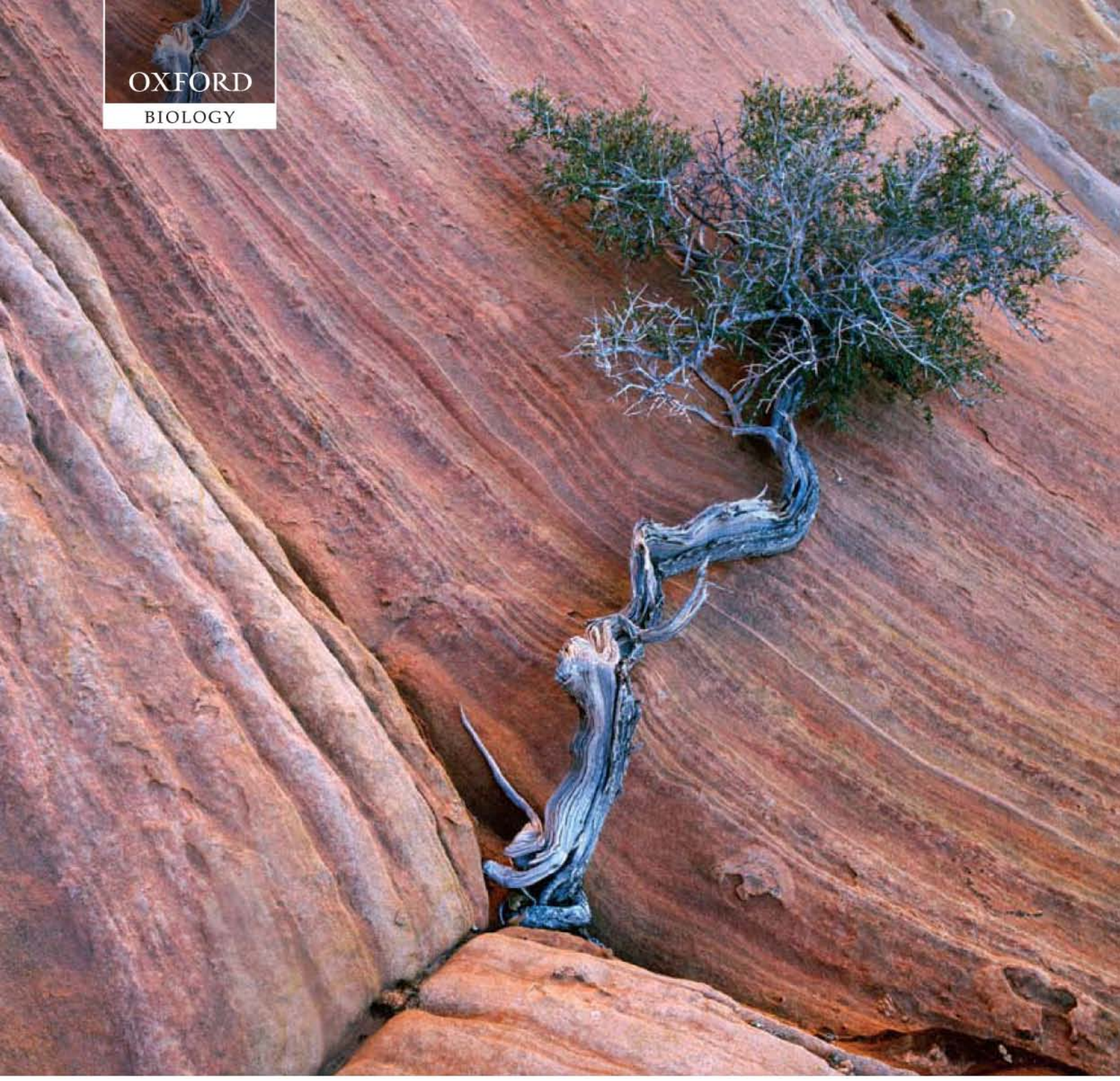

OXFORD
BIOLOGY

the TIMETREE *of* LIFE

edited by **S. BLAIR HEDGES** *and* **SUDHIR KUMAR**

foreword by James D. Watson

Snakes (Serpentes)

