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Snake phylogeny: evidence from nuclear and mitochondrial genes

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Abstract

We constructed phylogenies of snakes from the c-mos and cytochrome *b* genes using conventional phylogenetic methods as well as the relatively new method of Bayesian inference. For all methods, there was excellent congruence between the c-mos and cytochrome *b* genes, implying a high level of support for the shared clades. Our results agree with previous studies in two important respects: first, that the scolecophidians and alethinophidians are monophyletic sister clades; and second, that the Colubroidea is a monophyletic group with the Acrochordidae as its sister clade. However, our results differ from previous studies in the finding that *Loxocemus* and *Xenopeltis* cluster with pythons. An additional noteworthy result from our data is that the genera *Exiliboa* and *Ungaliophis*, often placed with *Tropidophis* (and *Trachyboa*, not included in the present study) in the Tropidophiidae, are in reality boids.

1. Introduction

Despite considerable recent interest in the evolution of snakes (Caldwell and Lee, 1997; Cohn and Tickle, 1999; Greene and Cundall, 2000; Lee et al., 1999; Scanlon and Lee, 2000; Tchernov et al., 2000), the phylogenies of snakes remain poorly known. An examination of recent morphological (Cundall et al., 1993; Kluge, 1991; Rieppel, 1998; Scanlon and Lee, 2000; Tchernov et al., 2000) and molecular (Dowling et al., 1996; Heise et al., 1995) snake phylogenies reveals considerable differences among these studies. The morphological studies (Cundall et al., 1993; Kluge, 1991; Rieppel, 1998; Scanlon and Lee, 2000; Tchernov et al., 2000) do agree in two respects: first, that the blind snakes, known as scolecophidians, are the sister clade to all other snakes, known collectively as the alethinophidians; and second, that within the alethinophidians, the Boidae, Pythonidae, Tropidophiidae, Bolyeriidae, Acrochordidae, and Colubroidea collectively form a monophyletic group to the exclusion of the Aniliidae, Anomochilidae, Uropeltidae, Xenopeltidae, and Loxocemidae. The molecular studies (Dowling et al., 1996; Heise et al., 1995) show little similarity to each other or to the morphological studies.

Given the continuing uncertainty about snake phylogeny, we inferred phylogenies from a broad sample of snakes using the mitochondrial cytochrome *b* and nuclear c-mos genes. Johnson (2001) has shown that the cytochrome *b* gene can be applied to divergences as old as the separation of passeriforms from other birds. Saint et al. (1998) have demonstrated the usefulness of the c-mos gene for the relationships of squamate reptiles. The data were analyzed with the conventional phylogenetic methods of maximum parsimony (MP), minimum evolution (ME), and maximum likelihood (ML), as well as the relatively new method of Bayesian inference (Yang and Rannala, 1997).

Being on different chromosomes, the mitochondrial cytochrome *b* and nuclear c-mos genes have had independent gene histories and form linkage partitions (Slowinski and Page, 1999). Data from independent genes can be analyzed either by combining all the data or by keeping the genes separate (de Queiroz et al., 1995), approaches that each have their advantages and disadvantages (Slowinski and Page, 1999). When the gene trees relating the sequences from two linkage partitions are identical, it is advantageous to combine the data, assuming that the data are consistent. On the other hand, if there is evidence that the gene trees relating the

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sequences from two linkage partitions are not identical, combining the data is contraindicated, primarily because it gives heavier weight to the gene with a larger number of nucleotides (Slowinski and Page, 1999). By analyzing linkage partitions separately, one gains the major advantage of the significance possessed by shared clades on the trees derived from the partitions: there is a high probability that shared clades correspond to real clades on the species phylogeny (Miyamoto and Fitch, 1995). The rationale behind this is very simple: the probability that two random trees will share any clades is quite low (Hendy et al., 1988). Hence, gene trees from two linkage partitions that share a number of clades are clearly non-random. And because these two partitions do not share the constraint of a linked gene history, the constraint must be the shared species phylogeny thereby revealing clades on the species phylogeny.

Because of evidence that the cytochrome *b* and c-mos sequences from our sample of snakes are not related by identical gene trees, we have analyzed the date separately. In doing so, we find excellent congruence, which we use as the basis for creating a robust, albeit incompletely resolved, phylogeny for the major groups of snakes.

2. Materials and methods

2.1. Specimens examined

We sequenced the mitochondrial cytochrome *b* and part of the nuclear c-mos genes using a sample of 42 snake species, representing two of the three extant scolecophidian families and 13 of the 15 extant alethinophidian families (Table 1). The Anomochilidae (Cundall et al., 1993) and Xenophidiidae (Wallach and Gunther, 1998) were not included because tissues were not available. For the purpose of tree rooting we chose the outgroup method (Watrous and Wheeler, 1981). Among extant lizards it is generally accepted that the sister taxon to the snakes lies among the anguinomorphs, though which is unknown and controversial (Bellairs, 1972; Bellairs and Underwood, 1951; Gorman and Gress, 1970). We have included two anguinomorph lizards (Table 1) as outgroups.

