Molecular Phylogenetics and Evolution
Vol. 16, No. 2, August, pp. 199–211, 2000
doi:10.1006/mpev.2000.0779, available online at http://www.idealibrary.com on IDEAL®

A Phylogeny of the *Trimeresurus* Group of Pit Vipers: New Evidence from a Mitochondrial Gene Tree

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Received June 14, 1999; revised February 9, 2000

The *Trimeresurus* group is an important radiation of over 40 Asian pit viper species. Once considered congeneric, four genera are generally currently recognized (*Trimeresurus sensu stricto, Ovophis, Protobothrops,* and *Tropidolaemus*) but relationships within and between these are still unclear. This study, based on mitochondrial cytochrome *b* sequences, is the first to include a large number of species (21) and demonstrates that the current taxonomy does not adequately represent either the relationships or the genetic diversity present in the complex. Although many deeper nodes are not strongly supported, the following novel conclusions are all well supported: (1) the paraphyly of *Trimeresurus sensu stricto,* (2) the presence of several divergent clades within *Trimeresurus sensu stricto,* (3) the paraphyly of some widespread, medically significant, species, (4) the nonmonophyly of *Ovophis,* and (5) the monophyly of *Protobothrops.* Mapping of morphological characters onto the mitochondrial tree further supports the four groups proposed for *Trimeresurus sensu stricto.*

INTRODUCTION

Pit vipers (Reptilia: Serpentes: Viperidae: Crotalinae) of the *Trimeresurus* complex are widely distributed across southern Asia and the Indo–Malayan archipelago. The complex represents a major evolutionary radiation, including circa 40 species (McDiarmid *et al.*, 1999; David and Ineich, 1999), which occupy a wide range of habitats and display a range of lifestyles and reproductive modes. New species are also being described on a regular basis. Originally all considered to be congeneric, *Trimeresurus sensu lato (s.l.),* the species are currently generally arranged in four genera, *Trimeresurus sensu stricto (s.s.), Ovophis, Protobothrops,* and *Tropidolaemus (Tro.).* However, there is considerable confusion and disagreement about the content of the four genera and, in particular, the removal of a few species to the latter three genera leaves *Trimeresurus s.s.* as a large and diverse, possibly paraphyletic, assemblage of over 30 species.

Despite their diversity in ecology and behavior, the species in the group are remarkably conservative in morphology. This marked phenotypic convergence among species is a feature of the crotaline radiation as a whole (Greene, 1992; Ogawa *et al.*, 1995; Kraus *et al.*, 1996). In the *Trimeresurus* complex, it is particularly well typified by the green pit vipers, or bamboo vipers. All initially considered a single species, Stejneger (1927) and Pope and Pope (1933) described scale and hemipenial characters that could distinguish between them. However, the presence of considerable geographic variation and sexual dimorphism in external color and scalation characters (Malhotra and Thorpe, 1997) contributes to frequent misidentification. All things considered, it is highly likely that a full resolution of their systematics has not yet been achieved and that a number of cryptic species may be undetected.

While molecular evidence is not free from the effects of homoplasy, it is likely to play a significant role in resolving the taxonomy of the *Trimeresurus* complex by providing an independent character system for phylogeny reconstruction. Although a number of molecular studies on crotalines have recently appeared (Kraus *et al.*, 1996; Vidal *et al.*, 1997; Parkinson *et al.*, 1997; Vidal and Lecointre, 1998; Parkinson, 1999), these focus largely on New World species, and few have included more than one species of *Trimeresurus.* The most comprehensive studies are Kraus *et al.* (1996) and Parkinson (1999), which included seven and eight species of *Trimeresurus s.l.,* respectively. Both these studies supported the paraphyly of *Trimeresurus s.l.* (see also Vidal and Lecointre, 1998) and the monophyly of *Trimeresurus s.s.* (three species) and *Protobothrops* (two or three species). The monophyly and affinities of *Ovophis* are still unresolved, as no study to date has included more than one species. All these studies admit that a more comprehensive study of *Trimeresurus s.s.* is required for a better understanding of the phylogenetic relationships within the *Trimeresurus* complex, as well as those among all Asian crotaline genera. This study is the first to include a significant number of species in the complex (21), allowing us to rigorously address the monophyly of the genera *Trimeresurus s.s,*

1055-7903/00 $35.00

Protobothrops, and *Ovophis* (the genus *Tropidolaemus* is only represented in this study by one species).

MATERIALS AND METHODS

Samples and Sequencing

An attempt was made to sample as many species as possible. This is a difficult task, not only because of the size of the group, but also because many of the species are secretive in habits and are found in remote areas. For the species in which accurate identification is problematic (i.e., most of the green species), only specimens from known localities, and which could be examined personally to verify the identification, were used. Given the strong possibility of cryptic species being present, we attempted to include multiple samples from across the range of widely distributed species. Comprehensive sampling has been achieved to any great extent only with *T. albolabris,* but we also have multiple samples for *T. popeiorum, T. purpureomaculatus,* and *T. stejnegeri.* Samples from single localities are available for *T. cantori, T. erythrurus, T. flavomaculatus, T. gracilis, T. gramineus, T. hageni, T. kanburiensis, T. malabaricus, O. monticola, P. mucrosquamatus, O. okinavensis, T. borneensis, T. trigonocephalus,* and *Tro. wagleri.* In total, 18 nominal species of the *Trimeresurus* complex, representing all four genera, were sequenced for this study (Table 1). Samples of *T. macrops* were also available, but failed to amplify with any of the primer pairs used (see below).

Samples were in the form of tail tip biopsies preserved in 80% ethanol, liver tissue in 80% ethanol, or 100–200 μl of blood taken from the caudal vein, placed in 1 ml 5% EDTA, and preserved in 2 ml SDS–Tris buffer (100 mM Tris, 3% SDS). Whole genomic DNA was extracted from 0.01–0.02 g of ethanol-preserved muscle, liver tissue, or 200–500 μl of blood/buffer, using standard protocols (Sambrook *et al.,* 1982). Various fragments of the cytochrome *b* gene were amplified using a slightly modified version of primer L14841 (5′-GCTTCCATCCAACATCTCAGCATGATG-3′[Kocher *et al.,* 1989]) and MVZ16 (5′-GGCAAATAGGAAGTATCATTCTG-3′ [Moritz *et al.,* 1992]). Some samples, which did not amplify well using this combination of primers, were amplified with modified versions of the primers Mt-A (5′-CTCCCAGCCCCATCCAACATCTCAGCATGATGAAACTTCG-3′ [Lenk and Wink, 1997]) and Mt-F (5′-AGGGTGGAGTCTTCTGTTTTTGGTTTACAAGACCAATG-3′ [Wink, 1995]). Reactions were conducted in 25-μl volumes and contained standard reagent concentrations (e.g., Hoelzel and Green, 1994). They were typically subjected to 35 cycles consisting of 30 s denaturation at 94°C, 30 s annealing at 50°C, and 30 s extension at 72°C, followed by a final 5-min extension step at 72°C. A negative (blank) control was always included to exclude the possibility of contamination. Unincorporated nucleotides and primers were removed using a variety of commercially available kits, e.g., Prep-a-gene (Bio-Rad), Wizard minicolumns (Promega), or QIAquick columns (QIAGen). The double-stranded product was then either manually sequenced using a modification of the Sequenase v2.0 protocol (Perkin–Elmer) or sequenced using dye-labeled terminators (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit) and subsequently run on an ABI Prism 377 DNA sequencer. Primer L14841 was primarily used for sequencing. The opposite strand was sequenced using the primers MVZ16 and H15149 (5′-CCCTCAGAATGATATTTGTCCTCA-3′ [Kocher *et al.,* 1989]), in most cases from several different PCR products.

