First publ. in: Molecular Phylogenetics and Evolution 44 (2007), 3, pp. 1017–1030

Nuclear gene phylogeny of narrow-mouthed toads (Family: Microhylidae) and a discussion of competing hypotheses concerning their biogeographical origins

Arie van der Meijden [a,e], Miguel Vences [b], Simone Hoegg [a], Renaud Boistel [c], Alan Channing [d], Axel Meyer [a,*]

[a] *Lehrstuhl für Zoologie und Evolutionsbiologie, Department of Biology, University of Konstanz, P.O. Box M618, 78457 Konstanz, Germany*
[b] *Division of Evolutionary Biology, Zoological Institute, Technical University Braunschweig, Spielmannstr. 8, 38106 Braunschweig, Germany*
[c] *Equipe Communications Acoustiques, NAMC, CNRS UMR 8620, IBAIC Bat 446 Université Paris Sud, F-91405 Orsay, France*
[d] *Department of Biodiversity and Conservation Biology, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa*
[e] *Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, P.O. Box 94062, 1090 GB Amsterdam, The Netherlands*

Abstract

The family Microhylidae has a large circumtropic distribution and contains about 400 species in a highly subdivided taxonomy. Relationships among its constituent taxa remained controversial due to homoplasy in morphological characters, resulting in conflicting phylogenetic hypotheses. A phylogeny based on four nuclear genes (*rag-1, rag-2, tyrosinase, BDNF*) and one mitochondrial gene (*CO1*) of representatives of all currently recognized subfamilies uncovers a basal polytomy between several subfamilial clades. A sister group relationship between the cophylines and scaphiophrynines is resolved with moderate support, which unites these endemic Malagasy taxa for the first time. The American members of the subfamily Microhylinae are resolved to form a clade entirely separate from the Asian members of that subfamily. *Otophryne* is excluded from the subfamily Microhylinae, and resolved as a basal taxon. The placement of the Asian dyscophine *Calluella* nested within the Asian Microhyline clade rather than with the genus *Dyscophus* is corroborated by our data. Bayesian estimates of the divergence time of extant Microhylidae (47–90 Mya) and among the subclades within the family are discussed in frameworks of alternative possible biogeographic scenarios.

Keywords: Amphibia; Microhylidae; Mirohylinae; Dyscophinae; Asterophryinae; Cophylinae; Scaphiophryninae; Hoplophryninae; Gastrophryninae; Phrynomerinae; Molecular phylogeny

1. Introduction

Amphibians have long been regarded as model organisms for the study of biogeography due to their alleged inability to disperse across salt water. Several recent molecular studies have shed new light on the biogeography of anurans (Bossuyt and Milinkovitch, 2001; Biju and Bossuyt, 2003; San Mauro et al., 2005; Van der Meijden et al., 2005). Although plate tectonics and terrestrial dispersal are generally assumed to be the dominant forces shaping large-scale patterns of anuran biogeography, transoceanic dispersal has been shown to have occurred in several instances (Hedges et al., 1992; Vences et al., 2003b; Measey et al., 2007).

The frogs of the family Microhylidae occur in the Americas, sub-Saharan Africa, Madagascar, India, and most of Southeast Asia to New Guinea and northernmost Australia, while the highest numbers of species are found in Southeast Asia and Madagascar. This wide distribution makes the Microhylidae a model case for biogeographical

* Corresponding author. Fax: +49 7531 883018.
E-mail address: Axel.Meyer@uni-konstanz.de (A. Meyer).

inference. A typical Gondwanan distribution suggests that their current distribution is primarily due to large-scale vicariance events. Savage (1973) proposed that the early microhylids were present on Gondwana, and their current distribution on Gondwanan continents is due to the break-up of the supercontinent. India and Australia subsequently were the origin of the Southeast Asian microhylids through dispersal from there, while some of the South American taxa spread northward to North America. More recently, Feller and Hedges (1998) proposed an "Out of Africa" pattern of dispersal for this family. They suggested dispersal from Africa to Asia and to North and South America as well as to Madagascar.

