THE HIGHER-LEVEL RELATIONSHIPS OF ALETHINOPHIDIAN SNAKES INFERRED FROM SEVEN NUCLEAR AND MITOCHONDRIAL GENES

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ABSTRACT.—Phylogenetic studies have defined two major groups of snakes, the Scolecophidia (ca. 340 species) and the Alethinophidia (ca. 2,640 sp.). Scolecophidians are burrowing snakes, whereas alethinophidians occupy a diversity of ecological niches. Here, we present new DNA-sequence data from several genes and analyze those together with available sequence data to examine the higher-level relationships of alethinophidian snakes, including boas and pythons. We find additional, significant support for a major split at the base of the evolutionary tree of alethinophidian snakes, between a South American clade (*Anilius* and Tropidophiidae) and all other species. Based on the fossil record and Earth history, we interpret this split as being the result of vicariance: the separation of South America and Africa in the mid-Cretaceous. We give the name Amerophidia to the South American clade and Afrophidia to the other clade.

INTRODUCTION

The order Squamata includes lizards (ca. 4770 species), snakes (ca. 3000 spp.) and amphisbaenians (ca. 170 spp.) (Uetz, 2006). According to recent molecular studies, the closest relatives of snakes are the anguimorphs and/or the iguanians. The presence of toxin-secreting oral glands is a shared derived character of this clade (named Toxicofera), demonstrating a single early origin of the venom system in squamates dating from the Jurassic (Fry et al., 2005; Vidal and Hedges, 2005).

Snakes are divided into two main groups. The fossorial scolecophidians (blindsnakes and threadsnakes, ca. 340 sp.) are small snakes with limited gape sizes that feed on small prey (mainly ants and termites) on a frequent basis. The alethinophidians (all other snakes, ca. 2640 sp.) are more ecologically diverse, and most species feed on relatively large prey, primarily vertebrates, on an infrequent basis (Cundall and Greene, 2000; Vidal and Hedges, 2002a). Among Alethinophidia, the caenophidians (advanced snakes, ca. 2470 sp.) widely use venom to subdue their prey, whereas the remaining alethinophidian snakes (ca. 170 sp.), which do not form a single (monophyletic) group, use constriction (secondarily lost by some fossorial species) (Greene and Burghardt, 1978; Greene, 1994; Vidal and Hedges, 2002a, b; Vidal and David, 2004).

According to recent molecular studies (Vidal and Hedges, 2002a; Vidal and David, 2004), the alethinophidian snakes include the following lineages: Aniliidae (genus *Anilius*, 1 sp.), Tropidophiidae (genera *Tropidophis* and *Trachyboa*, 32 spp.), Boidae (genera *Acrantophis*, *Boa*, *Candoia*, *Corallus*, *Epicrates*, *Eunectes*, *Sanzinia*, *Charina*, *Eryx*, *Gongylophis*, *Lichanura*, *Exiliboa*, *Ungaliophis*, 49 spp.), Calabariidae (genus *Calabaria*, 1 sp.), Pythonidae (genera *Aspidites*, *Antaresia*, *Apodora*, *Bothrochilus*, *Leiopython*, *Liasis*, *Morelia*, *Python*, 33 spp.), Loxocemidae (genus *Loxocemus*, 1 sp.), Xenopeltidae (genus *Xenopeltis*, 2 spp.), Bolyeriidae (genera *Bolyeria*, *Casarea*, 2 spp.), Cylindrophiidae (genus *Cylindrophis*, 10 spp.), Anomochilidae (genus *Anomochilus*, 2 spp.), Uropeltidae (genera *Brachyophidium*, *Melanophidium*, *Platyplectrurus*, *Plectrurus*, *Pseudotyphlops*, *Rhinophis*, *Teretrurus*, *Uropeltis*, 47 spp.), Xenophidiidae (genus *Xenophidion*, 2 spp.) and advanced snakes (Caenophidia). Because no evidence supports the contention that boas and pythons form a monophyletic group, the study of their evolution and phylogeny must involve the study of all major clades of alethinophidian snakes.

