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Phylogenetic Relationships of Elapid Snakes Based on Cytochrome *b* mtDNA Sequences

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Published molecular phylogenetic studies of elapid snakes agree that the marine and Australo–Melanesian forms are collectively monophyletic. Recent studies, however, disagree on the relationships of the African, American, and Asian forms. To resolve the relationships of the African, American, and Asian species to each other and to the marine/Australo–Melanesian clade, we sequenced the entire cytochrome *b* gene for 28 elapids; 2 additional elapid sequences from GenBank were also included. This sample includes all African, American, and Asian genera (except for the rare African *Pseudohaje*), as well as a representative sample of marine/Australo–Melanesian genera. The data were analyzed by the methods of maximum-parsimony and maximum-likelihood. Both types of analyses yielded similar trees, from which the following conclusions can be drawn: (1) *Homoroselaps* falls outside a clade formed by the remaining elapids; (2) the remaining elapids are divisible into two broad sister clades, the marine/Australo–Melanesian species vs the African, American, and Asian species; (3) American coral snakes cluster with Asian coral snakes; and (4) the "true" cobra genus *Naja* is probably not monophyletic as the result of excluding such genera as *Boulengerina* and *Paranaja*.

INTRODUCTION

Two recent molecular studies (Keogh, 1998 [partial 16S and cytochrome *b* mtDNA sequences]; Slowinski *et al.*, 1997 [amino acid sequences of the venom proteins PLA_2 and NXS]) have explored the phylogenetic relationships within the snake family Elapidae, a major group of venomous snakes containing nearly 300 species in approximately 60 genera (Golay *et al.*, 1993; herein we use Elapidae in the broad sense to include both terrestrial and marine species, whereas Golay *et al.* place the marine species in a separate family Hydrophiidae). The two studies agree that the marine and Australo–Melanesian species are collectively monophyletic. They further agree that the marine species are diphyletic: the "laticaudine" sea snakes have arisen from within an unidentified Australo–Melanesian oviparous clade (Keogh *et al.*, 1998), whereas "hydrophiine" sea snakes have arisen from within the viviparous Australian "*Notechis* group" (Keogh *et al.*, 1998; Slowinski *et al.*, 1997). These two conclusions had been previously reached in several other smaller studies (reviewed in Slowinski *et al.*, 1997), both morphological and molecular.

With regard to the remaining elapids, however, the studies of Slowinski *et al.* (1997) and Keogh *et al.* (1998) disagree. Whereas Slowinski *et al.* (1997) found evidence suggesting that all African, American, and Asian species are collectively monophyletic, Keogh *et al.* (1998) found kraits (*Bungarus*) to be the sister group of the marine and Australo–Melanesian clade, with the relationships of the African, American, and remaining Asian forms being unresolved. The motivation for the present study was to resolve the relationships of the African, American, and Asian species to each other and to the marine/Australo–Melanesian clade. Toward this end, we sequenced the entire cytochrome *b* gene from all African, American, and Asian genera (except for the rare African *Pseudohaje* [tree cobras]), as well as a sample of species from the marine/Australo–Melanesian clade. The data were analyzed by the maximum-parsimony and maximum-likelihood methods.

MATERIAL AND METHODS

Sequencing

Liver tissues or shed skins from all African, American, and Asian elapid genera were sampled for this study (Table 1), except for the rare African *Pseudohaje*. A representative sample of species from the marine/Australo–Melanesian clade was also included (Table 1). Additionally, two elapid cytochrome *b* sequences (*Micrurus fulvius*1, Accession No. U69846; *Elapsoidea semiannulata*, U80618) from GenBank were included in the data matrix. For outgroups, we sequenced the cytochrome *b* gene from the snakes *Coluber constrictor*, *Heterodon simus*, and *Acrochordus granulatus*. Two other snake cytochrome *b* sequences (*Morelia amethis-*