Although they are generally characterized as stout bodied mostly fossorial ant- and termite-specialists, microhylids are actually rather diverse in their biology. Most are fossorial or terrestrial, but arboreal forms also exist. The Asterophryinae and Genyophryninae of Southeast Asia and Australia undergo direct development, whereas all cophylines have endotrophic, non-free living tadpoles (McDiarmid and Altig, 1999). Both the subfamilies Microhylinae and Hoplophryninae contain species that have endotrophic larvae and species that have feeding and free swimming larvae. Although free exotrophic larvae are considered to be plesiomorphic for ranoid frogs, only about a third of the microhylid species retained this characteristic.

Approximately one in every five frog genera belongs to the Microhylidae. While this family encompasses 20% (64) of all frog genera, it includes only 8% (approximately 400) of the world's frog species (Amphibiaweb, 2006), a low average number of species per genus. There are on average only 5.2 species per genus, while families with a wide distribution like the Bufonidae and the Hyperoliidae have 13.8 and 13.4 species per genus, respectively. Of the 64 genera, 22 are monotypic.

Microhylids display a high level of morphological diversity. This high variability in morphological characters, especially of the cranium and pectoral girdle (Parker, 1934; Blommers-Schlosser, 1993), and high levels of homoplasy due to loss of pectoral girdle elements in members of all subfamilies has complicated the use of these characters for microhylid taxonomy (Zweifel, 1986). The high variability of these elements might be a by-product of miniaturization (as in Hanken and Wake, 1993), independently recurring in several clades. Wild (1995) attributed the high variability in their osteological characters to the effects of miniaturization and the inferred repeated evolution of fossoriality. This high diversity and frequent homoplasy of morphological characters (Zweifel, 1986; Blommers-Schlosser, 1993; Loader et al., 2004) is probably the cause of the severe taxonomic subdivision of the Microhylidae in eight subfamilies and the high genus to species ratio. Its biological and morphological diversity organized in a highly subdivided taxonomy, in combination with their puzzling distribution, has gained the Microhylidae a reputation of being systematically difficult (Duellman, 1979; Ford and Cannatella, 1993).

In this work, we will use the terms Microhylidae and microhylids sensu Frost et al. (2006) to refer to the monophyletic microhylid group excluding the African subfamily Brevicipitinae, which has been shown to be closer related to hyperoliids and arthroleptids than to the non-brevicipitine microhylids in previous studies (Darst and Cannatella, 2004; Loader et al., 2004; Van der Meijden et al., 2004; Frost et al., 2006).

Recent molecular phylogenetic studies (Sumida et al., 2000; Hoskin, 2004; Loader et al., 2004; Van der Meijden et al., 2004; Andreone et al., 2005) focussed on a comparatively small part of the circumtropic distribution of the Microhylidae, or concentrated on one or more subtaxa. Frost et al. (2006) presented the first broad phylogenetic hypothesis for the Microhylidae, but like Van Bocxlaer et al. (2006), omitted some taxa of controversial placement, like *Otophryne* and *Paradoxophyla*. In this paper, we present an inclusive molecular phylogeny of the Microhylidae, sampling all eight subfamilies. We also included a range of ranoid and arthroleptoid frogs as outgroups, to further establish the position of the Microhylidae relative to these taxa. We discuss the systematic and biogeographic implications of our findings.