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Abstract

Snakes have a Gondwanan origin and their early evolution occurred mainly on West Gondwana, the supercontinent comprising South America and Africa. New data from nine genes indicate that the divergence of Amerophidia and Afrophidia occurred 106 (116–97) million years ago (Ma), supporting their origin by continental breakup. Most (~85%) living snakes are afrophidians and are globally distributed now, but their initial radiation can be explained by dispersal out of Africa through Laurasia or India. Most basal afrophidian families (Henophidia) diverged in the Cretaceous, 104–70 Ma, while most advanced afrophidian families (Caenophidia), diverged in the early Cenozoic, 63–33 Ma.

Snakes are among the most successful groups of reptiles, numbering about 3070 extant species (*1*). They are divided into two main groups. The fossorial scolecophidians (~370 sp.) are small snakes with a limited gape size and feed on small prey (mainly ants and termites) on a frequent basis. The alethinophidians, or typical snakes (~2700 sp.), are more ecologically diverse and most species feed on relatively large prey, primarily vertebrates, on an infrequent basis (*2, 3*). According to most morphological studies, a distinctive evolutionary trend within living snakes is the increase of the gape size from fossorial scolecophidians (Typhlopidae, Leptotyphlopidae, and Anomalepididae) and fossorial alethinophidians (Aniliidae, Cylindrophiidae, Uropeltidae, and Anomochilidae) to ecologically diverse macrostomatan alethinophidian snakes such as boas, pythons, and caenophidians (advanced snakes) (*2*, but see *4*). Macrostomatans are able to ingest very large prey, often greater in diameter than the snake itself (*5*), and the monophyly of the macrostomatan condition is supported by several unambiguous shared-derived characters (*6*). All venomous snakes are found within Caenophidia, which includes the great majority of extant snakes (~2500 sp.) (*1*).

Previously, caenophidians were thought to comprise five families: the aquatic acrochordids, the atractaspidids (now a subfamily; some of them with a front-fanged venom system), the elapids, and the viperids (all of them with a front-fanged venom system), and the large and paraphyletic family Colubridae (now split into eight families), which includes rear-fanged snakes and the vast majority of caenophidians (~1900 sp.) (*7–12*). Here, the relationships and fossil record of snakes are reviewed and new data from nine nuclear protein-coding genes are analyzed, resulting in a timetree of snake families with new biogeographic implications.

Several higher-level snake phylogenies using nuclear genes, including some that incorporated mitochondrial genes, have been published since 2002 (*13–21*). They

Fig. 1 *Typhlops arator* from Cuba, Typhlopidae (upper left); *Rhinocheilus lecontei* from southwestern United States, Colubridae (upper right); *Cryptelytrops albolabris*, from southeastern Asia, Viperidae (lower left); and *Tropidophis feicki* from Cuba, Tropidophiidae (lower right). Credits: S. B. Hedges.

