# Phylogenetic Relationships of Amphibian Families Inferred from DNA Sequences of Mitochondrial 12S and 16S Ribosomal RNA Genes

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Nucleotide sequence comparisons were used to investigate ordinal and familial relationships within the class Amphibia. Approximately 850 base pairs of the mitochondrial 16S ribosomal RNA (rRNA) gene from representatives of 28 of the 40 families of extant amphibians were sequenced. Phylogenetic analyses of these data together with published data of the 12S rRNA gene for the same families and both genes for three more taxa (approximately 1,300 base pairs total for 35 taxa) support the monophyly of each of the three amphibian orders: Anura (confidence value with the interior-branch test: $P_c = 99\%$), Caudata ($P_c = 100\%$), and Gymnophiona ($P_c = 99\%$). An analysis using the four-cluster method cannot discriminate significantly between all three possible unrooted trees involving the three orders of amphibians and an outgroup. Within the Anura, there is support for the monophyly of the two suborders: Neobatrachia ($P_c = 100\%$) and Archaeobatrachia ($P_c = 97\%$); the latter was believed to be paraphyletic on the basis of morphology. Within the Archaeobatrachia, the following pairs of taxa cluster: Pelobatidae + Pelodytidae ($P_c = 99\%$), Pipidae + Rhinophrynidae ($P_c = 99\%$), *Ascaphus* + Leiopelmatidae ($P_c = 89\%$), and *Bombina* + Discoglossidae ($P_c = 99\%$). The latter six taxa cluster ($P_c = 94\%$) such that Pelobatidae + Pelodytidae forms a basal lineage within the Archaeobatrachia. Three major lineages are distinguished within the Neobatrachia: the superfamily Bufonoidea sensu Duellman ($P_c = 86\%$), the superfamily Ranoidea sensu Lynch ($P_c = 99\%$), and the Sooglossidae. Basal within the Bufonoidea, Myobatrachidae + Heleophrynidae cluster at $P_c = 96\%$. The enigmatic Dendrobatidae clusters with the bufonoid families ($P_c = 92\%$) and is excluded from the ranoid families ($P_c = 99\%$). The Microhylidae, considered by some to form a separate superfamily, clusters within the Ranoidea ($P_c = 99\%$). Within the Caudata, familial relationships are not resolved at significant confidence levels. We suggest that short divergence times among amphibian orders and among salamander families have contributed to the difficulty in fully resolving these relationships.

## Introduction

The living amphibians (Lissamphibia) have had a long evolutionary history dating back at least to the Triassic and include a myriad of forms found in all but the most inhospitable parts of the Earth (4,500 living species; Duellman 1993, p. 13). Despite long recognition of this diversity, many aspects of amphibian phylogeny remain poorly known, including the relationships of the three amphibian orders, Anura (frogs), Caudata (salamanders), and Gymnophiona (caecilians), as well as relationships among the families within each order. There are few characters that unequivocally distinguish the 40 extant amphibian families or that indicate relationships among them. Thus, extensive and intensive examinations of the morphology of both living and fossil amphibians still leave significant areas of disagreement (Lynch 1973; Duellman and Trueb 1986; Milner 1988; Trueb and Cloutier 1991; Ford and Cannatella 1993). Further conflicting phylogenies have been proposed based on cytological, developmental, and molecular data, or combinations thereof (Morescalchi 1973; Hillis 1991; Larson 1991; Hedges and Maxson 1993; Larson and Dimmick 1993).

Numerous studies in the past decade have compared nucleotide sequences to evaluate problematic phylogenies because sequence data generally are not affected by the complications of adaptive convergence, as are morphological characters (Hedges and Sibley 1994). Among tetrapods, molecular studies of nuclear and mitochondrial ribosomal genes demonstrated that the living amphibians form a monophyletic group (Hedges et al. 1990). Most morphological studies have not questioned that each order—Anura, Caudata, and Gymnophiona—is monophyletic, although such monophyly had been

neither confirmed nor refuted by molecular data. Relationships among the three orders are less apparent. Morphological analyses of living and fossil taxa generally group salamanders and frogs, whereas molecular analyses have associated salamanders with caecilians (Larson and Wilson 1989; Hedges et al. 1990; Larson 1991).

Hedges and Maxson (1993) demonstrated that a region of the mitochondrial 12S ribosomal RNA (rRNA) gene is useful for elucidating aspects of amphibian phylogeny. Although 32 families were sampled in that study, only 333 aligned nucleotide sites were examined. For phylogenetic analyses involving such a large number of lineages, it is important to increase the size of the data set to provide better statistical resolution of phylogenetic relationships. Accordingly, we now expand that earlier work (Hedges and Maxson 1993), analyzing those 12S rRNA sequences together with approximately 850 nucleotides of the mitochondrial 16S rRNA gene. Also, sequences of three anuran taxa (*Bombina*, Mantellidae, and Pseudidae) were added for both gene regions.

