Phylogeny and Classification of Poison Frogs (Amphibia: Dendrobatidae), Based on Mitochondrial 16S and 12S Ribosomal RNA Gene Sequences

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An analysis of partial sequences of the 16S ribosomal rRNA gene (582 bp) of 20 poison frog species (Dendrobatidae) confirmed their phylogenetic relationships to bufonid and leptodactylid frogs. Representatives of the ranoid families and subfamilies Raninae, Mantellinae, Petropedetinae, Cacosterninae, Arthroleptidae, Astylosternidae, and Microhylidae did not cluster as sister group of the Dendrobatidae. Similar results were obtained in an analysis using a partial sequence of the 12S gene (350 bp) in a reduced set of taxa and in a combined analysis. Within the Dendrobatidae, our data supported monophyly of the genus Phyllobates but indicated paraphyly of Epipedobates and Colostethus. Minyobates clustered within Dendrobates, contradicting its previously assumed phylogenetic position. Phobobates species clustered as a monophyletic unit within Epipedobates. Allobates was positioned in a group containing two Colostethus species, indicating that lack of amplexus, presence of skin alkaloids, and aposematic coloration evolved independently in Allobates and the remaining aposematic dendrobatids. © 2000 Academic Press

Key Words: Anura; Allobates; Colostethus; Dendrobates; Epipedobates; Minyobates; Phobobates; Phyllobates; Ranoina; molecular phylogeny; biogeography.

INTRODUCTION

The family Dendrobatidae is a Neotropical amphibian group of 186 species classified into 10 genera, according to Glaw et al. (1998). Many dendrobatid frogs share aposematic coloration and skin alkaloids. The presence of a cephalic amplexus is typical for the presumably more basal dendrobatid genera and, as far as is known, unique among anurans (Duellman and Trueb, 1986). Complex patterns of mating behavior and parental care are widespread among the different genera. These interesting traits make poison frogs very attractive for ethological and evolutionary studies (e.g., Weygoldt, 1987; Summers, 1992, 1999; Toft, 1995; Caldwell, 1996, 1997; Summers et al., 1997; Summers and Earn, 1999; Vences et al., 1998). Silverstone (1975, 1976) recognized three genera in the Dendrobatidae: Colostethus, Dendrobates, and Phyllobates. The generic partitioning of the family has been subject to major changes during the last 20 years. New genera were erected by Lynch and Ruiz-Carranza (1982), Myers (1987), Zimmermann and Zimmermann (1988), Myers et al. (1991), and LaMarca (1992, 1994). There has not been general acceptance of the validity of several of these genera (e.g., Myers et al., 1991; Toft, 1995; Jungfer et al., 1996).

So far, no comprehensive analysis of phylogenetic relationships among dendrobatid genera has been published. Limited hypotheses were based on few morphological, ethological, immunological, and skin toxin characters (Maxson and Myers, 1985; Myers et al., 1991, 1995; Toft, 1995; Caldwell, 1996). Recently, Summers et al. (1997, 1999) discussed relationships within Dendrobates based on DNA sequence data.

The Dendrobatidae are relatively well defined as a monophyletic group (Myers and Ford, 1986; Myers et al., 1991, 1995). In contrast, much debate exists with respect to the placement of the family within the major lineages of the Neobatrachia. Among their proposed sister groups are hyloid leptodactylids (Lynch, 1971, 1973; see also Noble, 1931 and Laurent, 1986), arthropodids (Ford, 1989, 1993), microhylids (Blommers-Schlosser, 1993), and petropedetines (Griffiths, 1959) and mantelline (Zimmermann, 1996) ranids. Recent molecular studies placed the dendrobatids within the hyloid lineage (composed of families including Bufonidae, Hylidae, Leptodactylidae, and Myobatrachidae; Hedges and Maxson, 1993; Hay et al., 1995; Ruvsinsky and Maxson, 1996) or failed to resolve unambiguously their phylogenetic position (Hillis et al., 1993). Since
neither petropedines nor arthroleptids were included in these studies, the position of the Dendrobatidae relative to them remains unknown from a molecular perspective. The same is true for coccosternids, which were often included in the Petropedetinae, and astylosternids, which were often included in the Arthroleptidae (see Dubois, 1992; Blommers-Schloesser, 1993).

The purpose of this paper is to test some of these assumed sister group relationships of the Dendrobatidae, using DNA sequences of the mitochondrial 16S and 12S ribosomal RNA genes. Additionally, our 16S data allow for statements on the status and phylogenetic position of some dendrobatid genera.