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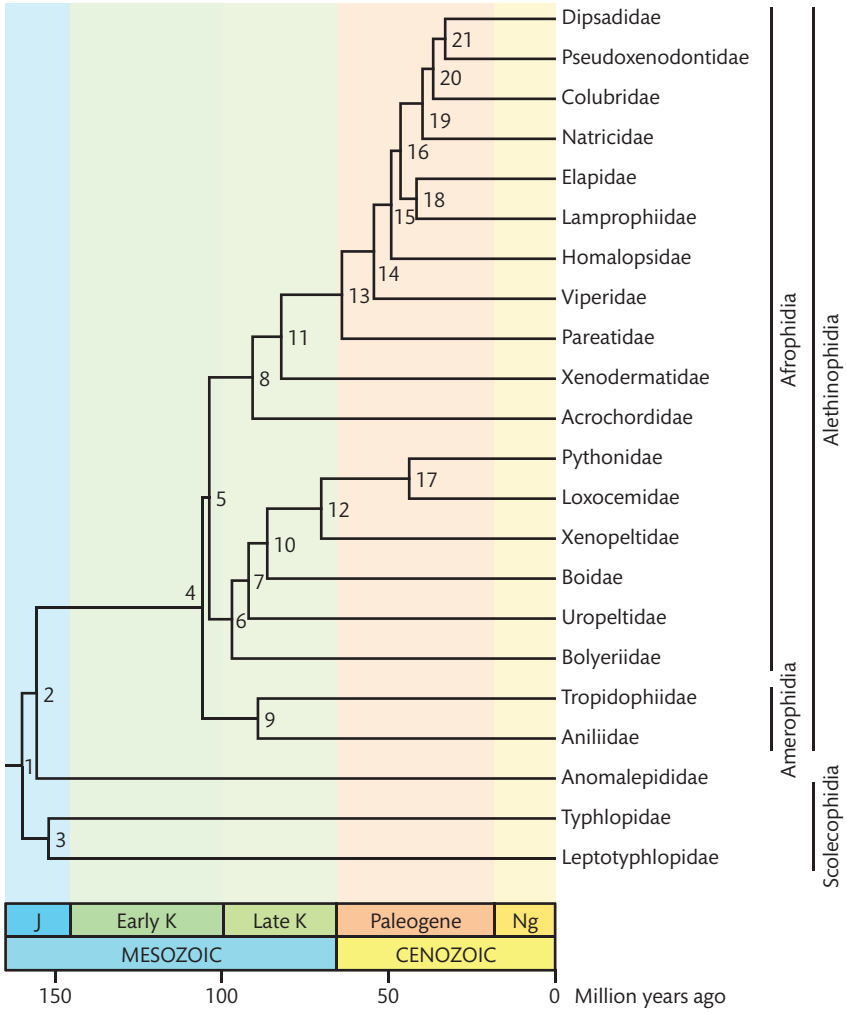

Fig. 2 A timetree of snakes. Divergence times are shown in Table 1. *Abbreviations*: J (Jurassic), Ng (Neogene), and K (Cretaceous).

all agree on the monophyly of alethinophidians, but a striking result is the paraphyly of the macrostomatan condition. The fossorial small-gaped Aniliidae (South American genus *Anilius*) and the terrestrial large-gaped (macrostomatan) Tropidophiidae (Neotropical genera *Trachyboa* and *Tropidophis*) cluster together, and form the most basal alethinophidian lineage (*13, 16, 17, 19, 20*). The genus *Anilius* is therefore not closely related to the Asian families formerly placed in "Anilioidea." We propose that Uropeltoidea Müller be used to describe the monophyletic group (*22*) that includes Cylindrophiidae, Uropeltidae, and Anomochilidae. Also, we provisionally use the taxon Henophidia Hoffstetter to describe all non-caenophidian Afrophidia, which usually form a monophyletic group in molecular phylogenetic analyses.

The alethinophidians were therefore primitively macrostomatan, and this condition was secondarily lost twice by Aniliidae and Uropeltoidea, in connection with burrowing (*13, 17, 20*). From a biogeographic point of view, the deep split between the Aniliidae–Tropidophiidae clade, which is of South American origin, and all remaining alethinophidians was recently hypothesized to represent a vicariant event: the separation of South America from Africa in the mid-Cretaceous. Accordingly, those two clades were named Amerophidia and Afrophidia (*20*). Among alethinophidians, the monophyly of the group including the Pythonidae, Xenopeltidae, and Loxocemidae is found in most molecular studies (*13, 15–17, 20*), with Loxocemidae as the closest relative to Pythonidae. Another large group

Table 1. Divergence times (Ma) and their confidence/credibility intervals (CI) among snakes (Serpentes).

