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Novel phylogenetic relationships of the enigmatic brevicipitine and scaphiophrynine toads as revealed by sequences from the nuclear *Rag-1* gene

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Owing to a general paucity of characters and an apparently high level of homoplasy, the systematics of frogs have remained disputed. A phylogeny based on the single-copy nuclear *Rag-1* gene revealed unexpected placements of scaphiophrynine and brevicipitine toads. The former have usually been considered as sister group to all other extant microhylids or are even classified as a separate family. Their basal position among microhylids was weakly indicated in our analysis; but they clearly are part of a strongly supported clade composed of representatives from five other microhylid subfamilies. By contrast, the brevicipitines, a group that hitherto was unanimously considered to belong to the Microhylidae, were highly divergent and placed as a sister group to the arthroleptoid clade. These novel phylogenetic placements are best reflected by a classificatory status of the Scaphiophryninae as a subfamily of the Microhylidae, whereas the brevicipitines may merit recognition as a distinct family. Our findings seem to corroborate a high degree of morphological homoplasy in frogs and suggest that even highly derived morphological states, such as the hydrostatic tongue of microhylids, hemisotids and brevicipitines, may be subject to convergent evolution, parallelism or character reversal.

Keywords: Amphibia; Microhylidae; Scaphiophryninae; Brevicipitinae; character reversal; homoplasy

1. INTRODUCTION

As a result of their pre-Gondwanan age and cosmopolitan distribution, amphibians are a good model system for the study of biogeography (Duellman & Trueb 1986; Feller & Hedges 1998). Their tolerance of salt water is limited; although they are capable of transoceanic dispersal (Vences *et al.* 2003), their distribution is likely to have been shaped in great part by vicariance (Duellman & Trueb 1986).

Application of molecular methods to the elucidation of amphibian phylogeny has revealed surprising instances of morphological homoplasy among regional radiations, for example of Madagascan and Indian tree frogs (Bossuyt & Milinkovitch 2000), or Indian and African burrowing frogs (Biju & Bossuyt 2003). These taxa belong to the Neobatrachia, a monophyletic group that contains the vast majority of the recent frogs (Feller & Hedges 1998; Hoegg *et al.* 2004).

Despite the renewed interest in the biogeographical and evolutionary history of anurans, one circumtropic neobatrachian family, the Microhylidae, has so far, to our knowledge, not comprehensively been studied through molecular phylogenetic analyses. Although single representatives of this family were included in some works (Feller & Hedges 1998; Biju & Bossuyt 2003), the intrafamilial relationships remain unstudied from a molecular perspective.

The Microhylidae contains 349 species in 67 genera (excluding scaphiophrynines), occurring in the Americas, sub-Saharan Africa, Madagascar, India and most of Southeast Asia to New Guinea and northernmost Australia (www.amphibiaweb.org, accessed 2003). Microhylids are defined by a uniquely derived tadpole morphology (type II of Orton 1952), by an osteological trend towards reduction of shoulder girdle elements, and by a specialized microphagous feeding behaviour with hydrostatic tongues (Meyers *et al.* 2004).

Among microhylids, the phylogenetic position of the eight species in the subfamily Scaphiophryninae from Madagascar is especially enigmatic. Scaphiophrynine tadpoles are intermediate between Orton's tadpole types II and IV (Orton 1952), the latter being the generalized neobatrachian type (Wassersug 1984). Scaphiophrynines were placed within the Ranidae until Guibé (1956) placed them into the Microhylidae. Savage (1973) suggested their inclusion in yet another family, the Hyperoliidae. Dubois (1992) raised them to family rank as Scaphiophrynidae. Another microhylid subfamily of uncertain affinities is the African Brevicipitinae, or rain frogs, composed of 18 species in five genera. Interestingly, these are the only microhylids in which direct development occurs, posing difficulties for an assessment of their larval features.

Here, we present data on the phylogenetic position of scaphiophrynine and brevicipitine toads using DNA sequences of a single-copy nuclear gene, *Rag-1*, which is known to provide an adequate resolution in the analysis of anuran relationships (Hoegg *et al.* 2004). Surprisingly our results indicate that brevicipitines might not belong to the microhylid lineage, whereas scaphiophrynines do, contrary to current classification and morphological evidence.