In this chapter we address the phylogeny of Alethinophidia, with emphasis on non-caenophidian taxa (including all boas and pythons), using DNA sequences obtained from seven nuclear and mitochondrial genes. This work builds upon a long history of research into snake classification, including landmark morphological studies such as those of Underwood (1967, 1976). However, our intent here is not to review the history of snake classification but rather to provide an update of the molecular evidence bearing on the higher-level relationships of the alethinophidians. Most of the data analyzed were collected in earlier published studies, although we present new data for a gene not previously analyzed (mitochondrial ND2), 13 new sequences of mitochondrial gene ND4, and one new sequence for the nuclear gene RAG1. For recent

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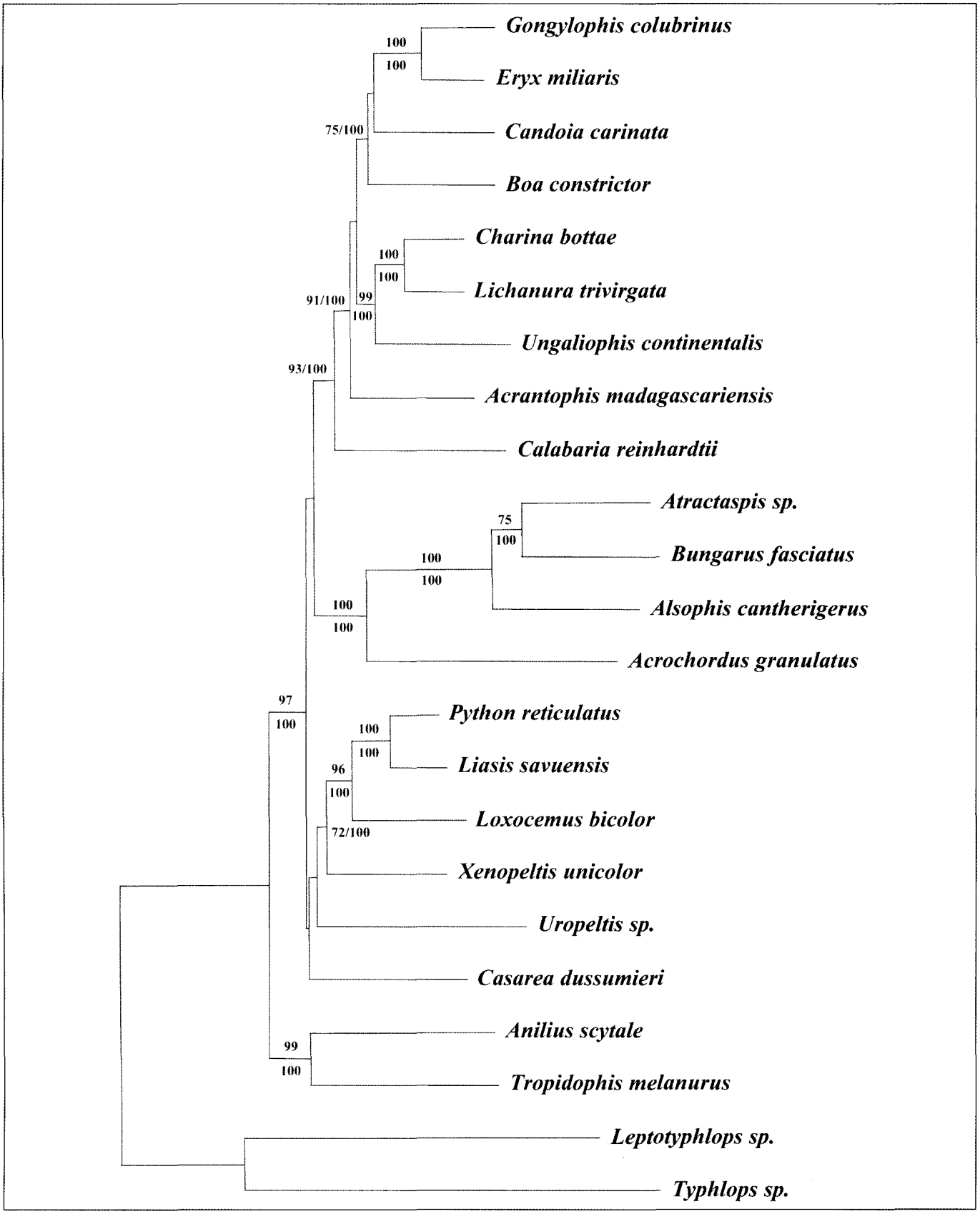

Fig. 1. ML tree obtained from the combined data set (4228 sites, log-likelihood, - 42612.33651). Values are bootstrap ML values above 70%, followed by Bayesian posterior probabilities above 95%. The only topological difference between the ML and Bayesian trees is the clustering of *Casarea* with *Uropeltis* in the Bayesian tree (very weakly supported, PP of 37%).