Timetree		Estimates													
Node	Time	This study		Ref. (*19*)		Ref. (*56*)		Ref. (*58*)	Ref. (*59*)		Ref. (*60*)		Ref. (*61*)	Ref. (*62*)	
		Time	CI	Time	CI	Time	CI	Time	Time	CI	Time	CI	Time	Time	CI
1	159.9	159.9	166–148	–	–	102.3	113–94	–	131.1	138–124	109.0	119–99	–	144.2	–
2	155.6	155.6	164–144	–	–	–	–	–	–	–	–	–	–	–	–
3	151.9	151.9	163–137	–	–	–	–	–	–	–	–	–	–	109.3	–
4	105.8	105.8	116–97	121	129–106	62.1	75–49	–	–	–	50.0	56–44	–	76.1	87–69
5	103.7	103.7	114–95	112	119–99	–	–	–	93.5	–	–	–	–	–	–
6	96.9	96.9	108–87	–	–	–	–	–	–	–	–	–	–	–	–
7	92.0	92.0	102–82	–	–	–	–	–	–	–	–	–	–	–	–
8	90.7	90.7	104–78	–	–	54.9	66–44	–	–	–	–	–	–	58.7	71–54
9	89.1	89.1	100–78	110	123–93	–	–	–	–	–	–	–	–	63.1	–
10	86.3	86.3	96–77	–	–	–	–	–	–	–	–	–	–	–	–
11	82.2	82.2	96–69	–	–	–	–	–	–	–	–	–	–	48.7	–
12	70.1	70.1	81–59	–	–	–	–	–	–	–	–	–	–	51.3	–
13	64.0	64.0	77–52	68	82–53	–	–	–	35.6	–	–	–	–	–	–
14	54.3	54.3	67–43	–	–	38.5	45–33	46.0	–	–	–	–	74.3	–	–
15	49.2	49.2	61–39	–	–	–	–	–	–	–	–	–	69.4	–	–
16	46.3	46.3	58–36	–	–	–	–	42.5	–	–	–	–	–	–	–
17	43.7	43.7	56–33	–	–	–	–	–	–	–	–	–	–	37.1	–
18	41.5	41.5	53–32	–	–	–	–	40.5	–	–	–	–	62.9	34.0	–
19	39.8	39.8	50–31	–	–	–	–	–	–	–	–	–	61.2	38.2	–
20	36.6	36.6	46–28	–	–	–	–	–	–	–	–	–	–	–	–
21	32.9	32.9	43–25	–	–	–	–	–	–	–	–	–	–	–	–

Note: Node times in the timetree are from the new analyses presented here. Other published estimates are also shown for comparison.

includes *Calabaria*, "boines," "erycines," and ungaliophiines (genera *Ungaliophis* and *Exiliboa*), with North American erycines and ungaliophiines as closest relatives (*13, 17, 19, 20*). Unfortunately, several higher-level henophidian relationships are still unresolved (*20*), a situation contrasting with our better state of knowledge of the interfamilial relationships among caenophidian snakes.

As recently as 2007, a study using seven nuclear protein-coding genes (C-*mos*, *RAG1*, *RAG2*, *R35*, *HOXA13*, *JUN*, and *AMEL*) resolved with strong support the relationships of all families of caenophidians (*21*). Caenophidians devoid of a front-fanged venom system were traditionally lumped into a large (~1900 sp.) family, "Colubridae," including several subfamilies. Because this family was shown to be paraphyletic, most of the subfamilies were elevated to a familial rank to reflect their evolutionary distinctiveness, and the name Colubridae was restricted to a less inclusive monophyletic group (*21*).

The caenophidian venom apparatus has experienced extensive evolutionary tinkering throughout its history. All traits, ranging from biochemical (specialization of the venoms) to dentition and glandular morphology, have changed independently, resulting in many kinds of toxins and diverse delivery systems (*12, 14, 23*). Rear-fanged—or more correctly defined, non-front-fanged—caenophidians possess complex venoms containing multiple toxin types, while the front-fanged venom system appeared three times independently: once early in caenophidian evolution with viperids, once within atractaspidines (a lamprophiid subfamily), and once with elapids. Further, a reduction of the venom system is observed in species in which constriction has been secondarily evolved as the preferred method of prey capture or dietary preference has switched from live prey to eggs or to slugs and snails (*12, 14, 23*).