2. MATERIAL AND METHODS

Taxa were selected to cover major clades among ranoid neobatrachians to which microhylids are known to belong (Biju & Bossuyt 2003; Hoegg *et al.* 2004). We included taxa of six out of the nine microhylid subfamilies accepted by Duellman & Trueb (1986), i.e. all except the Asterophryinae, Genyophryninae and Melanobatrachinae. The archaeobatrachian *Xenopus* and several hyloid neobatrachians were used as hierarchical outgroups. A list of taxa and GenBank accession numbers is given in table 1.

DNA was extracted from muscle tissue stored at −80 °C or fixed in 70% ethanol. Tissue samples were digested using proteinase K (final concentration 1 mg ml^{-1}), homogenized and subsequently purified following a standard salt extraction protocol. We used primers as in Hoegg *et al.* (2004). PCR was performed in 25 µl reactions containing 0.5–1.0 units of REDTaq DNA polymerase (Sigma,

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Table 1. Taxa included in this study, voucher specimens, and GenBank accession numbers of *Rag-1* sequences.
(Collection acronyms: MNHN, Muséum National d'Histoire Naturelle, Paris, France; UADBA, Université d'Antananarivo, Département de Biologie Animale, Madagascar; ZFMK, Zoologisches Forschungsinstitut und Museum A. Koenig, Bonn, Germany; ZSM, Zoologische Staatssammlung München, Germany. SIH and MV refer to frozen tissue collections of S. Hoegg and M.V.)

name	family; microhylid subfamily	general distribution	voucher specimen	GenBank accession numbers
Xenopus laevis	Archaeobatrachia: Pipidae	sub-Saharan Africa	voucher not collected	L19324
Arthroleptis variabilis	Arthroleptidae	sub-Saharan Africa	ZFMK 68794	AY571642
Bufo bufo	Hyloidea: Bufonidae	Europe	voucher not collected	AY323762
Bufo regularis	Hyloidea: Bufonidae	Africa	SIH-04	AY323763
Hyla cinerea	Hyloidea: Hylidae	North America	SIH-06	AY323766
Hyla meridionalis	Hyloidea: Hylidae	Europe	voucher not collected	AY571662
Heterixalus tricolor	Hyperoliidae	Madagascar	ZSM 700/2001	AY323768
Hyperolius viridiflavus	Hyperoliidae	sub-Saharan Africa	ZFMK 66726	AY323769
Kassina maculata	Hyperoliidae	sub-Saharan Africa	ZFMK 66445	AY571651
Leptopelis natalensis	Hyperoliidae	South Africa	ZFMK 68785	AY571654
Aglyptodactylus madagascariensis	Mantellidae	Madagascar	ZSM 183/2002	AY571640
Boophis doulioti	Mantellidae	Madagascar	ZSM 185/2002	AY571643
Laliostoma labrosum	Mantellidae	Madagascar	UADBA-MV2001.1466	AY571652
Dermatonotus muelleri	Microhylidae: Microhylinae	South America	ZFMK uncatalogued	AY571647
Kaloula pulchra	Microhylidae; Microhylinae	Southeast Asia	SIH-09	AY323772
Scaphiophryne calcarata	Microhylidae: Scaphiophryninae	Madagascar	ZSM 115/2002	AY571660
Phrynomantis annectens	Microhylidae; Phrynomerinae	sub-Saharan Africa	ZFMK 66771	AY571657
Breviceps fuscus	Microhylidae; Brevicipitinae	South Africa	ZFMK 66716	AY571644
Plethodontohyla alluaudi	Microhylidae; Cophylinae	Madagascar	ZSM 3/2002	AY571661
Dyscophus antongilii	Microhylidae; Dyscophinae	Madagascar	voucher not collected	AY571648
Cacosternum boettgeri	Ranidae	sub-Saharan Africa	ZFMK 66727	AY571645
Fejervarya sp.	Ranidae	Southeast Asia	ZFMK uncatalogued (MV-PBl1)	AY571649
Hoplobatrachus occipitalis	Ranidae	sub-Saharan Africa	ZFMK 65186	AY571650
Lankanectes corrugatus	Ranidae	Sri Lanka	MNHN 2000.616	AY571653
Nyctibatrachus major	Ranidae	India	ZFMK uncatalogued	AY571655
Petropedetes cf. *parkeri*	Ranidae	sub-Saharan Africa	ZFMK uncatalogued	AY571656
Ptychadena mascareniensis	Ranidae	sub-Saharan Africa	ZSM 190/2002	AY571658
Rana (*Amnirana*) *lepus*	Ranidae	sub-Saharan Africa	MV-Cam1	AY571641
Rana (*Rana*) *temporaria*	Ranidae	Europe	voucher not collected	AY323776
Chirixalus cf. *vittatus*	Rhacophoridae	Southeast Asia to India	ZFMK 65463	AY571646
Polypedates maculatus	Rhacophoridae	Bangladesh, Nepal, Sri Lanka, India	voucher not collected	AY323777
Rhacophorus [*Polypedates*] *dennysii*	Rhacophoridae	Southeast Asia	ZFMK 65461	AY571659