2.2. Molecular techniques

For some snakes, donors provided us with previously extracted total genomic DNA. However, in most instances, we extracted DNA from liver tissue or shed skin by the standard method of proteinase K digestion in lysis buffer, followed by phenol/chloroform extraction (see Burbrink et al., 2000, for details). Template DNA for the polymerase chain reaction (PCR) was also prepared as in Burbrink et al. (2000). We amplified the

Table 1
List of taxa sequenced for this study, GenBank Accession Numbers, and tissue sources

Taxon	GenBank Accession No.	Source
Scolecophidia		
Leptotyphlopidae		
Leptotyphlops humilis		CAS 190589
Typhlopidae		
Ramphotyphlops braminus		CAS 184353
Typhlops brachycephalus		CAS 200736
Alethinophidia		
Aniliidae		
Anilius scytale	/U69738	LSUMZ H-14435/GB
Cylindrophidae		
Cylindrophus ruffus		LSUMZ 12363
Uropeltidae		
Uropeltis phillipsi		LSUMZ H-5788
Xenopeltidae		
Xenopeltis unicolor		CAS 204861
Loxocemidae		
Loxocemus bicolor		LSUMZ H-6319
Pythonidae		
Antaresia childreni		No voucher
Python molurus		No voucher
Boidae		
Acrantophis dumerili	/U69735	No voucher/GB
Boa constrictor		No voucher
Candoia carinata		No voucher
Charina bottae		CAS 206040
Epicrates striatus	/U69799	No voucher/GB
Eryx johni		CAS 200907
Eunectes murinus	/U69808	No voucher/GB
Lichanura trivirgata		CAS 200649
Sanzinia madagascariensis	/U69866	No voucher/GB
Bolyeriidae		
Casarea dussumieri	/U69755	No voucher/GB
Tropidophiidae		
Exiliboa placata		UTACV R 44894
Tropidophis haetianus	/U69869	No voucher/GB
Ungaliophis continentalis	/U69870	No voucher/GB
Acrochordidae		
Acrochordus granulatus		No voucher
Colubroidea		
Colubridae		
Cerberus rhynchops		CAS 206574
Gonyosoma oxycephala		No voucher
Helicops angulatus		LSUMZ H-3346
Hydrops triangularia		LSUMZ H-3105
Hypsiglena torquata		CAS 206337
Leioheterodon modestus		LSUMZ H-1991
Meleya unicolor		No voucher
Oligodon cinereus		CAS 205028
Pareas macularius		CAS 206620
Phyllorhynchus decurtatus		No voucher
Pseudoxenodon karlschmidti		ROM 30627
Regina rigida		CAS 165994
Rhabdophis tigrinus		LSUMZ 37418
Xenochrophis punctulatus		CAS 201594
Viperidae		
Crotalus viridis		CAS 200713
Atheris nitschei		CAS 201709
Elapidae		
Bungarus fasciatus		CAS 207988

Table 1 (*continued*)

Taxon	GenBank Accession No.	Source
Notechis ater		SAM R 31604
Outgroups		
Anguidae		
Anguis fragilis		CAS 173016
Shinisauridae		
Shinisaurus crocodilurus		No voucher

Institutional abbreviations. CAS, California Academy of Sciences, Department of Herpetology; LSUMZ, Louisiana State University, Museum of Natural Science; Rom, Royal Ontario Museum; SAM, South Australian Museum; UTACV, University of Texas at Arlington, Department of Biology. In situations where two entries are given in a column, the left one corresponds to the c-mos sequence, the right one to the cytochrome *b* sequence.

entire mitochondrial cytochrome *b* gene using primers L14910 and H16064 (de Queiroz et al., 2002). Our cycling sequencing protocol for the cytochrome *b* gene was identical to that given in Burbrink et al. (2000). For sequencing we used primers L14919 and H16064 from that study as well as H15149 (Kocher et al., 1989), L15584 (de Queiroz et al., 2002), and H15716 (5′-TCT GGT TTA ATG TGT TG-3′). This combination of primers allowed us to sequence both strands of the approximately 1110–1116 nucleotides making up the cytochrome *b* gene of snakes and the 1113 nucleotides making up the cytochrome *b* gene of the lizard outgroups. In addition to the cytochrome *b* sequences generated by us, a number of sequences were obtained from GenBank (see Table 1).

For the c-mos gene, we previously developed the primers S77 and S78 (Lawson and Slowinski, in press), which allow the amplification and sequencing in snakes and some lizards of a 570–576 bp segment exclusive of the primers. PCR amplifications with these primers were done under standard conditions with negative controls.

Both cytochrome *b* and c-mos PCR products were purified using Promega Wizardprep PCR Preps DNA Purification System according to manufacturer's instructions. Cycle sequencing was performed on purified PCR products using the Perkin–Elmer Big Dye reaction premix for 50 cycles of 96 °C, 10 s; 45 °C, 5 s and 60 °C for 4 min. Nucleotide sequences were determined using an ABI model 310 Genetic Analyzer. We verified that our cytochrome *b* sequences were not nuclear pseudogenes by confirming that there were no internal stop codons. New sequences used in this study have been deposited in GenBank, Accession Nos. AY099961–AY099996.