Until now, the most comprehensive study to estimate divergence times among alethinophidian families used five nuclear genes (C-*mos*, *RAG1*, *BDNF*, *NT3*, *ODC*) and one mitochondrial gene (cyt *b*), and a Bayesian method (*19*). It showed that most interfamilial splits among alethinophidians occurred within the span of 25 million years in the early Cretaceous, 121–98 Ma, suggesting a radiation. Also, it suggested that dispersal and vicariant events associated with the fragmentation of the Gondwanan supercontinent have shaped the global distribution of alethinophidians. In that study (*19*), a scolecophidian was used as outgroup and the earliest snake divergences were therefore not dated. Also, one caenophidian exemplar (Acrochordidae) was used and interfamilial caenophidian splits were not dated.

Divergence times among all major groups of snakes are estimated here using nine nuclear protein-coding genes (C-*mos*, *RAG1*, *RAG2*, *R35*, *HOXA13*, *BDNF*, *JUN*, *AMEL*, and *NT3*). These were sequenced in 49 snake taxa representing all families with the exception of the Xenophidiidae, Anomochilidae, and Cylindrophiidae (Alethinophidia). Tissue samples were obtained from the tissue collections of N. V. and S. B. H. (see *13, 14, 16, 20, 24, 25* for details of the samples used). The taxa included Iguanidae: *Cyclura*, Helodermatidae: *Heloderma*, Anomalepididae: *Liotyphlops*, Typhlopidae: *Ramphotyphlops*, *Typhlops*, Leptotyphlopidae: *Leptotyphlops*, Aniliidae: *Anilius*, Tropidophiidae: *Tropidophis*, *Trachyboa*, Uropeltidae: *Rhinophis*, *Uropeltis*, Bolyeriidae: *Casarea*, Loxocemidae: *Loxocemus*, Xenopeltidae: *Xenopeltis*, Pythonidae: *Python*, *Liasis*, *Apodora*, Boidae: *Calabaria*, *Boa*, *Acrantophis*, *Candoia*, *Eryx*, *Gongylophis*, *Ungaliophis*, *Charina*, *Lichanura*, Acrochordidae: *Acrochordus*, Xenodermatidae: *Stoliczkaia*, Pareatidae: *Aplopeltura*, *Pareas*, Viperidae: *Bothriechis*, Homalopsidae: *Homalopsis*, Dipsadidae: *Leptodeira*, *Alsophis*, *Diadophis*, Colubridae: *Phyllorhynchus*, *Hapsidophrys*, *Calamaria*, *Grayia*, Pseudoxenodontidae: *Pseudoxenodon*, Natricidae: *Xenochrophis*, Elapidae: *Elapsoidea*, *Laticauda*, *Bungarus*, *Dendroaspis*, *Micrurus*, and Lamprophiidae: *Psammophylax*, *Leioheterodon*, *Lamprophis*, *Mehelya*, *Atractaspis*.

DNA extraction was performed using the DNeasy Tissue Kit (Qiagen). Amplification and sequencing was performed using sets of primers already described (*13, 19, 25*). The two strands obtained for each sequence were aligned using the BioEdit Sequence Alignment Editor program (*26*). The sequences produced for this work have been deposited in GenBank under Accession Numbers FJ433886-FJ434106. Sequence entry and alignment (51 taxa) were performed manually with the MUST2000 software (*27*). Amino acid properties were used, and ambiguous gaps deleted. This resulted in 561 bp for C-*mos*, 510 bp for *RAG1*, 708 bp for *RAG2*, 708 bp for *R35*, 408 bp for *HOXA13*, 669 bp for *BDNF*, 330 bp for the *JUN* gene, 378 bp for *AMEL*, and 519 bp for *NT3*. In all analyses, remaining gaps were treated as missing data.

Phylogenies were constructed using probabilistic approaches, with maximum likelihood (ML) and Bayesian methods of inference. ML analyses were performed with PAUP*4 (*28*). Bayesian analyses were performed with MrBayes 3.1 (*29, 30*). For ML methods, an appropriate model of sequence evolution was inferred

using ModelTest (*31*), for both separate and combined analyses. As we used only protein-coding nuclear genes, and because separate analyses showed no significant topological incongruence, we performed combined analyses, which are considered to be our best estimates of phylogeny. For the concatenated data set (4791 sites), the model selected was the TVM+I+G model. For the combined ML analysis, we used heuristic searches, with starting trees obtained by random addition with 100 replicates and nearest-neighbor interchange (NNI) branch swapping. For the bootstrap ML analysis, we performed 1000 replicates (NJ starting tree with NNI branch swapping). Bayesian combined analyses were run with model parameters estimated as part of the Bayesian analyses, with nine partitions corresponding to each gene (GTR model). 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.

