

Nuclear and mtDNA Phylogenies of the *Trimeresurus* Complex: Implications for the Gene versus Species Tree Debate

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Phylogenies based on mitochondrial DNA (mtDNA) may represent gene trees that may not be congruent with the equivalent species tree. One solution to this problem is to include additional, independent loci from the nuclear genome. Sequence data from the seventh intron of the β -fibrinogen gene were generated for 25 specimens of vipers, including 8 nominal species of the *Trimeresurus* complex of Asian pit vipers. Phylogenetic trees were generated using maximum-parsimony and maximum-likelihood methods. The taxonomic level at which the intron provided significant phylogenetic information was examined and the trees were compared to those produced from previously obtained mtDNA cytochrome b sequences. A variety of different approaches (separate analyses, conditional data combination, and consensus) were used in an attempt to provide a sound organismal phylogeny based on both nuclear and mtDNA data sets. We discuss the implications for the gene tree–species tree debate and its particular relevance to medically important organisms. © 2001 Academic Press

Key Words: gene tree; species tree; mitochondrial DNA; nuclear DNA; *Trimeresurus*; pit viper; phylogeny.

INTRODUCTION

The past decade has seen a huge number of phylogenetic studies on a wide range of taxa that have provided valuable insight into many aspects of evolutionary biology. The vast majority of these studies have been based on mitochondrial DNA (mtDNA) because of the rapid rate of sequence evolution (Brown *et al.*, 1979), the comparability of mtDNA genes across broad taxonomic boundaries (Brown, 1985), and the availability of universal primers (Kocher *et al.*, 1989). However, due to the lack of recombination, the 37 different genes of the animal mitochondrial genome are inherited as a single unit and hence phylogenies derived from several mtDNA genes are not independent estimates of organismal phylogeny (Moore, 1995; Page,

2000). Furthermore, a phylogeny produced from mtDNA represents a gene tree that may not be congruent with the species tree because of lineage sorting (Moore, 1995). Given that the major goal of phylogenetic studies is to infer the evolutionary history of species and populations, rather than genes, this poses a significant problem. The only apparent solution is to include additional, independent loci from the nuclear genome in phylogenetic studies (Wu, 1991). Recently, there have been several investigations of the utility of a range of intron sequences as a source of such loci. Exon-primed intron crossing conserved primers have been used in studies of actin in cetaceans (Palumbi and Baker, 1994) and fibrinogen (Prychitko and Moore, 1997) and aldolase, G3PD, α enolase, and lamin in birds (Friesen *et al.*, 1997).

For venomous snakes, attempting to determine the species tree is of more than esoteric interest for a number of reasons. First, snakebite is very common in tropical developing countries and treatment with antivenom remains the only specific treatment for envenomation by snakes (Theakston, 1997). If venom composition differs between snake taxa, then a monovalent antivenom against the venom of one taxa is unlikely to be effective in neutralizing the venom of another taxa. Hence, knowing which species are present in which geographic locations enables a sound basis for production and clinical application of antivenoms. Second, venom variation is of fundamental importance for biomedical and toxicological research (Chippaux *et al.*, 1991; Thorpe *et al.*, 1997; Theakston, 1997). However, any work on venom based on an incorrect taxonomic framework may be rendered valueless if the wrong name is attached to the model or if comparisons are based on incorrect material (Warrell, 1986).

This study focuses on the pit vipers of the *Trimeresurus* complex that are widely distributed across southern Asia and the Indo-Malayan archipelago. This complex represents a major evolutionary radiation and consists of over 40 species. Between three and five genera [*Trimeresurus sensu stricto* (*s.s.*), *Tropidolaemus*, *Ovophis*, *Protobothrops*, and *Ermia*] are generally recognized within this complex (McDiarmid *et al.*, 1999; David and Ineich,

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TABLE 1

List of Species Included in This Study, Their Origins, GenBank Accession Numbers, and Catalogue Numbers (Author's Personal Collection)

