Assessing the Phylogenetic Utility of Four Mitochondrial Genes and a Nuclear Intron in the Asian Pit Viper Genus, *Trimeresurus*: Separate, Simultaneous, and Conditional Data Combination Analyses

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A number of methods have been proposed for addressing how to optimize the analysis of multiple data sets from diverse mitochondrial and nuclear gene partitions in the pursuit of robust organismal phylogenies. The present study used separate, simultaneous, and conditional data combination methods to analyze 3,135 bp of data from four mitochondrial partitions and the seventh intron of the β fibrinogen gene in the Asian pit viper genus, *Trimeresurus sensu stricto*. The phylogenetic utility and homogeneity of all partitions were estimated via a combination of homogeneity partition tests, homoplasy indices, and partitioned Bremer support. Despite the detection of significant heterogeneity of phylogenetic signal between the mitochondrial and nuclear partitions, the simultaneous analysis represented the best-supported topology of all the data. The relatively slow rate (approximately one quarter of the rate of mtDNA) and functionally unconstrained molecular evolution of the intron resulted in much lower levels of homoplasy compared with the mitochondrial partitions. This was further shown via partitioned Bremer support, which, when considered throughout hierarchical clade levels, highlighted the phylogenetic strength and limitations of the intron at deeper and shallower phylogenetic levels, respectively. The simultaneous analysis helped to resolve the phylogenetic relationships of taxa that were unresolved throughout all individual gene trees and tentatively supports the existence of morphologically and genetically distinct clades within the genus. Topological appraisals of the mitochondrial gene partitions suggest that the cytochrome b and the NADH subunit 4 gene partitions are better estimators of phylogenetic relationships than are the 12S and 16S ribosomal RNA partitions at the taxonomic levels under consideration.

Introduction

Although mitochondrial DNA (mtDNA) trees are predicted to be good estimators of species trees, gene tree topologies may not reflect species tree due to the risk of historical lineage sorting and/or horizontal gene transfer (Pamilo and Nei 1988; Avise 1989; Moore 1995). Therefore, nuclear DNA gene trees can be used to corroborate mtDNA gene trees in the search of organismal, as opposed to gene, phylogenies (Moore 1995, 1997). Nuclear introns have the potential to fill this niche in the dynamic field of molecular markers. MtDNA normally evolves relatively rapidly at the sequence level (Vawter and Brown 1986), due in part to an elevated mutation rate resulting from the mitochondrial inefficiency in repairing replication errors and DNA damage (Brown, George, and Wilson 1979; Clayton 1984). Therefore, noncoding nuclear introns would be expected to have a greatly reduced rate of molecular evolution. However, in protein coding mtDNA sequences, there is a bias for transitions over transversions (Kimura 1980; Moritz, Dowling, and Brown 1987), and only the third, and sometimes first, position synonymous nucleotide sites are free from functional constraints. Thus, two thirds of all bases in protein-coding sequences are less likely to yield phylogenetic signal than the remaining third base, which is inherently prone to homoplasy (Allard, Farris, and Carpenter 1999; Björkland 1999). Conversely, introns are apparently free from functional constraints, and all bases have the potential to yield phylogenetically informative sites with lower levels of homoplasy, and lower transition:tranversion ratios (Slade et al. 1993; Palumbi

and Baker 1994; Slade, Moritz, and Heideman 1994; Prychitko and Moore 1997, 2000). Exon primed, intron crossing (EPIC) markers (Lessa 1992) rely on the design of degenerate primers either side of the intron in the adjacent, highly conserved exons (Friesen et al. 1997, 1999) and have been used at a number of systematic levels (Palumbi and Baker 1994; Slade et al. 1994; Pitra, Lieckfeldt, and Alonso 2000; Jenkins et al. 2001; Rockman, Rowell, and Tait 2001).

Thus, an increasing amount of data from a number of diverse mitochondrial and nuclear loci can be acquired in order to optimize phylogenetic signal and construct a more robust organismal phylogeny (Pamilo and Nei 1988; Baker and DeSalle 1997; Miller, Brower, and DeSalle 1997; Flynn and Nedbal 1998; Johnson and Clayton 2000; Pitra, Lieckfeldt, and Alonso 2000). Although the acquisition of large and diverse molecular data sets is undoubtedly beneficial to phylogenetic reconstruction, exactly how to analyze the data remains an ongoing debate. Essentially, three approaches have been suggested: (1) separate analysis, where trees are estimated separately from each partition, and the different estimates compared using taxonomic congruence (Miyamoto and Fitch 1995); (2) the total evidence approach, whereby all available data are combined in a simultaneous analysis (Kluge 1989); and (3) conditional data combination, whereby only homogenous data partitions (estimated by a statistical test of homogeneity) are combined in a simultaneous analysis (Bull et al. 1993; de Queiroz and Donoghue 1995). Furthermore, when combining data sets in simultaneous analyses, it is desirable to be able to evaluate how each data partition contributes towards the final tree topology. This can be done by comparing tree topologies of each data partition with the topology of the simultaneous analysis. In many situations however, relationships emerge in simultaneous analyses that are not supported by individual data

partitions (Baker and DeSalle 1997; Wenzel and Siddall 1999). Partioned Bremer support (PBS) (Baker and DeSalle 1997; Baker, Yu, and DeSalle 1998; Baker, Wilkinson, and DeSalle 2001) aims to divide Bremer support (i.e., the number of extra steps in tree length required before a node collapses [Bremer 1994]) of each node of a simultaneous analysis across the gene partitions that contribute towards the simultaneous analysis. The PBS values offer quantitative, either positive or negative, measures of support for each gene on each node of the simultaneous analysis tree, and the sum of the PBS values for all partitions will always equal the total Bremer support (TBS) for each node. Moreover, the sum of PBS for each partition offers a relative measure of support for each partition towards the final simultaneous analysis tree topology (Baker, Yu, and DeSalle 1998; Baker, Wilkinson, and DeSalle 2001).

A substantial range of analytical methods has therefore evolved in an attempt to comprehensively and accurately analyze molecular data sets and also to interpret how data sets interact with one another in simultaneous analyses. While critics and advocates for all methods exist (for review, see Huelsenbeck, Bull, and Cunningham 1996), only empirical tests can critically appraise the efficacy of each method (Miller, Brower, and DeSalle 1997). In order to test the phylogenetic utility of all the methods and also study the interaction of separate gene partitions in a simultaneous analysis, the present study will analyze four mitochondrial gene partitions and the seventh intron of the β fibrinogen gene (Prychitko and Moore 1997) in the Asian pit viper genus, *Trimeresurus sensu stricto* (*s.s.*).