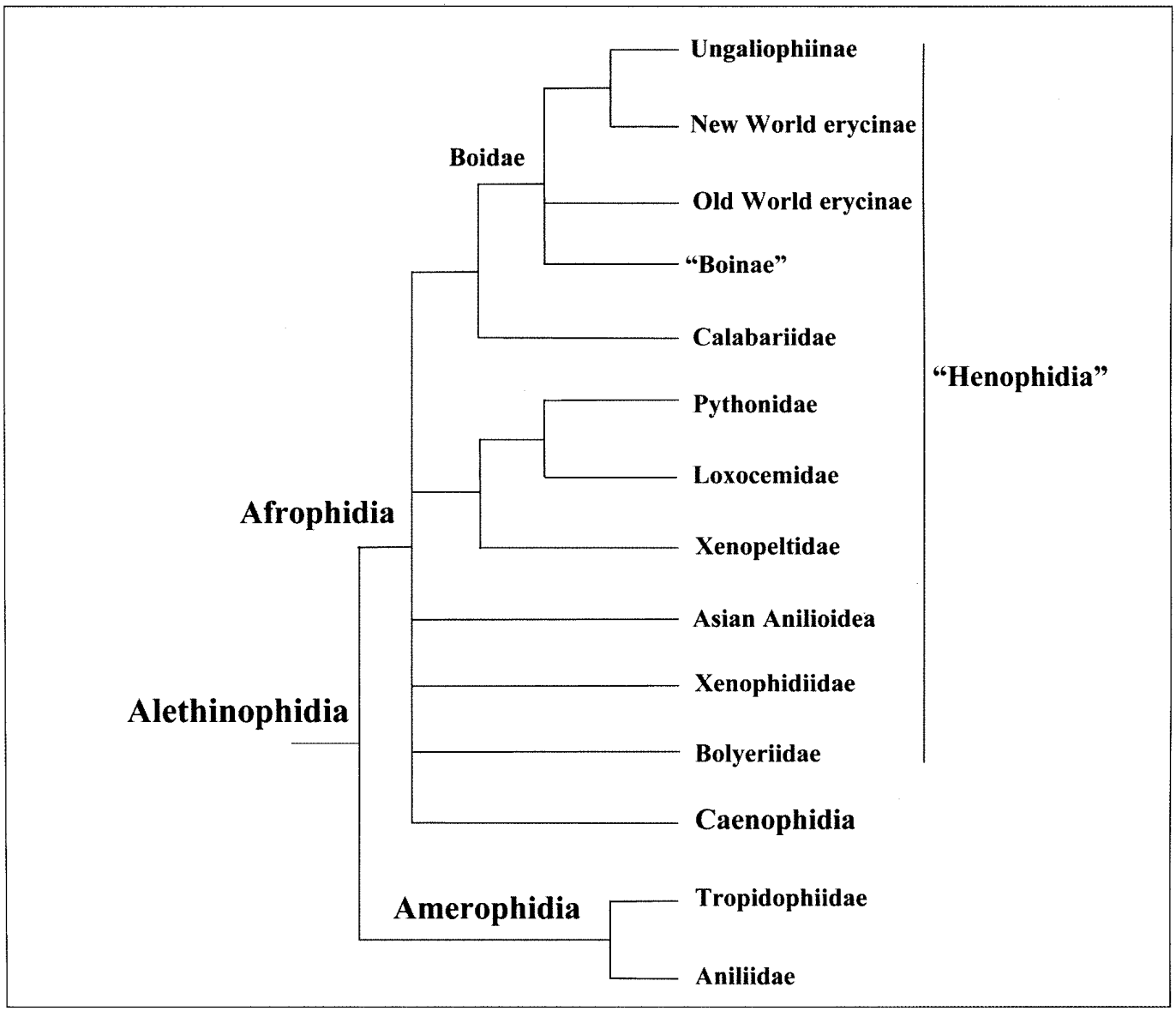

Fig. 2. A revised phylogenetic hypothesis and classification for the major groups of alethinophidian snakes. The phylogenetic status of "Henophidia" (all Afrophidia, excluding Caenophidia) remains uncertain

molecular phylogenetic studies on caenophidians, and additional discussion of snake classification, see Vidal (2002), Vidal and Hedges (2002a, b, 2004), Vidal and David (2004), and Lawson et al. (2005).