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TABLE 1

List of Taxa Sequenced for This Study

Taxon	Distribution	Museum voucher no. (tissue no.)
Elapids		
Australo–Melanesian and marine species		
Acanthophis antarcticus (common death adder)	Northern Territory, Australia	NTM R17880 (SAM S99)
Aspidomorphus muelleri (collared adder)	W. Sepik Prov., Papua New Guiniea	AM 135504 (SAM 40320)
Austrelaps superbus (copperhead)	S. Australia, Australia	SAM R19835
Drysdalia coronata (crowned snake)	Western Australia, Australia	SAM R22966
Hydrophis semperi (Garman's sea snake)	Philippines	USNM FS 56633
Laticauda colubrina (banded sea krait)	Indonesia	Unnumbered voucher in senior author's possession
Micropechis ikaheka (small-eyed snake)	W. Sepik Prov., Papua New Guinea	SAM 11800
Notechis ater (tiger snake)	S. Australia, Australia	SAM R31604
Pseudechis australis (king brownsnake)	S. Australia, Australia	SAM R31703
Toxicocalamus preussi (Papuan groundsnake)	W. Sepik Prov., Papua New Guinea	AM 135505 (SAM 40321)
African species		
Aspidelaps scutatus (shield-nose cobra)	Africa	LSUMZ 56251
Boulengerina sp. (water cobra)	Zaire	HLMD RA-1607
Dendroaspis polylepis (black mamba)	Africa	Unnumbered voucher in senior author's possession
Elapsoidea nigra (black garter snake)	Africa	LSUMZ 56273
*Hemachatus haemacha*tus (rinkals spitting cobra)	South Africa	No voucher
Homoroselaps lacteus (harlequin snake)	South Africa	LSUMZ 55386 (H 9582)
Naja nivea (cape cobra)	Africa	No voucher
Paranaja multifasciata (cobra)	Africa	No voucher
Walterinnesia aegyptia (black desert cobra)	North Africa	No voucher
Asian species		
Bungarus fasciatus (banded krait)	Ayeyarwady Div., Myanmar	CAS 207988 (RCD 12296)
Calliophis japonicus (Japanese coral snake)	Ryukyu Islands, Japan	CAS 204980 (JBS 1768)
Calliophis macclellandi 1 (Macclelland's coral snake)	Northern Vietnam	ROM 31158
Calliophis macclellandi 2 (Macclelland's coral snake)	Northern Vietnam	ROM 31159
Maticora bivirgata (long-glanded coral snake)	Southeast Asia	LSUMZ 37496 (HCD 2887)
Naja kaouthia (monocled cobra)	Ayeyarwady Div., Myanmar	CAS 206602 (RCD 12287)
Ophiophagus hannah (king cobra)	Ayeyarwady Div., Myanmar	CAS 206601
American species		
Micruroides euryxanthus (coral snake)	Arizona	AMNH 128233 (FT 961)
Micrurus fulvius 2 (coral snake)	Florida, USA	CAS 195959
Outgroups		
Acrochordus granulatus (wart snake)	Asia	No voucher
Coluber constrictor (racer)	Eastern USA	No voucher
Heterodon simus (hognose snake)	Florida, USA	CAS 195598

Note. Museum abbreviations are as follows: AM, Australian Museum; AMNH, American Museum of Natural History; CAS, California Academy of Sciences; HLMD, Hessisches Landesmuseum Darmstadt collection; LSUMZ, Louisiana State University Museum of Natural Science; NTM, Northern Territory Museum of Arts and Sciences; ROM, Royal Ontario Museum; SAM, South Australian Museum; USNM, United States National Museum.

tina, U69847; *Farancia abacura*, U69832) from GenBank were also included as outgroup sequences.

DNA extractions were performed by first digesting tissues in 2 ml lysis buffer (pH 8.0; 100 mM Tris–HCL; 50 mM EDTA; 10 mM NaCl; 0.5% SDS) containing proteinase K at a concentration of 0.06 mg/ml. Digestion was carried out for approximately 3 h at 65°C with constant motion, followed by two extractions in phenol–chloroform, followed by a final extraction in chloroform. DNA was then precipitated with ethanol and washed with 80% ethanol. The precipitated DNA was dissolved in TE buffer and diluted to an appropriate strength (200–400 ng/µl) prior to PCR.