Trimeresurus s.s. is one of six putative genera of the former *Trimeresurus sensu lato* (*s.l.*) complex. The currently accepted group consists of *Trimeresurus s.s.*, *Ovophis*, *Protobothrops*, *Tropidolaemus*, *Ermia* (David and Ineich 1999; McDiarmid, Campbell, and Touré 1999; Malhotra and Thorpe 2000), and *Triceratolepidophis* (Ziegler et al. 2000) comprising more than 40 species. Although the need for establishing sound phylogenies for a species radiation including many medically important species is clear (Tan, Armugam, and Tan 1989; Warrell 1995; Chippaux 1998), decades of research by numerous herpetologists has led to conflicting opinions regarding systematic relationships at almost all taxonomic levels (Tu et al. 2000). This is primarily due to a combination of a high degree of evolutionary convergence in potential morphologically diagnostic characters, confounded by pronounced geographic variation and sexual dimorphism (Malhotra and Thorpe 1997, 2000). Molecular data therefore plays a significant role in elucidating phylogenetic relationships in the *Trimeresurus* species group. To date, molecular data have been used at the intraspecific (Creer et al. 2001), interspecific (Giannasi, Thorpe, and Malhotra 2001), and intergeneric levels (Toda et al. 1999; Malhotra and Thorpe 2000; Tu et al. 2000). Based on a combination of a single mitochondrial gene and morphological observations, Malhotra and Thorpe (2000) recognized four distinct phylogenetic clades showing diagnostic morphological characters (hemipenis type and fusion of first upper labial and nasal scales) within

Trimeresurus s.s. These are referred to as the *T. albolabris* group, *T. stejnegeri* group, *T. popeorum* group, and the Indian subcontinent group (Malhotra and Thorpe 2000). With the present data (including five partitions from the mitochondrial and nuclear genomes), we aim to investigate the validity of these putative distinct clades and reconstruct the phylogenetic affinities of previously unresolved taxa.

Materials and Methods
Taxon Sampling and DNA Sequencing

Target operational taxonomical units (OTUs) were selected to optimize representation of the four species groups that represent the genus *Trimeresurus s.s.* Specifically, the *T. albolabris* group (including *T. albolabris*, *T. erythrurus*, *T. purpureomaculatus*, *T. septentrionalis* and *T. insularis*), the *T. popeorum* group (including *T. popeorum*, and *T. hageni*), the *T. stejnegeri* group (including *T. stejnegeri* and *T. vogeli*), and the Indian subcontinent group (including *T. trigonocephalus* and *T. puniceus*). Two additional Asian pit vipers, *Tropidolaemus wagleri* and *Deinagkistrodon acutus*, were assigned as outgroups, identified in the mitochondrial molecular systematic analysis of *Trimeresurus s.l.*, from here on referred to as the *Trimeresurus* group (Malhotra and Thorpe 2000). In the majority of taxa, gene regions were amplified from the same sample. When this was not possible, different gene regions were amplified from different samples of conspecifics from the same geographic locality where possible or, if not possible, the same species from a nearby location. A limited number of sequences obtained from GenBank were originally published in Kraus, Mink, and Brown (1996), Parkinson, Moody, and Ahlquist (1997), and Malhotra and Thorpe (2000).

Samples were in the form of tail tip biopsies or liver tissue preserved in 80% ethanol or 100 to 200 μl of blood (from the caudal vein), placed in 1 ml 5% EDTA and preserved in up to 5 ml SDS-Tris buffer (100 mM Tris, 3% SDS). Whole genomic DNA was extracted from 10 to 20 mg of ethanol-preserved muscle, liver tissue, or 200 to 500 μl of blood buffer, using a combination of standard protocols (Sambrook, Fritsch, and Maniatis 1989) and commercially available kits (SIGMA GenElute™ Mammalian Genomic DNA Kit). Fragments from four different mitochondrial genes (cytochrome b [CYTB], NADH subunit 4 [ND4], 12S ribosomal RNA [12S], and 16S ribosomal RNA [16S]) and fragments of differing lengths (due to the use of novel designed primers) of the entire seventh intron from the β fibrinogen gene (7IβFIB) were amplified using the following combinations of primers and PCR thermal cycling parameters. For CYTB, primers and thermal cycling were as in Malhotra and Thorpe (2000). For ND4, primers were as in Arévalo, Davis, and Sites (1994) with an initial denaturation of 94°C for 1 min, followed by 35 cycles of denaturation at 94°C, 1 min, annealing at 55°C, 1 min, extension at 72°C, 2 min, and a final extension of 72°C, 4 min. The 12S primers were as in Knight and Mindell (1993), and the 16S primers were as in Parkinson, Moody, and Ahlquist (1997), both with an initial denaturation of 94°C for 2 min, followed by 35

cycles of denaturation at 94°C, 30 s, annealing at 45°C, 30 s, extension at 72°C, 2 min, and a final extension of 72°C, 5 min. The 7IβFIB primers were as in Prychitko and Moore (1997) with an initial denaturation of 94°C for 3 min, followed by 33 cycles of denaturation at 93°C, 1 min, annealing at 50°C, 30 s, extension at 72°C, 2 min, and a final extension of 72°C, 2 min. The CYTB gene region would not amplify in *T. macrops* with modified versions of the primers Mt-A (Lenk and Wink 1997) and Mt-F (Wink 1995) and so the primers L14910 and H16064 (Burbrink, Lawson, and Slowinski 2000) were used to amplify a similar, largely overlapping CYTB gene region. A negative (upH₂O) was always included to exclude the possibility of contamination. Double-stranded PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining (Dowling et al. 1996). Target PCR products were then cleaned using a variety of commercially available kits, such as Prep-a-gene (Bio Rad), Wizard minicolumns (Promega), or Qiaquick columns (QIAGen). Single-stranded sequencing (one strand only) was carried out either using a modification of the Sequenase version 2.0 protocol (Perkin-Elmer) or using sequenced using dye-labeled terminators (ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit), and subsequently run on an ABI Prism 377 DNA sequencer. Due to differing levels of success of PCR amplification and sequencing for 7IβFIB, PCR products were amplified and sequenced using any successful combination of the Prychitko and Moore (1997) primers, and primers designed specifically for the *Trimeresurus* group (TRIMFIB-BI7L – internal, 50 bp: 5′-CCTTTTGGGATCTGGGTGTA-3′, and TRIMFIB-BI7LA – internal, 480 bp: 5′-GGAGACCAGTTGTCC-CAAAA-3′) using Primer3 (Rosen and Skaletsky 1998).