## Material and Methods
### Specimens Examined

Total DNA was extracted from fresh or frozen tissue samples (blood, liver, or muscle) representing 19 of 25 frog families, and 9 of 10 salamander families. Frozen samples were kept at −80°C prior to extraction. The taxa used are listed in the Appendix; family recognition follows Duellman (1993). Two subfamilies of caecilians (Caeciliainae and Typhlonectinae; families in Duellman [1993]) were included because they were part of the 12S rRNA analysis (Hedges and Maxson 1993) and were only recently synonymized into the Caeciliaidae (Hedges et al. 1993). Representatives of *Bombina* (Discoglossidae) and *Ascaphus* (Leiopelmatidae) were included in this analysis because recent studies have placed these genera in their own families (Bombinatoridae: Ford and Cannatella 1993; Ascaphidae: Green et al. 1989; Green and Cannatella 1993). For most taxa, sequences of both 12S and 16S rRNA genes were obtained from the same individual; exceptions are noted in the Appendix.

### DNA Amplification and Sequencing

Portions of the 16S rRNA gene were amplified using the polymerase chain reaction (PCR) and sequenced using previously described procedures (Hedges et al. 1991) with the following modifications: (1) the number of cycles of PCR required to generate a sufficient amount of template in double and single-stranded amplifications ranged from 25 to 45, (2) 30,000 molecular weight filters (Millipore) were used to purify the DNA template for sequencing, and (3) sequencing reactions were done using Taq (*Thermus aquaticus*) DNA polymerase.

Combinations of primers 16L1, 16L2, 16L2a, 16H1, 16H2, 16H3, 16H10 (Hedges 1994), and 16L10 (5′-AGT GGG CCT AAA AGC AGC CA-3′) were used to amplify and sequence approximately 850 base pairs (bp) of the 16S rRNA gene corresponding to sites 2205–3055 in the human sequence (Anderson et al. 1981). Complementary strands were sequenced in all taxa except for *Amphiuma tridactylum* and *Rhyacotriton olympicus* where a part of the 16S rRNA gene is represented only by the sequence from the light strand as repeated attempts to amplify the heavy strand were unsuccessful. For the additional 12S rRNA sequences of *Bombina orientalis, Pseudis paradoxa,* and *Mantella aurantiaca,* we used the same primers as Hedges and Maxson (1993) to amplify about 380 bp of that gene.

### Sequence Analysis

Sequences were read from autoradiograms using the digitizing program GELIN (S. W. Schaeffer, Pennsylvania State University). The following published sequences were added to the new 12S and 16S rRNA data: the 12S rRNA sequence of all taxa in the study of Hedges and Maxson (1993) the 12S and 16S rRNA sequences of the four caecilians (Hedges et al. 1993), and the anuran *Xenopus laevis* (Roe et al. 1985; GenBank accession no. X02890). Human (*Homo sapiens;* Anderson et al. 1981; V00662), domestic fowl (*Gallus gallus;* Desjardins and Morais 1990; X52392), and a reptile, tuatara (*Sphenodon punctatus;* Hedges 1994; L28076) were used as outgroups. All sequences were aligned by eye with the ESEE multisequence editing program (Cabot and Beckenbach 1989). As is common with multiple sequences of rRNA genes from diverse taxa, the alignment has large numbers of insertions, deletions, and variable regions where homology of sites could not be inferred with confidence. Therefore, we conservatively omitted 318 sites (25%) of uncertain alignment from further analyses. The alignment indicating sites omitted from the analysis has been deposited in the EMBL Nucleotide Sequence Database.

The DNA sequence analyses were conducted using the METREE program (Rzhetsky and Nei 1994) and the MEGA package (Kumar et al. 1993). All trees presented are neighbor-joining trees (Saitou and Nei 1987) constructed using Jukes-Cantor corrected distances (Jukes and Cantor 1969). For distance estimations, we excluded sites with gaps or ambiguous nucleotides (N) in each pairwise comparison of sequences. In order to obtain confidence estimates of the tree topology, the interior-branch test (Rzhetsky and Nei 1992) was applied. Interior-branch confidence values ($P_c$) are preferable to the bootstrap test ($P_b$; Felsenstein 1985), particularly when large numbers of sequences are used (Sitnikova et al. 1995).

Following the recommendations of Kumar et al. (1993), Jukes-Cantor distances were used for the analyses instead of more complicated distance corrections. This was because the Jukes-Cantor distances were low (0.1–0.4 with the majority of in-group distances less than 0.3), and neither the G+C content (about 43%) nor the transition/transversion ratios were strongly biased. Kumar et al. (1993) suggest that under these conditions the simplest distance correction method is preferred because more complex methods tend to give similar estimates but with greater variance. In fact, unnecessary corrections for unequal rates of substitutions in different lineages may decrease the consistency of the tree (Zharkikh and Li 1993). When phylogenies were constructed with Kimura (1980), Tajima-Nei (1984), Tamura (1992), Tamura-Nei (1993), and gamma-corrected distances, they were not appreciably different from the ones presented and only altered a few of the nodes that have low confidence values. Where confidence values are significant using the Jukes-Cantor distance correction ($P_c \geq 95\%$), we give the corresponding confidence value using the Kimura correction for transition-transversion bias ($P_{ck}$). We also conducted a parsimony analysis using PAUP 3.1.1 (Swofford 1993).