2.3. Phylogenetic analyses

Alignment by eye resulted in 573 c-mos sites and 1149 cytochrome *b* sites. Because the length of the cytochrome *b* gene varies considerably among snake species due to terminal indels, we only used the first 1110 sites for the analyses. Because application of the Templeton non-parametric test to the most parsimonious c-mos and cytochrome *b* gene tree (see Section 3) reveals that these two genes have non-identical histories, we analyzed the genes separately for all analyses. Likelihood tests (Goldman et al., 2000) were not applied to the likelihood trees because of the time constraint involved in simulating the null distribution. We constructed phylogenies of snakes using ME, MP, and ML, as well as the relatively new method of Bayesian inference (Yang and Rannala, 1997). ME, MP, and ML analyses were performed with PAUP*4 (Swofford, 2001). Bayesian analyses were performed with MrBayes (Huelsenbeck, 2001).

For the model-based methods (ME and ML), an appropriate model of sequence evolution was inferred for each gene using ModelTest (Posada and Crandall, 1998). For the c-mos data, this resulted in the selection of the HKY85 + G model with a ti/tv ratio 2.7886, a gamma parameter of 0.6846, and base frequencies as A = 0.2821, C = 0.2022, G = 0.2238, and T = 0.2920. For the cytochrome *b* data, this resulted in selection of the TVM + I + G model with substitution parameters as A–C = 0.1420, A–G/C–T = 2.7302, A–T = 0.2560, and C–G = 0.2841, a proportion of invariant sites of 0.2465, a gamma parameter of 0.4721, and base frequencies as A = 0.4017, C = 0.3551, G = 0.0508, and T = 0.1924. Because there was significant base compositional bias (see Section 3) for the cytochrome *b* data, we used log-determinant distances (Lake, 1994; Lockhart et al., 1994) for the cytochrome *b* ME analysis.

It has been customary to downweight sets of characters that experience higher rates of evolution relative to other sets of characters for MP analysis. This has led to the frequent practice of downweighting third-codon positions relative to the first- and second-codon positions in protein coding genes. However, characters that have experienced a higher rate of evolution relative to other characters have not necessarily experienced more homoplasy (Naylor et al., 1995). Moreover, several recent studies (e.g., Allard and Carpenter, 1996; Björkland, 1999; Savolainen et al., 2000) have shown that the downweighting of third-codon positions has a negative effect on the outcome. For these reasons, we chose to weight all nucleotide sites equally for all analyses.

The ME and MP analyses were done as 1000 successive heuristic searches with random stepwise addition followed by TBR branch-swapping. For the MP analyses, parsimony uninformative characters were eliminated. Because of the time constraints, ML analyses were done as a single heuristic search with ASIS stepwise addition followed by TBR branch-swapping. When multiple optimal trees were found for a method, they were condensed in a strict consensus tree.

Table 2
Number of shared clades between the optimal c-mos and cytochrome *b* maximum parsimony (MP); minimum evolution (ME); maximum likelihood (ML); and Bayesian inference (BI)

c-mos	Cytochrome *b*				
	MP	ME	ML	BI	Mean
MP	14	17	17	16	16
ME	16	19	18	19	18
ML	17	21	20	20	19.50
BI	18	20	19	20	19.25
Mean	16.25	19.25	18.5	18.75	

For the MP and ME analyses, bootstrapping was done with 100 replicates, each one run as 100 successive heuristic searches. For the ML analyses, bootstrapping was done with 100 replicates, each one done with a single stepwise addition analysis.

Bayesian analyses were performed with MrBayes (Huelsenbeck, 2001) by running 500,000 generations in four chains, saving the current tree every 10 generations. A six-parameter model was used with site-specific substitution rates for codon positions. For both genes, the likelihood sum scores had reached stationarity well before 500,000 generations and the last 5000 trees were used to construct a 50%-majority rule consensus tree.

To summarize the congruence between the two genes, we inferred a summary phylogeny from our analyses as follows: we first constructed a strict consensus tree from each of the 16 pairwise comparisons of cytochrome *b* and c-mos trees from the MP, ME, ML, and Bayesian analyses (see Table 2). We then combined these 16 strict consensus trees in a semistrict consensus tree containing only those clades that are not contradicted by any other clade on any tree. Hence, for the reasons discussed in the introduction, the summary phylogeny presents a robust picture of snake phylogeny.

3. Results

3.1. Sequences

The nucleotide sequence of the cytochrome *b* gene of the snakes examined in this study commences with the methionine codon ATG, as has been previously reported