Genus	Species	Geographic origin	Sequence code	Catalogue No.	GenBank Accession No. (β -fibrinogen)	GenBank Accession No. (Cyt b) ^a
<i>Calloselasma</i>	<i>rhodostoma</i>	West Malaysia	CAL1	A54	AF200622	AF171918
<i>Echis</i>	<i>ocellatus</i>	Garoua, Cameroon	ECHO567	WW567	AF200598	AF191579
<i>Ovophis</i>	<i>okinavensis</i>	Ryu-kyu Islands	B1	B1	AF200620	AF171915
<i>Protobothrops</i>	<i>mucrosquamatus</i>	Taiwan	TMUC1	A211	AF200621	AF171897
<i>Trimeresurus</i>	<i>albolabris</i>	Nepal	TAN2	A100	AF200614	AF171909
<i>Trimeresurus</i>	<i>albolabris</i>	North Thailand	T?2	A226	AF200612	AF171910
<i>Trimeresurus</i>	<i>albolabris</i>	Northeast Thailand	TAT4	A135	AF200618	AF171893
<i>Trimeresurus</i>	<i>albolabris</i>	South Thailand	TAT8	A134	AF200617	AF171894
<i>Trimeresurus</i>	<i>albolabris</i>	Hong Kong	TAHK1	A157	AF200615	AF171884
<i>Trimeresurus</i>	<i>albolabris</i>	West Java (1)	TAWJ1	A125	AF200613	AF171886
<i>Trimeresurus</i>	<i>albolabris</i>	West Java (2)	TAWJ3	A126	AF200616	AF171891
<i>Trimeresurus</i>	<i>albolabris</i>	East Java	TAEJ1	A115	AF200610	AF171887
<i>Trimeresurus</i>	<i>cantori</i>	Nicobar Islands	TCANT	A85	AF200606	AF171899
<i>Trimeresurus</i>	<i>erythrurus</i>	Rangoon, Myanmar	TERY1	A209	AF200607	AF171900
<i>Trimeresurus</i>	<i>gracilis</i>	Taiwan	TGRAC	A86	AF200619	AF171913
<i>Trimeresurus</i>	<i>gramineus</i>	South India	TG1	A219	AF200604	AF171905
<i>Trimeresurus</i>	<i>malabaricus</i>	South India	TMAL2	A217	AF200602	AF171901
<i>Trimeresurus</i>	<i>popeorum</i>	North Thailand	TPN1	A204	AF200600	AF171902
<i>Trimeresurus</i>	<i>popeorum</i>	South Thailand	TPS1	A202	AF200601	AF171904
<i>Trimeresurus</i>	<i>popeorum</i>	West Malaysia	TPM1	A196	AF200605	AF171888
<i>Trimeresurus</i>	<i>stejnegeri</i>	Northeast Thailand	TSL18	A181	AF200609	AF171898
<i>Trimeresurus</i>	<i>stejnegeri</i>	Taiwan (1)	TST4	A160	AF200608	AF171896
<i>Trimeresurus</i>	<i>stejnegeri</i>	Taiwan (2)	TST60	A161	AF200611	AF171880
<i>Trimeresurus</i>	<i>trigonocephalus</i>	Sri Lanka	TT1	A58	AF200603	AF171890
<i>Tropidolaemus</i>	<i>wagleri</i>	West Malaysia	TWAG1	A66	AF200599	AF171917

^a From Malhotra and Thorpe (2000).

1999). Certain species, such as *Trimeresurus albolabris*, are common and represent a significant problem to local people and an occupational hazard to agricultural workers. A recent phylogenetic study, based on cytochrome b mtDNA data, of 21 species in the complex (Malhotra and Thorpe, 2000) presented significant differences in species relationships that impinge on the previously accepted taxonomy. The main aims of this study were, first, to determine whether the previously identified seventh intron from the β -fibrinogen gene (Prychitko and Moore, 1997) was easily amplified across a range of medically important snake species for which mtDNA data exist; second, to determine at which taxonomic levels this intron provides significant phylogenetic information; and, third, to examine the phylogenetic resolution of different approaches (separate analyses, conditional data combination, consensus) when there is more than one data set. Finally, we hope to provide a sound organismal phylogeny for this medically and evolutionarily significant group of venomous snakes.

MATERIALS AND METHODS

Sampling

Twenty-three samples representing eight nominal species and four genera (*Tropidolaemus*, *Trimeresurus*

s.s., *Ovophis*, and *Protobothrops*) of the *Trimeresurus* complex were used in this study (see Table 1 for details). In addition, one other Asian pit viper (*Calloselasma rhodostoma*) was included since previous studies suggest that the *Trimeresurus* complex may not be monophyletic (Malhotra and Thorpe, 2000). Finally, a true viper (*Echis ocellatus*) was used as the outgroup. Samples were in the form of tail-tip biopsies or liver tissue in 80% ethanol or 100–200 μ l of blood taken from the caudal vein, placed in 1 ml of 5% EDTA, and stored in 2 ml SDS–Tris buffer (100 mM Tris, 3% SDS).