Sequence Analyses

Manually produced sequence autoradiographs were read directly, and chromatograms produced via automation were read using Chromas (Technelysium Pty. Ltd, 1988–2000). Where available, sequences were checked against published data (Knight and Mindell 1993; Parkinson, Moody, and Ahlquist 1997; Malhotra and Thorpe 2000; Parkinson, Zamudio, and Greene 2000). All mutations/indels were individually checked by eye at all sites across all taxa. The 12S and 16S mitochondrial gene sequences were aligned following Parkinson, Moody, and Ahlquist (1997), and the protein coding sequences (CYTB and ND4) were translated into amino acids to check for stop codons that might indicate the amplification of nuclear mitochondrial pseudogenes (Numts) (Sorenson and Fleischer 1996; Zhang and Hewitt 1996; Bensasson et al. 2001). If Numts were amplified using the original primers, it would be highly unlikely that the same nontarget sequence would be amplified using different primer pairs. In order to check this, a modified version of L14841 – 5′-GCTTCCATCCAACATCTCAGCAT-GATGA-3′ (Kocher et al. 1989) and a genus-specific primer H15947 – 5′-ATTGAGGCNAGTTGRCCRATTT CTGTG-3′ (C. Pook, personal communication) were used

to amplify and sequence (modified L14841) an internal CYTB fragment in a limited number of taxa. Areas of ambiguous alignment (due to minor indels) within the 12S and 16S partitions were treated as missing data. The 7IβFIB sequences (including 5′ and 3′ flanking exon sequences) were aligned by eye with the assistance of ClustalX (Higgins and Sharp 1988) to overcome indel-related alignment difficulties. The actual intron was located by aligning the nucleotide sequences (i.e., flanking exons plus intron) with the published cDNA exon sequence for the chicken fibrinogen β chain (Weissbach et al. 1991; Prychitko and Moore 1997). The peptide sequence GWWYNR (located approximately 50 amino acids from the carboxy terminus on the eighth exon) is conserved in all fibrinogen β chains sequenced to date (Weissbach et al. 1991) and was used to locate the seventh intron. The 5′ and 3′ flanking exon sequences were subsequently removed from the analysis, and only the intron sequence was used for phylogenetic analysis. Certain studies have shown that gaps in sequence alignments are phylogenetically informative (Giribet and Wheeler 1999), whereas others recommend the exclusion of any indels in analyses (Swofford et al. 1996; Swofford 1998). The inclusion of indels as a fifth character state in the present analyses resulted in tree topologies that made little or no taxonomic sense. Given the potentially controversial concept of the inclusion/exclusion of indels in analyses and the intuitive observations of analyses including indels, gaps were treated as missing data in the current data set.

Prior to the phylogenetic analyses, each gene partition was subjected to a number of preliminary sequence analyses. The presence of phylogenetic signal was tested using the $g1$ statistic (Hillis and Huelsenbeck 1992), and the substitutional model of molecular evolution was assigned according to distance analyses performed using the log likelihood function of Modeltest 3.06 (Posada and Crandall 1998) for ML searches. Saturation plots of pairwise differences due to transitions and transversions, transversions only, and transitions only were plotted (data not shown) against the probability model selected from the above procedure. Base composition was subjected to a G-test of heterogeneity (Sokal and Rohlf 1995). McDonald and Kreitman's (McDonald and Kreitman 1991), Tajima's D (Tajima 1989), and Fu and Li's D and F (Fu and Li 1993) test statistics were used to evaluate the possibility of nonneutral evolution.

All phylogenetic reconstructions were performed using PAUP 4.0b8 (Swofford 1998) with explicit assumptions replacing default parameters where highlighted below. To compare the similarity of phylogenetic signal between different gene partitions, partition homogeneity tests (1,000 replications) (Johnson and Clayton 2000) were conducted across the entire data set and between all pairwise combinations of individual partitions (Baker and DeSalle 1997). Phylogenetic analyses were performed for all gene partitions separately, a simultaneous (i.e., concatenated sequences) analysis of the mtDNA partition and nuclear partition was performed separately, and a simultaneous analysis of the entire data set was performed. Strict consensus trees were computed for individual gene partition parsimony searches, resulting in more than one

equally parsimonious tree. A further strict consensus of the above five trees provided a consensus of all gene partitions. Strict consensuses were also performed for permutations of gene partitions in which significantly different phylogenetic signal was detected. In order to check that concatenated gene regions from nonidentical ingroup individuals did not affect tree topologies, a series of further simultaneous searches were conducted, eliminating composite sequences from the analysis. Maximum-parsimony (MP) and maximum-likelihood (ML) analyses were conducted using a random addition heuristic search with tree-bisection-reconnection (TBR) branch swapping. Bootstrapping (1,000 replicates, with TBR branch swapping for MP searches or nearest neighbor interchange [NNI] branch swapping for ML searches) and Bremer support values (performed using SEPAL 1.4 [Salisbury 2000]) were calculated to obtain a relative measure of node support for the resulting trees (Felsenstein 1985). All data were equally weighted. This is in accordance with an increasing amount of theoretical and empirical evidence (Baker and DeSalle 1997; Kluge 1997; Miller, Brower, and DeSalle 1997; Allard, Farris, and Carpenter 1999; Björkland 1999; Wenzel and Siddall 1999) that suggests that equally weighted data actually represents the most severe test of the phylogenetic hypothesis (Baker, Wilkinson, and DeSalle 2001). Relative rates between the main lineages of the ingroup were tested using the two-cluster test of Takezaki, Rzhetsky, and Nei (1995) as implemented in PHYLTEST. A number of pairwise combinations of monophyletic lineages were compared against sister lineages throughout the hierarchical tree structure. Two-tailed Wilcoxon signed-ranks tests (Templeton 1983) and the Shimodaira-Hasegawa test (Goldman, Anderson, and Rodrigo 2000) were used to test whether alternative tree topologies were significantly different from the most parsimonious tree and the ML tree from the simultaneous analysis. For the two-tailed Wilcoxon signed-ranks tests, a tree was constructed to represent the alternative tree topology. This tree was then used as a constraint tree in a replicate parsimony analysis, constraining the analysis to retain only the most parsimonious trees compatible with the alternative tree topology to be tested. A strict consensus performed on these trees resulted in the alternative topology. Differences in tree length between the unconstrained parsimony analysis and the strict consensus of the constrained analyses were tested for significance using the Wilcoxon signed-ranks test, as implemented by PAUP (Templeton 1983). The Shimodaira-Hasegawa test (fully optimized model; Goldman, Anderson, and Rodrigo 2000) was performed on the ML tree and an edited tree to represent the alternative hypothesis.