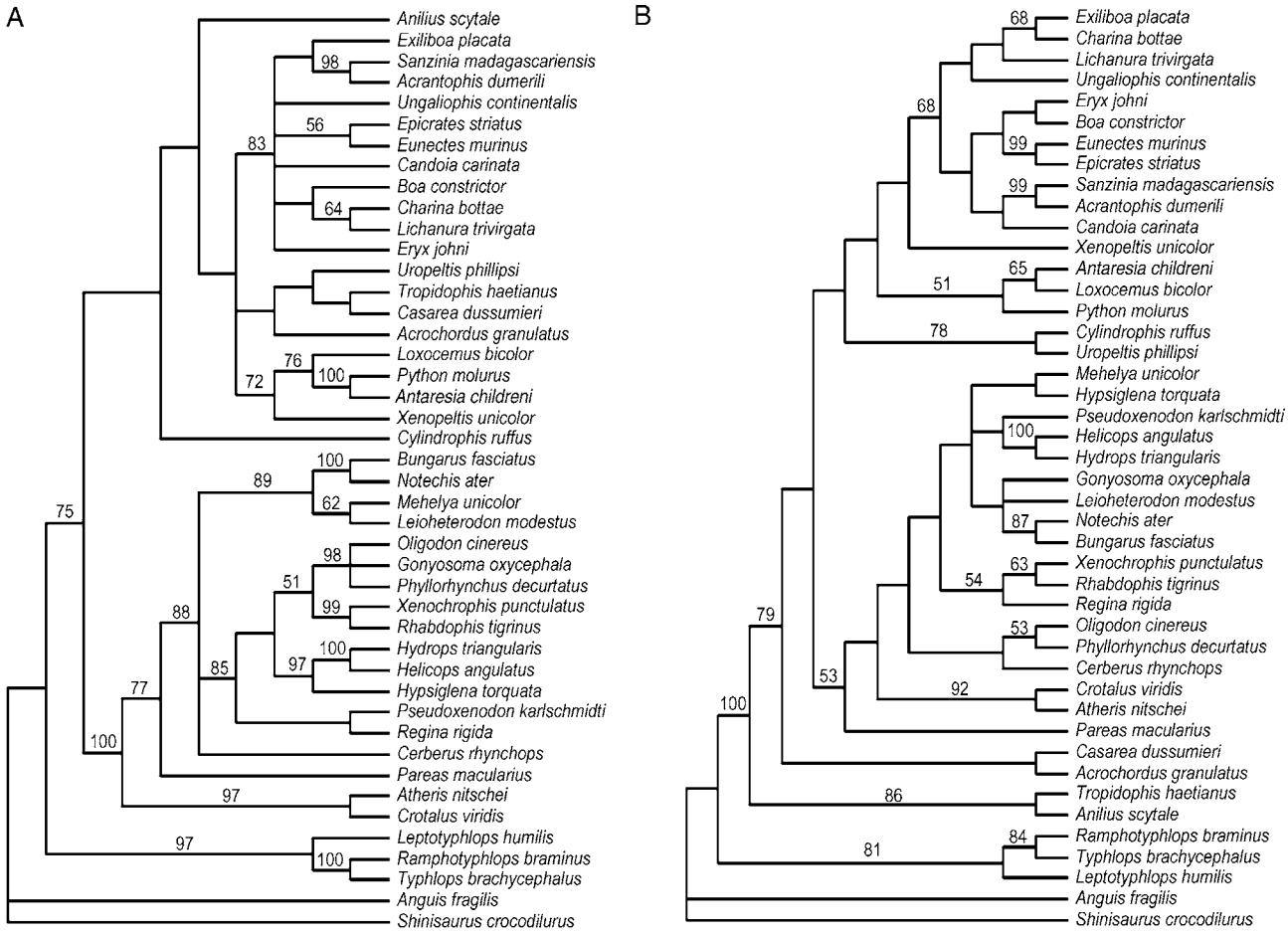

Fig. 1. (A) The strict consensus tree from the MP trees for the c-mos data, and (B) the strict consensus tree from the MP trees for the cytochrome *b* data. In both trees, bootstrap values > 50% are shown along the internodes.

for boid, elapid, and colubrid snakes (Burbrink et al., 2000; Campbell, 1997; Slowinski and Keogh, 2000) and which is apparently universal in squamates. In snakes, the signal for the termination of translation varies among taxa and is either a post-transcriptionally polyadenylated thymine or one of the mitochondrial stop codons (Campbell, 1997). With sequences that are terminated by a post-transcriptionally polyadenylated thymine, there is often ambiguity with regard to which thymine is the termination signal. For this reason, the exact length of the cytochrome *b* gene is often unclear. But in clear-cut cases, the cytochrome *b* gene is generally between 1110 and 1116 bp, similar to the finding of Slowinski and Keogh (2000) for elapids. The two lizard outgroup cytochrome *b* sequences were 1113 bp.

There are several hotspots for indels at the start of the cytochrome *b* gene and another one at the end of the gene. Near the start of the aligned data, there is a 9-bp gap at positions 10–18 in snakes not possessed by the lizard outgroups; further downstream, there is a 6-bp gap at positions 40–45 in all taxa, except *Ramphotyphlops braminus* and the lizard outgroups; and another 6-bp gap at positions 52–57 in all taxa, except *R. braminus* (which has a 3-bp gap at positions 55–57) and the lizard outgroups. Toward the end of the aligned cytochrome *b* sequences, there is a 3-bp gap at positions 1093–1095 in all althinophidians relative to the scolecophidians and the lizard outgroups. The aligned length of the snake cytochrome *b* sequences extends beyond the lizard sequences, which is either due to terminal deletions in the lizard sequences or terminal insertions in the snake sequences.

In the c-mos gene, there is a single hotspot for indels at positions 302–307 in the aligned data. At this spot, there is a 6-bp gap in the xenodontine, colubrine, and natricine colubrids, as well as in *Exiliboa* and *Acrochordus*; all other snakes except *Leptotyphlops* have a 3-bp gap between sites 302–304; *Leptotyphlops* and the lizard outgroups have no gap. There were no other gaps in the aligned c-mos data.