DNA Preparations, PCR Amplifications, and Sequencing

Whole genomic DNA was extracted using the protocol of Sambrook *et al.* (1989). The seventh intron from the β -fibrinogen gene was then amplified using the exon designed primers FIB-B17U and FIB-B17L (Prychitko and Moore, 1997). The cycling parameters used were as follows: denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min 30 s. A negative (blank) control was always included to monitor for possible contamination. PCR products were purified using the Wizard PCR purification system following the manufacturer's instructions. The

double-stranded purified PCR segment was then sequenced from both ends using BigDye Terminator cycle sequencing, and sequence was determined with an automated sequencer (Applied Biosystems 377) following the manufacturer's protocols.

Sequence Analysis

The nuclear DNA (nDNA) sequences were aligned with ClustalX (Thompson *et al.*, 1997), using default settings. Base composition was assessed using PAUP* Version 4.0b2a (Swofford, 1998). DnaSP Version 3 (Rozas and Rozas, 1999) was used to determine the number of monomorphic, polymorphic, and parsimony-informative sites and detect the presence of stop codons. The hypothesis that all mutations are selectively neutral (Kimura, 1983) was tested using Tajima's test as implemented in DnaSP Version 3 (Rozas and Rozas, 1999) based on the total number of mutations and including sites with gaps. A likelihood-ratio test (Felsenstein, 1988) was used to determine whether nucleotide substitutions are clock-like over the evolution of all sequences. Nucleotide saturation was assessed at each codon position by plotting numbers of transitions and transversions against the Tamura-Nei distance (plots not shown). We used DAMBE Version 3.5.14 (Xia, 2000) to further investigate saturation, since simulation studies show that phylogenetic information is essentially lost when the observed saturation is equal to, or larger than, half of full substitution saturation (Xia, 2000).

The alignment of the 25 sequences was tested for adequacy of phylogenetic signal using PAUP* Version 4.0b2a (Swofford, 1998) by plotting tree lengths of 100,000 random trees, with calculation of the g_1 statistic for the skewness of tree length distributions (Hillis and Huelsenbeck, 1992). 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). We also used g_1 in an iterative process to determine whether phylogenetic signal is evenly distributed throughout the branches of a tree, i.e., to assess the phylogenetic levels at which the data set has a significant signal-to-noise ratio. This was done by first defining a constraint tree in which only the well-supported tip clades found in the phylogenetic analysis were defined and recalculating the g_1 statistic. In subsequent tests, clades defined by successive levels of the tree were defined, and the point at which g_1 ceases to be significant indicates the limit of resolution of the data set (Hillis, 1991).

All phylogenetic analyses were executed using PAUP* Version 4.0b2a (Swofford, 1998). Different reconstruction methods were used to derive phylogenies as this allows the consistency of phylogenetic estimation to be evaluated (Avise, 1994). Phylogenetic trees

were reconstructed using maximum-parsimony (MP) (Swofford and Olsen, 1990) and maximum-likelihood (ML) (Felsenstein, 1981) methods. In the MP analysis, the heuristic search algorithm was employed with 10 random additions of taxa and tree bisection-reconstruction (TBR) branch swapping. The random addition of sequences increases the effectiveness of heuristic searches as they decrease the chance that a search will find suboptimal trees in "tree islands" other than those containing the most-parsimonious trees (Maddison, 1991). All other settings were left at default values. Maximum-likelihood analyses were performed using heuristic searches with the HKY model (Hasegawa *et al.*, 1985), with empirical base frequencies. The reliability of the trees produced by all phylogenetic reconstructions was tested by bootstrap analysis (Felsenstein, 1985) with 1000 replications. The Wilcoxon signed-ranks test (Templeton, 1983) was applied to compare the statistical significance of the best tree produced by each tree reconstruction method relative to one another.

MtDNA cytochrome b sequence data (660 bp) were also available for the 25 specimens used in the intron analysis (a subset of the specimens used in Malhotra and Thorpe, 2000). This allowed an assessment of the utility of the seventh intron from the β -fibrinogen gene at the between-genera taxonomic level. MP and ML trees were constructed for the mtDNA data to allow such a comparison. Again, the Wilcoxon signed-ranks test (Templeton, 1983) was applied to compare trees.