The choice of calibration points is a crucial step in dating analyses, and we therefore present a brief overview of the snake fossil record. Geologic times and boundaries of periods used here are from a recent update (*32*). Three localities, or group of localities, may be putatively the oldest snake-bearing site(s): Emery, Utah (*Coniophis* sp.) (*33*), In Akhamil, Algeria (*Lapparentophis defrennei*) (*34*), and El Kohol, Algeria (an indeterminate lapparentophiid-grade snake and a Serpentes *incertae sedis*) (*35*). They apparently all fall in the Albian–Cenomanian interval (112–94 Ma) (*5*), but an older age (Aptian; 125–112 Ma) cannot be ruled out for In Akhamil (P. Taquet, personal communication). Rage and Richter (*36*) reported a putative snake from the Barremian (early Cretaceous; 130–125 Ma) of Spain, but it is quite likely a lizard (*5*). Noonan and Chippindale (*37*) regarded *Dinilysia* as the earliest representative of the "Booidea," but there is no consensus about its phylogenetic relationships.

The oldest scolecophidian is from the Paleocene of Hainin, Belgium (early Selandian; 62–59 Ma) (*38*). However, the fossil record of scolecophidians is poor, which likely results from their small size and fragility of their bones. The oldest alethinophidians are an "acrochordoid" (*Nubianophis afaahus*, Nigerophiidae), a russellophiid (*Krebsophis thobanus*), and a caenophidian *incertae sedis* from Wadi Abu Ashim, Sudan (*39*), a locality that is regarded as Cenomanian (100–94 Ma). None of the known fossil snakes can be reliably assigned to the Aniliidae (restricted here to *Anilius)*. The extinct *Coniophis* was referred to the Aniliidae, or to the Uropeltoidea, but its monophyly is doubtful and its phylogenetic position is unknown. No fossil may be confidently assigned to the following lineages: Tropidophiidae, Uropeltoidea, Xenopeltidae, Loxocemidae, and Xenophidiidae. Concerning Bolyeriidae, only a subfossil is known.

The earliest pythonid was reported from the late early Miocene of Europe (*40*). In Australia, *Morelia riversleighensis* is present at Riversleigh in levels that may be either late Oligocene or early or middle Miocene (*41*). Older pythons may also be present in a middle Eocene locality of Europe; but this cannot be confirmed (*42*). The earliest Boidae are from the mid-Paleocene (62–59 Ma) of Itaboraí, Brazil (*43*). These boids are represented by the earliest "boines" (including the extant genus *Corallus*) and the earliest ungaliophiine. The locality comprises several fissure fillings; therefore it is difficult to correlate the locality with international stratigraphic charts based on marine beds, but it may be regarded as late Selandian. The fossil genus *Helagras* has been regarded as an "erycine," but it cannot be assigned to a taxon within the "Booidea" (*40*). The earliest known member of the North American erycine clade (*Charina*/*Lichanura*) is *Charina prebottae* from Wyoming (Aquitanian, 23–20 Ma) (*44*).

