naming new species until further work confirms these findings.

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