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

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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 Ruíz-Carranza (1982), Myers (1987), Zimmermann and Zimmermann (1988), Myers *et al.* (1991), and La Marca (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 hylodine leptodactylids (Lynch, 1971, 1973; see also Noble, 1931 and Laurent, 1986), arthroleptids (Ford, 1989, 1993), microhylids (Blommers-Schlösser, 1993), and petropedetine (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; Ruvinsky and Maxson, 1996) or failed to resolve unambiguously their phylogenetic position (Hillis *et al.,* 1993). Since

neither petropedetines 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 cacosternines, which were often included in the Petropedetinae, and astylosternids, which were often included in the Arthroleptidae (see Dubois, 1992; Blommers-Schlösser, 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 CTG GGA TTA GAT ACC CCA CTA T-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* (*leucomelas, 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

TABLE 1

Specimens Used for Analysis

| Family	Species	Origin	ZFMK	GenBank 16S	Gen Bank 12S
Discoglossidae	*Discoglossus pictus*		—	X86269*	X86235*
Bufonidae	*Bufo asper*		—	AF124109	U52733*
Leptodactylidae (Ceratophryinae)	*Ceratophrys ornata*	Pet trade (no locality)	66749	AF124111	U52739*
Leptodactylidae (Leptodactylinae)	*Leptodactylus mystacinus*	Paraguay	69878	AF124129	—
Leptodactylidae (Telmatobiinae)	*Eleutherodactylus* cf. *johnstonei*	Jamaica (captive bred)	—	AF124123	—
Arthroleptidae (Arthroleptinae)	*Arthroleptis variabilis*	Cameroon	68794	AF124107	—
Arthroleptidae (Arthroleptinae)	*Cardioglossa* cf. *leucomystax*	Comoe National Park, Ivory Coast	—	AF124110	—
Arthroleptidae (Astylosterninae)	*Trichobatrachus robustus*	Nkongsamba, Cameroon	66453	AF124136	AF124104
Arthroleptidae (Astylosterninae)	*Astylosternus schiotzi*	Edib, Cameroon	67733	AF124108	—
Ranidae (Raninae)	*Rana temporaria*	Koblenz, Germany	69883	AF124135	AF124103
Ranidae (Mantellinae)	*Mantella madagascariensis*	Madagascar	62732	AF124131	AF124101
Ranidae (Mantellinae)	*Mantella laevigata*	Madagascar	65637	AF124130	—
Ranidae (Petropedetinae)	*Petropedetes parkeri*	Nguti, Cameroon	—	AF124132	AF124102
Ranidae (Cacosterninae)	*Cacosternum boettgeri*	Bredell, South Africa	—	—	AF124096
Microhylidae (Dyscophinae)	*Dyscophus guineti*	Madagascar	64133	AF124122	AF124099
Dendrobatidae	*Colostethus bocagei*	South of Puyo, Ecuador	66400	AF124112	AF124097
Dendrobatidae	*Colostethus talamancae*	San Blas, Panama	—	AF124113	—
Dendrobatidae	*Colostethus* cf. *trilineatus*	Cobija, Bolivia	66794	AF124114	—
Dendrobatidae	*Allobates femoralis*	Brazil	69879	AF124106	—
Dendrobatidae	*Epipedobates azureiventris*	Tarapoto region, Peru	66730	AF124124	—
Dendrobatidae	*Epipedobates tricolor*	Captive bred (no locality)	—	AF124127	—
Dendrobatidae	*Epipedobates pictus*	Mataracú, Bolivia	66859	AF124126	—
Dendrobatidae	*Epipedobates parvulus*	Near Macas, Ecuador	66731	AF124125	—
Dendrobatidae	*Phobobates trivittatus*	Captive bred (Peru/Suriname)	69880	AF124128	—
Dendrobatidae	*Phobobates silverstonei*	Pet trade (no locality)	68763	AF124137	—
Dendrobatidae	*Phyllobates terribilis*	Río Saija, Colombia	—	AF124133	—
Dendrobatidae	*Phyllobates lugubris*	Panama	69881	AF124105	—
Dendrobatidae	*Phyllobates vittatus*	Costa Rica	69882	AF124134	—
Dendrobatidae	*Dendrobates auratus*	Pet trade (no locality)	66710	AF124115	—
Dendrobatidae	*Dendrobates tinctorius*	Pet trade (no locality)	66712	AF124121	—
Dendrobatidae	*Dendrobates leucomelas*	Venezuela	66711	AF124119	—
Dendrobatidae	*Dendrobates sylvaticus*	Cuba, Ecuador	—	AF124117	AF124098
Dendrobatidae	*Dendrobates pumilio*	Panama	—	AF124120	—
Dendrobatidae	*Dendrobates imitator*	Tarapoto region, Peru	—	AF124118	—
Dendrobatidae	*Minyobates fulguritus*	San Blas, Panama	—	AF124116	—

* Sequences obtained by Hay *et al.* (1995), Graybeal (1997), or Ruvinsky and Maxson (1996), not referring to the ZFMK voucher specimens.