Taufkirchen, Germany), 0.01 units of *Pwo* DNA polymerase (Roche, Mannheim, Germany), 50 ng of genomic DNA, 10 pmol of each primer, 15 nmol of each dNTP, 50 nmol of additional $MgCl_2$ and the REDTaq PCR reaction buffer (end concentrations: 10 mM of Tris–HCl, pH 8.3, 50 mM of KCl, 1.1 mM of $MgCl_2$ and 0.01% gelatine). Cycle conditions were adapted from a long-range PCR protocol (Barnes 1994), with an initial denaturation step at 94 °C for 5 min, followed by 10 cycles with 94 °C for 30 s, annealing temperatures increasing by 0.5 °C per cycle from 52 to 57 °C and extending for 3 min at 68 °C. An additional 20 cycles were performed with 94 °C for 10 s, 57 °C for 40 s and 68 °C for 3 min. The final extension was carried out at 68 °C for 5 min.

PCR products were purified with spin columns (QIAGEN). Sequencing was performed directly using the corresponding PCR primers (forward and reverse).

DNA sequences of both strands were obtained using the BigDye Terminator cycle-sequencing ready reaction kit (Applied Biosystems Inc.) on an ABI 3100 capillary sequencer using the manufacturer's instructions.

Maximum-parsimony (MP) and maximum-likelihood (ML) phylogenies were calculated using PAUP* (Swofford 2002). The best-fitting model of sequence evolution for ML analyses was obtained by MODELTEST v. 3.06 (Posada & Crandall 1998). Heuristic searches were performed using 10 replicates of a stepwise addition of taxa.

Robustness of the MP tree topology was tested by bootstrap analysis with 2000 replicates; 500 ML bootstrap replicates were calculated. Bayesian inference was conducted with MRBAYES v. 2.0 (Huelsenbeck & Ronquist 2001) using the general time-reversible model with one million generations, sampling trees every tenth generation (and calculating a consensus tree after omitting the first 5000 trees).