2. Materials and methods

2.1. Higher-level classification

Numerous classificatory schemes for anurans have been proposed but, although tending to incorporate novel phylogenetic information as it became available, they disagreed in the ranks assigned to family-group taxa. Van der Meijden et al. (2005) distinguished three monophyletic lineages within a superfamily Ranoidea: Arthroleptoidae, Microhyloidae and Ranoidae. These lineages were named "epifamilies" following Dubois (1992) but in a subsequent paper, Dubois (2005) suggested a different epifamily definition in which this rank applies to units above the superfamilial level. We disagree with the classification proposed by Dubois (2005) which sank a number of well-established and largely well-defined families such as Arthroleptidae, Astylosternidae, Hemisotidae, Hyperoliidae as subfamilies into a new family Brevicipitidae, but rather agree with Frost et al. (2006) to maintain these families. Likewise we consider the new taxonomic assignments of ranid subfamilies by Scott (2005) as premature and probably in many cases not reflecting the correct phylogeny. The high rates of discovery of new species as well as active work on the phylogenetic relationships of amphibians (Köhler et al., 2005) also require modifications of their classification and, in our opinion, warrant the recognition of additional new families rather than a reduction of the number of family group taxa. For the purpose of this paper we follow the scheme used in Van der Meijden et al. (2005) without referring to "epifamilial" taxa but rather will describe the clades, and accept the Brevicipitidae as a separate family (including the genera *Balebreviceps*, *Breviceps*, *Callulina*, *Probreviceps*, *Spelaeophryne*) following Frost et al. (2006).

2.2. Selection of taxa

Our sampling consisted of 34 microhylid species representing all currently recognized subfamilies. We included several outgroup taxa to resolve the relationships of the Microhylidae to its closest relatives. To represent the two major clades in the Ranoidea besides the Microhylidae we included (1) the arthroleptids *Arthroleptis* and *Trichobatrachus*, the brevicipitids *Breviceps* and *Callulina*, the hemisotid *Hemisus*, and the hyperoliids *Hyperolius* and *Leptopelis*, and (2) the ranid *Rana* and two mantellids of the genus *Mantidactylus*. The hylids *Litoria* and *Agalychnis*, and the pipids *Xenopus* and *Pipa* as well as two species of *Alytes* were included to further represent major groups in the anuran phylogeny. We further included the salamander *Lyciasalamandra*, a bird and a mammal to account for the synapsid–diapsid split for molecular clock calibration, and the lungfish *Protopterus* as outgroup.

2.3. Sequencing and alignment

DNA was extracted from toe clips fixed in 99% ethanol. Tissue samples were digested using proteinase K (final concentration 1 mg/mL), homogenized and subsequently purified following a high-salt extraction protocol (Bruford et al., 1992). Primers for *rag-1* and *rag-2* were from Hoegg et al. (2004). Primers for *tyrosinase* from Bossuyt and Milinkovitch (2000) were used as in Vences et al. (2003a). Primers were designed for an amplification of a 700 bp fragment of BDNF (Brain-derived neurotrophic factor) (BDNF.Amp.F1 ACCATCCTTTTCCTTACTATGG, BDNF.Amp.R1 CTA TCT TCC CCT TTT AAT GGT C). *CO1* primers were from Hebert et al. (2003, 2004) and used as in Vences et al. (2005). PCR was performed in 25 µl reactions containing 0.5–1.0 units of REDTaq DNA Polymerase (Sigma, Taufkirchen, Germany), 50 ng genomic DNA, 10 pmol of each primer, 15 nmol of each dNTP, 50 nmol additional $MgCl_2$ and the REDTaq PCR reaction buffer (in final reaction solution: 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$ and 0.01% gelatin). For *rag-1* and *rag-2* 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 ten 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. Additionally, 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 done at 68 °C for 5 min. PCR products were purified via 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. New sequences were combined with existing sequences taken from GenBank in the final dataset. New sequences were deposited in GenBank (for Accession numbers see Table 1).

Chromatograms were checked by eye using Sequencher (Gene Codes Corp., Ann Arbor, USA) or Chromas v.1.45 (Technelysium Pty Ltd., Tewantin, Australia) and the sequences were subsequently aligned using the Mega3 alignment editor (Kumar et al., 2004). The sequences were aligned using ClustalW (Thompson et al., 1994) with a gap opening penalty of 15 and a gap extension penalty of 6.66. The alignment was checked by eye based on the amino acid sequence. In spite of attempts to design specific primers, for a few taxa, not all gene sequences could be obtained (Table 1). Except a few outgroup taxa and two species where complete data sets were available from other, clearly congeneric species (*Stumpffia* and *Microhyla*), this mainly regards *Hoplophryne* (only partial *rag*-1 sequence, *COI* missing) and *Hamptophryne* (*tyrosinase* and *COI* missing). Missing genes were coded as "missing" (?) in the concatenated dataset. In Bayesian Inference (BI) and Maximum Parsimony (MP) analyses of large multigene data sets of hylid frogs, no relationship has been found between completeness of the sequence data of a taxon, and the support values the taxon receives (Wiens et al., 2005), suggesting that the limited amount of missing data in our concatenated alignment is unlikely to distort the phylogenetic results. Furthermore, the placement of taxa with missing data in our analysis was congruent between the single gene analyses in which the taxon was represented by a complete sequence, and the combined dataset, suggesting that omission of one or more genes from the combined set did not influence the placement of particular taxa.