In an attempt to assess different methods of combining independent data sets to derive a sound organismal phylogeny, we reconstructed phylogenies from concatenated nDNA and mtDNA sequences in a total evidence approach (Kluge, 1989). We then assessed the congruence of the underlying data by a partition homogeneity test, based on comparison of separate and combined tree lengths, with data randomization (Farris *et al.*, 1994). In this test, nDNA and mtDNA sequences from all 25 specimens were concatenated and designated as separate partitions, and congruence was assessed using 1000 heuristic search replicates. Subsequently, a variety of consensus approaches were explored [reviewed by Swofford (1991)]. The simplest of these is the strict consensus, which includes only the groups that appear on all of the competing trees (Sokal and Rohlf, 1981). Semistrict consensus trees (Bremer, 1990) are equivalent to strict consensus trees if all competing trees are fully dichotomous. However, they allow groups that are never contradicted, but may not appear in all the trees (e.g., if they are less than fully resolved), to be retained in the consensus tree. Majority-rule consensus trees (Margush and McMorris, 1981) are the most familiar and are less strict in that they allow groups that occur in more than a given proportion of all trees (typically a 50% cut-off is used) to be included. The Adams consensus tree (Adams,

1972, 1986) is also less strict in that it includes any groups shared by the competing trees regardless of whether they constitute completely uncontradicted components (Mickey and Platnick, 1989). It has been suggested to be useful when one or more taxa have very different positions on different trees, but when there is also a subset of sequences upon whose relationships the different trees agree upon. However, it may produce consensus trees that contain groups not in fact found in any of the competing trees, which complicates its interpretation.

RESULTS

Sequence Characteristics

We confirmed that we had amplified and sequenced the seventh intron from the β -fibrinogen gene by comparing the overlap of our putative exons with stretches of published chicken exon sequence (Weissbach *et al.*, 1991) that flanked the 5' and 3' ends of the intron. We determined the beginning and end of the intron sequence by locating the basepairs that form the 5' and 3' consensus splice sites that are highly conserved across introns (Bretahnach *et al.*, 1978). On average we found the seventh intron from the β -fibrinogen gene to be highly A-T rich (A = 29%, C = 21%, G = 16%, and T = 34%), which is in agreement with previous work on this intron by Pritchko and Moore (1997). The intron was found to be 927 bp in length. Over all sequences, there were 799 monomorphic sites, 108 polymorphic sites, and 47 parsimony-informative sites. Deletions were present in a few sequences but were short (maximum 3 bp in length) and were comparatively rare, with one present in *Trimeresurus cantori* and several different populations of *Trimeresurus albolabris* and two present in *Ovophis okinavensis*. Sequences have been deposited with GenBank (accession numbers are given in Table 1).

Tajima's test could not reject the hypothesis that all mutations are selectively neutral ($D = -1.62$, not significant, $0.10 > P > 0.05$). Furthermore, a likelihood-ratio test could not reject the action of a molecular clock, further indicating that the amplified intron was indeed conforming to neutral theory. The observed substitution saturation differed significantly ($T = 37.23$, $df = 29.8817$, $P < 0.0001$) from half of full substitution saturation, indicating that no significant saturation was present in the data set. A linear plot of transitions and transversions versus the Tamura-Nei distance (not shown) confirmed this. The presence of significant phylogenetic signal was indicated by the skewness parameter $g_1 = -1.23$, corresponding to $P < 0.001$ for the number of characters and taxa involved (Hillis and Huelsenbeck, 1992). Furthermore, the iterative g_1 analysis demonstrated that phylogenetic signal in the data set is evenly distributed

throughout the branches of the most parsimonious tree ($P < 0.001$ for all g_1 calculations).

Phylogenetic Relationships from β -Fibrinogen

A total of 2068 most parsimonious trees (length = 164) were produced with a consistency index (CI) of 0.896, a retention index (RI) of 0.915, and a homoplasy index (HI) of 0.104 (Fig. 1a). The ML analysis recovers an almost identical topology (Fig. 1b) with approximately the same degree of resolution. Indeed, using Templeton's test no significant differences were found between the two topologies ($z = -1.41$, $P = 0.157$). However, the ML analysis does not find *Tropidolaemus wagleri* and *C. rhodostoma* to be sister taxa.