To assess homoplasy levels, consistency index (CI), retention index (RI), and rescaled consistency (RC) index (Farris 1989; Swofford 1998) and data decisiveness (DD) values (Goloboff 1991) were calculated from each parsimony search. The DD values provide an index that serves as a measure of the information content of a given data set. It is calculated as $DD = (A - S)/(A - M)$, where A is the average length of a data matrix on all possible trees, S is the length of the most parsimonious tree (or trees), and M is the minimum possible number of steps for that data set (Baker, Wilkinson, and DeSalle 2001).

Partitioned Bremer Support

Working only with the combined data (with each partition defined in PAUP), the length of each partition (PL) on the simultaneous analysis (SA) tree was calculated by excluding all characters except one partition and executing the LENFIT command. TBS values were calculated for each node of the SA by constraining the analysis to only retain tree lengths not supporting the target node (CONSTRAINSA). The difference between the minimum length of CONSTRAINSA and SA gives TBS for each node, that is, the number of extra steps in tree length required before a node collapses (Baker and DeSalle 1997). To obtain PBS values, each individual partition was constrained using all the CONSTRAINSA trees to give the length of the constrained partition analysis (CONSTRPART) for each node and CONSTRPART − PL = PBS.

Results
Sequence Analysis

Summary sequence and tree statistics for individual and combined gene partitions are given in table 1 from 3,135 base pairs (bp) of sequence data for 19 taxa (GenBank accession numbers are given in table 1 of Supplementary Material online). No insertions, deletions, or stop codons were found in the protein-coding mtDNA sequences. Furthermore, identical sequences were obtained from different combinations of CYTB primers, suggesting that the tested partitions were target mtDNA and not Numts. The length of the intron sequence in *Trimeresurus s.s.* was 966 bp (table 1), including 15 indels between 1 and 74 bp long in the present alignment. Due to alignment ambiguities, the present 7IβFIB data has been sequenced from both directions and replaces that presented in Giannasi, Malhotra, and Thorpe (2001). The intron conforms to GT (5′ donor, start) and AG (3′ acceptor, finish) consensus splice site rules and has a characteristic pyridimine rich region preceding the 3′ splice site (Senapathy, Shapiro, and Harris 1990; Friesen 2000). The FIB-BI7U and FIB-BI7L primers (Prychitko and Moore 1997) amplified the intron in some OTUs of the *Trimeresurus* group. However, a large number of PCR reactions resulted in either complete failure, multiple products of equal intensity, double bands of very similar size, or single products of the wrong size. Some of the nontarget products were sequenced and subjected to the National Center for Biotechnology Information (NCBI), nucleotide Blast search engine (Altschul et al. 1997). These results confirmed that these were not homologous to 7IβFIB. Submitting the target 7IβFIB sequences to a nucleotide Blast search found between 95% and 98% sequence similarity (approximately 60 bp) with introns from *Trimeresurus* phospholipase A_2 inhibitor ''gPCI-I'' (Nobuhisa et al. 1997) and TATA-box binding protein ''gTgTBP'' (Nakashima et al. 1995) genes. Despite designing two internal primers for PCR amplification and sequencing, premature stops (likely to have been due to DNA secondary structure) were often encountered from both directions approximately between 320 and 380 bp

Table 1
Summary Sequence and MP Tree Statistics for the Separate Gene Partitions and Simultaneous Analysis Data Sets

Summary Statistics	7IβFIB	mtDNA 12S	16S	ND4	CYT B	Simultaneous mtDNA	Total Simultaneous
%G	17.0	17.1	18.2	11.6	12.1	13.9	15.2
%A	31.7	36.8	35.8	32.7	28.9	33.2	32.7
%T	35.9	20	25.0	25.4	27.8	25.2	28.1
%C	15.4	26.1	21.1	30.3	31.2	27.8	24.1
Size (bp)	966	423	512	662	572	2169	3135
Ts/tv ratio	1.601	3.548	2.441	4.392	4.132	3.632	3.642
Model	HKY	TrN	TrN	K3ST	GTR	GTR	GTR
PINVAR	—	0.533	0.569	0.546	—	0.528	0.605
Gamma	0.0103	0.521	0.655	1.305	0.223	0.865	0.856
PI	63	54	42	188	151	435	498
CI	0.902	0.596	0.699	0.513	0.521	0.537	0.569
(Strict)	0.902	0.566	0.663	0.511	0.503		0.564
RI	0.869	0.529	0.641	0.540	0.527	0.526	0.545
(Strict)	0.869	0.466	0.580	0.536	0.491		0.535
RC	0.783	0.315	0.449	0.2772	0.274	0.282	0.310
(Strict)	0.783	0.264	0.384	0.274	0.247		0.301
HI	0.098	0.404	0.303	0.487	0.479	0.463	0.431
(Strict)	0.098	0.434	0.337	0.489	0.498		0.436
DD	0.845	0.369	0.495	0.445	0.431	0.435	0.457
Resolved nodes	10	6	9	14	11	16	13
Congruent nodes	4	4	5	6	8	13	—

NOTE.—The models of molecular evolution represent the Hasegawa-Kishino-Yano (HKY) model (Hasegawa, Kishino, and Yano 1985), the Tamura and Nei (TrN) model (Tamura and Nei 1993), the Kimura three substitution (K3ST) model (Kimura 1981), and the general time-reversible (GTR) model (Swofford et al. 1996) either with or without the assumptions of proportions of invariable sites (PINVAR) or gamma shape correction parameters (Page and Holmes 1998; Swofford 1998). PI indicates number of parsimony informative base pairs. Measures of homoplasy (CI, RI, HI, RC, and DD values) are given for *n* equally parsimonious trees, followed by equivalent values for strict consensus trees of the equally parsimonious alternatives where applicable. Resolved nodes gives the number of completely resolved nodes, and congruent nodes shows the total number of resolved nodes, which are also present in the simultaneous analysis tree (Baker, Wilkinson, and DeSalle 2001).

along the intron. This left six taxa (*T. popeorum* from south Thailand, *T. hageni*, *T. insularis*, *T. macrops*, *T. stejnegeri*, and *T. vogeli*), with an approximately 60-bp gap within this region. The latter should not be confused with coincidental indels in samples that were sequenced fully from both directions (incorporating at least a 20-bp overlap).