Additional Sequences and Outgroups

A further three *Protobothrops* sequences were obtained from GenBank. These were for the species *P. tokarensis* (Accession No. AFO38884, from Vidal and Lecointre [1998]), and *P. flavoviridis* and *P. elegans* (unpublished, Accession Nos. D31622 and D31623, respectively). Only 548 bp of the *P. tokarensis* sequence aligned with the cytochrome *b* section sequenced for this study. The latter two sequences were substantially shorter, being only 286 bp long. The unresolved state of crotaline intergeneric relationships makes the choice of outgroup problematic, and the likely paraphyly of *Trimeresurus s.l.* necessitates the use of a variety of other crotaline genera to validate this. These included *Bothrops moojeni, Bothrops alternatus, Crotalus durissus terrificus* (provided by W. Wüster), *Crotalus cerastes* (GenBank, Accession No. U69733, unpublished), *Calloselasma rhodostoma,* and *Deinagkistrodon acutus* (Table 1). Recent studies (Milinkovitch and Lyons-Weiler, 1998) suggest that ingroup topology may be radically altered by use of different outgroups. A number of viperine genera were therefore used as outgroups, represented by *Daboia russelii, Macrovipera mauritanica* (provided by P. Lenk), and *Echis ocellatus,* as well as a representative of another family, *Naja siamensis* (the latter two sequences were provided by W. Wüster).

Sequence Analysis

The final analysis included 660 bp from 56 terminal taxa (including outgroups). Alignment was trivial, as there were no indels. Sequences were translated into amino acid sequences to check for the unexpected occurrence of stop codons, which might indicate that pseudogenes (Sorenson and Fleischer, 1996; Zhang and Hewitt, 1996) had been amplified. For distance analyses, the substitutional model which best fit the ingroup data was investigated by plotting the observed number of transversions against "time" (crudely measured by genetic distance). This is expected to be linear except at very large evolutionary distances, when sequences may begin to saturate due to multiple substitutions. Conversely, an exponential curve indicates that the

TABLE 1

List of Species Included in This Study, Their Origins, and GenBank Accession Nos.

Genus	Species	Geographic origin	Sequence code	CAT	GenBank Accession No.
Bothrops	*alternatus*	Parana, Brazil	—	WW59	AF191583
Bothrops	*moojeni*	Sao Paulo State, Brazil	WW17	IB55098	AF200222
Calloselasma	*rhodostoma*	West Malaysia	CAL1	A54	AF171918
Crotalus	*cerastes*	—	—	—	U69733
Crotalus	*durissus*	Sao Paulo State, Brazil	CROT136	IB 55601	AF200223
Deinagkistrodon	*acutus*	Taiwan	DACUTUS	A223	AF171919
Echis	*ocellatus*	Garoua, Cameroon	—	WW567	AF191579
Macrovipera	*mauritanica*	Morocco	—	—	AJ275714
Ovophis	*monticola*	Taiwan	TMONT1	A87	AF171907
Ovophis	*okinavensis*	Ryu-kyu Islands	B1	B1	AF171915
Protobothrops	*elegans*	—	—	—	D31623
Protobothrops	*flavoviridis*	—	—	—	D31623
Protobothrops	*mucrosquamatus*	Taiwan	TMUC1	A211	AF171897
Protobothrops	*tokarensis*	—	—	—	AFO38884
Trimeresurus	*albolabris*	Southeast Thailand	TAT1, TAT11	A127, A133	AF171885, AF171895
Trimeresurus	*albolabris*	Northeast Thailand	TAT4	A135	AF171893
Trimeresurus	*albolabris*	South Thailand	TAT5, TAT8	A130, A134	AF171923, AF171894
Trimeresurus	*albolabris*	West Thailand	TAT10	A139	AF171921
Trimeresurus	*albolabris*	North Thailand	T?1, T?2, TAT13	A225, A226, A149	AF171920, AF171910, AF171908
Trimeresurus	*albolabris*	Hong Kong	TAHK1	A157	AF171884
Trimeresurus	*albolabris*	Nepal	TAN2	A100	AF171909
Trimeresurus	*albolabris*	West Java	TAWJ1, TAWJ3	A125, A126	AF171886, AF171891
Trimeresurus	*albolabris*	East Java	TAEJ1, TAEJ2	A115, A111	AF171887, AF171892
Trimeresurus	*albolabris*	Pantar Is., Indonesia	TaPt1	WAM 107876	AF171881
Trimeresurus	*albolabris*	Alor Is., Indonesia	TaAl1	WAM 107905	AF171882
Trimeresurus	*albolabris*	Flores Is., Indonesia	TAF1	A119	AF171883
Trimeresurus	*albolabris*	Komodo Is., Indonesia	TAK1	A122	AF171924
Trimeresurus	*borneensis*	South Thailand	PUN1	A73	AF171912
Trimeresurus	*cantori*	Nicobar Islands	TCANT	A85	AF171889
Trimeresurus	*erythrurus*	Rangoon, Myanmar	TERY1	A209	AF171900
Trimeresurus	*flavomaculatus*	Philippines	B3	B3	AF171916
Trimeresurus	*gracilis*	Taiwan	TGRAC	A86	AF171913
Trimeresurus	*gramineus*	South India	TG1	A219	AF171905
Trimeresurus	*hageni*	South Thailand	THAG3	A224	AF171911
Trimeresurus	*kanburiensis*	South Thailand	TKT3	A241	AF171914
Trimeresurus	*malabaricus*	South India	TMAL2	A217	AF171901
Trimeresurus	*popeiorum*	West Malaysia	TPM1	A196	AF171888
Trimeresurus	*popeiorum*	South Thailand	TPS1	A202	AF171904
Trimeresurus	*popeiorum*	North Thailand	TPN1	A204	AF171902
Trimeresurus	*popeiorum*	West Thailand	TPN2	A205	AF171906
Trimeresurus	*purpureomaculatus*	West Malaysia	TP1	A82	AF171889
Trimeresurus	*purpureomaculatus*	Andaman Islands	TPA1	A76	AF171922
Trimeresurus	*stejnegeri*	Taiwan	TST4, TST60	A160, A161	AF171896, AF171880
Trimeresurus	*stejnegeri*	Northeast Thailand	TSL18	A181	AF171898
Trimeresurus	*stejnegeri*	North Vietnam	TPV1	ROM 7234	AF171903
Trimeresurus	*trigonocephalus*	Sri Lanka	TT1	A58	AF171890
Tropidolaemus	*wagleri*	West Malaysia	TWAG1	A66	AF17191

Note. Generic names are according to current usage and are listed in alphabetical order. Unless otherwise indicated, the catalogue numbers (CAT) given are for the authors' personal collection. ROM, Royal Ontario Museum; WAM, Western Australian museum; IB, Instituto Butantan, Sao Paulo, Brazil; WW, Wolfgang Wüster, personal collection.

genetic distance used, which incorporates positions that saturate much more quickly, has not adequately corrected this. For example, a plot of transversions against p-distances is substantially curved upward. Employing a correction such as the Jukes–Cantor (Jukes and Cantor, 1969) distance improves the fit to a linear regression (as judged by the R^2 values), but it is still obviously nonlinear. This process was repeated with the Kimura two-parameter (K2P) model (Kimura, 1980), the Hasegawa, Kishino, and Yano (HKY) model (Hasegawa *et al.*, 1985), and the General Time-reversible (GTR) model (Lanave *et al.*, 1984; Rodriguez *et al.*,

1990), all with, and without, gamma-distributed rates with various shape parameters. The best substitution model was judged to be the one involving the smallest increase in R^2 between the linear and the quadratic polynomial regressions. This model was then used for subsequent analyses.