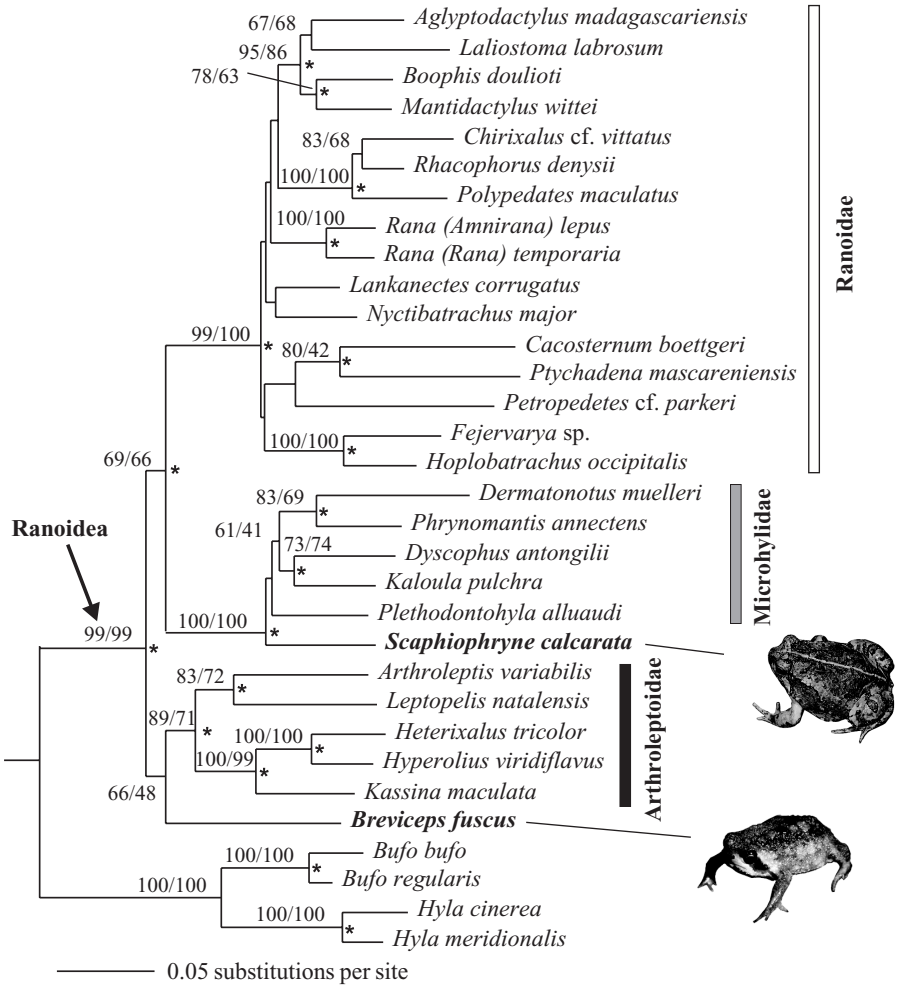

Figure 1. ML tree based on the analysis of 1566 bp of the *Rag-1* gene, highlighting the phylogenetic position of scaphiophrynines (*Scaphiophryne*) and brevicipitines (*Breviceps*) among ranoid neobatrachians. The numbers indicated on the branches are bootstrap support values in per cent of ML (100 replicates) and MP (2000 replicates) searches. Asterisks placed to the right of nodes indicate Bayesian posterior probabilities of greater than 95%. The tree was rooted with *Xenopus laevis* (not shown). The insert pictures show representatives of the genera *Scaphiophryne* and *Breviceps*.

We tested alternative phylogenetic hypotheses using Shimodaira–Hasegawa (SH) tests as implemented in PAUP*, with resampling estimated log-likelihood optimization and 1000 bootstrap replicates. To avoid biases by the previous selection of alternative topologies, we applied the SH test simultaneously to all possible unrooted trees in a reduced set of six taxa, containing *Breviceps*, *Scaphiophryne*, *Bufo regularis* as outgroup, and the microhylid, arthroleptoid and ranoid taxa with the shortest branch length each (*Plethodontohyla*, *Kassina*, *Lankanectes*), assuming that short branch lengths indicate a low number of autapomorphies that could mask phylogenetic affinities

3. RESULTS

The dataset consisted of 1566 DNA positions in 33 species. The trees obtained through MP, ML and Bayesian methods (figure 1) subdivide the ranoids into three well-supported major clades, corresponding to the epifamilies Ranoidae, Microhyloidae and the Arthroleptoidae as defined by Vences & Glaw (2001). The Ranoidae contained the families Rhacophoridae and Mantellidae, and the paraphyletic Ranidae. Within the Arthroleptoidae, the hyperoliid *Leptopelis* is a sister taxon of *Arthroleptis*, rendering Hyperoliidae paraphyletic with respect to *Arthroleptis*.