2.4. Data analysis

A homogeneity partition test (Farris et al., 1994) as implemented in PAUP* (Swofford, 2002) rejected homogeneity of the different markers ($P = 0.01$). Besides an analysis of the combined data set we therefore also performed separate analyses of each of the various genes. Transitions and transversions were plotted against F84 distances (Felsenstein, 1984) for the separate gene lignments. Of the nuclear genes, only *tyrosinase* showed slight deviance from a linear relationship between the number of transitions and the genetic distance for the most distantly related outgroup taxa. This gene was therefore excluded from the divergence time estimates.

Phylogeny reconstruction based on the separate and combined datasets was performed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. The best fitting models of sequence evolution were determined by the AIC criterion in Modeltest 3.7 (Posada and Crandall, 1998). ML tree searches were performed using PhyML, version 2.4.4 (Guindon and Gascuel, 2003). Bootstrap branch support values were calculated with 500 replicates. The Bayesian analyses of the combined and separate datasets were conducted with MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001), using models estimated with Modeltest under the AIC criterion, with 250,000 generations,

Table 1
Voucher specimens and accession numbers of taxa studied

Species	Family	Locality, Voucher	Accession number				
			rag-1	*rag-2*	*tyrosinase*	*BDNF*	*CO1*
Anodonthyla boulengerii	Microhylidae	Foulpointe, Madagascar, ZSM 264/2002	EF396072	EF396110	EF395959	EF395994	EF396036
Anodonthyla montana	Microhylidae	Andringitra, Madagascar, UADBA uncatalogued (fieldnumber MV 2001.530)	EF396071	EF396111	EF395960	EF395995	EF396037
Asterophrys turpicola	Microhylidae	Steve Richards, SJR5795	EF396074	EF396113	EF395961	EF395996	EF396038
Calluella guttulata	Microhylidae	Ubon province, Thailand, ZSM434/2002	EF396078	EF396115	EF395964	EF395999	EF396041
Chiasmocleis hudsoni	Microhylidae	French Guyana, MNHN uncatalogued	EF396079	EF396118	EF395967	EF396001	EF396043
Chiasmocleis shudikarensis	Microhylidae	French Guyana, personal collection Christian Marti, 119MC	EF396080	EF396117	EF395966	EF396002	EF396044
Cophixalus sp.	Microhylidae	Steve Richards, SJR3271	EF396081	EF396119	EF395968	EF396003	EF396045
Dermatonotus muelleri	Microhylidae	Paraguay, voucher not preserved	EF396082	EF396120	EF395969	EF396004	EF396046
Dyscophus antongilii	Microhylidae	Maroantsetra, Madagascar (no voucher)	EF396084	EF396122	EF395970	EF396005	EF396047
Dyscophus insularis	Microhylidae	Antsirasira, Madagascar, UADBA uncatalogued (fieldnumber MV 2001.38)	EF396083	EF396121	EF395971	EF396006	EF396048
Elachistocleis ovalis	Microhylidae	French Guyana, personal collection Christian Marti, 146MC	EF396085	EF396123	EF395972	EF396007	EF396049
Gastrophryne carolinensis	Microhylidae	Pet trade (no voucher)	EF396086	EF396124	EF395973	EF396008	EF396050
Glyphoglossus molossus	Microhylidae	Sagaing, Myanmar, CAS 210056	EF396087	EF396125	EF395974	EF396009	EF396051
Hamptophryne boliviana	Microhylidae	French Guyana, personal collection Michel Blanc, 42BM	EF396088	EF396126	Missing	EF396010	†Missing
Hoplophryne rogersi	Microhylidae	Bamba Ridge Forest Reserve, Tanzania	Missing	EF396128	EF395976	EF396012	†Missing
Hypopachus variolosus	Microhylidae	Nayarit, Mexico, MVZ 144018	EF396090	EF396129	EF395977	EF396014	EF396052