Some degree of saturation is likely to be present in the sequences. This was investigated by plotting the number of pairwise differences due to transitions (Ts) against the genetic distance produced from the model chosen by the procedure above (Milinkovitch *et al.*, 1996; Krajewski and King, 1996). This was done for transitions at first and second codon positions together and at third positions separately, as the latter are the most likely to show saturation effects.

Unless otherwise stated, analyses were performed using the beta test version (b2a) of PAUP* 4.0 (Swofford, 1998). The effect of using different combinations of noncrotaline outgroups was explored using neighbor-joining (NJ) and the model specified by the procedure described above. Once the best combination of outgroups had been selected, parsimony (MP) trees were constructed using a heuristic search. Tree bisection–reconnection (TBR) branch swapping was found to be computationally intractable and instead, subtree pruning–regrafting (SPR) branch swapping was used with 10 random addition replications. Four hundred bootstraps were also performed using the same settings, but with only one random sequence-addition replicate to reduce computational time. This was found to be superior to the alternative in which 1000 bootstraps were performed without swapping (the FASTSTEP option in PAUP* 4.0) in the amount of resolution present in the bootstrap 50% majority-rule consensus tree. Groups supported by less than 50% of the bootstraps were retained if they were compatible with the 50% majority-rule tree. Some form of weighting is often used in parsimony analysis to compensate for homoplasy due to the saturation of rapidly evolving positions. However, this has recently been shown to be ineffective by studies that have tested alternative weighting schemes (Allard and Carpenter, 1996; Philippe *et al.*, 1996; Milinkovitch and Lyons-Weiler, 1998; Vidal and Lecointre, 1998), with much phylogenetic information being discarded in the process. Thus, all the MP analyses presented in this paper are on unweighted data only.

A maximum-likelihood (ML) tree was also produced using the evolutionary model and shape parameter of the gamma distribution defined by the procedure described above. Transition:transversion ratios and base frequencies were estimated from the data. Starting trees for heuristic searches were obtained by neighbor-joining, with subsequent SPR branch swapping. Only 100 bootstraps were performed because of the computer-intensive nature of this method; in addition, nearest neighbor interchange (NNI) rather than SPR branch swapping was employed to further reduce the computational time required.

All phylogenetic methods incorporate implicit assumptions that, if violated, may lead to incorrect inference. Consequently, these assumptions should be explicitly tested where possible. First, the presence of phylogenetic signal in the data matrix was tested using the g_1 statistic (Hillis and Huelsenbeck, 1992) for the skewness of tree length distributions, estimated from 10,000 random trees. The critical values of g_1 are obtained from the table published in Hillis and Huelsenbeck (1992), and a significant result indicates that the length of the actual tree is significantly shorter than expected from random data (i.e., without any phylogenetic structure). A significant result may, however, also be obtained if the phylogenetic signal is confined to only part of the tree. As cytochrome *b* is a fast-evolving gene, it is possible that the phylogenetic signal is confined to the tips of the tree, and that at the deeper levels, the signal in this gene has been effectively randomized by saturation. Consequently, this was tested by holding together successive well-supported clades, and recalculating the g_1 statistic. The point at which it ceases to be significant indicates the limit of resolution of the dataset.

It is also possible that other sources of structure may exist. For example, two sequences with similar GC content biases will tend to appear to be more closely related in phylogenetic reconstructions. To evaluate this, the GC content of each sequence was calculated and the presence of significant heterogeneity between sequences was tested using a *G*-test (Sokal and Rohlf, 1981). The possibility of nonneutral evolution of the cytochrome *b* gene (Ballard and Kreitman, 1995) was tested using McDonald and Kreitman's (1991) test. This is a goodness-of-fit test that compares ratios of synonymous to replacement substitutions within and between species, with the expectation under neutrality that these should be the same. Eight sequences of *T. albolabris* were used (from those supported as monophyletic by the phylogenetic analysis), with a variety of outgroups from different levels of the tree.

The hypothesis of rate constancy was tested by the likelihood-ratio test (Felsenstein, 1988), which compares the likelihood of a tree using an assumption of clock-like evolution and a tree calculated without this assumption. However, this tests the overall "clockness" of the tree, whereas it would also be of interest to compare rates of substitution in subsets of the data (such as the clades revealed by phylogenetic analyses). Relative rate tests between the various lineages were therefore carried out using the two-cluster test of Takezaki *et al.* (1993) as implemented in PHYLTEST, which tests the null hypothesis that the average number of substitutions in two lineages, relative to that of an outgroup lineage, will be equal. It is similar to Li and Bosquet's (1992) test, but allows more than one

outgroup sequence to be used. Each of the clades (represented by clusters of monophyletic sequences) was tested against its sister clade, using the clade immediately interior to them as the outgroup (as the power of the test increases when the outgroup is more closely related).

Support from Morphology

MacClade 3.08a (Maddison and Maddison, 1995) was used to map various morphological characteristics (the type of hemipenis, the condition of the first upper labial and nasal scale, and the number of scale rows at midbody) onto our best-supported phylogeny for *Trimeresurus s.s.* to explore support for the clades identified in the tree and to resolve any conflicting arrangements. The linear parsimony option was used, with the number of possible reconstructions calculated using the equivocal cycling option, where appropriate. These characters have established taxonomic value (e.g., squamate hemipenes have repeatedly been shown to be valuable taxonomic characters in a number of different groups).

Hemipenial morphology. Hemipenes vary considerably throughout the group and provide one of the main characters for distinguishing between some of the green species (Pope and Pope, 1933). The main difference is in the presence or absence of hard spines, but two types of spinose hemipenes (referred to here as Type 1 and Type 2) can also be distinguished on the basis of the arrangement, shape, and number of the spines. A detailed description of these hemipenis types can be found in Pope and Pope (1933). While the ancestor of *Trimeresurus s.s.* is not well defined, neither Type 2 spinose or smooth hemipenes are found outside this clade, and Type 1 spinose would seem to be the most likely ancestral condition, being widespread in the more basal crotalines, viperines, and nonviperid taxa.

The condition of the labial and nasal scale. The first upper labial scale can either be distinctly separated from the adjacent nasal scale by a suture or they may be completely or partially fused (Stejneger, 1927). While the degree of fusion is variable in most species, a species was coded as having the fused state if any degree of fusion is present.

The number of scale rows at midbody. This varies between 19 and 29 in the species represented in this study and was coded as a discrete character.

The occurrence of green coloration. This was investigated to examine the relationship of the "green pit vipers" to each other. While there is no *a priori* reason to expect coloration to be a good indicator of evolutionary relationships, as it is likely to be subject to strong selection, many authors use this term as if it implies a close relationship. We distinguished between species that are never green, those that have the typical green pit viper pattern (a plain green background, usually with a white lateral stripe, which may be edged below with red and sometimes extends onto the head, and a reddish tail), and those that have a green component but with other dominant patterns present (e.g,. purple-brown bands in *T. kanburiensis*).