## Results and Discussion

Of a total of 1,293 nucleotide sites (908 in the 16S rRNA data set and 385 in the 12S rRNA data set), we aligned with confidence 975 sites (644 sites in the 16S rRNA data set and 331 sites in the 12S rRNA data set of Hedges and Maxson [1993] and this study) yielding 634 variable sites. The phylogeny constructed from these data (fig. 1) provides stronger support for several familial groupings suggested earlier (Hedges and Maxson 1993) and indicates other relationships that were not resolved by the 12S data set alone. A single most parsimonious tree (3,753 steps; tree not shown) was obtained that differed very little from the neighbor-joining tree (the minor differences in topology are noted throughout the article).

### Amphibian Phylogeny

The monophyly of each of the three orders of amphibians is significantly supported by this data set (fig. 1): Anura ($P_c = 99\%$, $P_{ck} = 100\%$), Caudata ($P_c = 100\%$, $P_{ck} = 100\%$), and Gymnophiona ($P_c = 99\%$, $P_{ck} = 99\%$). This is in agreement with most morphological analyses (Trueb and Cloutier 1991) except that of Rage and Janvier (1982), who suggested that the Caudata may be paraphyletic with respect to the Anura. Previous molecular studies that included more than a single representative of the Anura, Caudata, and Gymnophiona were unable to demonstrate conclusively the monophyly of these orders because the sequences examined were either too
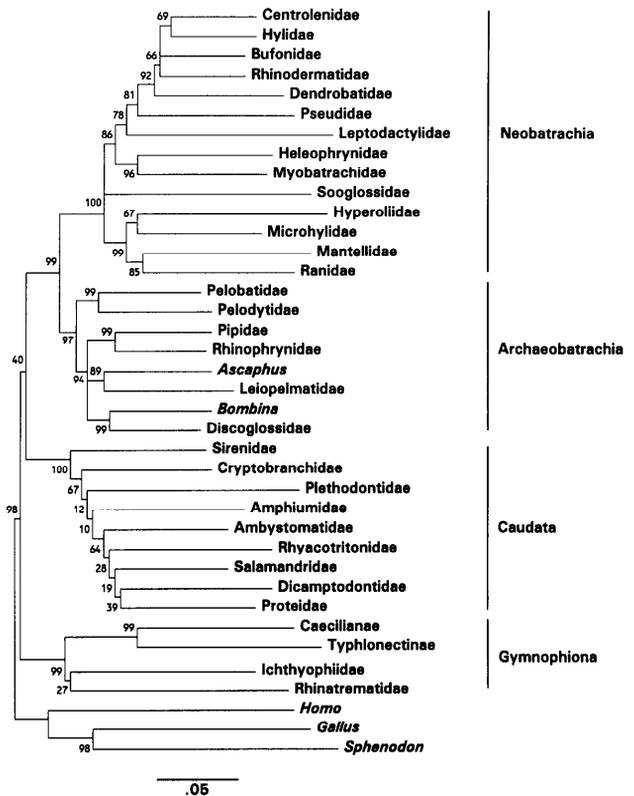


FIG. 1.—Lissamphibian relationships inferred by a neighbor-joining analysis (Jukes-Cantor distance with pairwise deletion) of the combined mitochondrial 12S and 16S rRNA gene sequences (975 aligned sites, 634 variable). Human, domestic fowl, and tuatara sequences were used as outgroups. Numbers on the tree represent confidence values from the interior-branch test.

conserved (Hedges et al. 1990) or too short (Hedges and Maxson 1993).

A close relationship of Anura and Caudata is suggested by the combined 12S + 16S data set, although not significantly ($P_c = 40\%$). Morphological analyses generally associated salamanders with frogs (Rage and Janvier 1982; Benton 1990; Trueb and Cloutier 1991; Milner 1993; Trueb 1993), and molecular analyses have grouped salamanders with caecilians (Larson and Wilson 1989; Hedges et al. 1990; Larson 1991; Hedges et al. 1993; Hedges and Maxson 1993). Bolt's (1991) examination of the morphology of living and fossil amphibians also supported the latter relationship, as do some morphological characteristics of a recently discovered Jurassic caecilian (Jenkins and Walsh 1993) and soft anatomy of Lissamphibians alone (Trueb and Cloutier 1991). Of the molecular studies, only Hedges et al. (1990), in analyses of 18S and 28S rRNA data, obtained high bootstrap confidence values for the relationships of the amphibian orders ($P_b = 94\%$ for Caudata with Gymnophiona).