Kaloula pulchra	Microhylidae	Pet trade (no voucher)	EF396091	EF396130	EF395978	EF396015	EF396053
Microhyla butleri	Microhylidae	Vinh Phu Province, Vietnam, MVZ 223728	EF396094	EF396132	Missing	EF396019	EF396054
Microhyla heymonsi	Microhylidae	Hainan province, China, MVZ 236751	EF396095	EF396133	EF395979	EF396020	EF396055
Microhyla pulchra	Microhylidae	Vinh Phu Province, Vietnam, MVZ 223797	EF396093	EF396134	EF395980	EF396021	EF396056
Micryletta inornata	Microhylidae	Pai near Chiang Mai, Thailand (voucher not preserved)	EF396096	EF396135	EF395981	EF396022	EF396057
Otophryne pyburni	Microhylidae	French Guyana, personal collection Christian Marti, 1116MC	EF396097	EF396136	EF395982	EF396023	EF396058
Paradoxophyla palmata	Microhylidae	Ranomafana, Madagascar, ZSM 792/2003	EF396098	EF396137	EF395983	EF396024	EF396059
Phrynomantis annectens	Microhylidae	Ongongo, Namibia, ZFMK 66771	EF396099	EF396139	EF395985	EF396025	EF396060
Phrynomantis bifasciatus	Microhylidae	Coast province, Kenya, MVZ 234047	EF396100	EF396138	EF395984	EF396026	EF396061
Platypelis grandis	Microhylidae	Mantadia, Madagascar, ZSM 163/2002	EF396101	EF396140	EF395986	EF396027	EF396062
Plethodontohyla alluaudi	Microhylidae	Andasibe, Madagascar, ZSM 3/2002	EF396102	EF396141	EF395988	EF396028	EF396063
Plethodontohyla brevipes	Microhylidae	Ranomafana, Madagascar, ZSM 649/2003	EF396103	EF396142	EF395987	EF396029	EF396064
Ramanella cf. *obscura*	Microhylidae	Kandy, Sri Lanka, MNHN 2000.628	EF396104	EF396143	EF395989	EF396030	EF396065
Rhombophryne testudo	Microhylidae	Nosy Be, Madagascar, ZSM 475/2000	EF396105	EF396144	EF395990	EF396031	EF396066
Scaphiophryne calcarata	Microhylidae	Madagascar, ZSM 115/2002	EF396106	EF396145	EF395991	EF396032	EF396067
Stumpffia psologlossa	Microhylidae	Nosy Be, Madagascar (tissue 2001f42)	EF396107	Missing	EF395992	EF396033	EF396068
Stumpffia pygmaea	Microhylidae	Nosy Be, Madagascar (tissue 2001f21)	EF396108	EF396146	EF395993	EF396034	EF396069
Callulina kreffti	Brevicipitidae	Tanga region, Tanzania, MVZ 234046	EF396077	EF396116	EF395965	EF396000	EF396042
Breviceps fuscus	Brevicipitidae	Big Tree, South Africa, ZFMK 66716	EF396075	DQ019520	EF395962	EF395997	EF396039
Breviceps mossambicus	Brevicipitidae	Kwambonambi, South Africa, ZFMK 68849	EF396076	EF396114	EF395963	EF395998	EF396040
Hemisus marmoratus	Hemisotidae	Coast province, Kenya, MVZ 233793	AY364216	EF396127	EF395975	EF396011	EF396070
Hyperolius viridiflavus	Hyperoliidae	Barberton, South Africa, ZFMK 66726	AY323769	AY323789	AF249161	EF396013	Missing
Arthroleptis variabilis	Arthroleptidae	Cameroon, ZFMK 68794	EF396073	EF396112	AY341756	Missing	Missing
Trichobatrachus robustus	Arthroleptidae	Nkongsamba, Cameroon, ZFMK 66453	EF396109	EF396147	AY844192	EF396035	Missing
Leptopelis natalensis	Hyperoliidae	Mtunzini, South Africa, ZFMK 68785	EF396092	EF396131	AY341755	EF396016	Missing
Mantidactylus wittei	Mantellidae	Madagascar, ZSM 405/2000	AY323774	AY323795	AY341751	EF396018	Missing
Mantidactylus sp. "Comoros"	Mantellidae	Mayotte, ZSM 652/2000	AY323775	AY323794	AY341750	EF396017	Missing
Rana (temporaria)	Ranidae	Voucher not collected	AY323776	AY323803	AF249182	EF407507	Missing
Agalychnis callidryas	Hylidae	Pet trade (no voucher)	AY323765	AY323780	AY844153	EF407508	Missing
Litoria caerulea	Hylidae	Pet trade (no voucher)	AY323767	AY323793	AY844131	EF407509	Missing