RESULTS

Testing Evolutionary Assumptions

DNA extracts amplified using different primer sets always gave identical sequences, as did different PCR products amplified with the same primer sets. No deletions, insertions, or stop codons were found in the data, which suggests that paralogous nuclear insertions have not been amplified. The base frequency distribution (A = 29.4%, C = 30.8%, G = 11.3%, T = 28.4%) and pattern of substitutions were as expected for coding mitochondrial DNA (mtDNA) sequences. The Ts:tv ratio (estimated by maximum-likelihood from the NJ tree) was 3.72. A conservative test for the significance of the g_1 statistic indicated that there was significant structure in the data ($g_1 = -0.2980$). There was no significant difference between the base frequency distribution of the different sequences ($G_H = 16.875$, $P < 0.99$). The McDonald–Kreitman neutrality test failed to reject the null hypothesis of neutral evolution for any comparison.

The substitution model which best fit the data (by the procedure described above) was the HKY model with gamma-distributed rates (shape parameter = 0.30), in which the lines fitted by linear and polynomial regression were exactly coincident. This substitution model was then used for subsequent distance-based analyses. Maximum corrected sequence divergence in the ingroup under this model was 0.4467, between *T. gracilis* and *T. albolabris.* However, *T. gracilis, T. borneensis, P. mucrosquamatus,* and *O. okinavensis* all have distances over 0.4 with a number of species. When number of transitions are plotted against this distance, they are found to be substantially saturated, but as expected, most of this is due to third position transitions only as transitions at second and third positions lie substantially on the same line as the transversions (Fig. 1). There are few distances between 0.14 and 0.18, and this discontinuity marks the boundary between those distances that are largely unsaturated and those in which saturation is marked. Most of the between-species distances in the dataset are above this boundary, and this indicates the presence of substantial homoplasy at this position, which may bias the parsimony analysis.

Testing the influence of the use of different outgroups on NJ trees showed that the tree is sensitive to different combinations, although this variability was

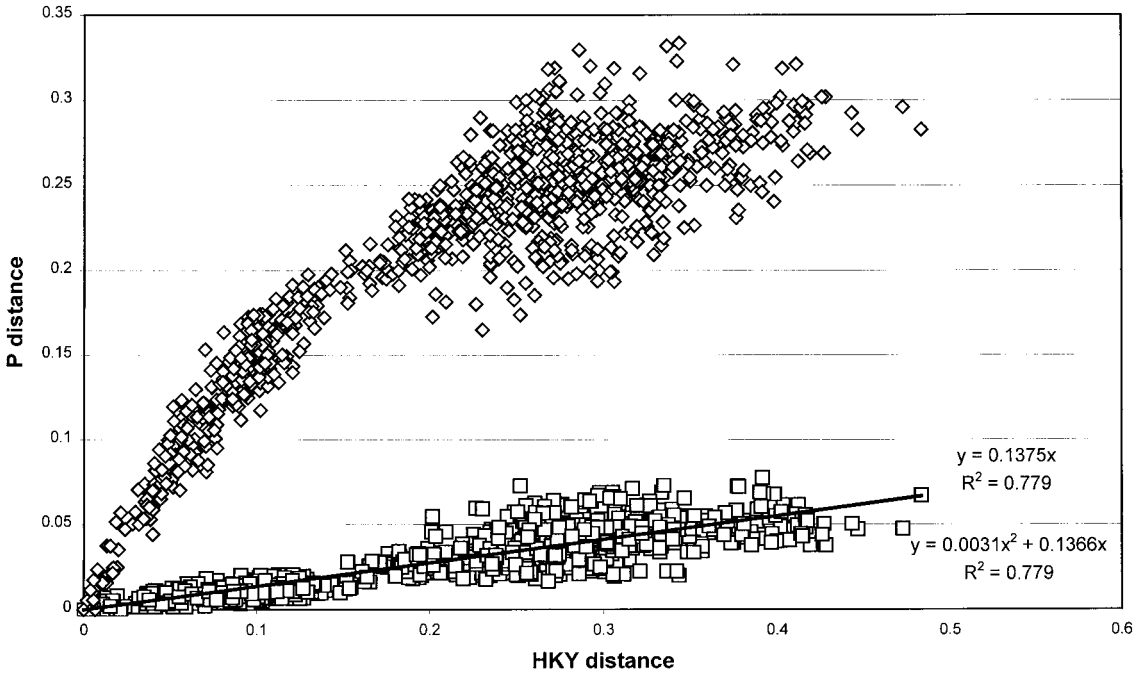

FIG. 1. Observed proportion (p-distance) of transversions (squares) plotted against the Hasegawa–Kishino–Yano (HKY) distance with gamma-distributed rates (shape parameter = 0.3). The equations and R^2 for the linear and quadratic regressions are given. Saturation at third codon positions (diamonds) is indicated by a decline in the rate of accumulation of observed proportion of transitions. Observed proportion of transitional changes at first and second codon positions (not shown) lie on a largely similar line to the transversions (y = 0.1375×, R^2 = 0.779).

confined to the deeper levels of the tree. Removing one outgroup at a time always changed the relative position of the clades below a certain level of the tree and in some cases also the composition of those clades. However, it seemed clear that the use of both *D. russelii* and *M. mauritanica* was redundant, as the results did not change if only one of these was deleted. In addition, using only *M. mauritanica* and *E. ocellatus* gave results identical to those using all four outgroups, and so they were retained as the outgroups.

Sequence Analysis

Among the 54 remaining sequences there were 283 parsimony-informative sites. Unweighted MP produced 16 equally parsimonious trees, which differ in only minor aspects involving closely related (intraspecific) terminal taxa (Fig. 2). A number of species groups have been indicated in Fig. 2 for ease of discussion. However, the bootstrap support for many of the basal nodes defining these species groups is low. The hypothesis that this is due to the randomization of the phylogenetic signal through saturation over greater periods of evolutionary time was tested using the g_1 statistic. This was done by holding together clades which were supported in the bootstrap analysis (see Fig. 2), i.e., the *albolabris* group, the *stejnegeri* group, *T. popeiroum,* the Indian subcontinent species (not including *T. borneensis*), the *Protobothrops* species, and a few other highly supported sister taxa relationships between the remaining species, and collapsing the rest of the ingroup branches. Once these relationships are accounted for, the g_1 statistic should be nonsignificant if there is no remaining phylogenetic structure in the rest of the tree, and in this case there would be no justification for attempting further resolution of the tree. However, g_1 is similar to that of the whole dataset at -0.2991 ($P < 0.01$), and it would appear that, despite the rapid evolution of cytochrome *b,* it retains enough signal to justify its use at the intergeneric level.

