Higher-Level Snake Phylogeny Inferred from Mitochondrial DNA Sequences of 12S rRNA and 16S rRNA Genes

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Portions of two mitochondrial genes (12S and 16S ribosomal RNA) were sequenced to determine the phylogenetic relationships among the major clades of snakes. Thirty-six species, representing nearly all extant families, were examined and compared with sequences of a tuatara and three families of lizards. Snakes were found to constitute a monophyletic group (confidence probability [CP] = 96%), with the scolecophidians (blind snakes) as the most basal lineages (CP = 99%). This finding supports the hypothesis that snakes underwent a subterranean period early in their evolution. Caenophidians (advanced snakes), excluding *Acrochordus,* were found to be monophyletic (CP = 99%). Among the caenophidians, viperids were monophyletic (CP = 98%) and formed the sister group to the clapids plus colubrids (CP = 94%). Within the viperids, two monophyletic groups were identified: true vipers (CP = 98%) and pit vipers plus *Azemiops* (CP = 99%). The elapids plus *Atractaspis* formed a monophyletic clade (CP = 99%). Within the paraphyletic Colubridae, the largely Holarctic Colubrinae was found to be a monophyletic assemblage (CP = 98%), and the Xenodontinae was found to be polyphyletic (CP = 91%). Monophyly of the henophidians (primitive snakes) was neither supported nor rejected because of the weak resolution of relationships among those taxa, except for the clustering of *Calabaria* with a uropeltid, *Rhinophis* (CP = 94%).

Introduction

Snakes arose from lizards approximately 125 million years ago (Rage 1984; Carroll 1988, p. 218). The more than 2,500 extant species currently recognized occupy nearly every habitat on each continent (except Antarctica) as well as many oceanic islands. Despite this ecological diversity and a long evolutionary history, snakes are remarkably conservative morphologically. This paucity of informative morphological characters has, in part, hindered resolution of the higher-level relationships among snakes. Although several molecular studies have addressed this problem, there is still no consensus regarding relationships among snake families.

Snakes traditionally are divided into three major lineages: the Scolecophidia (blind snakes), the Henophidia ("primitive" snakes), and the Caenophidia ("advanced" snakes) (Cope 1864; Nopsca 1923; Hoffstetter 1955). Scolecophidians comprise three families (Anomalepididae, Leptotyphlopidae, and Typhlophidae) and are characterized by slender, cylindrical bodies with blunt heads, smooth shiny scales, reduced eyes and pigment, and fossorial lifestyles. The Scolecophidia generally is considered to be the most basal group of snakes, although there is disagreement as to whether it is monophyletic (Underwood 1967; Groombridge 1979*b;* Rieppel 1988*b*).

Henophidians consist of a large number of taxa exhibiting a wide range of morphologies. Included in this group are the boas and pythons as well as many less familiar forms, such as the sunbeam and shield-tailed snakes. Numerous classification schemes for these taxa have been proposed (Underwood 1967; Smith et al. 1977; Dowling and Duellman 1978; McDowell 1987). As with the scolecophidians, disagreement exists as to whether this group is monophyletic (Underwood 1967; Groombridge 1979*c;* Dessauer et al. 1987).

The majority of snakes are caenophidians. These "advanced" snakes include many harmless forms as well as all known venomous species. Caenophidians include three major groups: (1) the colubrids, typical harmless species (e.g., rat snakes and racers), (2) the elapids, front-fanged species with neurotoxic venom (e.g., cobras and coral snakes), and (3) the viperids, species with movable front fangs and hemotoxic venom (e.g., vipers and rattlesnakes). Caenophidians are assumed to be monophyletic (Underwood 1967; Dessauer et al. 1987; Cadle 1988). However, the taxa included in this group as well as their relationships have been debated

Key words: Reptilia, serpentes, phylogeny, 12S and 16S ribosomal RNA.

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Mol. Biol. Evol. 12(2):259–265. 1995.

0737-4038/95/1202-0008$02.00

(Groombridge 1979*a*, 1984; McDowell 1979, 1987; Cadle 1988).