For the cytochrome b gene there was significant nucleotide compositional bias among taxa ($X^2 = 2215.06$, df = 129, $P = 0.000003$). But there was no significant bias for the c-mos gene ($X^2 = 56.42$, df = 129, $P = 1.00$).

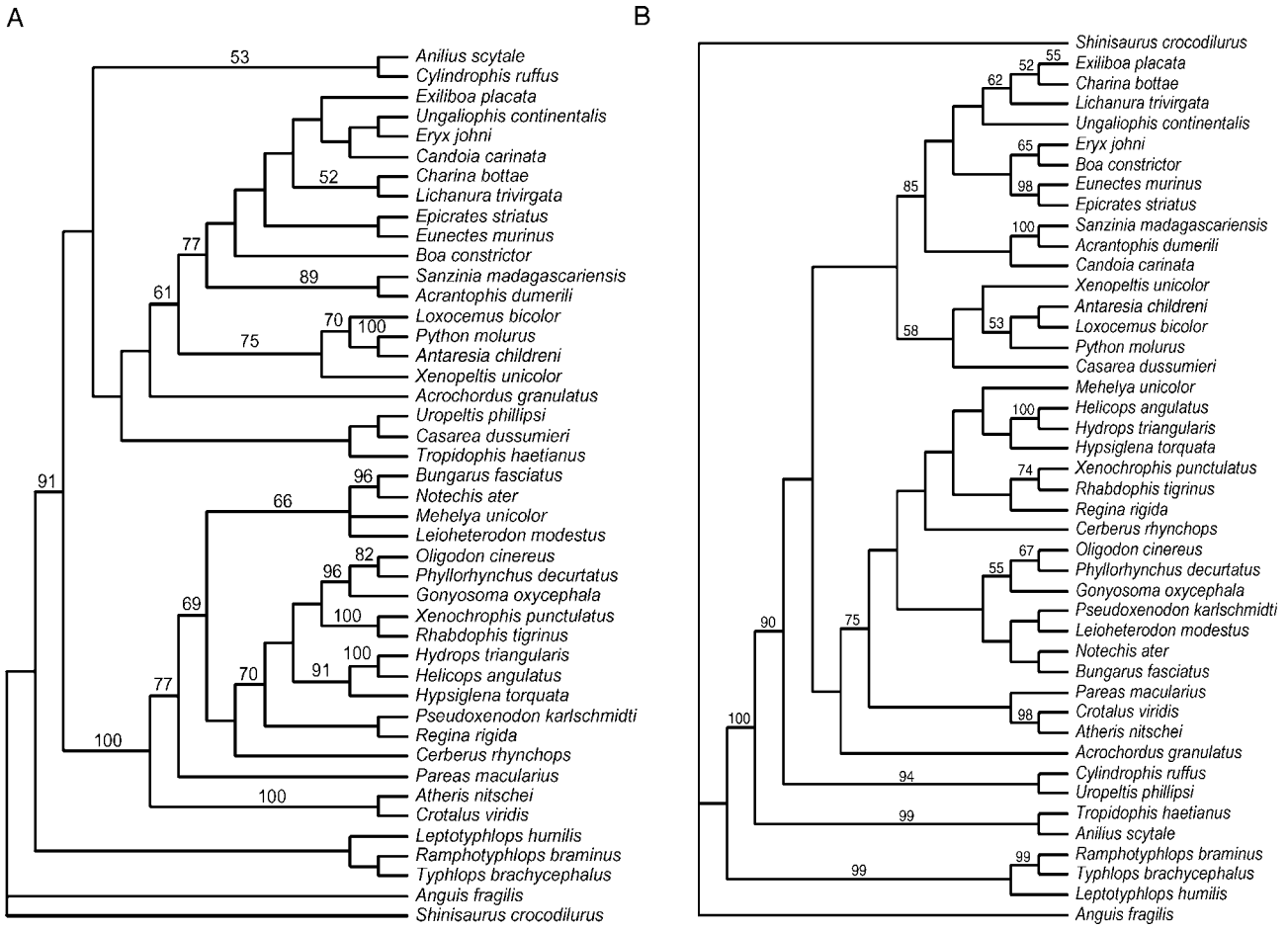

Fig. 2. (A) The strict consensus tree from the ME trees for the c-mos data, and (B) the strict consensus tree from the ME trees for the cytochrome *b* data. In both trees, bootstrap values > 50% are shown along the internodes.

3.2. Phylogenetic analyses

Figs. 1A and B show the strict consensus trees from the most parsimonious trees for the c-mos (216 trees of length = 516 steps; RI = 0.734) and cytochrome *b* (five trees of length = 6116 steps; RI = 0.341) genes. Application of the Templeton non-parametric test to the trees of Figs. 1A and B for the c-mos ($P < 0.0001$) and cytochrome *b* ($P < 0.0001$) data reveals that these trees are significantly different for both data sets. Figs. 2A and B show the strict consensus trees from the best ME trees for the c-mos (two trees of length = 1.28941 steps) and cytochrome *b* (one tree of length = 5.57919) genes. Figs. 3A and B show the strict consensus trees from the best ML trees for the c-mos (one tree of $-\ln L = 4321.32314$) and cytochrome *b* (one tree of $-\ln L = 23759.64177$) genes. Figs. 4A and B show the 50%-majority rule consensus trees from the last 5000 Bayesian trees for the c-mos and cytochrome *b* genes.