Instead, the low bootstrap support for some interior nodes may be caused by a few "floating" sequences (e.g., *T. borneensis, T. kanburiensis,* and *O. monticola*), which are not closely related to any species represented in the tree and therefore are particularly variable in their position. To test this, these taxa were excluded, and the bootstraps repeated to see whether these were responsible for lowering the bootstrap values for nodes which otherwise would be reasonably well supported. Although this does seem to be the case, the increase in support was not large (Fig. 2) and does not increase the overall resolution of the 50% consensus tree. Nevertheless, it is likely that the addition of species, which would reduce the branch lengths lead-

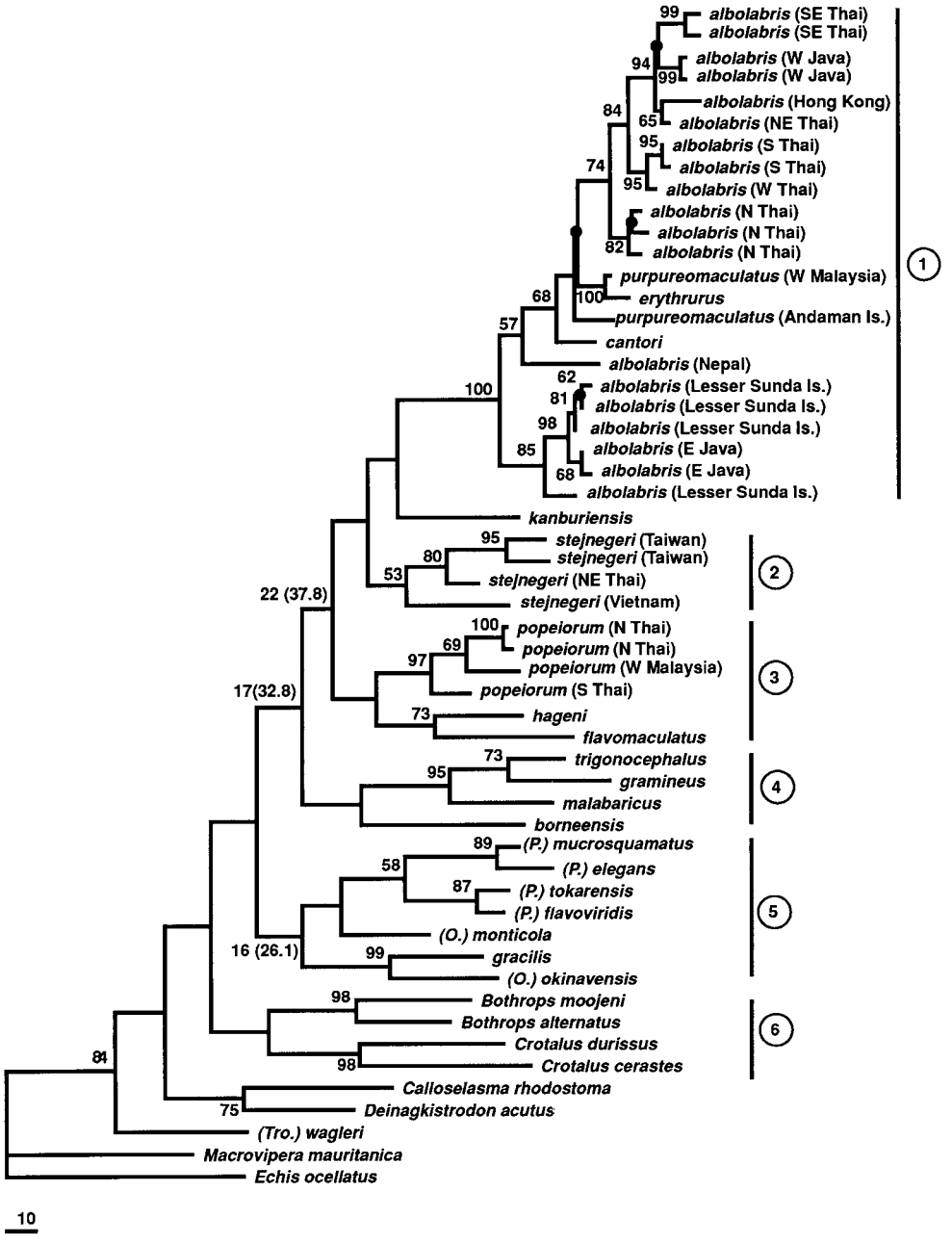

FIG. 2. The 50% consensus tree of 16 equally parsimonious trees of length 1782. Full generic names are given for species that do not belong to the *Trimeresurus s.l.*, whereas only the specific name is given for species belonging to *Trimeresurus s.s.* Finally, species allocated to one of the three other genera contained within *Trimeresurus s.l.* are indicated by generic abbreviations in parentheses. Filled circles indicate nodes that collapse in the strict consensus tree. The figures below nodes are the bootstrap support values (above 50%) from 400 replications. Figures in parentheses after bootstrap values at three nodes indicate the increase in bootstrap support when some sequences that are particularly variable in position (see text for full list) were eliminated from the analysis. The species groups referred to in the text are indicated on the tree. These are (1) *albolabris* species group, (2) *stejnegeri* species group, (3) *popeiorum* species group, (4) Indian subcontinent species group, (5) *Protobothrops/Ovophis* species group, and (6) New World species.

ing to these problematic taxa, would help to resolve this problem.

The ML (Fig. 3) tree is extremely similar to the MP tree; in fact the likelihood scores of the two trees ($-\ln L = 8281.13458$ and 8282.20308 for the MP and ML trees, respectively) are not significantly different ($P = 0.9$) in a Kishino–Hasegawa test (Kishino and Hasegawa, 1989). Both trees are most consistent above, and including, the Indian subcontinent/*T. borneensis* clade. This coincides with *Trimeresurus s.s.* (with the excep-