Phylogenetic Relationships from Cytochrome b

A single most-parsimonious tree (length = 947) was produced with a CI of 0.466, a RI of 0.541, and a HI of 0.534 (Fig. 2a). The ML analysis (Fig. 2b) produced a topologically almost identical tree, which is not significantly different ($z = -2.00$, $P = 0.055$) from the MP tree. However, both mtDNA trees show greater resolution than the intron trees and are significantly different from the intron trees (intron MP vs mtDNA MP, $z = -5.11$, $P < 0.001$; intron MP vs mtDNA ML, $z = -9.19$, $P < 0.001$; intron ML vs mtDNA MP, $z = -5.11$, $P < 0.001$; intron MP vs mtDNA ML, $z = -5.10$, $P < 0.001$).

Phylogenetic Relationships from Total Evidence

Using a partition homogeneity test, the concatenated data set was found to be significantly incongruent ($P = 0.001$). Nevertheless, a total evidence approach advocates combining data sets regardless (Kluge, 1989). Three equally parsimonious trees (length = 1145) were produced from concatenated sequences, with a CI of 0.514, a RI of 0.573, and a HI of 0.486. The MP and ML analyses resulted in almost identical topologies (Fig. 3). The degree of significant differences between all topologies is summarized in Table 2.

Phylogenetic Relationships from Consensus

Approaches

Consensus trees resulting from different approaches are given in Fig. 4. The strict consensus tree retains virtually no phylogenetic information (Fig. 4a). The semistrict consensus approach (Fig. 4b) provides a greater degree of resolution, recognizing (among others) groups corresponding to *Trimeresurus stejnegeri* and *Trimeresurus popeorum* and suggesting that *T. albolabris* may contain more than one species. The majority-rule tree (Fig. 4c) provides a similar level of resolution as the semistrict tree, recognizing most of the same groups and also suggesting the paraphyly of *T. albolabris*. However, the sister relationship between *Calloselasma* and *Tropidolaemus* is lost, as is the grouping of *Protobothrops* with the *O. okinavensis*