Significant phylogenetic signal was detected from all gene partitions ($P < 0.01$). The partition homogeneity tests showed significant heterogeneity throughout the comparison, including all five gene partitions ($P = 0.001$), and between the 7IβFIB partition and all mtDNA partitions ($P < 0.01$ in all pairwise comparisons). No heterogeneity in phylogenetic signal was detected between any of the mtDNA partitions. The 7IβFIB sequence was the most AT rich (67.6%), had the lowest transition:transversion ratio

(1.6), and had the lowest proportion of phylogenetically informative positions (6.5%) compared with the mtDNA sequences (table 1). The ratio between 7IβFIB and the total mtDNA average pairwise uncorrected sequence divergence between all ingroup taxa ranged from 0.13 to 0.35, with a mean of 0.26 (table 2). Varying degrees of saturation were found in plots incorporating transitions (i.e., transitions only and transitions plus transversions) in all gene partitions except 16S. No saturation was detected in any of the gene partition transversion-only plots (saturation plots for all partitions are shown in fig. 1 of Supplementary Material online). There were no significant differences between the base frequency distributions in any of the tested gene partitions. Combinations of the McDonald and Kreitman and the Tajima, and, Fu and Li tests failed to reject the null hypothesis of neutral evolution

Table 2
Mean Pairwise Uncorrected Sequence Divergence (%) Between the Species Groups and OTUs Involved in the Phylogenetic Analyses

	albolabri	macrops	popeorum	stejnegeri	hageni	puniceus	Trigonocephalus
albolabris		1.28	2.07	2.17	2.50	3.66	3.62
macrops	9.85		2.25	2.38	2.68	3.55	3.87
popeorum	9.48	9.22		1.34	2.47	2.32	2.57
stejnegeri	9.62	9.29	8.58		2.07	2.83	2.96
hageni	10.35	10.42	9.21	8.80		2.95	3.13
puniceus	12.03	11.59	11.92	11.36	12.04		3.39
Trigonocephalus	10.46	11.30	10.58	10.58	11.30	11.91	

NOTE.—The species groups correspond to *albolabris* (*T. albolabris*, *T. erythrurus*, *T. purpureomaculatus*, *T. septentrionalis*, and *T. insularis*), *popeorum* (*popeorum* OTUs only), and *stejnegeri* (*T. stejnegeri* and *T. vogeli*). The upper right matrix is the 7IβFIB gene partition. The lower left matrix is the combined mtDNA partition.
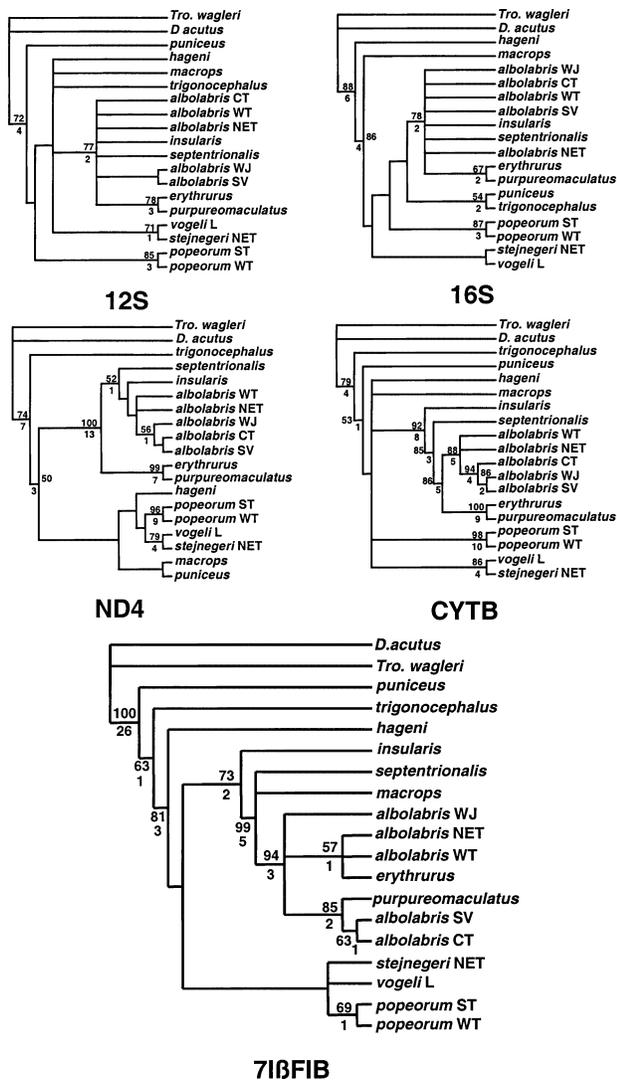
Fig. 1.—Strict consensus MP trees with bootstrap (above) and Bremer support (below) values shown adjacent to corresponding nodes for the four mtDNA (12S, 16S, ND4, and CYTB) and the 7IβFIB gene partitions. Suffixes to species names indicate geographical localities of conspecific samples: N = north, E = east, S = south, W = west, C = central, J = Java, L = Laos, T = Thailand, and V = Vietnam.

in any gene partition. The relative rate tests failed to detect significant variation between relative rates of any tested combination of lineages. Considerably lower levels of homoplasy (measured by all indices, CI, RI, RC, HI, and DD) were detected in the 7IβFIB partition than in all of the equivalently homoplasious mtDNA partitions (table 1).