Dessauer et al. (1987) reviewed the published molecular evidence for snake relationships and presented some additional data. For higher-level relationships, the immunological techniques have provided the most information, although the quantitative method of microcomplement fixation (primarily of serum albumin) reaches its upper limit of usefulness with such ancient divergences. Qualitative information from enriched Ouchterlony double-diffusion tests, however, has offered some insights. These data have supported the following conclusions: (1) snakes are monophyletic, made up of two very ancient lineages (scolecophidians, and henophidians plus caenophidians); (2) while the scolecophidians and caenophidians each are monophyletic, the henophidians very likely are not; (3) pythons and boids are not each others' closest relatives; (4) viperids are the sister group to the elapids and colubrids; and (5) sea snakes are members of the elapid lineage. Some of the unresolved questions from this analysis included the possible monophyly of the colubrids and the phylogenetic positions of *Acrochordus* and *Atractaspis*.

Cadle (1988) examined the phylogenetic relationships of advanced snakes, using micro-complement fixation. Four major clades were recognized: viperids, elapids, colubrids, and *Atractaspis*. Xenodontine colubrids were shown to comprise at least two lineages as divergent from one another as are other major colubrid lineages. The front-fanged delivery systems present in viperids and elapids were interpreted as the result of convergent evolution. As in Dessauer et al. (1987), the Viperidae was identified as the sister group to the other advanced snakes, and the monophyly of the colubrids could not be resolved.

No previous amino acid or DNA sequence studies have included a sufficient diversity of taxa to reach significant conclusions regarding the higher-level phylogeny of snakes. Knight and Mindell (1994) sequenced portions of the mitochondrial 12S and 16S ribosomal RNA genes to address the relationships of the Colubrinae, Elapidae, and Viperidae; however, only a single species from each taxon was sampled. Other published studies utilizing DNA sequence data have focused on intrafamilial questions (Knight and Mindell 1993; Knight et al. 1993).

To determine the phylogenetic relationships among the major groups of living snakes, we sequenced portions of the mitochondrial (mt) 12S and 16S ribosomal RNA (rRNA) genes from 36 species of snakes representing 13 of the 16 families (sensu McDowell 1987). The following questions in snake phylogenetics were addressed: Which lineage or lineages are the most basal? Are the scolecophidians, henophidians, and caenophidians monophyletic groups? Which of the three major caenophidian groups is the sister taxon to the other two? What are th phylogenetic positions of several enigmatic taxa such a *Acrochordus, Atractaspis,* and *Azemiops?*

Material and Methods

Tissue samples (liver, blood, or tissue homogenate were obtained for the following species (laboratory at breviations: HGD, Herndon G. Dowling; LM, Linda F Maxson; RH, Richard Highton; SBH, S. Blair Hedges *Acrochordus javanicus,* RH 52795 ("Thailand"); *Agki trodon contortrix,* RH 54411 (North Carolina, Unio Co.); *Atractaspis corpulenta,* RH 65710 ("Africa"); *Bit arietans,* RH 58157 ("Africa"); *Boa constrictor,* RI 55193 ("South America"); *Boiga cynodon,* RH 5724 ("Thailand"); *Bungarus fasciatus,* RH 63881 ("Soutl east Asia"); *Calabaria reinhardtii,* HGD 145803 ("We Africa"); *Chironius carinatus,* RH 68227 ("Sout America"); *Crotalus horridus,* RH 60210 (New Jerse Burlington Co.); *Dipsas catesbyi* (12S sequence), SBI 171139 (Peru: Pasco; 1.5 km NW Cacazu); *Dipsas ca tesbyi* (16S sequence), LM 1968 (Peru: Madre de Dio Cuzco Amazónico); *Elaphe obsoleta,* RH 60213 (Mary land, Montgomery Co.); *Enhydris enhydris,* RH 6503 ("Thailand"); *Farancia abacura,* RH 53660 (Georgi Liberty Co.); *Gonyosoma oxycephalum,* RH 5634 ("Thailand"); *Lamprophis fuliginosus,* RH 62688 ("A rica"); *Leptotyphlops columbi,* SBH 192936 (Bahama San Salvador; Little Fortune Hill); *Liotyphlops albiro tris,* SBH 172151 ("Venezuela"); *Loxocemus bicolo* HGD 145976 ("Mexico"); *Lycodon laoensis,* RH 6503 ("Thailand"); *Micruroides euryxanthus,* RH 5253 ("Arizona"); *Micrurus diastema,* RH 52446 (Mexic Quintana Roo; Coba); *Naja naja,* RH 58101 ("Southea Asia"); *Nerodia rhombifera,* HGD 76973 (South Car lina, Jasper Co.); *Ophiophagus hannah,* RH 6081 ("Southeast Asia"); *Psammophis condenarus,* RH 5601 ("Thailand"); *Python reticulatus,* RH 57242 ("Tha land"); *Rhamphiophis oxyrhynchus,* RH 52866 ("A rica"); *Rhinophis drummondhayi,* SBH 194102 (S Lanka: Pindarawatta; north of Namunukula); *Trime esurus tokarensis,* RH 63874 ("Ryukyu Islands"); *Tr pidophis wrighti,* SBH 191157 (Cuba: Guantánamo; km N La Munición); *Typhlops lumbricalis,* SBH 19101 (Cuba: Guantánamo; La Fangosa); and *Xenodon severu* RH 68185 ("South America").