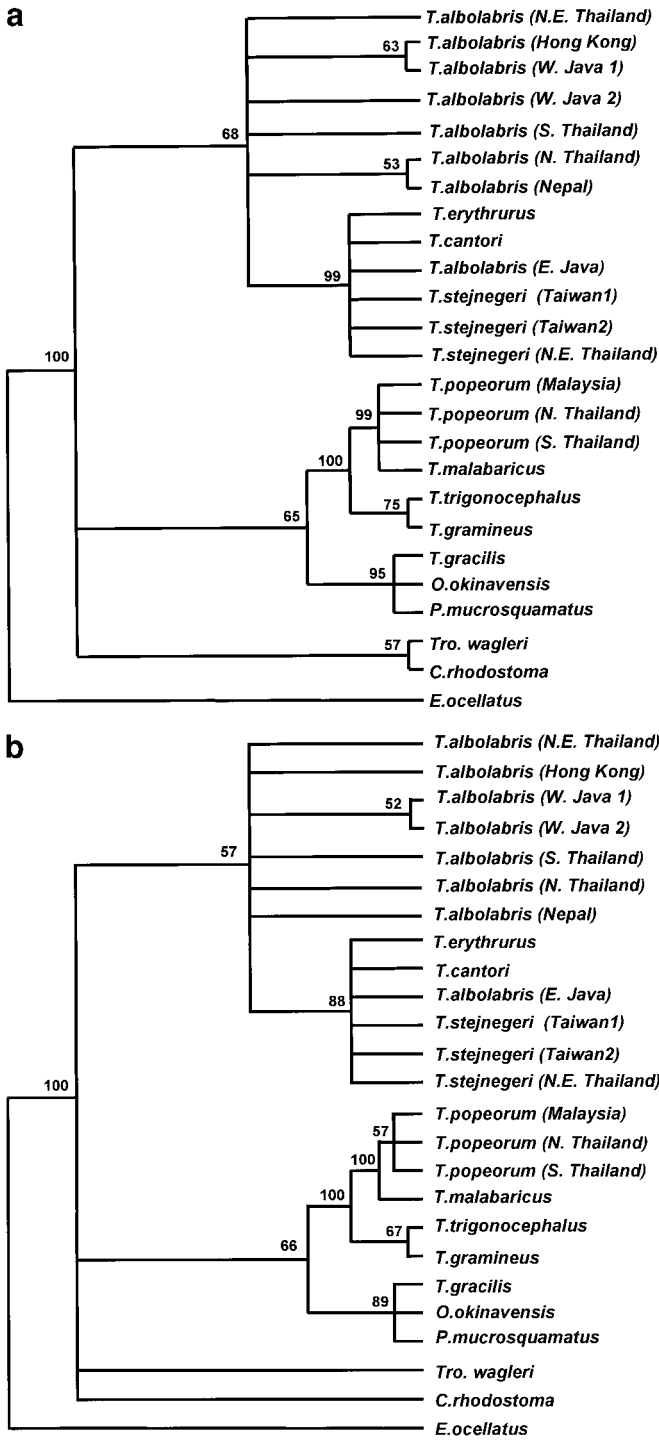


FIG. 1. Phylogenetic relationships based on sequence data from the seventh intron of the β -fibrinogen gene. Bootstrap percentages (above 50%) are indicated below the nodes to which they refer, where appropriate (a) Maximum-parsimony tree. (b) Maximum-likelihood tree.

Trimeresurus gracilis cluster. Finally, the Adams consensus tree (Fig. 4d) also provides similar information concerning the taxonomy of *Trimeresurus*.

DISCUSSION

Using EPIC PCR amplification, the seventh intron from the β -fibrinogen gene appears to be relatively

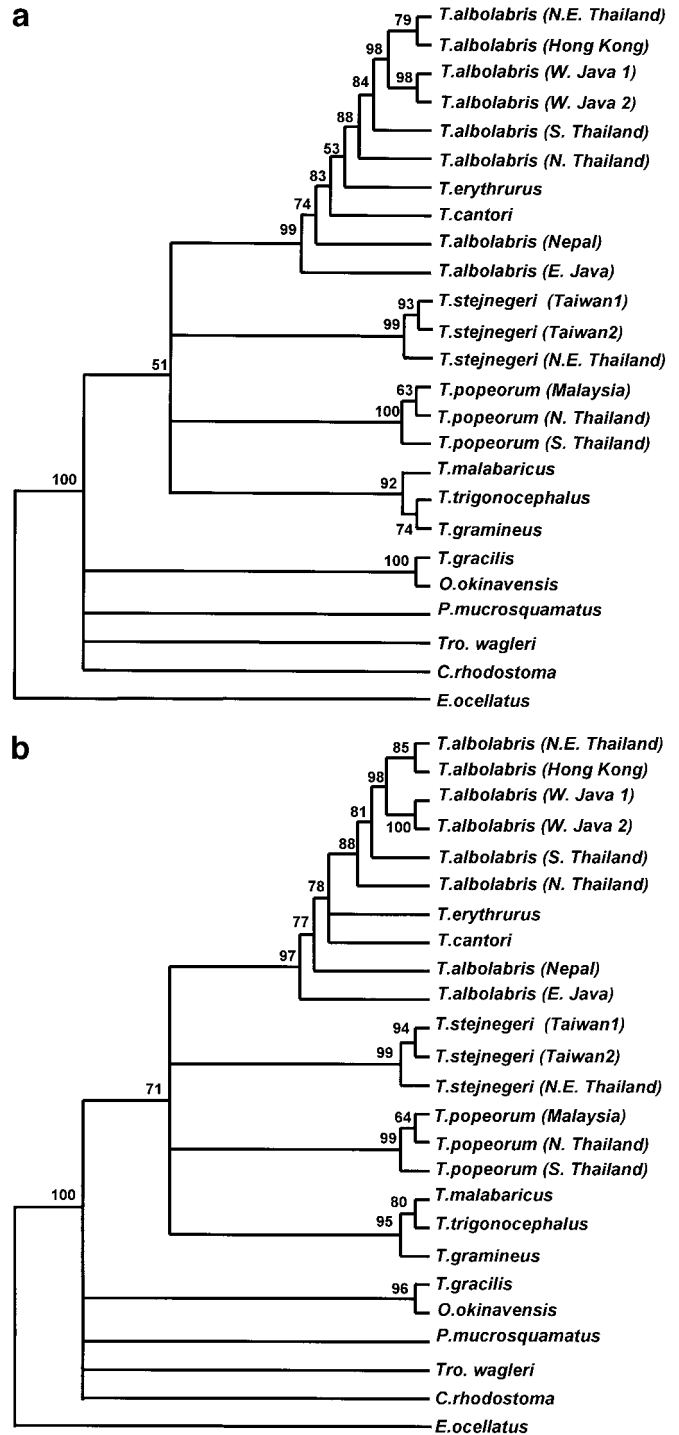


FIG. 2. Phylogenetic relationships based on a subset of cytochrome b mtDNA sequence data from Malhotra and Thorpe (2000). Bootstrap percentages (above 50%) are indicated where appropriate. (a) Maximum-parsimony tree. (b) Maximum-likelihood tree.