Gene Partition, Simultaneous Analysis, and Conditional Data Combination Phylogenies

Figure 1 shows strict consensus MP trees for the four mtDNA and the 7IβFIB gene partitions (ML trees are shown in fig. 2 of Supplementary Material online). Figure 2A shows the single most parsimonious tree resulting from the simultaneous analysis of all five partitions. The sequential removal of each of the concatenated partitions

from nonidentical ingroup individuals had no major effect on the simultaneous analysis tree structure. The concatenated mtDNA MP tree topology (see fig. 3A in Supplementary Material online) only differed from the total simultaneous analysis in showing complete resolution below node six. Supplementary Material fig. 3B shows the concatenated mtDNA ML tree topology. The strict consensus tree for all gene partition trees (not shown) resulted in a single monophyletic group comprising the two *T. popeorum* OTUs, with the rest of the ingroup taxa forming an unresolved polytomy. All gene trees varied in topology and resolution (between partitions, individual and simultaneous analyses, and MP and ML trees), but all supported the monophyly of the *T. albolabris* group (Malhotra and Thorpe 2000). Topological congruence with the simultaneous analysis tree also differed throughout the gene trees, with CYTB sharing the highest percentage of nodes (62%) with the simultaneous analysis (table 1). The *T. stejnegeri*, *T. vogeli*, and *T. popeorum* representatives were identified as monophyletic groups throughout all mtDNA MP gene trees and as a single monophyletic group in the 7IβFIB MP tree. The precise phylogenetic relationships between the Indian subcontinent group and the *T. popeorum* and *T. stejnegeri* groups remained ambiguous throughout the gene trees as well as between the total simultaneous MP and ML trees (total simultaneous ML tree shown in fig. 4 of Supplementary Material online). The *T. albolabris*, *T. stejnegeri*, and *T. popeorum* (without *T. hageni*) groups were all very strongly supported in the single most-parsimonious simultaneous analysis. However, even though the simultaneous MP and ML (simultaneous analysis ML tree shown in fig. 4 of Supplementary Material online) analyses resulted in completely resolved phylogenies, the nodes between the *T. stejnegeri* group and *T. popeorum* group OTUs were only poorly supported. The placement of *T. hageni* outside of the *T. popeorum* group contradicted previous molecular and morphological findings (Malhotra and Thorpe 2000). Therefore, the two-tailed Wilcoxon signed-ranks test and the Shimodaira-Hasegawa test were performed on alternative tree topologies supporting all existing clades, except with the inclusion of *T. hageni* with *T. popeorum*. Both tests failed to reject the null hypothesis of no difference between the original parsimony and likelihood trees, with the constrained and edited versions of the trees, respectively. These relationships remained unresolved in a strict consensus of the phylogenetically incongruent concatenated mtDNA partitions and the 7IβFIB partition trees (fig. 2B).

Partitioned Bremer Support

The results of the PBS analysis are presented in table 3, showing PBS values for 13 nodes across all partitions and summed PBS scores for each partition to evaluate the contribution of a given partition to the overall support of the simultaneous analysis tree (Baker, Wilkinson, and DeSalle 2001). In order of total level of support, CYTB (64) > ND4 (58) > 7IβFIB (24) > 12S (22) > 16S (17) represents the hierarchical degree of support for the simultaneous analysis tree.

Discussion

The Seventh Intron of the β Fibrinogen Gene in *Trimeresurus*

The size of the 7IβFIB sequence varies considerably throughout taxonomic groupings. In the current *Trimeresurus* alignment, the intron is 966 bp in length, compared with between 476 and 1,141 bp in pigeons and doves (Johnson and Clayton 2000) and 876 bp woodpeckers (Prychitko and Moore 1997). The rationale for the design of the 7IβFIB primers (Prychitko and Moore 1997) adhered to the rules of "universal" EPIC primers, but PCR amplifications varied considerably from total failure to amplification of multiple products. The latter products were most likely paralogous intronless pseudogenes or nonhomologous products (Slade et al. 1993; Slade, Moritz, and Heideman 1994). Complete PCR failures were probably the result of primer-binding site mismatches, and future directions may benefit from the design of more genus-specific primers (Lessa 1992). The intron is characteristically A-T rich (68%). Although the precise function (Slade et al. 1993; Friesen 2000; Prychitko and Moore 2000) and origins (Gilbert 1978; Cavalier-Smith 1985) of introns are still not clear, for phylogenetic purposes they are regarded as selectively neutral, non-coding regions of DNA. Based upon work on pseudo-genes, Li and Graur (1991) explained that due to the tendency of some nucleotides to mutate more frequently than others, 64.5% of all mutations that occurred in noncoding regions of DNA tend to result in either A or T (Prychitko and Moore 1997). After advances in sequencing technology, gene and genome sequencing projects, and the pursuit of more molecular phylogenetic markers, empirical studies may reveal more about the origin, function, and molecular evolution of introns. The discovery of an almost identical 60-bp fragment in two unrelated introns suggests a common origin of at least parts of the introns in the 7IβFIB, TATA-box binding protein "gTgTBP" (Nakashima et al. 1995), and phospholipase A_2 inhibitor "gPCI-I" (Nobuhisa et al. 1997) gene sequences.

Comparative Molecular Evolution of the Mitochondrial and the Intron Gene Partitions

Although the 7IβFIB gene sequence has only been used in a limited number of phylogenetic studies, comparable modes and rates of molecular evolution have been observed throughout bird families (Prychitko and Moore 1997, 2000; Johnson and Clayton 2000) and in the
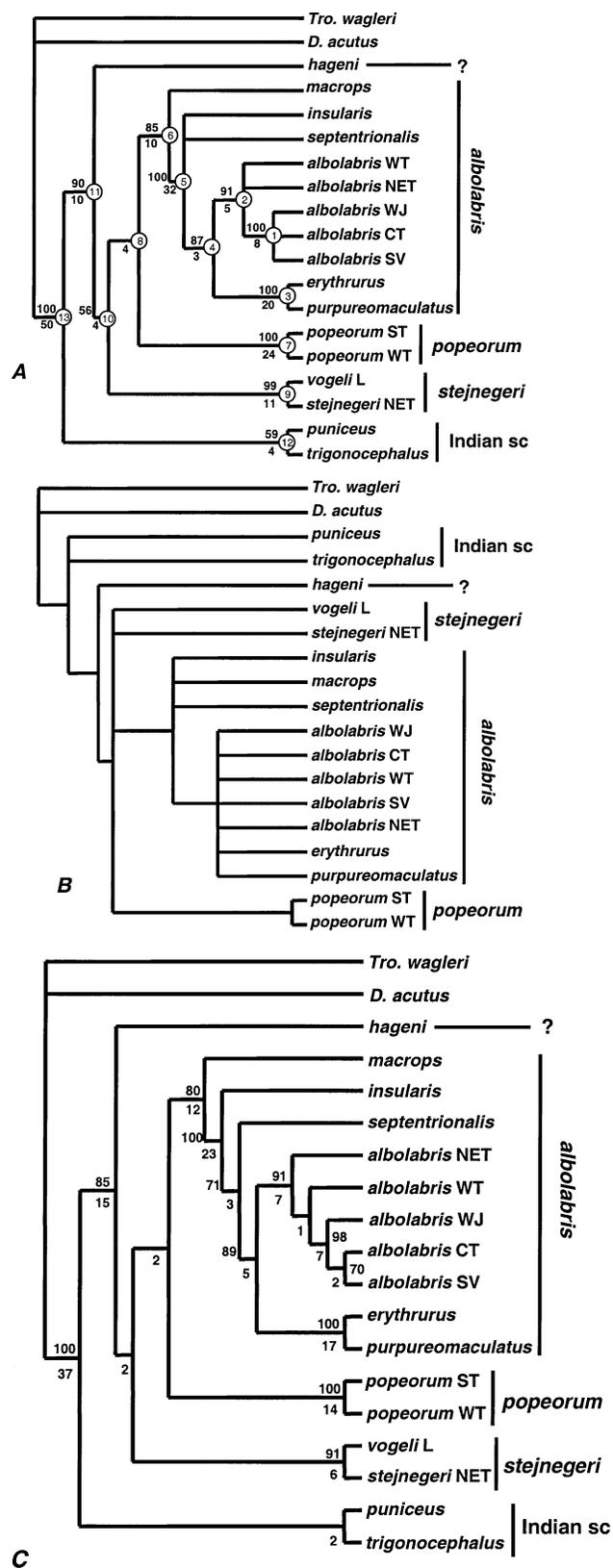