DNA extraction, amplification, and sequencin followed protocols previously described (Hedges et a 1991; Hedges and Bezy 1993). Two regions of the m 12S and 16S rRNA genes were sequenced, correspondin to sites 1092–1477 and 2607–3055, respectively, of th complete human mitochondrial genome (Anderson e al. 1981). The primers used (12L1, 12H1, 16L1, 16H1 are described elsewhere (Kocher et al. 1989; Hedge 1994). Sequences were read from autoradiographs an aligned by eye using the multisequence editing progra

ESEE (Cabot and Beckenbach 1989). Data for *Azemiops feae* (accession numbers L01763–L01764), *Coluber constrictor* (L01765, L01770), *Sceloporus undulatus* (L28075), *Sphenodon punctatus* (L28076), and *Vipera ammodytes* (L01768–L01769) were obtained from GenBank. Shorter sequences were available for representatives of two additional lizard families, the Teiidae (*Ameiva auberi*) and Xantusiidae (*Cricosaura typica*) (Hedges and Bezy 1993).

The *Sphenodon* sequence was used as a reference for alignment. Aligned sequences were analyzed using MEGA (Kumar et al. 1994). Phylogenetic trees were reconstructed with the neighbor-joining (NJ) algorithm (Saitou and Nei 1987) and by maximum parsimony. A lizard, *Sceloporus undulatus* (Iguanidae), was included, and the trees were rooted with a tuatara, *Sphenodon punctatus*.

Neighbor-joining analyses were performed with the following distance measures: Jukes-Cantor (Jukes and Cantor 1969), Kimura two-parameter correction for transition/transversion bias (Kimura 1980), and Tamura three-parameter correction for transition/transversion bias and base composition bias (Tamura 1992). Sites with missing data or gaps were not included in the NJ analyses. Confidence levels of the NJ trees were assessed by calculating the confidence probability (CP) of each branch length (Kumar et al. 1994). The underlying mathematical basis of the CP value (Rzhetsky and Nei 1992, 1993) is better understood than that of the bootstrap *P* value (Felsenstein 1985; Zharkikh and Li 1992*a*, 1992*b*). Furthermore, recent computer simulations suggest that CP values are better estimators of statistical reliability of branches than are bootstrap *P* values (Sitnikova et al., 1995).