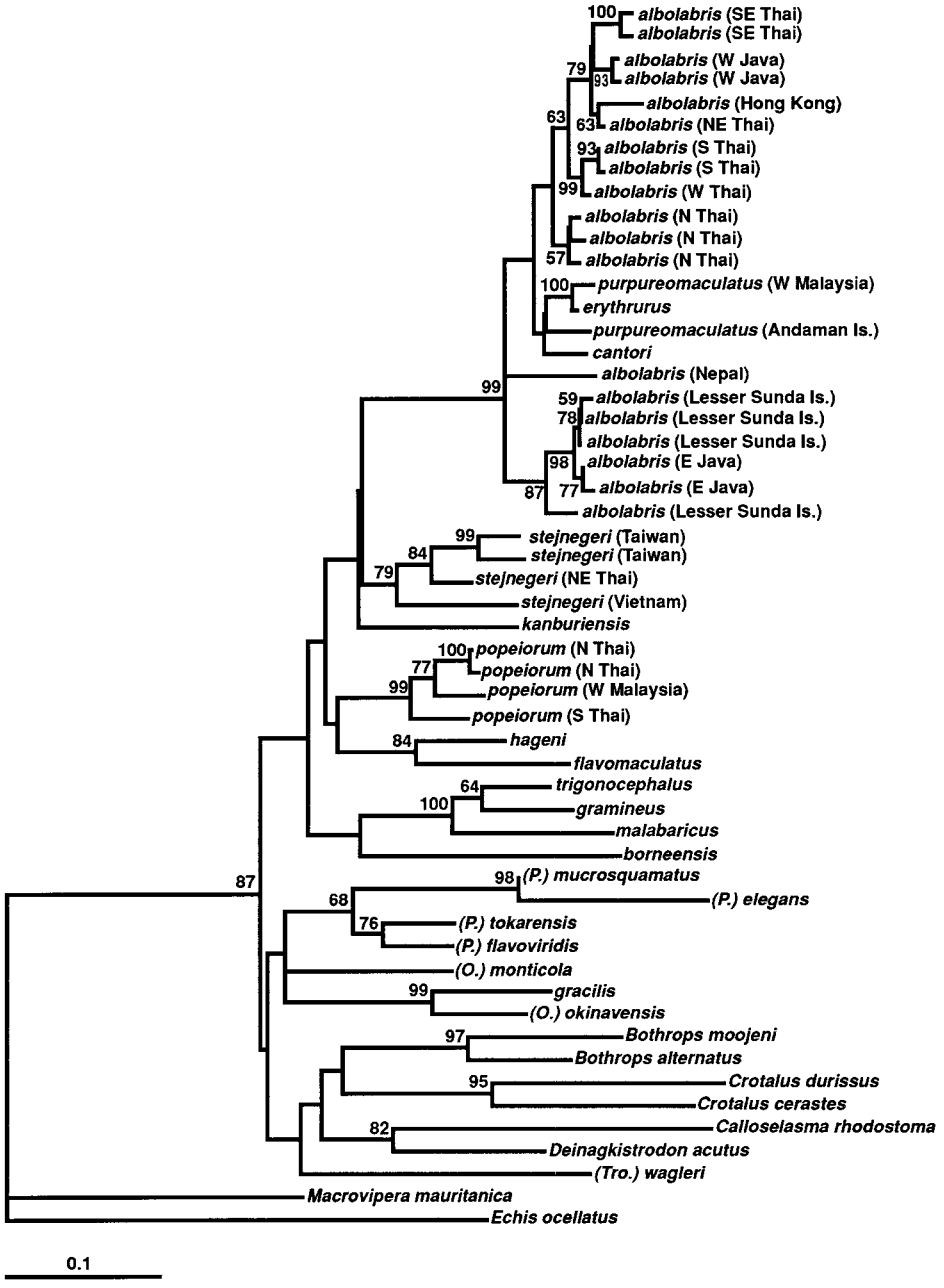

FIG. 3. Maximum-likelihood tree, showing bootstrap support values (above 50%) from 100 bootstrap replications. Species notations are described in the legend to Fig. 2.

tion of *T. gracilis;* see below). The region of the tree with the best support, as in the MP tree, is the clade containing *T. albolabris, T. purpureomaculatus, T. cantori,* and *T. erythrurus* (i.e., the *albolabris* species group in Fig. 2). Both trees show that the species *T. albolabris,* as currently defined, is paraphyletic, consisting of at least three clades (an exclusively Indonesian clade with populations from East Java and the Lesser Sunda Islands, a Nepalese clade, and a clade containing all other mainland populations, as well as West Java) which are split by a group of clearly distinct species (*T. erythrurus, T. cantori,* and *T. purpureomaculatus*). However, there are small differences between the topologies produced by the two methods in the relative positions of *T. cantori, T. erythrurus* and mainland and island populations of *T. purpureomaculatus.* These form a distinct clade in the ML tree, but are sequential in the MP tree. Also, although the Nepalese *T. albolabris* falls in exactly the same place in both trees, the branch length is so short in the ML tree that it appears unresolved in the phylogram. The final difference within this subsection of the tree is the po-

sition of *T. kanburiensis,* which falls between the *stejnegeri* and the *albolabris* groups in the MP tree, but between the *stejnegeri* and the *popeiorum* groups in the ML tree.

At deeper levels of the trees, the main groups defined by both methods are still remarkably consistent, and the points of major interest are all drawn from the well-supported aspects of both trees. The species assigned to *Protobothrops* are supported as a monophyletic group in both trees. However, the *Ovophis* species represented in this study are not monophyletic; instead, *O. okinavensis* is grouped together with a species of *Trimeresurus s.s., T. gracilis,* making both *Ovophis* and *Trimeresurus s.s.* paraphyletic. Both trees show that *Tropidolaemus* is quite distinct from other *Trimeresurus s.l.,* in agreement with all recently published molecular studies (Kraus *et al.,* 1996; Vidal and Lecointre, 1998; Parkinson, 1999). It is worth noting, however, that the monophyly of the New World pit vipers is not strongly supported. It may be that previous studies have not sampled the Asian pit viper radiation sufficiently well to firmly establish their reciprocal monophyly.

A likelihood ratio test to test for the action of a molecular clock, which compares a ML tree calculated with and without the assumption of a molecular clock, rejected the null hypothesis of equal rates operating across the tree. However, the relative rate test did not reject the null hypothesis of equal rates between any of the major clades defined above. Most of the rate variation would therefore appear to be within rather than between clades.

Support from Morphology

As the trees are essentially similar for the subsection corresponding to *Trimeresurus s.s.,* only this part of the tree was used for character mapping. The minor disagreements between the two trees were explored by examining each alternative dichotomous resolution rather than by collapsing these into polytomies. We show only the results using the ML tree in Fig. 3, with alternative reconstructions on the MP tree being reported in the text.

Hemipenis type (Fig. 4A). There are three types of hemipenes represented within *Trimeresurus s.s.,* which are consistent with the species groups defined by the DNA analysis. Three steps are required with a rescaled consistency index (RC) of 1.0. The *albolabris* and *popeiorum* species groups and *T. kanburiensis* all have smooth hemipenes. The distribution of Type 1 and Type 2 spinose hemipenes also appears very consistent. The former are restricted to members of the *stejnegeri* group, whereas the latter support the placement of *T. borneensis* with the Indian subcontinent species group, a clade with low bootstrap support. Note, however, that the latter are not entirely unambiguous; whereas the distribution of the spines are similar in both *T. borneensis* and Indian subcontinent species, the sizes of the spines are different. The alternative placement of *T. kanburiensis* on the MP tree, between the *stejnegeri* and the *albolabris* groups, involves the same number of steps; so, this character does not favor one arrangement over the other.

Condition of the first upper labial and nasal scale (Fig. 4B). Species having a nasal scale that is fully or partially fused with the first upper labial mostly belong to the *albolabris* species group, which is a well-supported clade in the mtDNA trees. The exception, in the ML tree, is *T. kanburiensis.* In this version, two steps are required to explain the distribution of this character (with two possible reconstructions) and it has a RC of 0.4. In fact, the branch length separating the *stejnegeri* clade and *T. kanburiensis* in the ML tree is so short that it is effectively a polytomy. The alternative position of this taxon shown in the MP tree (Fig. 2), in which it is basal to the *albolabris* clade, requires one less step and the RC increases to 1.0. This character, therefore, strongly supports the alternative position for this taxa given in the MP tree.

The number of scale rows at midbody (Fig. 4C). This character appears to be extremely conservative in the *Trimeresurus* group, being 21 in a large number of species (which adds to the difficulties of distinguishing between the green pit vipers, as this is character is commonly used in keys). One change to a higher number of scales has occurred in the *albolabris* species group (in the species *T. purpureomaculatus, T. cantori* and *T. erythrurus*). Altogether, five steps are required, and the RC = 0.6. In the MP tree, in which the species split sequentially from the common ancestor, at least one subsequent reversal would also be required to have occurred, making it the less parsimonious alternative. Evolution of a lower number of scale rows (19) has also occurred in some species, notably in three of four members of the Indian subcontinent/*T. borneensis* clade.

Green coloration (Fig. 4D). In contrast to the previous three characters, the distribution of green coloration shows little consistency between clades. At least nine steps are required, with eight possible reconstructions. The rescaled consistency index is only 0.25. Many species are rather polymorphic; for example, *T. flavomaculatus* displays all three character states. Again, one less step is required for the *T. purpureomaculatus, T. erythrurus,* and *T. cantori* arrangement in the ML tree than in the MP tree. Overall, there are a much larger number of steps (a minimum of nine) required to plot the evolution of this character than required of the other three characters, and the consistency index is correspondingly much lower.