A recent method for analyzing selected branching patterns among sequences from a large number of taxa that is "particularly useful for determining the branching patterns of a deep phylogeny using a large number of species" (Rzhetsky et al. 1995, p. 163) provided an opportunity to test directly the three pairs of hypotheses of phylogenetic relationships among the three amphibian orders Anura (F), Caudata (S), and Gymnophiona (C) where the outgroup (O) included *Homo, Gallus,* and *Sphenodon.* The hypotheses considered were ([F,S],[C,O]) versus ([F,C],[S,O]); ([F,S],[C,O]) versus ([C,S],[F,O]); ([C,S],[F,O]) versus ([F,C],[S,O]). The total (12S + 16S) mitochondrial data set from all species in figure 1 was used. Monophyly of each of the three orders as well as that of the outgroup was assumed as they were well supported by the neighbor-joining analysis (fig. 1). The analysis requires no information on branching order within each monophyletic lineage. Our phylogenetic tree (fig. 1) suggests that frogs share a most recent common ancestry with salamanders, although statistical support for this is low ($P_c$ = 40%). We used the Rzhetsky et al. (1995) algorithm to test the hypothesis that frogs and salamanders are closest relatives (F,S) and found that this tree ([F,S],[C,O]) was not significantly better at the 5% level than the alternative arrangements: frogs + caecilians ($P > 25\%$) or salamanders + caecilians ($P > 58\%$). Similarly, testing the hypothesis of the second topology commonly proposed to account for the ordinal relationships of amphibians ([C,S],[F,O]), we found this association was not preferred to the frogs + caecilians topology ($P > 17\%$). Thus, based on these mitochondrial sequence data, none of the three possible unrooted trees can be rejected with confidence.

To further evaluate the relationships among the three amphibian orders with a larger data set, we selected from our data the same four members of these lineages that were used in earlier analyses of relationships based on sequence data from nuclear ribosomal genes. We combined our mitochondrial 12S and 16S rRNA data for a caecilian (*Typhlonectes natans*), a salamander (*Siren intermedia*), an anuran (*Xenopus laevis*), and a mammal (*Rattus norvegicus;* Gadaleta et al. [1989]; GenBank accession no. X14848) with the corresponding 18S rRNA sequences aligned in Hedges et al. (1990) and the 28S rRNA sequences of *Typhlonectes compressicauda, Siren intermedia, Xenopus laevis, Rattus rattus* (18S and partial 28S), and *Mus domesticus* (partial 28S) from Larson (1991), yielding a total of 5,397 aligned sites with 719 variable sites. The tree constructed (fig. 2) agrees with previous molecular analyses in grouping salamanders and caecilians, although this result still is not statistically significant ($P_c$ = 84%).

The lack of a statistically significant resolution of ordinal relationships within the Lissamphibia suggests
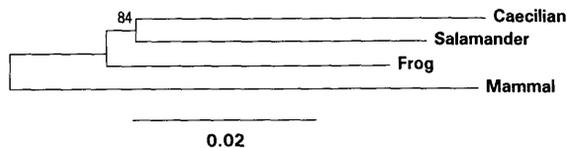


FIG. 2.—Relationships among representatives of the three amphibian orders inferred from a neighbor-joining analysis (Jukes-Cantor distance with pairwise deletion) of the data set comprising 12S, 16S, 18S, and 28S rRNA gene sequences (5,397 aligned sites, 719 variable). A mammal was used as an outgroup. The number on the tree is the confidence value from the interior-branch test. Caecilian, *Typhlonectes natans* (12S, 16S, 18S) and *T. compressicauda* (28S); salamander, *Siren intermedia;* frog, *Xenopus laevis;* mammal, *Rattus norvegicus* (12S, 16S), *R. rattus* (18S, partial 28S), and *Mus domesticus* (partial 28S).

the need for yet additional data from these and other genes, as well as additional representatives within each group. It is possible that all three orders diverged from a common ancestor within a relatively short period of time, resulting in branch lengths so short as to be very difficult to define with certainty without large amounts of sequence data. Problems associated with determining branching order for rapidly diversifying lineages are exemplified by the case of humans, chimpanzees, and gorillas first addressed in the late 1960s by Sarich and Wilson (1967) which required decades of study and kilobases of sequence to resolve (Ruvolo et al. 1994). The major groups of amniotes also diverged in a relatively short period of time in the late Paleozoic and early Mesozoic, and those relationships are requiring a large amount of sequence data to obtain statistically significant resolution (Hedges et al. 1990; Hedges 1994).

## Anura: Archaeobatrachia

Traditionally, anurans were divided into the "archaic" frogs, the Archaeobatrachia, and the "advanced" frogs, the Neobatrachia (Reig [1958]; Archaeobatrachia redefined in Duellman [1975]). Laurent (1979) erected the suborder Mesobatrachia for those archaeobatrachians (Pelobatidae, Pelodytidae, Pipidae, and Rhinophrynidae) whose morphology seemed transitional between the two suborders. From studies of morphological characters, Ford and Cannatella (1993) went a step further and made the Mesobatrachia a sister group to the Neobatrachia (to form the Pipanura) and abolished the Archaeobatrachia as a taxonomic entity. They proposed that the Discoglossidae, Bombinatoridae, *Leiopelma,* and *Ascaphus* successively fall outside of the rest of the anurans, rather than forming a monophyletic unit. However, statistical tests were not applied, so the significance of these findings is unclear. Most morphological systematists considered the Archaeobatrachia to be paraphyletic with respect to the Neobatrachia, but Discoglossidae and *Bombina* generally were placed in one

monophyletic family (Discoglossidae), as were *Leiopelma* and *Ascaphus* (Leiopelmatidae). The families Bombinatoridae and Ascaphidae were proposed on the basis of inferred paraphyly and differences in morphology, karyology, or allozyme genetic distance (Bombinatoridae: Ford and Cannatella [1993]; Ascaphidae: Green et al. [1989], Green and Cannatella [1993]). In the molecular study of Hillis et al. (1993), nucleotide sequence of 28S rRNA of eight families of anurans did not support the validity of Archaeobatrachia or Neobatrachia. The latter group was found when morphological data were added to the molecular data, but no estimates of statistical confidence of branches within the phylogenies were given.