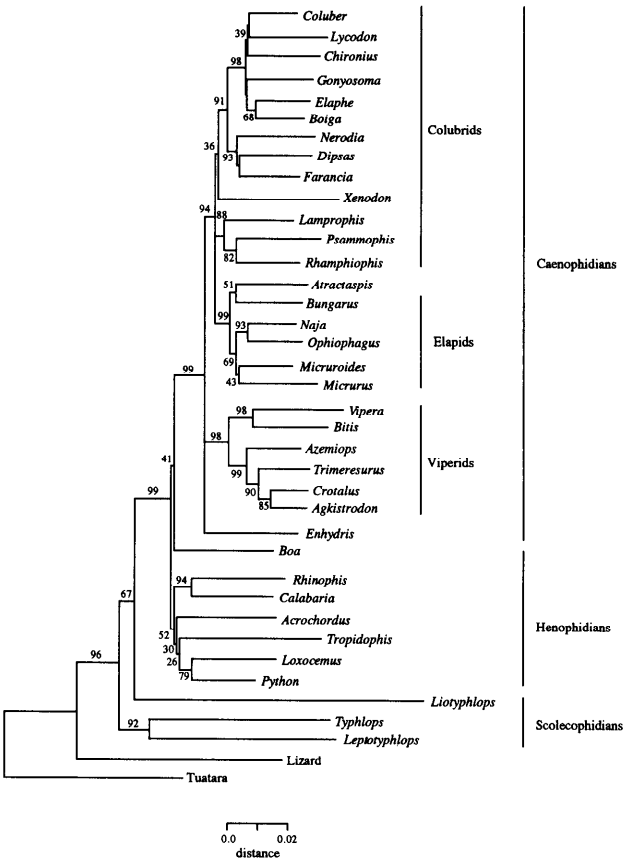

FIG. 1.—Phylogenetic tree of snakes based on mitochondrial 12S and 16S rRNA sequence data (818 aligned sites). An iguanid lizard (*Sceloporus undulatus*) was included, and the tree was rooted with a tuatara (*Sphenodon punctatus*). The tree was constructed using neighbor-joining with the Jukes-Cantor distance (scale bar). Confidence probability values are given for each branch.

Results and Discussion

For the 12S rRNA fragment, there were 416 aligned sites, 287 of which were variable (214 informative under the conditions of parsimony). For the 16S rRNA fragment, there were 473 aligned sites, of which 242 were variable (175 informative under the conditions of parsimony). For analysis, the sequence data were combined. Two sections, corresponding to sites 1–36 and 658–692 of the tuatara sequence, were unalignable and not included in the analysis, resulting in 818 aligned sites, of which 461 were variable (327 parsimony sites).

Tree reconstruction with neighbor-joining produced nearly identical topologies regardless of the distance measure utilized (fig. 1). Maximum-parsimony analysis produced a tree (not shown) with the same branching order for the major groups of snakes (scolecophidians (henophidians (viperids (elapids, colubrids)))). Trees constructed with one or all of the available lizard sequences resulted in snake monophyly, with the same branching order (scolecophidians (henophidians, caenophidians)).

Scolecophidia

Scolecophidians are identified as the most basal lineages of living snakes (fig. 1), and there is strong statistical support for this placement (CP = 99%). Bellairs and Underwood (1951) suggested, on the basis of morphological data, that the scolecophidians represent two lineages which arose at the base of the ophidian phylogenetic tree (i.e., the scolecophidians are paraphyletic). Similar conclusions were reached by List (1966), examining osteology, and by Langebartel (1968), who characterized the hyoid and its associated musculature. However, numerous authors have disagreed, proposing that the scolecophidians are descended from a single ancestor (Underwood 1967; Rieppel 1979; Smith and MacKay 1990).

Our tree suggests that these snakes do not constitute a monophyletic clade, although statistical support for

this hypothesis is not strong (CP = 67%). The relationships among the scolecophidians, as indicated by sequence data, are that the typhlopids and leptotyphlopids are more closely related to each other (CP = 92%) than either is to the anomalepids (represented here by *Liotyphlops*). This pattern is concordant with some morphological data (Langebartel 1968; McDowell 1974, 1987; Groombridge 1979*b*), but a number of osteological features suggest that the leptotyphlopids are the sister taxon to a typhlopid-anomalepid clade (List 1966; Underwood 1967; Rieppel 1988*b*; Zug 1993, p. 465). Previously, some morphological data (McDowell and Bogert 1954; Robb 1960) had been interpreted to suggest that *Typhlops* should not be classified as a snake at all but considered a lineage distinct from both lizards and snakes. This hypothesis is not widely accepted, and our tree offers strong support (CP = 96%) that typhlopids indeed are snakes.