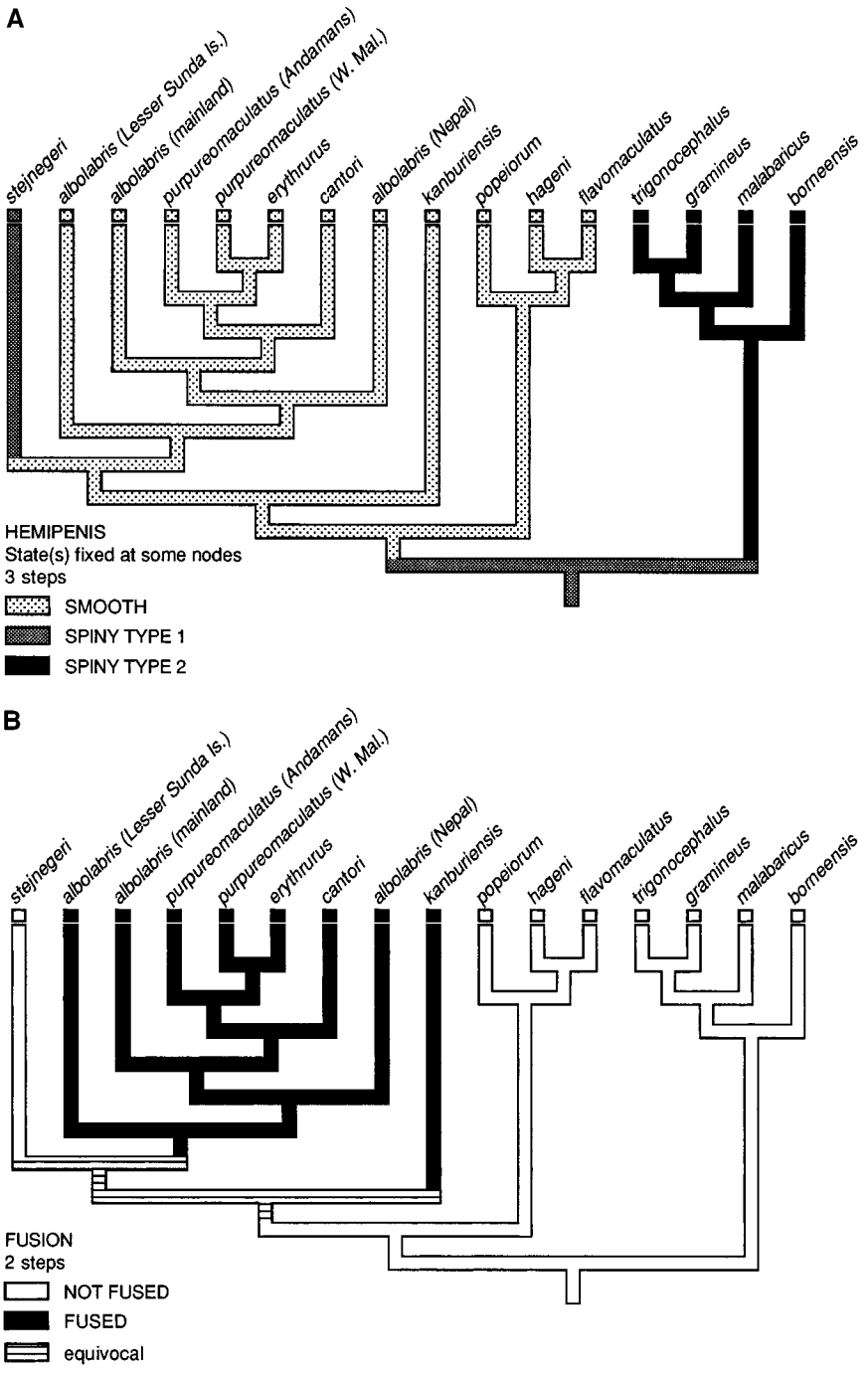

FIG. 4. Morphological characters mapped onto the maximum-likelihood tree (with morphologically conservative clades condensed to a single tip) for *Trimeresurus sensu stricto* only. (A) Hemipenial morphology, (B) fusion of first upper labial scale and nasal scale, (C) number of scale rows at midbody, and (D) green coloration. See text for further explanation.

DISCUSSION

Deeper Crotaline Relationships

The monophyly of the two species assigned to *Ovophis* (*O. monticola* and *O. okinavensis*) is not supported, and as *O. okinavensis* is unambiguously more closely related to *T. gracilis,* a species currently assigned to *Trimeresurus s.s.*, both genera are clearly paraphyletic as currently defined. In view of the major systematic rearrangement suggested by the mitochondrial gene tree, it is particularly noteworthy that the monophyly of *Protobothrops* continues to