MATERIALS AND METHODS

Tissue samples were obtained from the tissue collections of N. Vidal and S. B. Hedges (see Vidal and Hedges, 2004 for details of the samples used). DNA extraction was performed using the DNeasy Tissue Kit (Qiagen). Amplification and sequencing was performed using the following sets of primers: L2408, 5'-TG-CACTGTGACATTGGCAA-3' (Vidal and Hedges, 2004), H2928, 5'-GACTGCYTG GCATTCATTT-3' (Vidal and Hedges, 2004) and H2920, 5'-GC-CATTCATTTTYCGAA-3' (Vidal and Hedges, 2004) for the RAG1 gene; ND4, 5'-TGA-CTA-CCA-AAA-GCT-CAT-GTA-GAA-GC-3' (Forstner et al., 1995) and LEU, 5'-TAC-TTT-TAC-TTG-GAT-TTG-CAC-CA-3' (Forstner et al., 1995) for the ND4 gene; and L4437b, 5' -CAG-CTA-AAA-AAG-CTA-TCG-GGC-CCA-TAC-C-3' (Kumazawa et al., 1996), H5382, 5'-GTG-TGG-GCR-ATT-GAT-GA-3' (de Queiroz et al., 2002), and tRNA-trpR, 5'-GGC-TTT-GAA-GGC-TMC-TAG-TTT-3' (de Queiroz et al., 2002) for the ND2 gene. Both strands of the PCR products were sequenced using the BigDye sequencing kit (Applied Biosystems, Foster City, California) in the ABI Prism 3100-Avant Genetic

Analyser. The two strands obtained for each sequence were aligned using the BioEdit Sequence Alignment Editor program (Hall, 1999). The ND4 and ND2 sequence data used in this work, including sequence data obtained from GenBank or the references, are listed in the Appendix. Except as mentioned in the Appendix, the C-mos and RAG1 sequences were obtained from Vidal and Hedges (2002a, b) and Vidal and Hedges (2004); the 12S and 16S rRNA sequences were obtained from Vidal et al. (2000) and Vidal and Hedges (2002a, b); the cytochrome b (cyt-*b*) sequences were obtained from Slowinski and Lawson (2002), Lawson et al. (2004), and Lawson et al (2005).

Sequence entry and alignment were performed manually with the MUST2000 software (Philippe, 1993). Alignment of the protein coding sequences was straightforward. For the 16S rRNA sequences, alignment was ambiguous in three highly variable areas corresponding to loops that we have deleted from analyses. In order to align the 12S rRNA sequences, we used the secondary structure model described by Hickson et al. (1996) (see Vidal et al., 2000 and Gower et al., 2005).

This resulted in 534 bp for the C-mos gene, 510 bp for the RAG1 gene, 1101 bp for the cyt-*b* gene, 353 bp for the 12S rRNA gene, 374 bp for the 16S rRNA gene, 672 bp for the ND4 gene, and 684 bp for the ND2 gene. In all analyses, remaining gaps were treated as missing data.

We built phylogenies using probabilistic approaches, with Maximum Likelihood (ML) and Bayesian methods of inference. ML analyses were performed with PAUP*4 (Swofford, 1998). Bayesian analyses were performed with MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003; Nylander et al., 2004). For ML methods, an appropriate model of sequence evolution was inferred using ModelTest (Posada and Crandall, 1998), both for nuclear, mitochondrial, and combined analyses. As trees resulting from separate nuclear and mitochondrial analyses were almost identical and showed no significant topological incongruence (no conflicting clades that were supported in alternative ML analyses by bootstrap values greater than 50%, data not shown), we also performed combined analyses, which are considered to be our best estimates of phylogeny. For the nuclear data set (1044 sites), the model selected is the TrN+G model (base frequencies: A (0.2986), C (0.1936), G (0.2362), T (0.2716); substitution parameters: A-G: 4.4965, C-T: 6.5997; gamma distribution shape parameter (G): 0.5071). For the mitochondrial data set (3184 sites), the model selected is the GTR+I+G model (base frequencies: A (0.4321), C (0.3158), G (0.066), T (0.1861); substitution parameters: A-C: 0.3332, A-G: 4.303, A-T: 0.5636, C-G: 0.321, C-T: 5.4627; proportion of invariable sites (I): 0.1529; G: 0.3043). For the concatenated data set (4228 sites), the model selected is the GTR+I+G model (base frequencies: A (0.4038), C (0.2897), G (0.1148), T (0.1917); substitution parameters: A-C: 1.2744, A-G: 3.3927, A-T: 1.4322, C-G: 0.37, C-T: 11.8926; proportion of invariable sites (I): 0.2424; G: 0.4011). Bayesian analyses were run with model parameters estimated as part of those analyses, and the best-fit models as inferred by Modeltest (GTR for the nuclear analysis; GTR for the mitochondrial analysis; GTR/GTR for the combined (nuclear/mitochondrial) analysis). For the ML analyses, we used heuristic searches, with starting trees obtained by random addition with 100 replicates and nearest-neighbor interchange (NNI) branch swapping. For bootstrap ML analyses, we performed 1000 replicates (NJ starting tree with NNI branch swapping). Bayesian analyses were performed by running 2,000,000 generations in four chains, saving the current tree every 100 generations. The last 18,000 trees were used to construct a 50% majority-rule consensus tree.