Alytes muletensis	Bombinatoridae	Mallorca, Spain (no voucher)	AY323755	AY323781	AY341747	EF407510	Missing
Alytes dickhilleni	Bombinatoridae	Parejo, Spain (no voucher)	DQ019494	DQ019517	EF407506	EF407511	Missing
Pipa parva	Pipidae	Pet trade (no voucher)	AY874304	AY323799	AY341762	EF407512	Missing
Xenopus sp.	Pipidae	GenBank	AY874355	L19325	AY333967	BC082887	Missing
Lyciasalamandra	Salamandridae	GenBank	AY456261	AY323797	AY341765	AF497712	Missing
Gallus gallus	Aves: Phasianidae	GenBank	XM 421090	AY443150	D88349	XM 419645	Missing
Homo sapiens	Mammalia: Hominidae	GenBank	AY130302	NM 000536	U01873	AF411339	Missing
Protopterus	Dipnoi: Protopteridae	GenBank	AY442928	AF369086	Missing	U93369	Missing

Localities and voucher specimens refer to sequences obtained in this study. Some other sequences from Genbank refer to other conspecific individuals. Collection acronyms are as follows: MNHN, Muséum National d'Histoire Naturelle, Paris; MVZ, Museum of Vertebrate Zoology, University of California at Berkeley, USA; UADBA, Université d'Antananarivo, Département de Biologie Animale, Madagascar, numbers being fieldnumbers of M. Vences of specimens deposited in UADBA; ZFMK, Zoologisches Forschungsinstitut und Museum A. Koenig, Bonn, Germany; ZSM, Zoologische Staatssammlung München, Germany.
Accession numbers marked with an asterisk indicate sequences of congeneric species, except for *Lyciasalamandra* which we combined with a *tyrosinase* sequence of a different salamander genus, *Hynobius* and a *BDNF* sequence of *Tylototriton*, and *galychnis*, which was combined with a *tyrosinase* sequence of *Phyllomedusa hypochondrionalis*.

sampling trees every 10th generation (and calculating a consensus tree after omitting the first 3000 trees).

In addition to analyzing the dataset produced for this study, we analyzed the molecular data of Frost et al. (2006) for the Microhylidae and Brevicipitidae, using sequences of *Rana* and *Litoria* as outgroups. This dataset consisted of nuclear DNA sequences of histone *H*3, *rhodopsin*, *seven in absentia* (*SIA*), *tyrosinase*, the 28*S* ribosomal subunit and a section of mitochondrial DNA spanning 12*S*, The *Val tRNA* and 16*S* sequences. Sequences for 34 species, including five outgroup species, were downloaded from GenBank and aligned using ClustalW (Thompson et al., 1994) with a gap opening penalty of 15 and a gap extension penalty of 6.66. Hypervariable and gapped regions of the rRNA sequences were removed from the alignment. The amphibian origin of all downloaded sequences was confirmed by blasting them against the GenBank database using the NetBlast program, version 2.2.15 for win32 (NCBI). The best fitting models of sequence evolution were determined by the AIC criterion in Modeltest 3.7 (Posada and Crandall, 1998). ML and MP phylogenetic reconstructions were performed based on the combined alignment of 4045 basepairs using PhyML version 2.4.4 (Guindon and Gascuel, 2003) and Paup* (Swofford, 2002), respectively.