Microhylids formed a highly supported clade that contained *Scaphiophryne* but not *Breviceps*. Within this clade, relationships were poorly resolved owing to very short basal branch lengths, suggesting the possibility of a rapid lineage formation early in the evolution of this group. The ML and Bayesian analyses placed *Scaphiophryne* as a sister group to the remaining microhylids. However, this placement did not receive strong support, and the differentiation of *Scaphiophryne* within the clade of the remaining microhylids was small as indicated by the short branch lengths between splits in this clade. *Breviceps* did not group with other microhylids but instead was the sister group of the Arthroleptoidae.

All alternative tree topologies reflecting current classification, i.e. *Breviceps* is part of the Microhylidae whereas *Scaphiophryne* is not, were significantly rejected by the SH tests in the reduced set of taxa: *Plethodontohyla* was the sister group of *Scaphiophryne* and not of *Breviceps* in the reduced set of taxa analysed ($p < 0.001$). However, maintaining a sister-group relationship of *Scaphiophryne* and *Plethodontohyla*, alternative positions of *Breviceps* could not be significantly excluded; this also applied to its placement as the sister group to the (*Scaphiophryne*, *Plethodontohyla*) clade.

4. DISCUSSION

Larvae of *Scaphiophryne* are characterized by morphological characteristics that are considered to be

plesiomorphic relative to the highly specialized, suspension-feeding microhylid type (Wassersug 1984; Haas 2003). Their derived larval traits define microhylids as monophyletic group to the exclusion of *Scaphiophryne*. The most parsimonious phylogeny based on this character complex therefore would predict this genus to occupy a distinctly basal position relative to other microhylids. However, what seems clear from the tree shape (figure 1) is that scaphiophrynines did not diverge particularly early in microhylid evolution but were one of the major lineages in the initial radiation of these frogs. This indicates a fast evolutionary transition from the *Scaphiophryne*-like tadpole morphology to a derived microhylid tadpole. Also, the tree presented by Biju & Bossuyt (2003) was unambiguous in suggesting a sister-group relationship between scaphiophrynines and other microhylids. From a classificatory point of view, this phylogenetic pattern would best be reflected by a status as a subfamily of the Microhylidae rather than as separate family.

The family Microhylidae, according to our analysis, contains endemic genera from South America (*Dermatonotus*), Asia (*Kaloula*), Africa (*Phrynomantis*) and Madagascar (*Dyscophus*, *Scaphiophryne*, *Plethodontohyla*). The Madagascan taxa were not a monophyletic clade. Instead, *Dyscophus* grouped with the Asian *Kaloula*, indicating possible intercontinental relationships parallel to those of the rhacophorid (Asia) and mantellid (Madagascar) tree frogs (Bossuyt & Milinkovitch 2000; Biju & Bossuyt 2003).

Surprisingly, the African *Breviceps* (Brevicipitinae) was resolved not as being part of the microhylid clade, but grouped with the Arthroleptoidae. This placement differs from conclusions based on morphological and mitochondrial characters (e.g. Emerson *et al.* 2000). Our data were not sufficient significantly to exclude all alternative phylogenetic hypotheses, but the SH tests did significantly exclude the classical hypothesis in which the brevicipitines are part of the Microhylidae to the exclusion of *Scaphiophryne*. If confirmed by further datasets, the grouping favoured by our analysis would suggest that the specialized hydrostatic tongue that is characteristic for microhylids, including brevicipitines (Meyers *et al.* 2004), was reversed back to a more generalized state in the Arthroleptoidae, or, possibly even more interestingly, evolved convergently or in parallel at least twice (in brevicipitines and in microhylids). This hypothesis is further supported by the finding that *Hemisus*, another taxon characterized by a hydrostatic tongue, groups with arthroleptoids rather than with microhylids as usually thought (Biju & Bossuyt 2003). The separate phylogenetic placement of brevicipitines from other microhylids, together with the possession of several striking morphological specializations shared only with the Microhylidae and Hemisotidae, might justify a change in their classificatory assignment, i.e. inclusion in their own family.