RESULTS AND DISCUSSION

Aniliidae and Tropidophiidae

Our results (Fig. 1) support the close relationship of these two families which otherwise are not similar in morphology or ecology. Aniliidae (genus *Anilius*) has a small gape (non-macrostomatan), whereas Tropidophiidae (genera *Trachyboa* and *Tropidophis*) has a larger gape (macrostomatan), although not as large as that of many boas and pythons. This close relationship was found in earlier studies using nuclear genes (Vidal and Hedges, 2002a; Vidal and Hedges, 2004; Vidal and David, 2004; Noonan and Chippindale, 2006), but not in studies using one or two mitochondrial genes (Wilcox et al., 2002; Lawson et al., 2004). Moreover, the clade comprising Aniliidae and Tropidophiidae forms the most basal alethinophidian lineage. The genus *Anilius* is therefore only distantly related to the remaining anilioids, which are all Asian and form a monophyletic group including Cylindrophiidae, Uropeltidae, and Anomochilidae (Gower et al., 2005). Our findings therefore support the inference made by Vidal and Hedges (2002a) and Vidal and David (2004) that the alethinophidians were primitively macrostomatan and that this condition was secondarily lost twice by Aniliidae and Asian anilioids, in connection with burrowing.

Because Aniliidae is restricted to South America, and Tropidophiidae is believed to have originated in South America (Hedges, 1996), this clade is probably of South American origin. Considering this, and the deep split between this clade and other alethinophidian snakes, one obvious implication is that it represents a vicariant event: the separation of South America from Africa in the mid-Cretaceous. In turn, this would imply that most alethinophidian snakes had their roots in Africa, a hypothesis that is in agreement with the fossil record (Rage and Werner, 1999) and that could be further tested by dating of divergence events with molecular clocks.

Xenopeltidae, Loxocemidae, and Pythonidae

The monophyly of the group including the Pythonidae, the Xenopeltidae, and the Loxocemidae, which was found in most recent molecular studies (Slowinski and Lawson, 2002; Vidal and Hedges, 2002a; Wilcox et al., 2002; Vidal and David, 2004; Vidal and Hedges, 2004), is here strongly supported, with Loxocemidae as the closest relative to Pythonidae. Noonan and Chippindale (2006) differed in clustering *Xenopeltis* with Asian anilioids and Caenophidia. Within Pythonidae, the Australian/New Guinean taxa appear to form a monophyletic group according to Lawson et al. (2004).

Asian anilioids, Bolyeriidae, and Xenophidiidae

Among Asian anilioids, the genus *Anomochilus* is rooted within Cylindrophiidae, this clade being in turn the sister-group to the monophyletic Uropeltidae (Bossuyt et al., 2004; Gower et al., 2005). According to analyses of CYT-*b* sequences (Lawson et al., 2004), the enigmatic Malaysian Xenophidiidae may be the closest relative of the Bolyeriidae from Round Island in the Indian Ocean.