Henophidia

Of the remaining snake lineages, two groups are evident: the henophidians and the caenophidians. Relationships among the henophidians are not well resolved. Different classifications have divided this group into as few as four families (Langebartel 1968; Dowling and Duellman 1978, p. 100.1; Rage 1984) or as many as nine (McDowell 1987). Aniliids and uropeltids often are assumed to be sister taxa, forming the most basal clade of henophidians (Underwood 1967; Rage 1984; Rieppel 1988*a*). Our data do not support the monophyly of this group, although the separation of *Boa* from other henophidians is weakly supported (CP = 41%), and the remaining henophidians cluster in a single group (CP = 52%). *Rhinophis*, a uropeltid, has affinities with the henophidians (CP = 99%) rather than the scolecophidians as previously suggested (Dowling and Duellman 1978, p. 100.1).

Much interest has centered on the classification of booid snakes (boas, pythonids, tropidophiids, and bolyeriids). The monophyly and status of the members of this group have been discussed extensively. While some authors believe that these snakes are similar enough to be assigned subfamilial status within the single family Boidae (Underwood 1967; Rage 1984), others separate them into two or more lineages, often considered distinct families (Underwood 1976; Dowling and Duellman 1978; Dessauer et al. 1987; McDowell 1987). Our data suggest that these snakes are not each others' closest relatives. *Boa* is placed outside of a group containing the other "boid" taxa (*Calabaria, Tropidophis,* and *Python*). *Loxocemus* appears more closely related to *Python* (CP = 79%) than does *Tropidophis*. This relationship has been suggested by morphological data (Underwood 1976; Groombridge 1979*c*; Underwood and Stimson 1990). *Calabaria* is shown to be more closely related to the uropeltid *Rhinophis* (CP = 94%) than to either *Python* or *Boa*, in contrast to Kluge's (1993) classification. While not conclusive, our data do suggest that the Boidae (sensu Underwood 1967) should be reevaluated from both the molecular and morphological perspectives with additional taxonomic sampling.

The three families we could not sample in this study (Aniliidae, Bolyeriidae, and Xenopeltidae) are commonly considered henophidians. The bolyeriids (with their unique intramaxillary joint), have been associated with the "higher henophidians" (i.e., booids and tropidophiids of Cundall and Irish 1989). Aniliids may be most closely related to uropeltids, with xenopeltids as their sister group (Underwood 1967; Groombridge 1979*c*; Kluge 1993; but see also McDowell 1987; Underwood and Stimson 1990).

The phylogenetic position of *Acrochordus* remains unclear. This taxon has been considered a henophidian (Underwood 1967; Hardaway and Williams 1976; Smith et al. 1977; McDowell 1987), a caenophidian (Dowling and Duellman 1978; Dowling et al. 1983; Groombridge 1984), or the sister taxon to both henophidians and caenophidians (McDowell 1975, 1979). Our data exclude *Acrochordus* from the caenophidians (CP = 99%) and place it within the henophidian clade. However, little resolution is offered as to its relations with these taxa. It is possible that *Acrochordus* is basal to the caenophidians and should not be classified as a henophidian. Further investigation of this enigmatic taxon is warranted.

Caenophidia

The advanced snakes are well defined, and their monophyly is strongly supported (CP = 99%). Within this clade, three main groups are apparent: the viperids, the elapids, and the colubrids. Both the viperids and the elapids are shown to be monophyletic groups (CP = 98% and 99%, respectively). The branching order within the caenophidians is resolved, with the viperids separating prior to an elapid-colubrid divergence (CP = 94%). Within the viperid clade, true vipers form the sister group to the pit vipers and *Azemiops*. Little agreement exists for the phylogenetic placement of *Azemiops*. It has been classified as either the most primitive viperid (Liem et al. 1971; Dowling 1975; Underwood 1979), a true viper (Underwood 1967; Smith et al. 1977), or placed as the sister taxon to the pit vipers (Cadle 1992; Knight and Mindell 1993). In our analysis (fig. 1), *Azemiops* clusters strongly with the pit vipers (CP = 99%).

Disagreement has existed as to whether the elapids form a monophyletic group. While some authors assert that the elapids are monophyletic (Cadle and Sarich 1981; McCarthy 1985), others accept the proposal that New World coral snakes are more closely related to xenodontine colubrids than to other elapids (Duellman 1979; Laurent 1979). Our data support the monophyly

of the elapids (CP = 99%) and include *Atractaspis* in this clade. This taxon has been classified as a viperid (Underwood 1967), a colubrid (Hardaway and Williams 1976; Smith et al. 1977; Dowling and Duellman 1978), or an independent lineage allied with the elapids (McDowell 1986, 1987; Dessauer et al. 1987; Cadle 1988). Sea snakes were not included in this study, but on the basis of molecular and morphological data, they are most closely related to Australasian elapids (Mao et al. 1983; McCarthy 1986; Dessauer et al. 1987).