TABLE 2

Summary of Topological Differences between All Trees Assessed by the Wilcoxon Signed-Ranks Test (Templeton, 1983)

	nMP	nML	mtMP	mtML	Combined MP
nML	-1.41 ^{NS}				
mtMP	-5.11 ^{NS}	-5.11***			
mtML	-9.19***	-5.11***	-2.01 ^{NS}		
Combined MP	-3.37***	-3.14***	-1.15 ^{NS}	-2.18*	
Combined ML	-3.37***	-3.14***	0.00 ^{NS}	-0.89 ^{NS}	-3.24*

Note. n represents a nuclear DNA tree, mt represents a mtDNA tree, combined represents a combined nDNA/mtDNA tree, MP represents maximum-parsimony, and ML represents maximum-likelihood.

*** $P < 0.0001$; ** $P < 0.001$; * $P < 0.05$; ^{NS}not significant.

These include the partition homogeneity test (Farris *et al.*, 1995), the Wilcoxon signed-ranks test (Templeton, 1983), the Kishino–Hasegawa test (Kishino and Hasegawa, 1989), level of nodal support (Flynn and Nedbal, 1998), and the likelihood heterogeneity test (Huelsenbeck and Bull, 1996). In this study, we use the partition homogeneity test and find the concatenated intron and mtDNA data sets to be significantly incongruent ($P = 0.001$). Hence, following the CDC approach it is not appropriate to combine the two data sets and hence we are still no closer to resolving a species tree from our two gene trees.

A third approach is to unconditionally combine all data sets in a “total evidence” analysis as advocated by Kluge (1989). The main attraction of combining all data into a single analysis is that it makes use of all the available data. Indeed, MP and ML analyses on the combined intron and mtDNA data result in almost identical topologies and exhibit the highest degree of resolution and support of all the trees considered (i.e., MP and ML intron, MP and ML mtDNA). This is despite the significance of the partition homogeneity test that advocates keeping the two data sets separate. This observation provides support for Sullivan’s (1996) suggestion that while tests of homogeneity provide evidence to keep data partitions separate in phylogenetic analyses, combining certain heterogeneous partitions can lead to a more robust phylogeny in some details. The resulting total evidence trees seem to provide “sensible” taxonomic information that corresponds to morphology (Malhotra and Thorpe, 2000). Among the species represented by multiple samples, *T. stejnegeri* and *T. popeorum* are recognized as monophyletic groups, but *T. albolabris s.l.* is seen to be paraphyletic. Hence, at first glance it appears that the total evidence approach may provide the best method of producing a species tree from more than one gene tree and therefore allow us to make taxonomic suggestions concerning these medically important snakes.

However, it has been suggested that some data sets can be “positively misleading” (Bull *et al.*, 1993) and incorporation into a total evidence analysis can pro-

duce incorrect estimates of phylogeny (e.g., Flynn and Nedbal, 1998). As a result we advocate caution and agree with Flynn and Nedbal (1998) that it would prove advantageous to have a more accurate measure of heterogeneity between data partitions that can differentiate between compromising the integrity of existing phylogenetic signal and sustaining and/or increasing phylogenetic signal. A further weakness of the total evidence approach is that it assumes that all the data reflect the same evolutionary history. This may not be the case since in many species (e.g., humpback whales) one may expect mtDNA and nuclear genes to have different, conflicting histories that may represent gender-biased migration (Palumbi and Baker, 1994). For example, if males move more than females and breed more successfully than females, then nuclear genes may be mixed more thoroughly than the maternally inherited mtDNA sequences.

Moreover, different genes may have different histories for a variety of other reasons, such as gene duplication (resulting in paralogous genes), lineage sorting, and horizontal transfer (Page, 2000). The reconstruction of the history of a gene family is interesting in its own right and has benefited from the application of reconciled trees (Goodman *et al.*, 1979; Page, 2000). However, the original problem of inferring species trees from one or more gene trees also would benefit substantially from the application of reconciled tree concepts. The optimal species tree is simply the tree in which all the gene trees can be embedded with the least cost. The program GeneTree (Page, 1998) is currently available to do such an analysis. Unfortunately, we were not able to implement this method, as it requires fully resolved gene trees. Furthermore, weakly supported and highly supported nodes are given equal weighting. These limitations are currently being investigated (Page, 2000) and may provide a novel and practical way to infer organismal phylogenies from one or more gene trees. This study suggests that these developments are likely to be very important in order to produce organismal phylogenies from multiple gene trees and thus facilitate the practical implementation