Two primers (R. Lawson, unpublished) in the tRNAs flanking the cytochrome *b* gene were used to amplify the entire cytochrome *b* gene. PCR was carried out using the hot start method in 100-µl volumes as follows: the lower layer contained 16 µl H_2O, 5 µl 10× buffer with 1.5 mM Mg^{2+}, 2 µl dNTPs (10 mM each dNTP), and 1 µl of each primer (25 µM). This was overlayered with paraffin wax and then 57 µl H_2O, 5 µl 10× buffer, 3 µl *taq* (1 u/µl), and 10 µl template. PCR amplifications were carried out with the following cycle parameters: initial denaturation at 94°C for 7 min; followed by 40 cycles of 94°C, 30 s; 46°C, 30 s; 72°C, 1 min; with a final 7-min extension at 72°C followed by ramping to 4°C. PCR products were cleaned of remaining dNTPs, primers, and primer dimers using the Promega Corporation

Wizard PCR preps DNA purification system according to the manufacturer's protocol.

Cycle sequencing was performed with 5′-CCCTCAGAATGATATTTGTCCTCA-3′ (Kocher *et al.*, 1989), four other sequencing primers (R. Lawson, unpublished), and Perkin–Elmer Big-Dye reaction premix. Cycle parameters were the following: 50 cycles of 96°C, 10 s; 45°C, 5 s; 60°C, 4 min; with final ramping to 4°C. The sequenced products were separated using ABI Model 377 or 310 automated sequencers. The output sequences contained in the ABI files were assembled and analyzed using Sequencer 2.0. The sequences were placed in GenBank (AF217812-217842).

Phylogenetic Analyses

The 35 (30 elapid, 5 outgroup) complete cytochrome *b* sequences were aligned by eye and the aligned sequences assembled into a data matrix. Because the coding sequences varied from 1101 to 1122 bp (Table 2), only the first 1100 bp were used in the analyses. For the parsimony analyses, uninformative characters were excluded. Two types of phylogenetic analyses were employed using PAUP* 4.0 (Swofford, 1998): maximum-parsimony and maximum-likelihood. Maximum-parsimony analyses were run with all sites weighted equally (see Results for justification). Two hundred sequential heuristic searches were performed with the starting trees obtained via random stepwise addition, followed by TBR branch swapping. The reliability of the clades on the shortest tree(s) was assessed using bootstrapping (Felsenstein, 1985) performed with 100 replicates, each executed as 20 sequential heuristic searches done as above. Skewness values (g_1; Hillis, 1991) were estimated from random samples of 10,000 trees generated by PAUP* 4.0.

For the maximum-likelihood searches, it was first necessary to choose an appropriate model of sequence evolution (Swofford *et al.*, 1996). This was done using likelihood ratio tests (Huelsenbeck and Rannala, 1997) comparing nested, successively more-parameter-rich models. Three models were compared using PAUP* 4.0: the F81, HKY85, and GTR models (Swofford *et al.*, 1996). Because it is well understood that the three codon sites in protein-coding genes experience different substitution rates, rate heterogeneity was assumed and rate variation was modeled as a discrete gamma distribution (Yang, 1993, 1996) with four rate categories. Fixing the most-parsimonious tree as the tree for the tests, the gamma GTR model was found to be superior to the gamma HKY85 model ($\chi^2 = 134$, $df = 4$, $P \ll 0.001$), which was much superior to the gamma F81 model ($\chi^2 = 2376$, $df = 1$, $P \ll 0.001$). A maximum-likelihood heuristic search (ASIS stepwise addition followed by TBR branch swapping) was then run under the gamma GTR model using the estimated substitution parameters and the estimated gamma parameter (0.297).