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 azureiventris 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. quinquevittatus* groups (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),
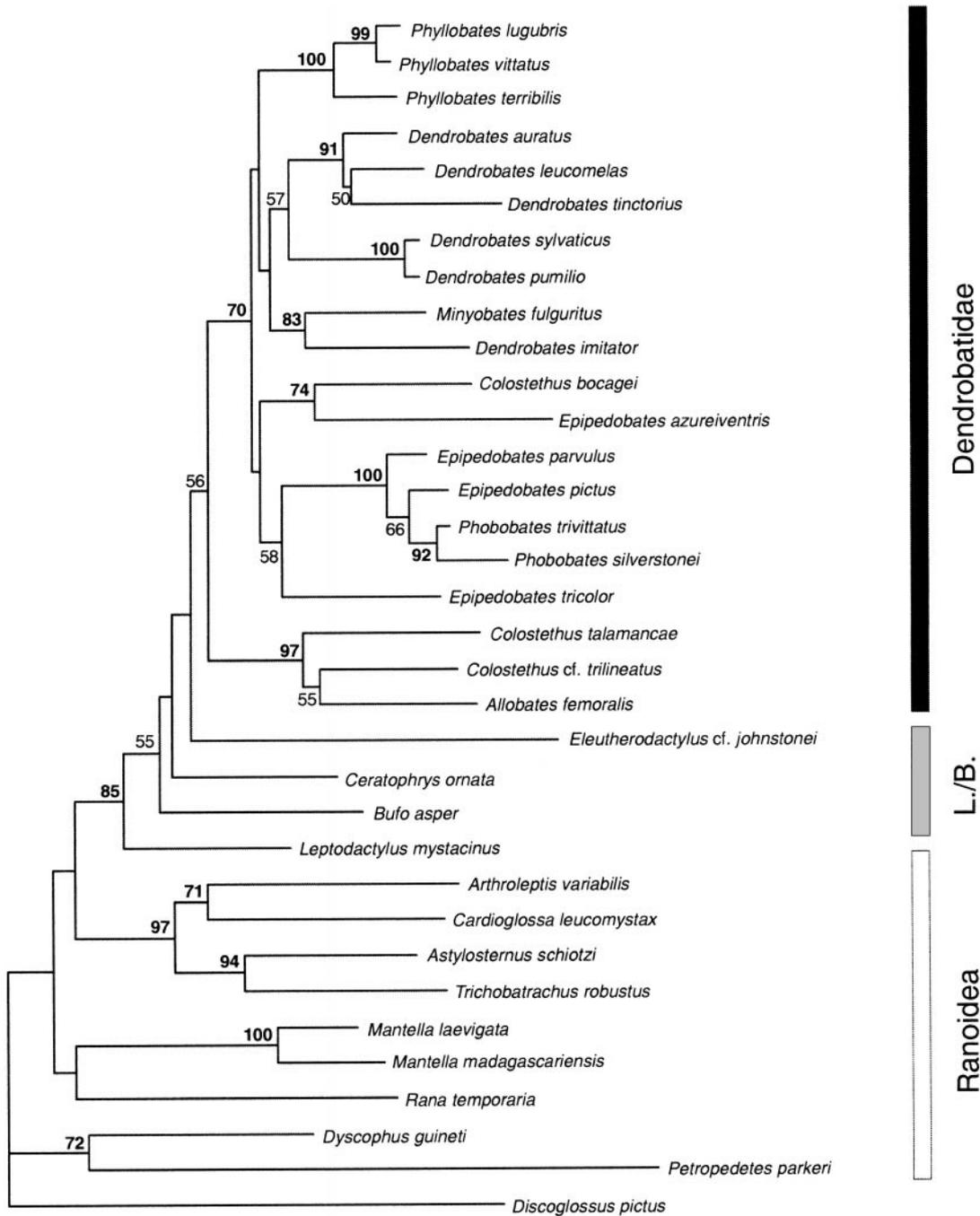
FIG. 1. Neighbor-joining tree based on 518 bp of the mitochondrial 16S ribosomal RNA gene sequences, including representatives of the Dendrobatidae and of families and subfamilies which in the past were considered as their potential sister groups. Numbers are bootstrap values in percentage (2000 replicates; values below 50% not given; values of more than 70% in boldface). *Discoglossus pictus* was used as outgroup. Families Leptodactylidae and Bufonidae are abbreviated (L., B.).