Table 2 shows the results of our congruence analysis comparing the c-mos and cytochrome *b* trees. The mean number of shared clades between the c-mos and cytochrome *b* trees was 18.19 for all methods. The parsimony analyses underperformed the other methods (mean number of shared clades for c-mos MP trees = 16; mean number of shared clades for cytochrome *b* MP trees = 16.25).

A semistrict consensus of the 16 strict consensus trees represents the comparisons from Table 2 in a tree (Fig. 5) with 23 clades, two more than on any of the pairwise strict consensus trees (Table 2). This tree is our summary tree and shows the relationships that are supported by both genes. MP and ME bootstrap values from Figs. 1–4 mapped onto this tree; ML bootstrap support values were not included because they were calculated from stepwise addition only replicates and are thus likely to be underestimated.

4. Discussion

Our results (Fig. 5) agree with recent morphological studies, (Cundall et al., 1993; Kluge, 1991; Rieppel, 1998; Scanlon and Lee, 2000; Tchernov et al., 2000) in

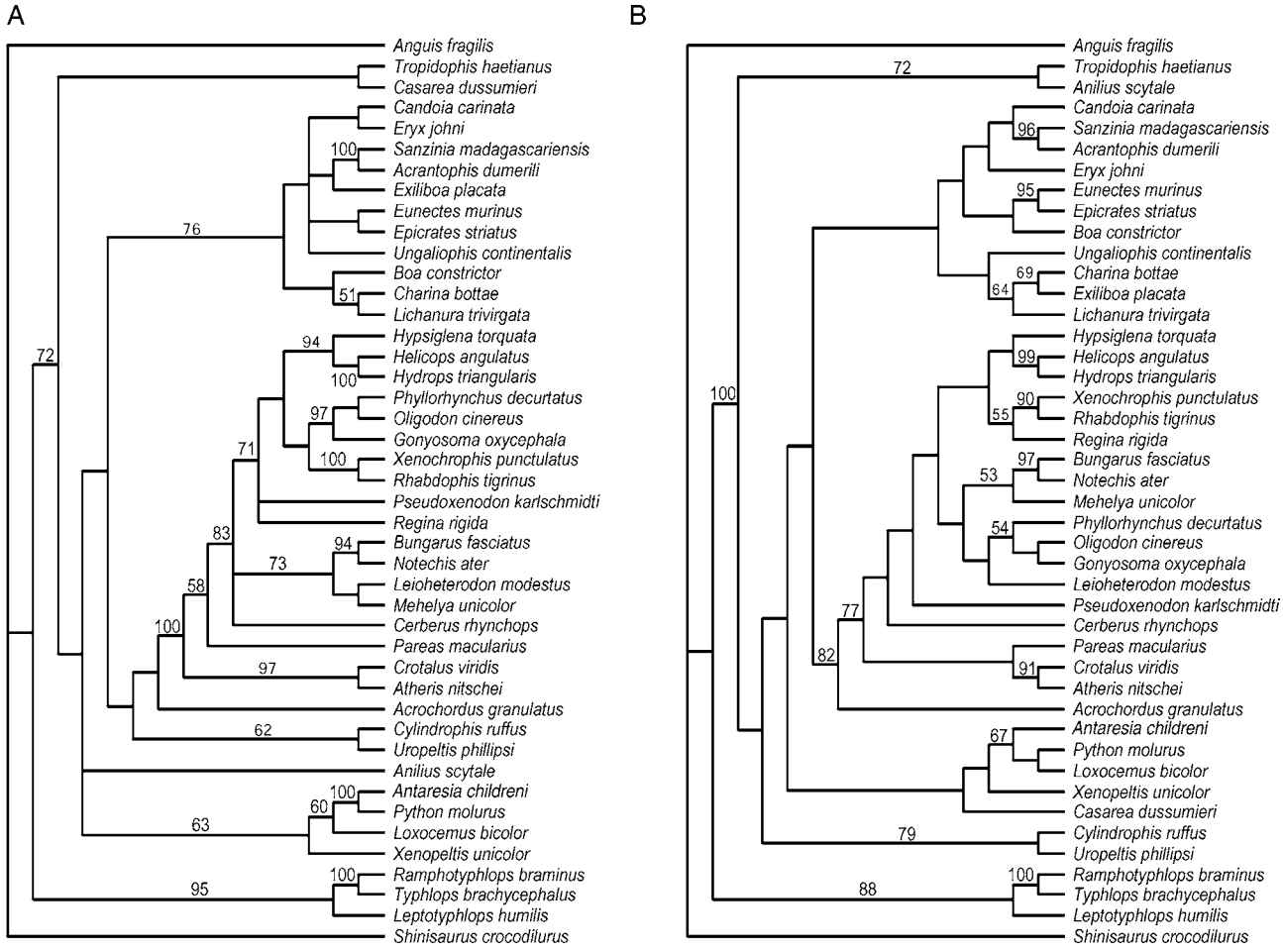

Fig. 3. (A) The ML tree for the c-mos data, and (B) the ML tree for the cytochrome *b* data. In both trees, bootstrap values > 50% are shown along the internodes.