Our molecular data (fig. 1) differ from the morphological phylogenies by finding the Archaeobatrachia (sensu Duellman 1975) to form a monophyletic unit with a confidence value of $P_c = 97\%$ ($P_{ck} = 99\%$), disputing a monophyletic Mesobatrachia or Pipanura. This also was found by Hedges and Maxson (1993) but not with statistical significance.

Within the Archaeobatrachia (fig. 1), four well-defined pairs of taxa are evident: (1) Pelodytidae + Pelobatidae ($P_c = 99\%$, $P_{ck} = 96\%$; superfamily Pelobatoidea of Duellman [1975]), (2) Pipidae + Rhinophrynidae ($P_c = 99\%$, $P_{ck} = 99\%$; superfamily Pipoidea of Duellman [1975]), (3) *Bombina* + Discoglossidae ($P_c = 99\%$, $P_{ck} = 93\%$; family Discoglossidae), and (4) *Ascaphus* + Leiopelmatidae ($P_c = 89\%$; family Leiopelmatidae). The latter three groups form a monophyletic cluster ($P_c = 94\%$). The parsimony analysis resulted in (((Pelobatoidea + Pipoidea) Discoglossidae) Leiopelmatidae), separating Laurent's (1979) Mesobatrachia from the more "archaic" frogs. Neither analysis supports the Discoglossoidea (Discoglossidae + Leiopelmatidae) of Duellman (1975). The clustering of *Bombina* with Discoglossidae and *Ascaphus* with Leiopelmatidae in figure 1 is in agreement with currently established anuran taxonomy based on morphology (Duellman 1975, 1993) rather than with the proposed families Bombinatoridae and Ascaphidae.

## Anura: Neobatrachia

The families comprising the Neobatrachia generally are considered to form a clade, and we found significant support ($P_c = 100\%$, $P_{ck} = 100\%$) for the monophyly of this group (fig. 1). We recognize three major lineages within this suborder. The Bufonoidea (sensu Duellman 1975) and Ranoidea (sensu Lynch 1973) are already defined, and the Sooglossidae forms a separate lineage.

In this study, the Bufonoidea includes the traditionally recognized bufonoid families of Duellman (1975): Centrolenidae, Hylidae, Bufonidae, Rhinodermatidae, Dendrobatidae, Pseudidae, Leptodactylidae, Heleophrynidae, and Myobatrachidae ($P_c = 86\%$). We find significant support ($P_c = 96\%$, $P_{ck} = 88\%$) for the clustering of the South African Heleophrynidae with the Australian Myobatrachidae, and as the most basal lineage of the Bufonoidea. Lynch (1973) placed these Old World taxa as separate subfamilies within the Myobatrachidae to distinguish them from the South American Leptodactylidae. However, Lynch placed the Myobatrachidae with the Sooglossidae as primitive members of a ranoid series, and Heleophrynidae as a primitive member of a bufonoid series which included the Leptodactylidae. Tyler (1979) placed the Myobatrachinae and Heleophryninae within the Leptodactylidae on the assumption that all three groups had a common Gondwanan origin and were distinguished by current geographic distribution rather than diagnostic morphological characters. Our phylogenetic tree (fig. 1) groups the Heleophrynidae with the Myobatrachidae separately from the Leptodactylidae (which is more closely allied to other New World bufonoids), whereas the parsimony analysis groups Leptodactylidae with Rhinodermatidae further within the bufonoids. Lynch (1973) suggested that the leptodactylids and the myobatrachids each are paraphyletic assemblages, and he identified four subfamilies of Leptodactylidae and three of Myobatrachidae (including Heleophryninae). Sequences of representatives from all of these subfamilies are needed to clarify their relationships to one another and to the bufonoids.

The phylogenetic position of the dart-poison frogs (Dendrobatidae) has been a long-standing controversy in anuran systematics (Ford 1993). Some authors have placed this family with the ranoids (Griffiths 1959; Duellman and Trueb 1986, pp. 473, 475; Ford and Cannatella 1993; Trueb 1993) while others have associated dendrobatids with the microhyloids (Blommers-Schlosser 1993) or the bufonoids (specifically among the leptodactylids; Lynch 1971, 1973; Morescalchi 1973; Duellman 1975). Ford (1993) evaluated the data on which the competing hypotheses of phylogenetic placement of the Dendrobatidae were made and concluded that many of the characters used in those studies conflicted and new characters would be needed to resolve this phylogenetic question. Our molecular results (fig. 1) show the Dendrobatidae to be associated with bufonoid families ($P_c = 92\%$) and excluded from the cluster of ranoid families ($P_c = 99\%$, $P_{ck} = 99\%$). Bogart (1991) compared the karyotypes of dendrobatids, ranids (Ranoidea), and leptodactylids (Bufonoidea) and found that dendrobatid karyotypes can be more easily derived from those of the leptodactylids, thereby also supporting a bufonoid relationship for the dendrobatids. This also conforms to biogeography as the ranoids are widely dis-

tributed throughout the Old World, whereas dendro-batids and bufonoids apparently originated in the New World (Reig 1972).