and lack of (cephalic) mating amplexus evolved independently from the *Epipedobates–Phyllobates–Dendrobates* 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-Schlösser, 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 Hyloidea) 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 Hyloidea
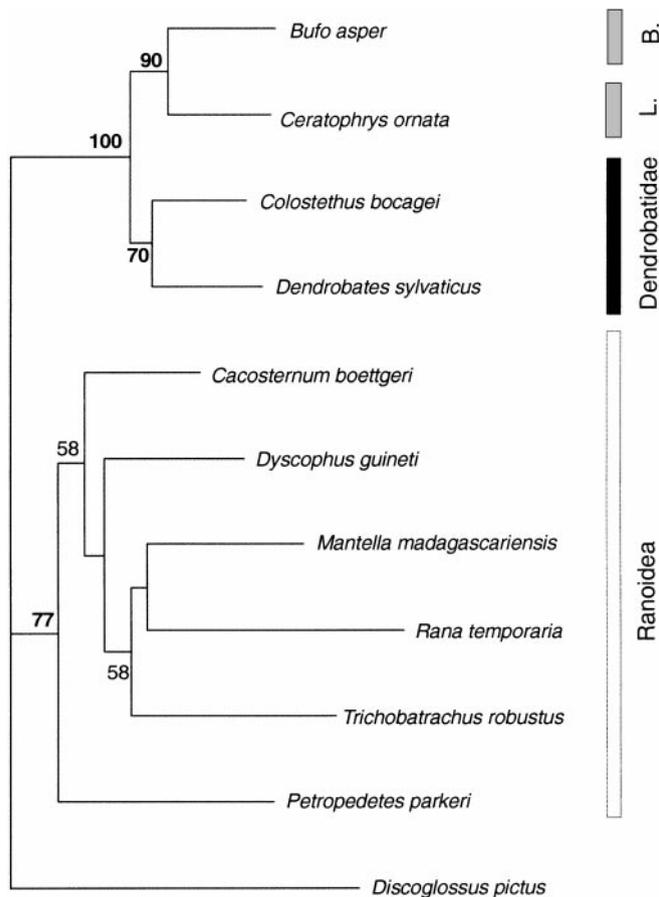
FIG. 2. Neighbor-joining tree based on 350 bp of the mitochondrial 12S ribosomal RNA gene sequences, including representatives of the Dendrobatidae and of families and subfamilies which in the past were considered as their potential sister groups. See Fig. 1 for additional explanations.
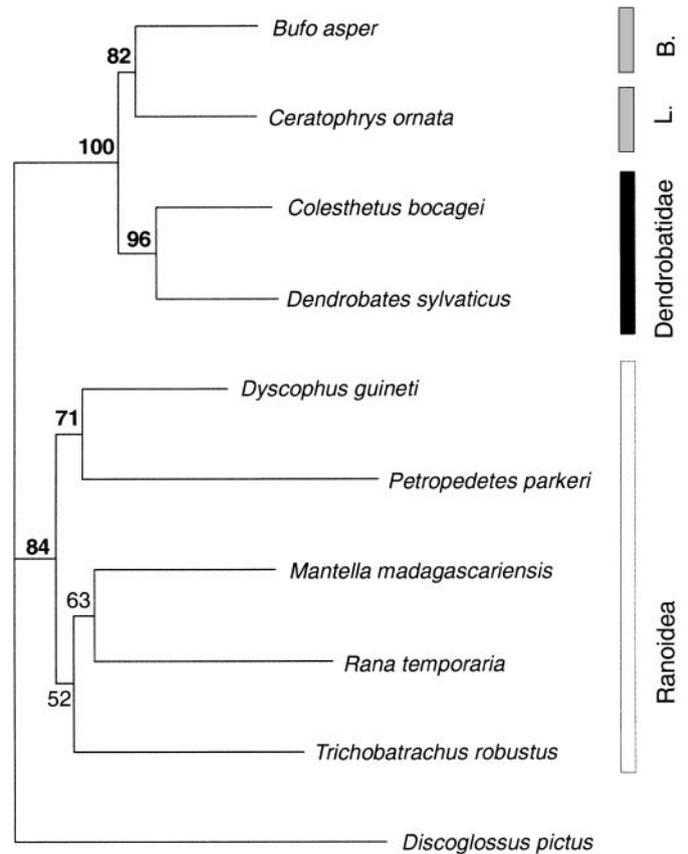
FIG. 3. Neighbor-joining tree based on a combined analysis of 518 bp of the mitochondrial 16S ribosomal RNA gene sequences and 350 bp of the mitochondrial 12S ribosomal RNA gene sequences. See Fig. 1 for additional explanations.

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

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 *Otophryne* (Zweifel, 1986; 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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