TABLE 2

Summary of Cytochrome *b* Gene Lengths and Termination Signals for the Elapid Sequences Generated in This Study

Taxon	Gene length (bp)	Termination signal
Elapids		
Acanthophis antarcticus	1116	T
Aspidelaps scutatus	1116	T
Aspidomorphus muelleri	1116	T
Austrelaps superbus	1101	Stop codon (TAA)
Boulengerina sp.	1116	T
Bungarus fasciatus	1110	T
Calliophis japonicus	1116	T
Calliophis macclellandi 1	1113	T
Calliophis macclellandi 2	1113	T
Dendroaspis polylepis	1116	T
Drysdalia coronata	1113	Stop codon (TAA)
Elapsoidea nigra	1113	T
Hemachatus haemachatus	1116	T
Homoroselaps lacteus	1113	T
Hydrophis semperi	1116	T
Laticauda colubrina	1113	T
Maticora bivirgata	1122	T
Micropechis ikaheka	1110	Stop codon (TAA)
Micruroides euryxanthus	1113	T
Micrurus fulvius 2	1113	T
Naja kaouthia	1116	T
Naja nivea	1116	T
Notechis ater	1104	Stop codon (TAA)
Ophiophagus hannah	1113	T
Paranaja multifasciata	1116	T
Pseudechis australis	1116	T
Toxicocalamus preussi	1113	T
Walterinnesia aegyptia	1116	T
Outgroups		
Acrochordus granulatus	1116	T
Coluber constrictor	1113	T
Heterodon simus	1116	T

RESULTS

Cytochrome b Sequences

Characteristics of complete snake cytochrome *b* sequences have previously been considered by Campbell (1997). Drawing on cytochrome *b* sequences from a sample of mostly henophidian snakes, Campbell reported that the gene in snakes is between 1113 and 1116 bp long. Using an alignment against other tetrapods, he demonstrated that the reason why the gene is shorter in snakes than in other vertebrates is due to several codon deletions near the ends of the gene. In our study, we found greater variation in the length of the cytochrome *b* gene in elapids than was reported by Campbell, with the gene ranging between 1101 and 1122 bp long (Table 2). This variation in elapids is due to deletions and/or insertions at the 3′ end, rather than internal insertions/deletions.

In approximately half of the snakes examined by Campbell (1997), the cytochrome *b* gene was termi-

nated by a stop codon. The remaining sequences were terminated by a T, which is presumably polyadenylated posttranscriptionally to form a functional stop codon (Campbell, 1997), a mechanism that has been reported in other vertebrates (e.g., carp: Chang *et al.*, 1994). In our study, we found that only 14% of the elapid sequences end with a stop codon, which was always TAA (Table 3). The remaining sequences end with a terminal T (Table 2).

Nucleotide compositional bias manifested itself in several ways (Table 3). First, compositional bias existed among the three codon sites, with A's dominating at first sites, T's at second sites, and A's at third sites, which possessed very few G's (Table 3). This is similar to the pattern found by Campbell (1997) in other snakes and is generally similar to the pattern found in other vertebrates (e.g., mammals: Irwin *et al.*, 1991). Second, there was significant compositional bias among the species at the third sites (Table 3) but not at the first and second sites. Comparison of the third site nucleotide frequencies across species reveals that, in the context of the preferred trees (see *Phylogenetic Analyses*), there is little conservatism to the frequencies, indicating the lack of a strong phylogenetic component to the third site compositional bias.

Phylogenetic Analyses

A single shortest tree of 3993 steps (Fig. 1; CI = 0.250; RI = 0.339) was found from the parsimony searches. The g_1 statistic for this data was −0.797, indicating highly structured data. The g_1 statistic was also measured separately for the three sets of codon sites: the first sites contributed 827 steps to the overall length and had a RI of 0.343 and a g_1 of −0.454; the second sites contributed 317 steps to the overall length and had a RI of 0.419 and a g_1 of −0.280; the third sites contributed 2849 steps to the overall length and had a RI of 0.328 and a g_1 of −0.693. These RI and g_1 data indicate that all three sets of codon sites retain phylogenetic signal and that there is little justification for downweighting third sites relative to the first and second sites, despite the fact that the third sites contribute the majority of the substitutions.