We obtained both 12S and 16S rRNA sequence for the paradoxical frog, *Pseudis paradoxa,* so named because the tadpole (up to 25 cm) is enormous relative to the adult (2–7.5 cm). *Pseudis* was placed in the Hylidae or Leptodactylidae until it was assigned familial rank by Savage and Carvalho (1953). In morphological analyses the Pseudidae is grouped together with the Hylidae and Centrolenidae (Lynch 1973; Duellman and Trueb 1986, p. 473; Ford and Cannatella 1993). In our analysis, Pseudidae is a member of the bufonoid lineage apparently (although not significantly) closer to the Leptodactylidae than to the Hylidae or the Centrolenidae.

The Hyperoliidae, Microhylidae, Mantellidae, and Ranidae clustered at $P_c = 99\%$ ($P_{ck} = 99\%$), in accordance with families and subfamilies in the Ranoidea as defined by Lynch (1973). The topology of our tree (fig. 1) supports the grouping of Microhylidae within the Ranoidea (Ford and Cannatella 1993; Hedges and Maxson 1993) rather than in a distinct superfamily Microhyloidea (Duellman 1975; Laurent 1979; Blommers-Schlosser 1993). We have a partial sequence of the 16S rRNA region for *Rhacophorus pardalis* (Rhacophoridae), which groups with the ranoids (tree not shown) in agreement with traditional systematics (Duellman 1975). The "Rhacophoridae" in Hedges and Maxson (1993) was found to be a South American hylid, *Smilisca phaeota,* which was sequenced in error due to a coincidence of identifier numbers of tissue samples from different collections.

The Sooglossidae previously has been placed in the Ranoidea (Duellman 1975), the Bufonoidea (Laurent 1979), the Microhyloidea (Blommers-Schlosser 1993), and as a sister group with the myobatrachines (Lynch 1973; Duellman and Trueb 1986, p. 473; Ford and Cannatella 1993). Morphologically the Sooglossidae is primitive with respect to other neobatrachians (Duellman and Trueb 1986, p. 474). Here we identify the Sooglossidae as a distinct major lineage in the Neobatrachia with no closer affinities to one superfamily than another (fig. 1). The parsimony analysis places the Sooglossidae as the most basal lineage of the Neobatrachia (tree not shown). The Sooglossidae consists of three species found only on the Seychelle Islands, and it may have been isolated since the islands separated from the Indian continent approximately 64 Mya (Dickin et al. 1986; Mart 1988). There are no known fossil sooglossids.

## Caudata

The relationships of the salamander families have been addressed in several recent molecular studies. A study based on nuclear rRNA sequences (Larson and Wilson 1989) of 7 of the 10 salamander families found the Plethodontidae to be the most basal family followed by Amphiumidae and the lineage consisting of the Ambystomatidae + Proteidae to be the most derived. This is a markedly different phylogeny than the (more traditional) phylogeny based on morphological characters (Duellman and Trueb 1986, p. 466) that has the Plethodontidae as one of the most derived lineages (as sister group to the Ambystomatidae), with the Sirenidae as the most basal family. Hillis (1991) reanalyzed both morphological and molecular data sets individually, and together, and constructed several trees that differed from one another and from the original studies. In none of the trees in which Sirenidae was included did it form the basal lineage.

Larson (1991) used parsimony methods to analyze an expanded nuclear rRNA data set including representatives of all recognized salamander families and again found the Plethodontidae + Amphiumidae to be the most basal lineage. Other researchers Hedges and Maxson (1993) evaluated relationships among salamander families using sequences from the mitochondrial 12S rRNA gene and concluded that the Sirenidae was the most basal family ($P_b = 47\%$) as traditionally interpreted by morphology (Duellman and Trueb 1986, p. 466), but none of their bootstrap values was significant. In that study (Hedges and Maxson 1993) Larson's (1991) 28S rRNA data were reexamined using a neighbor-joining analysis with gaps coded as informative characters, and a different tree than in Larson (1991) was found in which the Sirenidae was the most basal lineage.