Fig. 2.—(*A*) Strict consensus MP tree for the simultaneous analysis of all five gene partitions. Bootstrap (above) and Bremer support (below) values are shown adjacent to corresponding nodes. Circled node numbers refer to nodes used in calculating the total and PBS values (table 3). Suffixes to species names are as in figure 1. (*B*) Strict consensus MP tree for the phylogenetically incongruent simultaneous mtDNA and 7IβFIB gene partitions. (*C*) Single most parsimonious tree resulting from the simultaneous analysis of the CYTB, ND4, and 7IβFIB partitions only. Delimited groups in the phylogenetic trees correspond to the four morphologically distinct species groups (*T. albolabris*, *T. stejnegeri*, *T. popeorum*, and the Indian subcontinent [sc]) discussed in the text.

Table 3
PBS for the Simultaneous Analysis Parsimony Tree

Node Number	Total Bremer Support	Gene Partition				
		12S	16S	ND4	CYTB	7IβFIB
1	8	1	2	2	4	−1
2	5	0	−1	0	5	1
3	20	3	2	9	8	−2
4	3	0	0	−1	6	−2
5	32	5	4	18	8	−3
6	10	−1	1	5	−1	6
7	24	7	4	2	11	0
8	4	1	−1	1	6	−3
9	11	6	0	2	5	−2
10	4	−2	0	3	1	2
11	10	−2	−3	10	2	3
12	4	2	0	1	3	−2
13	50	2	9	6	6	27
Total	185	22	17	58	64	24

NOTE.—All gene partition columns show the PBS for each partition for the corresponding nodes on the simultaneous analysis tree shown in figure 2*A*.

present study. Although the mitochondrial partitions mutate, on average, four times faster than the 7IβFIB partition, a slower rate of molecular evolution in the latter does result in less homoplasy at the phylogenetic level addressed in this study. Data with reduced levels of homoplasy are desirable for phylogenetic reconstruction, but the rate of character change must be fast enough to track speciation events in rapidly evolving clades (Moore 1995, 1997). This is partly exemplified in the PBS analysis. Despite displaying the lowest levels of homoplasy, the 7IβFIB partition scored relatively poorly in the PBS analysis. This was the cumulative result of a number of negative scores for nodes numbered 1 to 5, corresponding to the *T. albolabris* group. Further subdividing the PBS only between nodes 1 to 5 results in a support hierarchy of CYTB (31) > ND4 (28) > 12S (9) > 16S (7) > 7IβFIB (−7). Mean uncorrected pairwise sequence divergence within the *T. albolabris* group is 4.17% for the total mtDNA partition and only 0.87% for the 7IβFIB partition. Thus, phylogenetic resolution (as measured by nodal support) in the *T. albolabris* group is high for the mtDNA partitions, but the phylogenetic utility of the 7IβFIB partition appears to be limited with lower levels of divergence. Conversely, when PBS scores are summed for nodes 6 to 13, the order of level of support changes to CYTB (33) > 7IβFIB (31) > ND4 (30) > 12S (13) > 16S (10). The shift is primarily the result of a very large PBS value (27) for the 7IβFIB partition at node 13 (fig. 2*A* and table 3 [supporting the monophyly of the *Trimeresurus s.s.* relative to the outgroups]), reinforcing the hypothesis that nuclear partitions are more suited to the analysis of deeper phylogenetic divergences (Friesen 2000; Weibel and Moore 2002).

Phylogenetic Reconstruction Using Heterogenous Mitochondrial and Nuclear Data Sets

The separate analyses, followed by taxonomic congruence (implemented by a strict consensus of all separate partitions) method of resolving phylogenetic relationships from multiple partitions (Miyamoto and Fitch 1995), resulted in an almost total loss of phylogenetic signal. One of the key criticisms of consensus approaches is evidently the loss of some resolution (Eernisse and Kluge 1993; Nixon and Carpenter 1996; Allard, Farris, and Carpenter 1999), and here a strict consensus yielded essentially no information. Although a separate analysis of each partition leads to an understanding of the relative merits and shortcomings of each data set (de Queiroz and Donoghue 1995; Miller, Brower, and DeSalle 1997), topological differences between gene partitions have the potential to seriously compromise the utility of the taxonomic congruence method in the current example.

Alternatively, the total simultaneous parsimony analysis resulted in a highly resolved tree with high support values for the maximum number of nodes, compared with all other trees. The addition of the phylogenetically heterogenous 7IβFIB to the total mtDNA partition resulted in a reduction in resolution at three nodes within the *T. albolabris* group, had negligible effect on remaining node support, and added support to previously unsupported nodes. It must be acknowledged that greater levels of resolution in tree structure may sometimes convey unwarranted confidence. However, if the best estimate of phylogenetic relationships is the one that offers the simplest, or most parsimonious, explanation of all the relevant data (Miller, Brower, and DeSalle 1997; Baker, Wilkinson, and DeSalle 2001), the total simultaneous analysis would be the preferred method of analysis with the present data. Similar results have been observed in other empirical studies (Flynn and Nedbal 1998; Baker, Wilkinson, and DeSalle 2001), which highlight the fact that although the phylogenetic signal in data is additive (i.e., the addition of more phylogenetically informative characters), noise is actually averaged over all data partitions (Wenzel and Siddall 1999).