MATERIALS AND METHODS

DNA was extracted using QIAmp tissue extraction kits (Qiagen) from tissue samples (hindleg muscle, either fresh or preserved in 98% pure ethanol). We used the primers 16SA (light chain; 5'-CGC CTG TTT ATC AAA AAC AT-3') and 16SB (heavy chain; 5'-CCG GTC TGA ACT CAG ATC ACG T-3') of Palumbi et al. (1991) to amplify a section of the mitochondrial 16S ribosomal RNA gene. The PCR cycling procedure was as follows: initial denaturation step, 90 s at 94°C; 33 cycles, denaturation 45 s at 94°C, primer annealing for 45 s at 55°C, and extension for 90 s at 72°C. Additionally, we used the primers L25195 (light chain; 5'-AAA AAC AT-3') and H2916 (heavy chain; 5'-GAG GGT GAC GGG CGG TGT GT-3') to amplify a section of the mitochondrial 12S ribosomal RNA gene. The cycling procedure was as follows: 35 cycles, denaturation 45 s at 94°C, primer annealing for 60 s at 50°C, and extension for 120 s at 74°C. PCR products were purified using QIAquick purification kits (Qiagen). We sequenced single-stranded fragments using an automatic sequencer (ABI 377). The obtained sequences (lengths referring to the aligned sequences including gaps) comprised 582 bp (16S) and 350 bp (12S) homologous to the base pair positions 3995–4550 (16S) and 2581–2831 (12S) of the Xenopus laevis mitochondrial genome (Roe et al., 1985). Voucher specimens were deposited in the Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn (ZFMK; see Table 1). Sequences have been submitted to GenBank (Accession Nos. AF124096–AF124137).

Sequences were aligned manually using the computer program SEQUENCE NAVIGATOR (Applied Biosystems). We omitted two short sections (together 64 bp) from the original 16S data set which were too variable to be reliably aligned.

Sequences were analyzed using PAUP, version 4 beta (Swofford, 1998). We calculated maximum-parsimony (MP) trees with gaps treated as a fifth character and neighbor-joining (NJ) trees based on the Jukes–Cantor distance (Jukes and Cantor, 1969), with gaps treated as missing data. The treatment of gaps as fifth characters in MP analyses followed a conservative all-evidence approach, as their treatment as missing data generally increases the statistical support of most nodes, according to our personal observations. Two thousand bootstrap replicates (Felsenstein, 1985) were run in all analyses, following Hedges (1992). Only topologies with a bootstrap support of 70% and higher were considered in taxonomic and phylogenetic conclusions, according to Hillis and Bull (1993), who found a 95% probability of correct topology in branches supported by such bootstrap values. Discoglossus pictus (Discoglossidae) was used as outgroup.

RESULTS AND DISCUSSION

Intrafamilial Phylogeny and Classification of Dendrobatids

Of a total of 518 analyzed 16S nucleotide sites, 273 were variable and 192 of these were phylogenetically informative. Six equally most-parsimonious trees (1094 steps) were obtained. The strict consensus of these (not shown) grouped dendrobatids as monophyletic, as did the NJ analysis (bootstrap support 56%) (see Fig. 1). MP and NJ analyses differed in the phylogenetic arrangement of dendrobatid genera, but most bifurcations were not supported by relevant bootstrap values. A maximum-likelihood tree (not shown) was almost identical to the NJ tree in topology and bootstrap values. Results of additionally performed analyses excluding all characters with gaps showed no relevant differences.

The three included species of Phyllobates clustered as a monophyletic unit (bootstrap support 100%), confirming Myers et al. (1978, 1995) and Maxson and Myers (1985). The subclade of the two Central American species (P. lugubris and P. vittatus) also was supported (99%).

Within Dendrobates, the groups (auratus (leucemias, tinctorius)) and (sylvaticus, pumilio) were well supported (91 and 100%). Minyobates fulguritus was the sister group of Dendrobates imitator (83%). According to the results of a log likelihood test, as implemented in PHYLIP (Felsenstein, 1993), two alternative topologies (Minyobates being the sister group of a clade comprising Dendrobates and Phyllobates; and Minyobates being the sister group of Dendrobates) were significantly worse. Only one alternative arrangement (Minyobates being the sister group of Phyllobates) could not be significantly excluded. Minyobates does not appear to be the sister group of the Dendrobates-Phyllobates clade, as assumed by Myers (1987), but rather a subgroup and thus synonym of Dendrobates (see also Jungfer et al., 1996 and phylogenetic data of Summers et al., 1997).

The two included representatives of Phobobates were
grouped together (92%) and included in a clade with two species of *Epipedobates* (100%). Recognition of Phobobates would impose a further subdivision of *Epipedobates* which is not warranted by the current data. We agree with Myers et al. (1991), Toft (1995), Caldwell (1996), and Vences et al. (1998) to consider Phobobates as synonym of *Epipedobates*.

*Epipedobates azureiventeri* was grouped with *Colostethus bocagei* (74%), indicating that the two speciose genera *Epipedobates* (including Phobobates) and *Colostethus* may not be monophyletic. Both are defined mainly by plesiomorphic characters, according to Myers (1987), Zimmermann and Zimmermann (1988), and Myers et al. (1991). Allobates femoralis was grouped with two species of *Colostethus* (97%). This contradicts the hypothesis of Myers et al. (1991, p. 18), who positioned femoralis in the genus *Epipedobates* and at the "basal part of the lipophilic alkaloid producing group." Classification of femoralis in the monotypic *Allobates* is therefore warranted (see also Hillis et al., 1993; Caldwell, 1996; Vences et al., 1998).