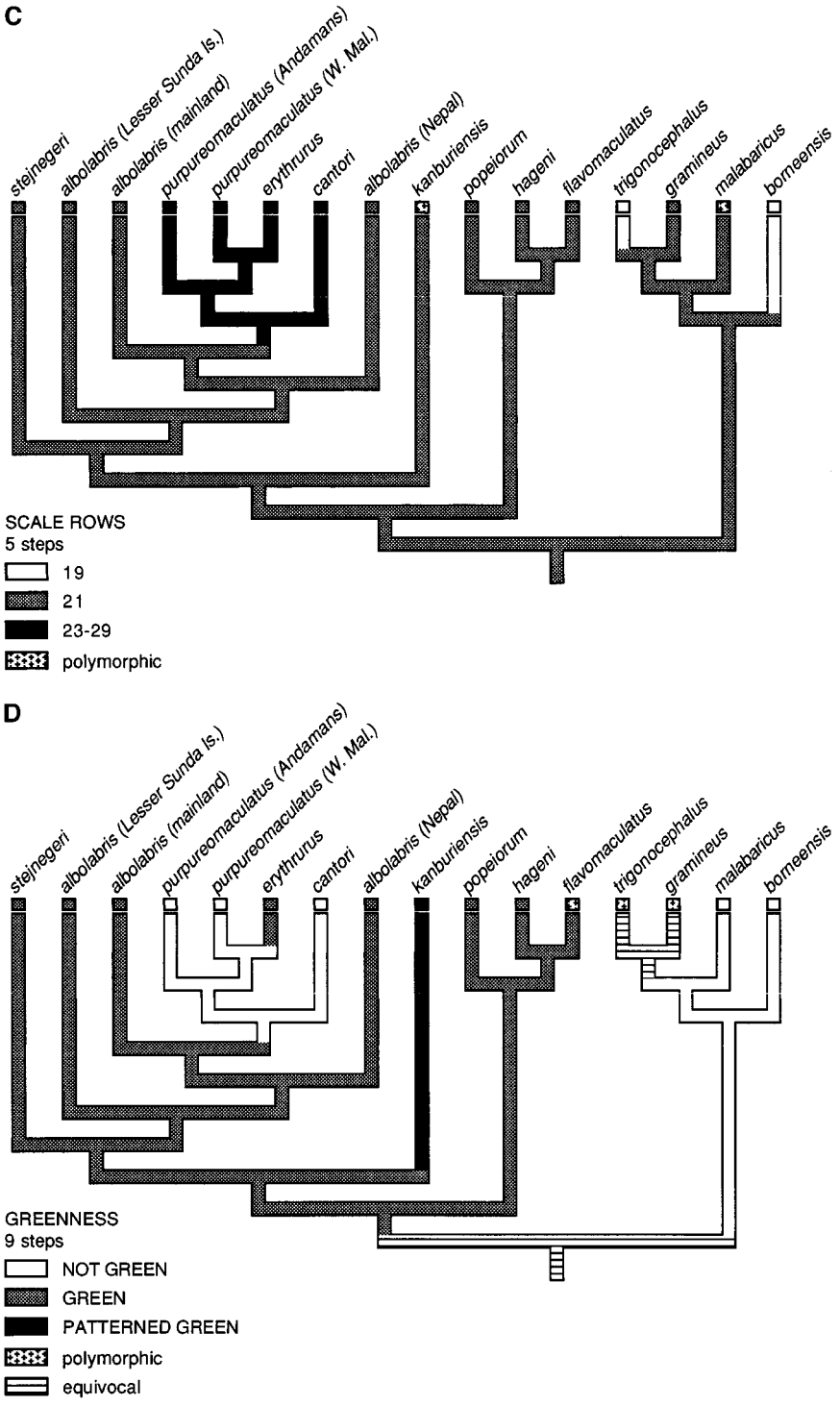

FIG. 4—*Continued*

be supported. However, extrapolation beyond the species actually included in this study is unwise. The content of *Ovophis* and *Protobothrops* currently owes much to species groups defined in an earlier work (Maslin, 1942), which we have shown elsewhere to be rife with errors. Clearly, more sampling of the species involved is required to fully address the status and validity of these genera. In addition, low bootstrap values supporting the critical nodes do not allow firm conclusions to be made about the relationships of these groups at deeper levels. The addition of more sequence data (particularly from slower-

evolving mitochondrial genes) may be useful to provide further resolution at this level.

Implications for the Taxonomy of Trimeresurus s.s.

Trimeresurus s.s. appears to be sufficiently structured to warrant further subdivision into genera or subgenera. However, the unique transmission characteristics of mitochondrial genes compared to nuclear genes imply that it may be unwise to base a major systematic revision on a single mitochondrial gene. Work is in progress on a corroborating nuclear-based phylogeny. However, the congruence between the morphological characters and the mtDNA gene tree not only lends support to the hypothesis of relationships proposed here, but also suggests that we could infer the relationships of other species for which genetic data is not available. For example, *T. strigatus,* an Indian subcontinent species, has a Type 2 spinose hemipenis in common with the other Indian subcontinent species, and its affinities are almost certainly with this group rather than with *Protobothrops,* as suggested by Kraus *et al.* (1996).

A combination of two morphological characters, hemipenis type and fusion of labial/nasal scale, defines four major clades within *Trimeresurus s.s.* It is possible that closer examination will be able to distinguish additional variation in the hemipenes (e.g., there appears to be variation in the presence of soft papillate processes among species with smooth hemipenes). This character may therefore be of crucial importance in resolving the taxonomy of the group, and it is unfortunate that so many new descriptions of species still fail to mention the hemipenis. The number of scale rows at midbody shows more changes (and particularly, more reversals) than the former two characters. However, this character also provides support for clades identified in the analysis where other characters are invariable. Finally, the lack of congruence of coloration with the DNA topology suggest that green coloration is not indicative of close evolutionary relationship but of evolutionary convergence. This convergence has reached a quite remarkable degree of perfection, as indicated by the fact that some species can still be reliably distinguished only by their hemipenial morphology (e.g., *T. popeiorum* and *T. stejnegeri*). This coloration is not restricted to *Trimeresurus s.s.,* having apparently been independently derived in the very distinct *Tropidolaemus wagleri.* All the green pit vipers are predominately arboreal and nocturnal, but other, nongreen, arboreal species exist. Further discussion regarding this convergence must await the resolution of the taxonomy of the bamboo vipers, which is among the most confused in the whole group.

Paraphyly of T. albolabris and T. purpureomaculatus

This study underlines the fact that, despite decades of study, the taxonomy of the green pit vipers is still not fully resolved, and there are still cryptic species present. The results show that *T. albolabris* is clearly paraphyletic, consisting of at least three clades. These correspond to the clades reported in Malhotra and Thorpe (1997), but their paraphyly was not detected in that study, as *T. purpureomaculatus* was erroneously assumed to be an outgroup. One of these clades, the largest "mainland" clade, appears to contain further substructure (Figs. 2 and 3) which may have systematic implications (currently under investigation). *T. purpureomaculatus* is also paraphyletic, with mainland populations (*T. p. purpureomaculatus*) being more closely related to *T. erythrurus* than to the Andaman island populations (*T. p. andersoni*).

The fact that the only species in which extensive sampling has been carried out across its range shows considerable substructuring and polyphyly raises the possibility that by the time other widespread species have been studied in detail, there may be even more species of *Trimeresurus.* This, together with the relatively deep divergence detected, even among the very similar green pit vipers, which are commonly not distinguished in the medical and biochemical literature, underlines the fact that arriving at a stable taxonomy for the *Trimeresurus* group will be of more than esoteric interest.

ACKNOWLEDGMENTS

We are grateful to the large numbers of people who have assisted us in the field or supplied us with tissue samples for analysis. These include Dr. Jennifer Daltry, and Dr. Wolfgang Wüster, Dr. Nicholas Giannasi, Nicholas Cockayne (University of Wales, Bangor), Professor David Warrell (University of Oxford), Romulus Whitaker, Dr. Indraneil Das, and Gerry Martin (Madras Crocodile Bank), Mr. S. S. Ramachandra Raja (Wildlife Association of Ramnad District, India), Dr. Michihisa Toriba (Japan Snake Institute), Anslem de Silva (Peridinaya University, Sri Lanka), Professor Sangkot Marzuki (Eijkmann Institute, Indonesia), Dr. Aucky Hinting, Rick Hodges, Vincen Khartono, Pak Harwono, and his staff (Surabaya Zoo), Dr. Wen-hao Chou (National Museum of Natural Science, Taiwan), Jean-Jay Mao (Taipei City Zoo, Taiwan), Tanya Chan-ard, Jarujin Nabhitabhata (National Science Museum of Thailand), Dr. Lawan Chanhome (Queen Savoabha Memorial Institute, Thailand), Dr. Kumthorn Thirakhupt and Dr. Peter Paul van Dijk (Chulalongkorn University, Thailand), Dr. Cheelaprabha Rangsiyanon (Chiang Mai University), Merel J. Cox, Jonathan Murray, Galen Valle, Dr. James D. Lazell (the Conservation Agency, USA), Dr. Bob Murphy (Royal Ontario Museum, Canada), Dr. Trinh Xuan Kiem (Cho-Ray Hospital, Vietnam), Michel Guillod, and Vincent Morier (Ophiofalm, Lausanne, Switzerland). We also acknowledge the National Science Council of Thailand for permission to carry out fieldwork in Thailand. We are grateful to Dale Taneyhill for supplying his program for carrying out the *G* test for heterogeneity. This study was funded by the Leverhulme Trust (F174/I) and the Darwin Initiative (162/6/65), with additional support for fieldwork from the Royal Society, the Percy Sladen Trust, the Bonhote Trust, and the Carnegie Trust.

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