A single tree of -log likelihood 16427.039 was found from the maximum-likelihood search (Fig. 2). This tree is very similar to the maximum-parsimony tree (Fig. 1) but differs in several ways which are discussed below.

TABLE 3

Compositional Bias for the Sequences Generated in This Study

Mean compositional bias for all species	A	C	G	T	χ^2 test among all species (*P*)
First positions	0.3363	0.2376	0.1721	0.2525	27.73 (1.000)
Second positions	0.1984	0.2888	0.1114	0.4000	7.88 (1.000)
Third positions	0.3790	0.3765	0.0427	0.2000	269.99 (0.000)
All positions	0.3045	0.3009	0.1088	0.2843	109.81 (0.281)

DISCUSSION

In the following, we summarize the salient features of elapid phylogeny as presented in Figs. 1 and 2. In doing so, we compare our results with those of earlier studies. Congruence is probably the best arbiter of the accuracy of phylogenetic results (Penny *et al.*, 1982; Miyamoto and Cracraft, 1991; Slowinski, 1993).

Homoroselaps

Prior to McDowell (1968), *Homoroselaps* (as *Elaps*) was considered an elapid because of its possession of the proteroglyphous maxilla. Based on morphological evidence, McDowell (1968) argued for the removal of *Homoroselaps* from the Elapidae and transfer to the Colubridae, but Underwood and Kochva (1993), in a phylogenetic analysis of morphological characters in representatives of *Atractaspis*, *Homoroselaps*, African aparallactine colubrids, African elapids, and the South American colubrids *Apostolepis* and *Elapomorphus*, found support for a relationship between *Homoroselaps* and elapids and returned *Homoroselaps* to the Elapidae. Our data indicate that *Homoroselaps* falls outside a clade formed by the remaining elapids, a conclusion supported by Keogh *et al.* (unpublished). To determine whether *Homoroselaps* shares a close relationship to elapids will require additional work within the context of a large sample of colubroid snakes.

Elapines vs Hydrophiines

Both the parsimony and the likelihood trees (Figs. 1 and 2) support a division of the remaining elapids into two broad clades: the marine and Australo–Melanesian species on the one hand vs the African, American, and Asian species on the other hand. The monophyly of the former clade can hardly be contested; in phylogenetic studies of elapids, this is the one result that all studies agree on (see Keogh, 1998; Keogh *et al.*, 1998; Slowinski *et al.*, 1997, and references therein). Relationships within the marine and Australo–Melanesian clade have been explored in a number of studies (see Keogh *et al.*, 1998, and references therein) and will not be discussed further here.

Less well corroborated is the hypothesis that the African (sans *Homoroselaps*), American, and Asian elapids are collectively monophyletic. Keogh (1998), in a phylogenetic analysis of partial 16S and cytochrome *b* sequences, found *Bungarus* to be the sister taxon to the marine/Australo–Melanesian clade, with the relationships of the African, American, and remaining Asian forms left unresolved at the base of the tree. Yet, within the context of a very limited number of elapids, the molecular studies of Mao *et al.* (1983) and Guo *et al.* (1987) have linked *Bungarus* with the cobra genus