The oldest caenophidian fossils are mentioned above under the heading Alethinophidia. They are an "acrochordoid" (*N. afaahus*, Nigerophiidae), a russellophiid (*K. thobanus*), and a caenophidian incertae sedis. They come from Wadi Abu Ashim (Cenomanian). The oldest acrochordid is *Acrochordus* recovered from southern Asia (Aquitanian, 23–20 Ma) (*45, 46*). No fossil may be assigned to the following families: Xenodermatidae, Pareatidae, Homalopsidae, Pseudoxenodontidae, and Lamprophiidae. The earliest Viperidae are from Germany (earliest Aquitanian, 23–20 Ma) (*47*). In its present understanding, no fossil may be assigned to the Family Colubridae with certainty. Various fossils were assigned to the genus *Coluber*, including fossils from the Oligocene. But the referral to *Coluber* is only symbolic because it is not possible to distinguish this genus from several other genera (that are perhaps not all Colubridae) on the basis of the available material (vertebrae). The oldest Dipsadidae would be *Paleoheterodon arcuatus* from Sansan, France, implying a dispersal from the New World (early Serravallian, 14–12 Ma) (*48*). The earliest natricid is *Natrix mlynarskii* from the early Oligocene (Rupelian, 34–28 Ma) of France (*49*). The oldest ascertained elapids come from Spain and France (late Burdigalian, 20–16 Ma) (*50*). However, in Australia, an elapid (close to the hydrophiine *Laticauda*) was recorded from RSO Site of Godthelp Hill, whose age may be either

latest Oligocene or more probably early Miocene (*51*). This snake may therefore be the earliest elapid, but this cannot be confirmed.

Bayesian timing analyses were conducted with Multidivtime T3 (*52, 53*). The assumed topology was from the ML analysis, with *Heloderma* used as outgroup. PAML 3.14 (*54*) was used to estimate model parameters. Multidivtime requires prior estimates for rttm, rttmsd, bigtime, rtrate, rtratesd, brownmean, and brownsd. We followed recommendations accompanying the software and adjusted the last four priors based on the rttm setting. The prior for the rttm (ingroup root) parameter, which is not a calibration point and does not have a major affect on posteriors, was set at 100 Ma (oldest fossil snake), 166 Ma (oldest anguimorph, ref. *55*), and 130 Ma (intermediate). The three rttm resulted in less than 1% difference in time estimates, so the intermediate rttm was used in the primary ("best") analysis. The prior rttmsd was set at one-half of rttm based on recommendations accompanying the software. Analyses were performed treating the nine-gene data set as one partition and as nine partitions. The average deviation between the unpartitioned and the partitioned analyses is −0.06 Ma, and the partitioned analysis was chosen as our primary analysis. The prior bigtime (a value larger than an expected posterior), which is not a calibration point and has little affect on posteriors, was set at 200 Ma (Triassic–Jurassic boundary). Analyses were run for 1,100,000 generations, with a sample frequency of 100 after a burnin of 100,000 generations.

The fossil calibration points used here as minimum dates are the oldest elapid (20.4 Ma), the oldest natricid (28.4 Ma), the oldest *Charina* (20.4 Ma), the oldest ungaliophiine (58.7 Ma), the oldest pythonid (20.4 Ma), and the oldest caenophidian (93.5 Ma). As the use of the latter calibration has been discussed by Sanders and Lee (*56*), we performed analyses with and without it. The oldest anguimorph (166 Ma) was used as a maximum date for the snake node. We performed analyses using one additional geological calibration point. Because there is no evidence for continuous emergent land in the Antilles before the late Eocene (*57*), we assigned this date (37.2 Ma) as a maximum constraint for the split between *Trachyboa* and *Tropidophis* (maximum divergence times among species of West Indian *Tropidophis* are similar to the divergence time of *Tropidophis* and *Trachyboa*; S. B. H., unpublished data).

To examine the effect of the geologic calibration, we performed analyses with and without it. In all analyses, the posterior times obtained for 18 out of the 21 nodes discussed here fell within the credibility intervals derived from the primary partitioned analysis using all eight original calibrations. The three exceptions are Pythonidae vs. Loxocemidae node (extreme value: 62.2 Ma instead of 43.7 Ma in the primary analysis), the Xenopeltidae vs. Pythonidae/Loxocemidae node (extreme value: 85.9 Ma instead of 70.1 Ma), and the Boidae vs. Xenopeltidae/Loxocemidae/Pythonidae node (extreme value: 97.5 Ma instead of 86.3 Ma). In any case, these differences do not alter the following results and discussion that are based on the analysis performed with all eight calibration points and rttm set at 130 Ma (Fig. 2). As noted, until now, the only study having estimated divergence times among snake families using several nuclear genes is by Noonan and Chippindale (*19*). Other studies have used one or two nuclear genes or mitochondrial genes (*56, 58–62*), and those reported time estimates are presented in Table 1, for comparison.