Microhylids are characterized by a high variability in their osteological characters owing to the repeated evolution of fossoriality and the effects of miniaturization (Wild 1995). Osteological characters are usually more conservative in anurans and are therefore considered to be informative features for higher-level taxonomy (Duellman & Trueb 1986). The unexpected molecular phylogenetic placement of scaphiophrynine and brevicipitine toads, if further confirmed, could provide a vivid example for the high level of homoplasy in morphological characters in anurans and indicates that, in these organisms, convergent evolution and reversals may be possible even in seemingly highly derived morphological traits.

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Barnes, W. M. 1994 PCR amplification of up to 35 kb DNA with high fidelity and high yield from λ bacteriophage templates. *Proc. Natl Acad. Sci. USA* **91**, 2216–2220.

Biju, S. D. & Bossuyt, F. 2003 New frog family from India reveals an ancient biogeographical link with the Seychelles. *Nature* **425**, 711–714.

Bossuyt, F. & Milinkovitch, M. C. 2000 Convergent adaptive radiations in Madagascan and Asian ranid frogs reveal covariation between larval and adult traits. *Proc. Natl Acad. Sci. USA* **97**, 6585–6590.

Dubois, A. 1992 Notes sur la classification des Ranidae (amphibiens anoures). *Bull. Mens. Soc. Linn. Lyon* **61**, 305–352.

Duellman, W. E. & Trueb, L. 1986 *Biology of amphibians*. New York: McGraw-Hill.

Emerson, S. B., Richards, C., Drewes, R. C. & Kjer, K. M. 2000 On the relationships among ranoid frogs: a review of the evidence. *Herpetologica* **56**, 209–230.

Feller, A. E. & Hedges, S. B. 1998 Molecular evidence for the early history of living amphibians. *Mol. Phylogenet. Evol.* **9**, 509–516.

Guibé, J. 1956 La position systématique des genres *Pseudohemisus* et *Scaphiophryne* (Batraciens). *Bull. Mus. Natn. Hist. Nat. Ser.* **228**, 180–182.

Haas, A. 2003 Phylogeny of frogs as inferred from primarily larval characters (Amphibia: Anura). *Cladistics* **19**, 23–89.

Hoegg, S., Vences, M., Brinkmann, H. & A. Meyer, A. 2004 Phylogeny and comparative substitution rates of frogs inferred from sequences of three nuclear genes. *Mol. Biol. Evol.* (In the press.)

Huelsenbeck, J. P. & Ronquist, F. 2001 MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755.

Meyers, J. J., O'Reilly, J. C., Monroy, J. A. & Nishikawa, K. C. 2004 Mechanism of tongue protraction in microhylid frogs. *Exp. Biol.* **207**, 21–31.

Orton, G. L. 1952 Key to the genera of tadpoles in the United States and Canada. *Am. Midl. Nat.* **47**, 382–395.

Posada, D. & Crandall, K. A. 1998 MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Savage, J. M. 1973 The geographic distribution of frogs: patterns and predictions. In *Evolutionary biology of the Anurans: contemporary research on major problems* (ed. J. L. Vial), pp. 351–445. Columbia, MO: University of Missouri Press.

Swofford, D. L. 2002 *PAUP*. Phylogenetic analysis using parsimony (*and other methods)*, v. 4beta10. Sunderland MA: Sinauer.

Vences, M. & Glaw, F. 2001 When molecules claim for taxonomic change: new proposals on the classification of Old World treefrogs. *Spixiana* **24**, 85–92.

Vences, M., Vieites, D. R., Glaw, F., Brinkmann, H., Kosuch, J., Veith, M. & Meyer, A. 2003 Multiple overseas dispersal in amphibians. *Proc. R. Soc. Lond.* B **270**, 2435–2442. (DOI 10.1098/rspb.2003.2516.)

Wassersug, R. J. 1984 The *Pseudohemisus* tadpole: a morphological link between microhylid (Orton type 2) and ranoid (Orton type 4) larvae. *Herpetologica* **40**, 138–149.

Wild, E. R. 1995 New genus and species of Amazonian microhylid frog with phylogenetic analysis of New World genera. *Copeia* **1995**, 837–849.