Recent data suggest parallel evolution of several characteristic patterns within dendrobatids. Summers et al. (1999) concluded that female parental care in the *Dendrobates histrionicus* group (here included: *D. sylvaticus* and *D. pumilio*) and biparental care in the *D. quinquemaculatus* group (here included: *D. imitator*) evolved independently from male care (see also Summers and Earn, 1999). Our data are in accordance with this hypothesis. The possible paraphyly of *Epipedobates* as indicated by our data also makes the convergent evolution of skin alkaloid accumulation (or its loss in some species) very probable. According to the phylogenetic position of *Allobates* femoralis as suggested by our results, its slightly aposematic coloration (Silverstone, 1976), traces of skin alkaloids (Daly et al., 1987),

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* Sequences obtained by Hay et al. (1995), Graybeal (1997), or Ruvinsky and Maxson (1996), not referring to the ZFMK voucher specimens.
and lack of (cephalic) mating amplexus evolved independently from the Epipedobates–Phyllobates–Dendrobatid clade.

Terrestrial egg deposition and tadpole transport appear to be plesiomorphic in dendrobatids. The same is true for diurnal activity patterns (except, perhaps, Aromobates; see Myers et al., 1991). These features may have been important prerequisites in the dendrobatid radiation, allowing complex mating and parental care systems (involving optical stimuli and largely depending on the independence from larger water bodies) to evolve several times separately. Also, diurnality may have been important in the evolution of aposematic coloration, which probably is related to
microphagous specialization with skin alkaloid accumulation from ant prey (Caldwell, 1996; Vences et al., 1998).

Higher-Level Phylogenetic Relationships

In the MP and NJ analyses of the 16S data, ranoids (i.e., Raninae, Mantellinae, Petropedetinae, Cacosterninae, Arthroleptidae, Astylosternidae, and Microhylidae; see Dubois, 1992; Blommers-Schloesser, 1993) were not arranged as monophylum, but none of the nodes in the cladogram advocating their paraphyly was significantly corroborated. The Bufonidae and Leptodactylidae (superfamily Hylidea) were grouped with the dendrobatid clade (85%). NJ and MP analyses of 12S sequences for a reduced set of taxa (Fig. 2) supported the monophyly of the ranoid clade (77%) and the position of dendrobatids as sister group to the leptodactylid/bufonid clade (100%). The combined analysis of 16S and 12S sequences for the reduced taxa set confirmed these groupings both in the NJ (Fig. 3) and MP analysis (84 and 100%).

The inclusion of dendrobatids in the Hylidea confirms Hay et al. (1995) and Ruvinsky and Maxson (1996). These authors, however, did not include arthroleptids, astylosternids, petropedetines, nor cacosternines in their analysis. Our data therefore allow for the first time the opportunity to exclude a relationship of dendrobatids with any of these groups, as opposed to the hypothesis of Ford (1989, 1993), who assumed an arthroleptid/dendrobatid sister group relationship.

This information adds relevant perspectives on the biogeographic relationships among South America, Madagascar, Africa, and Asia. Ranoids are mainly an Old World group, with only microhylids and ranids of the genus *Rana* being present in North and South America. Dendrobatids are restricted to Central and South America. Several Malagasy animal groups, such as boas, oplurine iguanas, and podocnemine turtles among reptiles, have sister group relationships to South American rather than to African groups (Kluge, 1991; Schulte et al., 1998; Georges et al., 1998). Our data indicate that a parallel situation does not exist in amphibians; apparently, the ranoid radiation is monophyletic relative to the mainly South American leptodactylids and dendrobatids (as also supported by the data.

**FIG. 2.** Neighbor-joining tree based on 350 bp of the mitochondrial 12S ribosomal RNA gene sequences, including representatives of the Dendrobatidae and of families and subfamilies which in the past were considered as their potential sister groups. See Fig. 1 for additional explanations.

**FIG. 3.** Neighbor-joining tree based on a combined analysis of 518 bp of the mitochondrial 16S ribosomal RNA gene sequences and 350 bp of the mitochondrial 12S ribosomal RNA gene sequences. See Fig. 1 for additional explanations.
of Hedges and Maxson, 1993; Hay et al., 1995; Ruvinsky and Maxson, 1996). It is rather plausible at the current time to consider the (relatively few) New World Rana species as a monophyletic group (Hillis and Davis, 1986). The same is true for the New World microhylids, except for the enigmatic genus Otophyne (Zweifel, 1966; Wassersug and Pyburn, 1987). It can therefore be hypothesized that the origin and main radiation of the ranoid clade took place in the Old World, with subsequent (relatively recent) dispersal of the ancestors of New World Rana and microhylids to North and South America (Feller and Hedges, 1998).

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