A more cautious appraisal however, would favor the consensus of the heterogenous total mtDNA and 7IβFIB data sets (Bull et al. 1993; de Queiroz and Donoghue 1995). Both analyses are broadly congruent with each other and also with the larger CYTB gene analysis in Malhotra and Thorpe (2000), but all differ in the actual phylogenetic relationships between the *T. stejnegeri*, *T. popeorum* (including *T. hageni*), and Indian subcontinent groups. Based on diagnostic morphological characters (hemipenis type and of the first upper labial and nasal scales) and the results of the Wilcoxon signed-ranks and Shimodaira-Hasegawa tests, it could be suggested that *T. hageni* does share close systematic affinities with the *T. popeorum* group and that *T. puniceus* and *T. trigonocephalus* form a subset of the monophyletic Indian subcontinent group (Malhotra and Thorpe 2000). However, these data suggest that unequivocally assigning *T. hageni* to a monophyletic group with *T. popeorum* remains questionable. Further morphological and phylogenetic appraisals may assist in clarifying these phylogenetic relationships.

Due to PCR problems (thought to be the result of oligonucleotide primer sequence-binding site mismatches for the CYTB gene fragment), this is the first time *T. macrops* has been included in a molecular analysis of

the genus *Trimeresurus*. The individual gene trees are incongruent regarding the position of *T. macrops*. Nonetheless, the high ND4 partition PBS value at node six, the 7IβFIB gene tree and the simultaneous analysis all show high levels of support to suggest that *T. macrops* is part of the *T. albolabris* group. This placement is also supported by AFLP analysis (Giannasi, Thorpe, and Malhotra 2001) and by morphologically diagnostic characters. Both *T. macrops* and the rest of the *T. albolabris* group have a smooth rather than spiny hemipenis and fused first upper labial and nasal scales (Malhotra and Thorpe 2000).

The relationships between the *T. stejnegeri* and *T. popeorum* groups lack support, or are poorly defined throughout all analyses, incorporating over 3,000 bp of DNA data from a combination of five mitochondrial and nuclear data partitions. Appraising this particular phylogenetic anomaly suggests that it is more likely to represent a hard rather than a soft polytomy (Hillis, Mable, and Moritz 1996; Page and Holmes 1998). In the case of a hard polytomy, the lack of phylogenetic support between taxa represents a relatively rapid speciation event, as opposed to uncertainty about relationships due to a lack of confidence in the data. This phenomenon is certainly disheartening, given the quantity of data involved, but other studies working on similar data sets have also concluded that some nodes may be inherently difficult to resolve (Baker, Wilkinson, and DeSalle, 2001). Most aspects of the four distinct clades delineated in Malhotra and Thorpe (2000) are supported by a combined mtDNA and nuclear DNA analysis. However, certain phylogenetic relationships (e.g., *T. hageni*) will require corroboration from further analyses incorporating similar combinations of partitions but with the inclusion of more representative taxa from *Trimeresurus s.s.*

Which are the Best Genes to Use for Phylogenetic Reconstruction at the Present Taxonomic Levels?

The application of DNA data to phylogenetic reconstruction has led to the collection of diverse molecular data sets to test taxonomic hypotheses (Flynn and Nedbal 1998; Johnson and Clayton 2000; Parkinson, Zamudio, and Greene 2000; Baker, Wilkinson, and DeSalle 2001). This practice evidently leads to well-supported, highly resolved trees, compared with individual partitions. However, the acquisition of such data sets is an expensive and time-consuming process. If it is accepted that the best estimate of phylogeny is taken to be the simplest explanation of all the relevant data (i.e., the simultaneous analysis) (Miller, Brower, and DeSalle 1997), the PBS allows an evaluation of phylogenetic accuracy of all partitions. According to this estimate, the CYTB and the ND4 mtDNA partitions were better predictors of phylogenetic accuracy than the 12S and 16S mtDNA partitions. The CYTB and ND4 partitions also gave the highest supported and most resolved topologies. Furthermore, notwithstanding the sizes of the mtDNA partitions, both the CYTB and ND4 yielded much higher percentages of parsimony informative sites than did the 12S and 16S partitions (CYTB, 26.4%; ND4, 28.4%; 12S, 12.7%; and 16S, 8.2%). Thus, although the CYTB and ND4 partitions were saturated and displayed the highest levels of homoplasy, these partitions would be the preferred mtDNA molecular markers for the current example. Therefore, the phylogenetic analysis of CYTB and/or ND4 mtDNA partitions, in addition to a variable nuclear intron, would approach an optimal but more streamlined approach to estimating organismal pit viper phylogenies for the present levels of divergence. Indeed, a simultaneous MP analysis run on the CYTB, ND4, and the 7IβFIB partitions resulted in a single, fully resolved tree (fig. 2C) with very similar branch support and a fully congruent topology to the total simultaneous analysis.

Future Directions

This work represents an investigative study aiming to identify the phylogenetic utility of nuclear gene partitions in achieving a robust organismal phylogeny for the largest radiation of Old World pit vipers (David and Ineich 1999; McDiarmid et al. 1999; Tu et al. 2000) containing many medically relevant species (Tan, Armugan, and Tan 1989; Warrell 1995; Chippaux 1998). After the assessment of phylogenetic signal in the 7IβFIB partition, we aim to screen and locate further nuclear gene partitions to corroborate existing mitochondrial phylogenies at the interspecific and the intergeneric levels. The future work will contribute towards resolving taxon relationships (Malhotra and Thorpe 1997; Malhotra and Thorpe 2000) and will have consequences for antivenom production strategies, biodiversity assessments, identification of evolutionary significant conservation units (Brooks, Mayden, and McLennan 1992; Moritz 1995), and understanding venom evolution in this diverse radiation of venomous snakes.

Supplementary Material

GenBank accession numbers and locality details for the five gene partitions, in addition to cited unpublished phylogenetic trees, are available as supplementary information on the MBE Web site.

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