Our ML and Bayesian topologies are virtually identical, differing only in the position of Bolyeriidae and Uropeltidae (in the Bayesian tree, Bolyeriidae and Uropeltidae cluster together and form the sister group to Caenophidia). Whatever the method used, these positions are not supported statistically, and we consider them to be unresolved. Similarly, the paraphyly of Scolecophidia is weakly supported (ML BP: 53%, Bayesian PP: 56%), and we conservatively follow the strong morphological evidence available and consider scolecophidians to be monophyletic (*63*). The remaining interfamilial relationships confirm previously obtained results (*20, 21*).

The timetree of snakes supports a Gondwanan origin for the group, based on the distribution of the basal lineages (Scolecophidia, Aniliidae, Tropidophiidae, Boidae, Bolyeriidae, and Uropeltoidea, whether the last two lineages are basal to henophidians or to caenophidians). According to the same data, snakes most probably evolved on West Gondwana (South America and Africa), which drifted from East Gondwana from 166 to 116 Ma (*64*). The earliest divergences among living lineages occurred in the late Jurassic between 152 (163–137) Ma and 156 (164–144) Ma. Among toxicoferans, the relative positions of snakes, anguimorphs, and iguanians are still unresolved, but if the traditional clustering of snakes with anguimorphs (that are of Laurasian origin) is confirmed, it would mean that the Jurassic split (166 Ma) may correspond to the breakup of Pangaea (*25*).

Another major result is the split between the group formed by Aniliidae and Tropidopiidae and all remaining Alethinophidia that is estimated here as 106

(97–116) Ma. This date corresponds to the opening of the Atlantic Ocean, and supports the inference that this deep alethinophidian split is a vicariant event (*20*). Furthermore, this result is in agreement with the fossil record, because the oldest known diverse snake fauna is from Africa (Sudan) (*39*).

Among Henophidia (Afrophidia to the exclusion of Caenophidia), all interfamilial splits except one (the divergence between Pythonidae and Loxocemidae) took place in the Cretaceous between 104 (95–114) Ma and 70 (59–81) Ma. Those dates are similar to those of Noonan and Chippindale (112–98 Ma) (*19*) and suggest that most interfamilial splits among non-caenophidian alethinophidians (Amerophidia and Henophidia) occurred in the middle to late Cretaceous. Among Caenophidia, all interfamilial splits except the two most basal ones occurred during the Paleogene between 63 (52–77) Ma and 33 (25–43) Ma. In contrast, three recent molecular clock analyses using one or two genes obtained much younger time estimates for most divergences (*56, 60, 62*). However, the two studies using several nuclear genes, ours and the one of Noonan and Chippindale (*19*) have similar, older estimates that are probably more reliable.

Geological and paleobiogeographical data show that the isolation of Africa was broken intermittently during the Cretaceous by contact with Laurasia. Therefore, the initial radiation and dispersal of Afrophidia can be explained by dispersal out of Africa through Laurasia or India or both (*64, 65*). In turn, the early biogeographic history of Caenophidia is firmly rooted in Asia based on the successive branching, in a ladder-like fashion (basal to derived) of these Asian or mostly Asian families: acrochordids, xenodermatids, pareatids, viperids (partly Asian), and homalopsids (*21*). Among Henophidia, the relationships between Bolyeriidae, Uropeltoidea, Boidae, and the Xenopeltidae/Loxocemidae/Pythonidae clade are still not well resolved. Thus, their biogeography is more difficult to interpret and probably involves both dispersal and vicariant events (*19*).

Acknowledgments

Assistance with taxonomic issues was provided by P. David and with paleontological issues by L. P. Bergqvist. DNA samples from *Pseudoxenodon bambusicola* and *Calamaria pavimentata* were from R. Lawson. Support was provided by the Service de Systématique moléculaire du Muséum National d'Histoire Naturelle to N.V., by the U.S. National Science Foundation and National Aeronautics and Space Administration (NASA Astrobiology Institute) to S.B.H., and by the Consortium National de Recherche en Génomique, Genoscope.

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