Calabariidae and Boidae

The Calabariidae forms the sister-group to the Boidae, as was found by Vidal and Hedges (2002a), Vidal and David (2004), and Lawson et al. (2004). The Boidae then includes all genera belonging to the "boines," the "erycines," and the ungaliophiines. Noonan and Chippindale (2006), however, rooted *Calabaria* within the Boidae (sister-group to African boids). The Neotropical genera *Ungaliophis* and *Exiliboa*, which have been shown to be sister-groups (Vidal and Hedges, 2002a), are the closest relatives to the North American erycines (genera *Charina* and *Lichanura*) (Vidal and Hedges, 2002a; Lawson et al., 2004). The Old World erycines (genera *Eryx* and *Gongylophis*) form a monophyletic group. Finally, we do not retrieve the clear-cut division between Old World and New World "boines" found by Lawson et al. (2004) and Burbrink (2005) using cyt-*b* sequences. Actually, our results suggest that "boines" are not monophyletic, as was previously found by Noonan and Chippindale (2006) using five nuclear genes and one mitochondrial gene.

Taxonomic Implications

A conservative phylogenetic hypothesis for the main snake lineages, as inferred from our molecular data set, is summarized on the consensus tree displayed in Figure 2; accordingly, a revised classification is proposed.

SERPENTES
  SCOLECOPHIDIA
  ALETHINOPHIDIA
    AMEROPHIDIA *nomen novum*

Includes the most recent common ancestor of Aniliidae and Tropidophiidae and all descendants. The name refers to their geographic origin.

    AFROPHIDIA *nomen novum*

Includes the most recent common ancestor of Boidae, Calabariidae, Pythonidae, Loxocemidae, Xenopeltidae, Bolyeriidae, Xenophidiidae, Cylindrophiidae, Anomochilidae, Uropeltidae, and Caenophidia, and all descendants. The name refers to their geographic origin.

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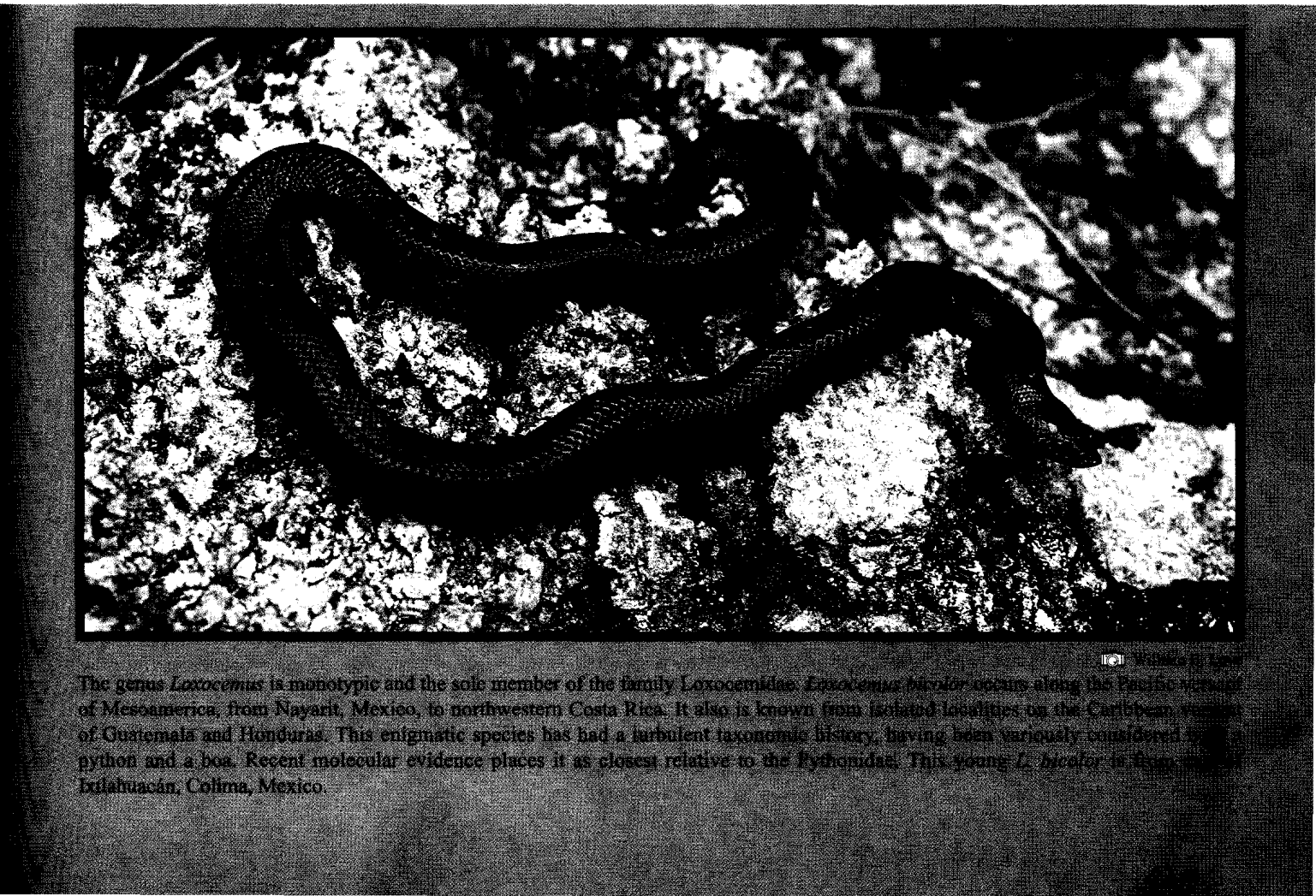
APPENDIX.