Our phylogeny based on the combined 12S and 16S rRNA data (fig. 1) also places the Sirenidae basally among the salamanders ($P_c = 67\%$) followed by the Cryptobranchidae ($P_c = 12\%$). We did not obtain 16S sequence data for Hynobiidae, but it generally is considered to be the closest relative of the Cryptobranchidae. Members of these three families fertilize eggs externally, which is thought to be more primitive than internal fertilization as is practiced by all other families. Our data place Plethodontidae with the other internal-fertilizers in a derived position on the salamander phylogeny. However, apart from the monophyly of the Caudata, none of the nodes in the caudate portion of this phylogeny is statistically significant. Therefore, we added our mitochondrial rRNA sequences to Larson's (1991) 18S and 28S rRNA sequence data using the taxa Larson and Dimmick (1993) selected for a condensed sampling of the Caudata. We included a frog (*Xenopus*) and a caecilian (*Typhlonectes*) and used *Homo* as an outgroup (3,703 aligned sites, 808 variable). In contrast to the mitochondrial data set alone (fig. 1), this tree (not shown) places the Plethodontidae then the Amphiumidae as the

basal lineages within the salamanders ($P_c$ = 99%, $P_{ck}$ = 99%) and clusters Ambystomatidae + Dicamptodontidae ($P_c$ = 99%, $P_{ck}$ = 99%), as seen by Larson (1991). These arrangements appeared to be derived from the nuclear data, so we constructed trees from the nuclear (2,746 aligned sites, 313 variable) and mitochondrial (957 aligned sites, 495 variable) data sets separately, applying the same analytical methods used throughout this study to ensure that we were examining comparable data analyses. The nuclear data set gave the same results as the combined data set and also grouped Salamandridae with Ambystomatidae and Dicamptodontidae ($P_c$ = 99%, $P_{ck}$ = 91%). The tree from the mitochondrial data set had no significant nodes. The lack of support for most of the nodes leaves the question of salamander relationships little more resolved than the morphological studies where convergence of morphological characters due to paedomorphosis or adaptations to terrestrial versus aquatic environments has complicated phylogenetic inference (Duellman and Trueb 1986, p. 465). In addition, the long terminal branches but short internal branches of the molecular-based phylogenies suggest that the caudate families radiated and diverged within a short span of time. Sequence data from additional genes will be needed to obtain a robust estimate of salamander phylogeny.

## Gymnophiona

In this analysis the Caeciliainae clusters with the Typhlonectinae ($P_c$ = 99%, $P_{ck}$ = 99%). Hedges et al. (1993) synonymized the former Typhlonectidae within the Ceciliaidae to maintain the monophyly of the latter, in accordance with their nucleotide sequence data, immunological data (Hass et al. 1993), and morphological data (Nussbaum and Wilkinson 1989). We are unable to resolve the positions of Ichthyophiidae and Rhinatrematidae but have no reason to disagree with a basal position for Rhinatrematidae as observed in the caecilian phylogeny based upon an expanded analysis of mitochondrial DNA sequence data (Hedges et al. 1993).

## Conclusions

Our analyses of mitochondrial DNA sequence data from 32 families of amphibians provide statistically significant molecular evidence that each of the three orders of amphibians is monophyletic. Within the Caudata, the externally fertilizing families (Sirenidae and Cryptobranchidae) are weakly resolved as basal lineages, in agreement with long-standing interpretation of salamander phylogeny. However, combining the mitochondrial data with published nuclear data of salamanders (Larson 1991) places two internally fertilizing families (Plethodontidae and Amphiumidae) as basal.

Significant support is presented for the monophyly of each of the anuran suborders Archaeobatrachia and Neobatrachia (sensu Duellman 1975), in contrast to the usual understanding that the former is paraphyletic. Within the Archaeobatrachia, significant support was found for the groupings of Pelobatidae + Pelodytidae, Pipidae + Rhinophrynidae, and Bombina + Discoglossidae, and strong support for Ascaphus + Leiopelmatidae. The latter three pairs clustered to the exclusion of the first group, disputing the validity of the taxa Mesobatrachia (Laurent 1979) and Pipanura (Ford and Cannatella 1993) and unexpectedly placing the Pelobatoidea as the most basal archaeobatrachian lineage. Within the Neobatrachia, three major lineages are distinguished: the Bufonoidea of Duellman (1975), the Ranoidea of Lynch (1973), and the Sooglossidae.

Some questions are not yet clearly answered, such as the relationships among amphibian orders and among salamander families. Even when we combined our mitochondrial data set with previously published sequences of nuclear rRNA genes, we were unable to fully resolve these relationships. This might be attributed to short divergence times both among modern amphibian orders and among salamander families, resulting in small internal branches on the phylogenetic tree that are difficult to define conclusively.

Despite these areas of uncertainty, this study proposes a working hypothesis for a molecular phylogeny for the Lissamphibia containing some of the most strongly supported nodes to date. We are optimistic that it should be possible to resolve the relationships among the three amphibian orders, and the enigmatic lineages within them, when sufficient lengths of nucleotide sequences with the appropriate level of variation are analyzed.

APPENDIX

Voucher specimens for the taxa surveyed in the present study are deposited in the following collections: American Museum of Natural History (AMNH), California Academy of Science (CAS), Chicago Field Museum of Natural History (FMNH), University of Illinois Museum of Natural History (UIMNH), University of Michigan Museum, Ann Arbor (UMMZ), Smithsonian National Museum of Natural History (USNM), and Western Australia Museum (WAM). The specimens marked LM, RH, and SBH are from the frozen tissue collections of Linda R. Maxson (Pennsylvania State University), Richard Highton (University of Maryland), and S. Blair Hedges (Pennsylvania State University), respectively.