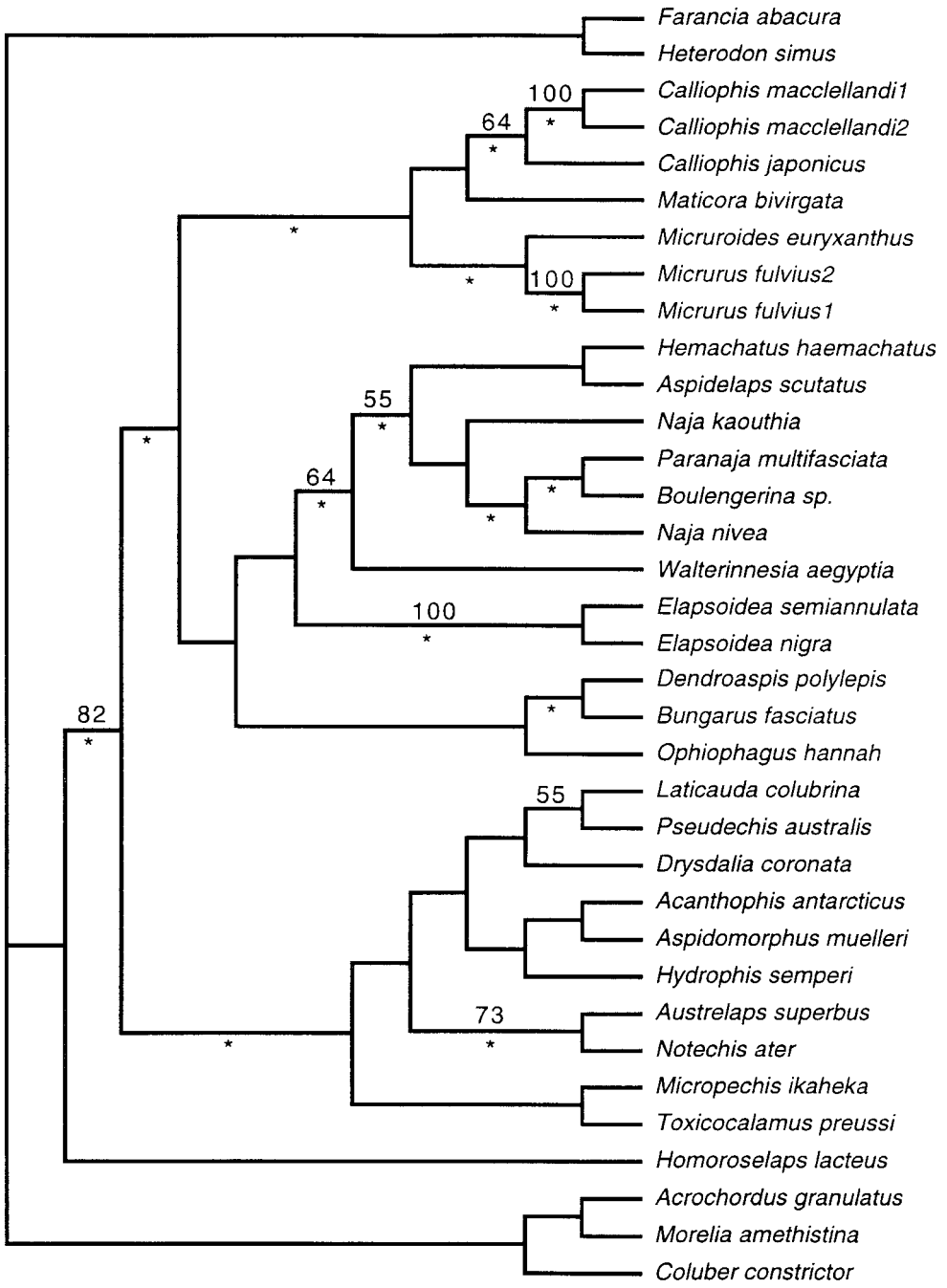

FIG. 1. The most-parsimonious tree (L = 3993 steps, CI = 0.250, RI = 0.339) resulting from unweighted analysis of the cytochrome *b* sequences. Bootstrap values greater than 50% are shown above the branches. Asterisks indicate the clades that were also present on the ML tree (Fig. 2).

Naja to the exclusion of marine/Australo–Papuan forms. Further, in a study based on the amino acid sequences of two venom protein genes, Slowinski *et al.* (1997) found the African and Asian species (no American species were sampled) to be monophyletic, a result supported by both genes. Thus, the balance of evidence supports monophyly for the African, American, and Asian species.