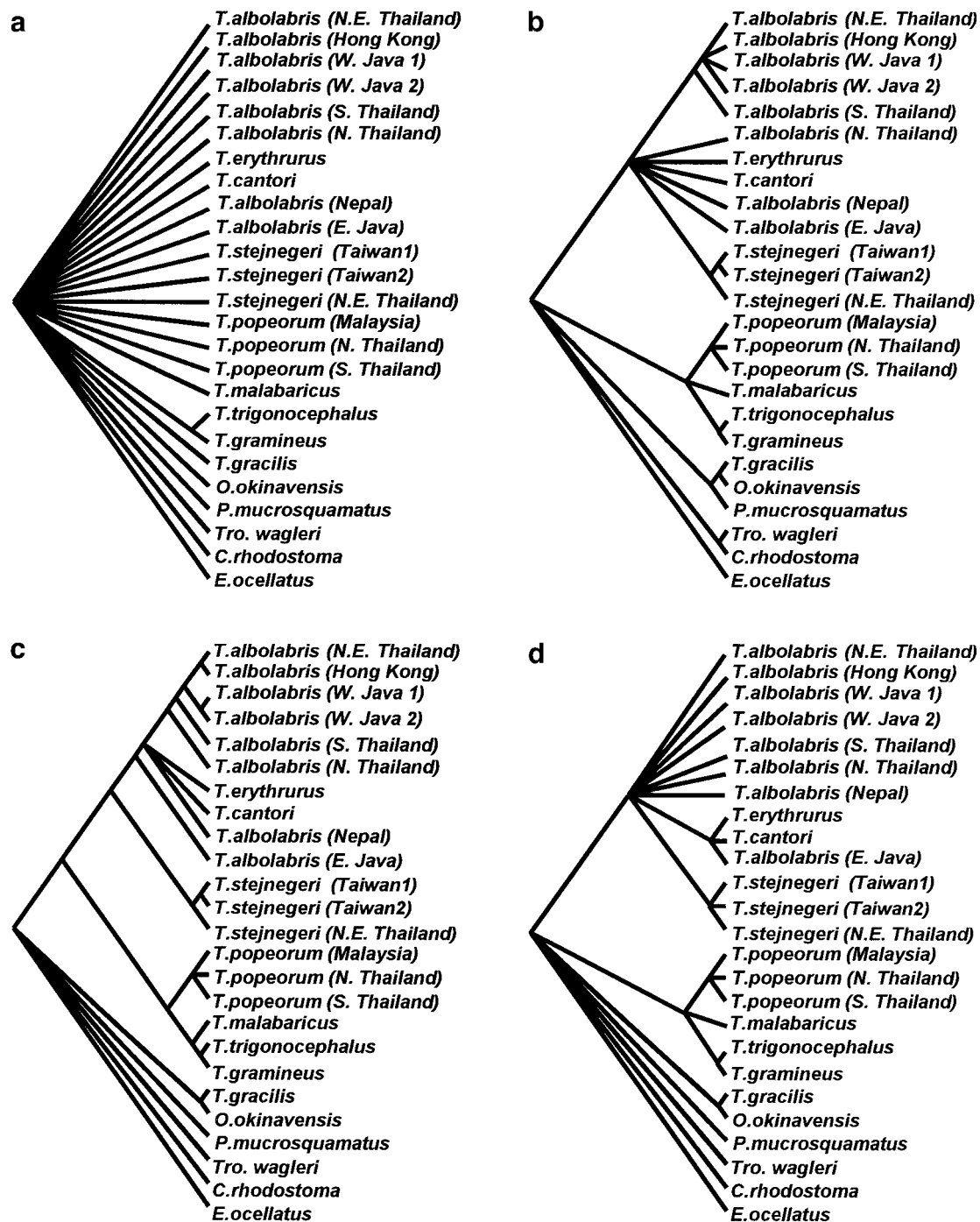


FIG. 4. Consensus trees of all six constructed trees (MP and ML trees from intron, mtDNA, and combined sequences). (a) Strict consensus (b) Semistrict consensus (c) 50% majority-rule consensus (d) Adams consensus.

of molecular phylogenetics in providing a stable taxonomy for medically significant organisms.

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