**Table A1**
**Taxa Examined**

| Taxa | Species and Locality |
|---|---|
| Anura: | |
| Ascaphus .......... | *Ascaphus truei* (UIMNH 94103-06)—Oregon, Wallowa Mountains |
| Bombina .......... | *Bombina orientalis* (LM 3153)—dealer, locality unknown |
| Bufonidae ......... | *Bufo valliceps* (UIMNH 95424)—Louisiana, Allen Parish |
| Centrolenidae ...... | *Centrolene geckoideum* (LM 85)—Ecuador |
| Dendrobatidae ...... | *Dendrobates speciosus* (UIMNH 94442-99)—Panama, 12S rRNA; *D. auratus* (LM 721)—Panama, 16S rRNA |
| Discoglossidae ....... | *Discoglossus pictus* (LM 2352-53)—Tunisia |
| Heleophrynidae ..... | *Heleophryne natalensis* (LM 1001)—South Africa, Natal, 12S rRNA; *H. purcelli* (LM 2964)—locality unknown, 16S rRNA |
| Hylidae ........... | *Hyla cinerea* (RH 57458)—Maryland, Dorchester County |
| Hyperoliidae ....... | *Hyperolius argus* (CAS 161016)—Kenya, Kilifi District |
| Leiopelmatidae ...... | *Leiopelma hamiltoni* (LM 3174)—New Zealand, Maud Island |
| Leptodactylidae ..... | *Eleutherodactylus cuneatus* (SBH 172809)—Cuba, Cienfuegos Province, Soledad |
| Mantellidae ........ | *Mantella aurantiaca* (AMNH 123693-129695)—Madagascar |
| Microhylidae ....... | *Gastrophryne carolinensis* (RH 55501)—South Carolina, Aiken County |
| Myobatrachidae ..... | *Neobatrachus pelobatoides* (LM 2779)—Western Australia, Beverley, 12S rRNA; *N. pelobatoides* (WAM 101147)—Western Australia, Jerramungup, 16S rRNA |
| Pelobatidae ......... | *Scaphiopus holbrookii* (LM 3070)—North America |
| Pelodytidae ......... | *Pelodytes punctatus* (LM 731)—Spain, Cadiz |
| Pipidae ........... | *Xenopus laevis*—GenBank accession no. X02890 |
| Pseudidae ......... | *Pseudis paradoxa* (LM 3072)—Bolivia, Santa Cruz |
| Ranidae ........... | *Rana pipiens* (UIMNH 95421)—dealer, locality unknown |
| Rhinodermatidae .... | *Rhinoderma darwinii* (LM 3172)—Chile |
| Rhinophrynidae ..... | *Rhinophrynus dorsalis* (UIMNH 94144)—Mexico |
| Sooglossidae ........ | *Nesomantis thomasseti* (LM 2549)—Seychelles, Silhouette Island |
| Caudata: | |
| Ambystomatidae ..... | *Ambystoma mexicanum* (UIMNH 95430)—bred at the Indiana University Amphibian Facility |
| Amphiumidae ....... | *Amphiuma tridactylum* (LM 2594)—dealer, locality unknown |
| Cryptobranchidae .... | *Cryptobranchus alleganiensis* (UIMNH 94035)—dealer, locality unknown |
| Dicamptodontidae ... | *Dicamptodon ensatus* (LM 445)—Washington, Wahkiakam County, 12S rRNA; *D. ensatus* (LM 441)—Oregon, Oak Springs, 16S rRNA |
| Plethodontidae ...... | *Plethodon yonahlossee* (RH 69670-72)—North Carolina, Buncombe County |
| Proteidae .......... | *Necturus lewisi* (UIMNH 94301)—North Carolina, Johnston County, 12S rRNA; *N. alabamensis* (UIMNH 94297-99)—Florida, Okaloosa County, 16S rRNA |
| Rhyacotritonidae .... | *Rhyacotriton olympicus* (LM 384)—Washington, Mason County, 12S rRNA; *R. olympicus* (RH 43510-12)—Washington, Spirit Lake, 16S rRNA |
| Salamandridae ...... | *Notopthalmus viridescens* (LM 2660)—Pennsylvania, Centre County, 12S rRNA; *N. viridescens* (LM 3147)—Pennsylvania, Centre County, 16S rRNA |
| Sirenidae .......... | *Siren intermedia* (LM 2531)—Illinois, Alexander County |
| Gymnophiona: | |
| Caeciliaidae: | |
| Caeciliainae ....... | *Caecilia* sp. (UMMZ 190146)—Ecuador, Cotapaxi |
| Typhlonectinae .... | *Typhlonectes natans* (UMMZ 186672)—dealer, locality unknown |
| Ichthyophiidae ...... | *Ichthyophis bannanicus* (UMMZ 189122)—China, Yunnan |
| Rhinatrematidae ..... | *Epicrionops* sp. (UMMZ 190478)—Ecuador, Cotopaxi |

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