Slowinski *et al.* (1997) recommended adopting a modified version of Smith *et al.*'s (1977) classification for elapids, wherein all marine and Australo–Melanesian species are placed in the subfamily Hydrophiinae, while all African, American, and Asian species are placed in the subfamily Elapinae. This classification differs from Smith *et al.*'s only in including the marine *Laticauda* within the hydrophiines. The present study

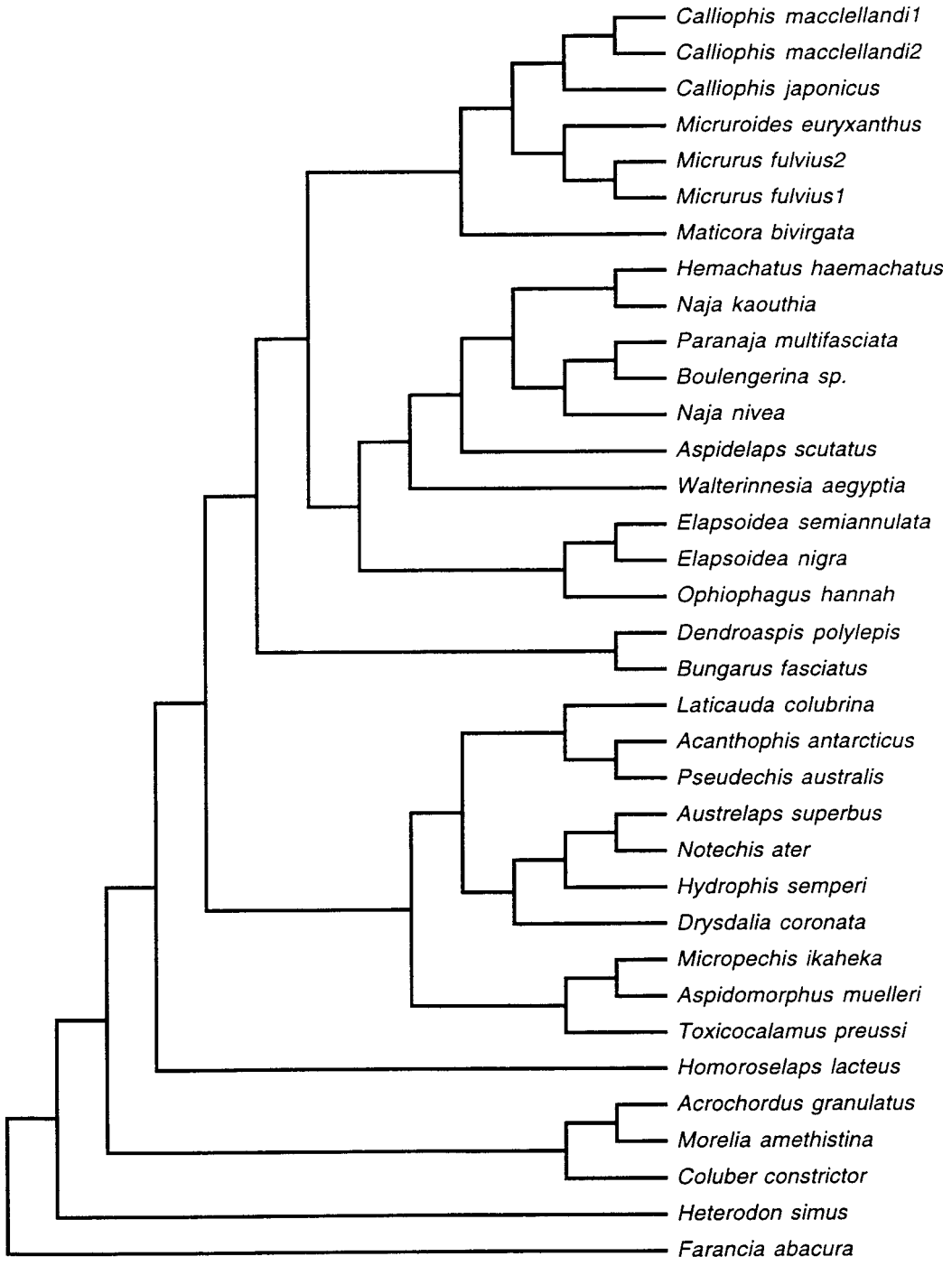

FIG. 2. The maximum-likelihood tree (log likelihood = 16427.039) resulting from analysis of the cytochrome *b* sequences under the gamma GTR model.

provides additional support for Slowinski *et al.*'s subfamilial classification.

Elapines

The parsimony analysis (Fig. 1) found elapines to be divided into two clades: coral snakes vs cobras, *Bungarus, Elapsoidea,* and *Dendroaspis.* The term "cobra" has traditionally been applied to the genera *Aspidelaps, Boulengerina, Hemachatus, Naja, Ophiophagus, Paranaja, Pseudohaje,* and *Walterinnesia,* a mostly African group generally characterized by the ability to flatten the neck into a "hood" when threatened. The African mambas (*Dendroaspis*) also have the ability to spread a hood when threatened (Broadley, 1983; Greene,

1997), albeit more weakly than many members of the aforementioned group. Our study found significant bootstrap support for a core cobra group consisting of *Naja*, *Boulengerina*, *Paranaja*, *Aspidelaps*, *Hemachatus*, and *Walterinnesia*. Oddly, the Asian king cobra, *Ophiophagus hannah*, was not part of this clade, clustering instead with a group including *Dendroaspis* and *Bungarus* on the most-parsimonious tree (Fig. 1) or with *Elapsoidea* on the maximum-likelihood tree (Fig. 2). This result calls into question the monophyly of cobras and underscores the uncertainty of the homology of the hood spreading behavior in cobras and mambas. The relationships of *Dendroaspis*, *Ophiophagus*, and *Bungarus* differed between the parsimony and likelihood analyses, suggesting that more work is necessary to resolve the relationships of these problematic taxa.