All C-mos and RAG1 sequences were obtained from Vidal and Hedges (2002a, b) and Vidal and Hedges (2004), with the exception of the *Atractaspis corpulenta* (NV; Rabi, Gabon) RAG1 sequence, which is original.

ND4 sequences (672 bp) used for this study (13 out of 23 are original). Typhlopidae: *Typhlops punctatus* (Forstner et al. (1995)); Leptotyphlopidae: *Leptotyphlops dulcis* (Forstner et al. (1995)); Aniliidae: *Anilius scytale* (original); Tropidophiidae: *Tropidophis melanurus* (original); Uropeltidae: *Uropeltis melanogaster* (original); Bolyeriidae: *Casarea dussumieri* (original); Calabariidae: *Calabaria reinhardtii* (original); Loxocemidae: *Loxocemus bicolor* (original); Xenopeltidae: *Xenopeltis unicolor* (original); Pythonidae: *Python reticulatus* (original), *Liasis savuensis* (original); Boidae: *Boa constrictor* (Forstner et al. (1995)), *Acrantophis madagascariensis* (Forstner et al., 1995)), *Candoia carinata* (original), *Eryx miliaris* (AF302942), *Gongylophis colubrinus* (original); *Ungaliophis continentalis* (original), *Charina bottae* (AF302945), *Lichanura trivirgata* (AF302944); Acrochordidae: *Acrochordus granulatus* (U49296); Elapidae: *Bungarus fasciatus* (U49297); Lamprophiidae: *Atractaspis bibroni* (U49314); Dipsadidae: *Alsophis cantherigerus* (original).

ND2 sequences (684 bp) used for this study (all are original). Typhlopidae: *Typhlops lumbricalis*; Leptotyphlopidae: *Leptotyphlops asbolepis* (SBH 160211; Dominican Republic: Barahona Province; 0.3 km S and 13.5 km E (airline distance) of Canoa); Aniliidae: *Anilius scytale*; Tropidophiidae: *Tropidophis melanurus*; Uropeltidae: *Uropeltis melanogaster*; Bolyeriidae: *Casarea dussumieri*; Calabariidae: *Calabaria reinhardtii*; Loxocemidae: *Loxocemus bicolor*; Xenopeltidae: *Xenopeltis unicolor*; Pythonidae: *Python reticulatus*, *Liasis savuensis*; Boidae: *Boa constrictor*, *Acrantophis madagascariensis*, *Candoia carinata*, *Eryx miliaris*, *Gongylophis colubrinus*, *Ungaliophis continentalis*, *Charina bottae*, *Lichanura trivirgata*; Acrochordidae: *Acrochordus granulatus*; Elapidae: *Bungarus fasciatus*; Lamprophiidae: *Atractaspis corpulenta*; Dipsadidae: *Alsophis cantherigerus*.



The genus *Loxocemus* is monotypic and the sole member of the family Loxocemidae. *Loxocemus bicolor* occurs along the Pacific versant of Mesoamerica, from Nayarit, Mexico, to northwestern Costa Rica. It also is known from isolated localities on the Caribbean versant of Guatemala and Honduras. This enigmatic species has had a turbulent taxonomic history, having been variously considered a colubrid, a python and a boa. Recent molecular evidence places it as closest relative to the Pythonidae. This young *L. bicolor* is from near Tulahuacán, Colima, Mexico.