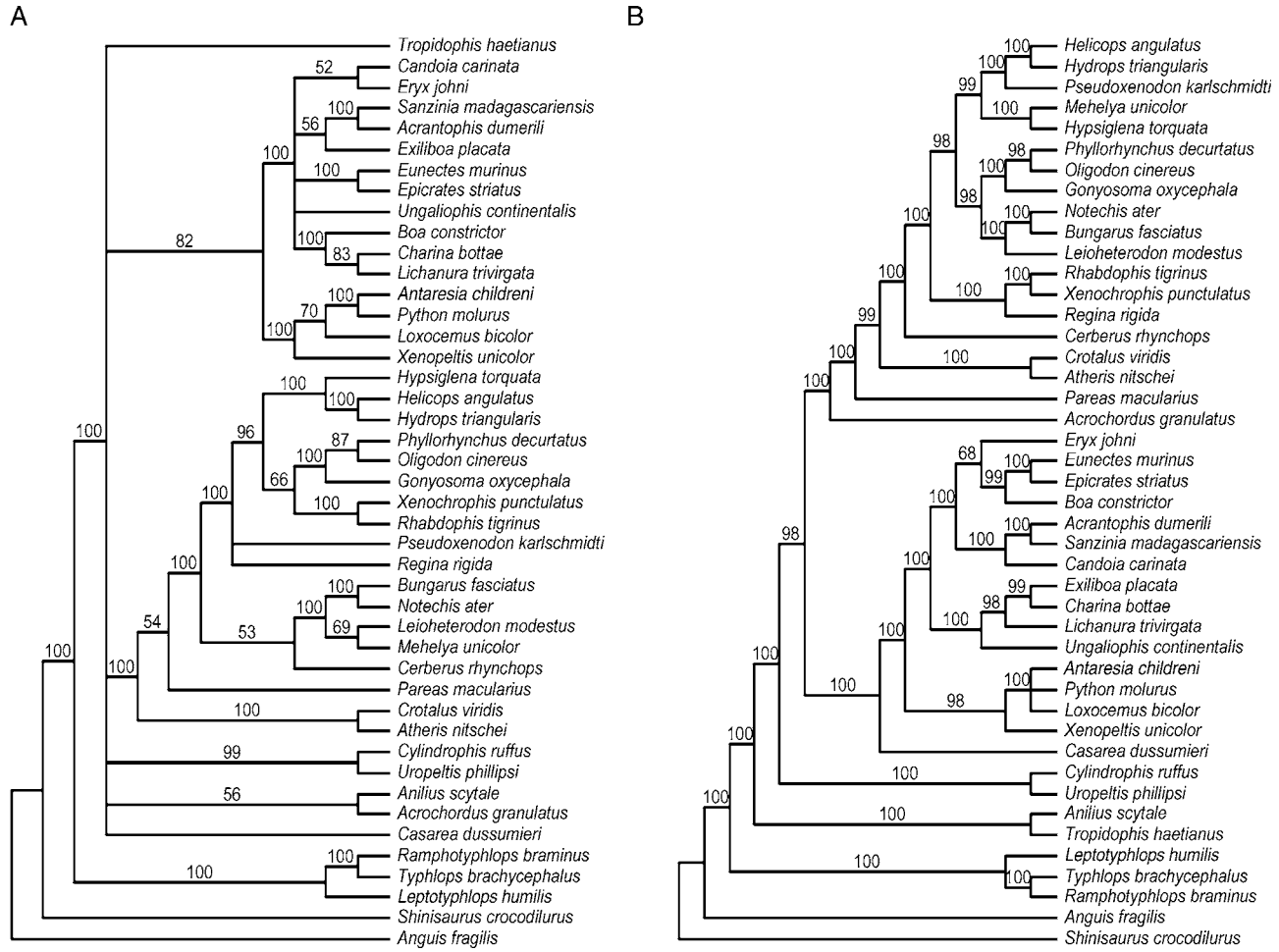

Fig. 4. (A) The Bayesian c-mos tree, and (B) the Bayesian cytochrome *b* tree. In both trees, the numbers along the internodes are the posterior clade probabilities.

two respects: first, that the scolecophidians and alethinophidians are monophyletic sister clades; and second, that the Colubroidea is a monophyletic group with the Acrochordidae, though not strongly supported by our data, as its sister clade. We find that uropeltids and cylindrophiids are closely related, which agrees with some studies (Cadle et al., 1990; Kluge, 1991; Tchernov et al., 2000), but contrary to others (Cundall et al., 1993; Scanlon and Lee, 2000). Examination of cytochrome *b* sequences from other lizards reveals that the gaps in the gene (see Section 3) correspond to deletion events in the early history of snakes. The 3-bp gap at positions 1093–1095 in all alethinophidians relative to the scolecophidians and the lizards is thus a synapomorphy for althinophidians, providing additional corroboration for the sequence data.