Our analysis did not support the monophyly of the largest family, the Colubridae. The majority of the species examined formed two groups that clustered with the elapids. The position of *Enhydris* (a homalopsine) was not resolved, being placed in a trichotomy with the viperids and the clade formed by the elapid and remaining colubrid taxa. A basal position for homalopsines has been proposed based on morphological data (McDowell 1986). Three Old World genera (*Lamprophis, Psammophis,* and *Rhamphiophis*) were grouped together (CP = 88%) and placed in a trichotomy with the elapids and remaining colubrid taxa. A monophyletic group comprised of representatives of the Colubrinae was strongly supported (CP = 98%), which largely agrees with previous molecular studies (Dowling et al. 1983; Cadle 1984*b;* Dessauer et al. 1987). Another monophyletic group consisted of two xenodontines (*Dipsas* and *Farancia*) and a natricine (*Nerodia*) (CP = 93%). A third xenodontine, *Xenodon,* was placed outside of this clade (CP = 91%). These results agree with immunological data which suggested that xenodontines comprise at least two lineages as distinct from each other as from other colubrid lineages (Cadle 1984*a,* 1984*b*).

Venom and venom delivery systems in snakes often have been investigated in attempts to discern phylogenetic patterns (Minton 1986; Kochva 1987; Minton and Weinstein 1987). All elapids and viperids possess venom, and many colubrids also are venomous. The manner in which venom is injected differs in the three groups: viperids have fangs located on short, rotating maxillae in the front of the mouth; elapids have fixed fangs, often followed by several teeth, on elongate maxillae; and venomous colubrids have fixed fangs at the posterior end of elongate maxillae which usually are preceded by several teeth. Our findings suggest that viperids diverged prior to the separation of elapids and colubrids (CP = 94%). To account for the present phylogenetic distribution of front-fanged venom delivery systems, one can propose that such a system evolved early in the evolutionary history of the advanced snakes and later was lost in the colubrid lineage (Underwood and Kochva 1993). An alternative explanation is that viperids and elapids independently evolved front-fanged systems (McDowell 1986; Cadle 1988; Knight and Mindell 1994). While sequence data cannot reject either of these hypotheses, they do reject the hypothesis that the colubrids represent the basal lineage among the advanced snakes (Bogert 1943; Johnson 1956; Kardong 1980; Minton 1986) and that venom delivery systems utilizing front fangs are a shared, derived characteristic of elapids and viperids.

It has been hypothesized that the early snakes were fossorial (Mahendra 1938; Walls 1940; Underwood 1967). Present-day snakes have a suite of characters indicative of this behavior: loss of limbs, eyelids, and external ear openings, and eyes which appear to have undergone severe reduction and have been largely reevolved (Walls 1940). Our results are concordant with this early-fossorial hypothesis. Scolecophidians comprise the basal lineages of the snake phylogenetic tree and are characterized by reduced eyes and a burrowing lifestyle. One can propose a scenario in which early snakes, living underground, underwent morphological modification prior to a lineage (or lineages) reemerging to live on the surface. Subsequent radiation aboveground would have given rise to the henophidians and caenophidians.

Sequence Availability

The nucleotide sequence data (accession numbers Z46433, Z46443-Z46502, Z46524-Z46525, Z46597, Z46738-Z46739) and alignment (accession number DS19842) reported here have been deposited in the EMBL Nucleotide Sequence Database.

Acknowledgments

We thank C. Gans, R. Highton, R. Thomas, and D. Reynolds for providing some of the tissue samples. This research was supported by grants from the U.S. National Science Foundation (BSR 8507847 to Richard Highton, BSR 8906325 and BSR 9123556 to S.B.H., and BSR 8918926 to L.R.M. and S.B.H.).

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Naoyuki Takahata, reviewing editor

Received September 22, 1994

Accepted October 25, 1994