Figures 1 and 2 call into question whether the widespread African and east Asian *Naja* is monophyletic. Szyndlar and Rage (1990) and Szyndlar and Zerova (1990) reviewed some osteological characters of the skull in living and fossil members of *Naja* and concluded that a suite of synapomorphies supported the monophyly of Asian *Naja* but that African *Naja* might be paraphyletic. Our study sampled only one African *Naja* (*N. nivea*) and therefore cannot address the monophyly or nonmonophyly of this group but does indicate that, collectively, *Naja* is nonmonophyletic because of the exclusion of *Boulengerina* and *Paranaja*.

Our study found American (*Micruroides* and *Micrurus*) and Asian (*Calliophis* and *Maticora*) coral snakes to be related, a result also found by Keogh (1998). A close link between these groups is hardly surprising, given the degree of morphological similarity (McDowell, 1986; Slowinski and Boundy, unpublished). Both the maximum-parsimony and the maximum-likelihood analyses (Figs. 1 and 2) suggest that the New World coral snakes are monophyletic, with *Micrurus* and *Micruroides* clustering as sister genera. This result is significant because Slowinski and Boundy (unpublished), in an exploration of the relationships among American and Asian coral snakes based on morphological characters, found that there is no compelling morphological evidence linking *Micruroides* and *Micrurus* to the exclusion of the Asian forms. Both the maximum-parsimony and the maximum-likelihood analyses (Figs. 1 and 2) suggest that the subtropical *Calliophis* (represented here by *C. macclellandi* and *C. japonicus*) are monophyletic, in accordance with Slowinski and Boundy. Further, the maximum-likelihood tree (Fig. 2) suggests that the subtropical *Calliophis* and New World coral snakes are sister clades, to the exclusion of other Asian coral snakes, also in accordance with Slowinski and Boundy.

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