Our results differ from recent studies in firmly linking the "primitive" *Loxocemus* and *Xenopeltis* with pythons. Recent morphological studies (Cundall et al., 1993; Kluge, 1991; Rieppel, 1998; Scanlon and Lee, 2000; Tchernov et al., 2000) have all found the Boidae, Pythonidae, Tropidophiidae, Bolyeriidae, Acrochordidae, and Colubroidea to be monophyletic to the exclusion of the Xenopeltidae and Loxocemidae as well as other snakes and this seems to have become generally accepted (Cundall and Greene, 2000; Pough et al., 1998). However, these studies (Cundall et al., 1993; Kluge, 1991; Rieppel, 1998; Scanlon and Lee, 2000; Tchernov et al., 2000) share many of the same characters and can therefore be expected to share some clades based on this fact alone. Our finding of a link between xenopeltids and loxocemids with pythonids, on the other hand, is based on the congruence between two sets of independent data. Further, there is support from an earlier morphological study (Underwood, 1976), for a link between loxocemids and xenopeltids with pythonids, and from a molecular study (Heise et al., 1995) for a link between loxocemids and pythonids.

An additional noteworthy result from our data is that the genera *Exiliboa* and *Ungaliophis*, often placed with *Tropidophis* (and *Trachyboa*, not included in the present study) in the Tropidophiidae (Kluge, 1991), are in

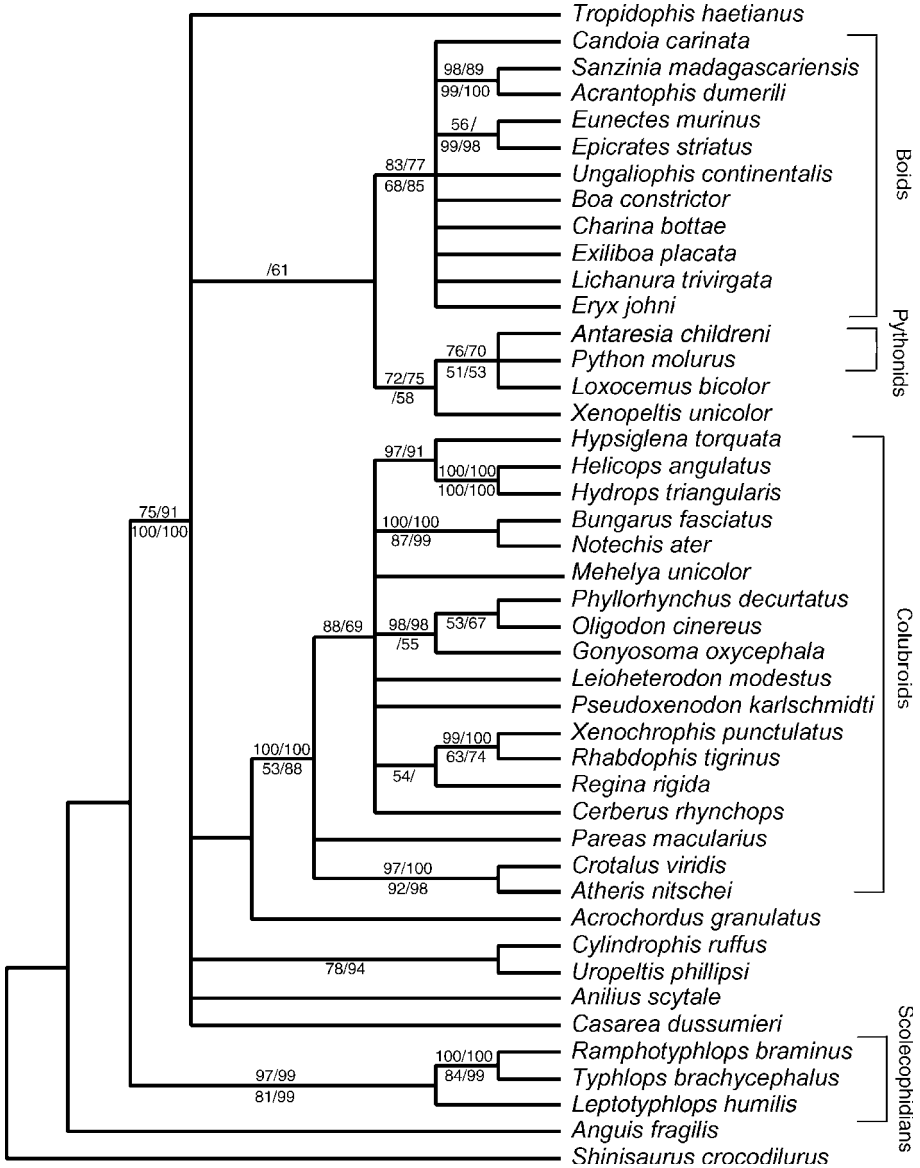

Fig. 5. The semistrict consensus constructed from the 16 pairwise strict consensus trees from the pairwise c-mos/cytochrome *b* comparisons in Table 2. The MP and ME bootstrap values from Figs. 1–4 are mapped onto the clades (MP/ME c-mos above branch; MP/ME cytochrome *b* below branch).

reality boids. Again, there is evidence from other sources, both morphological (Zaher, 1994) and molecular (Dessauer et al., 1987), supporting our placement. Unfortunately, we are not able to resolve the relationships of Aniliids, Tropidophiids (sensu stricto as *Tropidophis*), and Bolyeriids.

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