2.5. Divergence time estimation

Bayesian divergence time estimates were conducted using the software packages PAML (Yang, 1997) and Multidivtime (Thorne and Kishino, 2002) using a dataset consisting of only the nuclear genes *rag-1*, *rag-2*, and *BDNF* (2919 bp). The following calibration points were used: (1) minimum age of the frog–salamander split at 230 Mya (fossil record of frog ancestor *Triadobatrachus*; (Sanchiz, 1998)); (2) minimum age of the split between *Agalychnis* and *Litoria* at 42 Mya (last connection between Australia and South America; (Seddon et al., 1998)); (3) maximum age of the split between *Mantidactylus wittei* and *Mantidactylus* sp. from the Comoro islands at 15 Mya (volcanic origin of the oldest Comoro island Mayotte; (Vences et al., 2003b)); (4) minimum age of the *Alytes muletensis–Alytes dickhilleni* split at 5 Mya (Mediterranean salinity crisis: Fromhage et al., 2004); (5) age interval of the split between diapsids and synapsids at 338–288 Mya (Graur and Martin, 2004); (6) a minimum age of 338 Mya for the divergence between Lissamphibia and Amniota based on the aïstopod fossil, *Lethiscus stocki* (Ruta et al., 2003); (7) minimum age for the divergence of the South American *Pipa* and the African *Xenopus* of 110 Mya, corresponding to the final separation of South America from Africa (Sanmartin and Ronquist, 2004).

3. Results

The concatenated alignment with a total of 4143 bp consisted of *rag-1*, *rag-2*, *BDNF*, *tyrosinase* and *CO1* with 1380, 819, 720, 651, and 573 bp, respectively. *Rag-1* showed 773 variable and 637 parsimony-informative sites, whereas *rag-2* had

593 variable and 516 parsimony-informative positions. *BDNF* appeared to have a relatively slow mutation rate, with only 221 variable and 114 parsimony-informative sites. *Tyrosinase* had 399 variable sites of which 333 were parsimony-informative. The mitochondrial *CO1* alignment had 267 variable sites and 241 were parsimony-informative positions.

No well supported incongruencies among the results of the separate gene analyses and the combined analysis were found. The ML phylogeny based on the complete dataset (Fig. 1), rooted with *Protopterus, Homo, Gallus*, *Alytes* and the pipids (removed from Fig. 1 for better graphical representation) shows a highly supported clade composed of the Hylidae as represented by *Agalychnis* and *Litoria* sister to the Ranoidea clade. Resolution at the base of the Ranoidea clade is low, but the three major clades within the Ranoidea (see Van der Meijden et al., 2005) are highly supported. *Leptopelis* is placed as sister to the astylosternid *Trichobatrachus* and separated from the hyperoliid *Hyperolius*. Sister to the clade formed by the arthroleptids, astylosternids and hyperoliids, the brevicipitids form a clade as sister group to *Hemisus* (Hemisotidae), providing further evidence that they are not part of the Microhylidae as shown earlier using only *rag-1* (Van der Meijden et al., 2004) and using both mitochondrial and nuclear sequences as well as morphological data by Frost et al. (2006).

Within the Microhylidae, basal resolution is low, but there are several well resolved clades. All phylogenetic hypotheses from the separate and combined analyses resolve the cophylines as a clade irrespective of the phylogenetic analyses used. Another highly resolved clade contains the included representatives of the South American Gastrophryninae, which was also supported as a clade in the separate analyses of all nuclear genes except those based on the *tyrosinase* gene alone. Notable in this clade is the highly nested position of the North American taxa *Hypopachus* and *Gastrophryne*. The Asian microhylines included form a separate clade. The Malagasy dyscophines are placed as sister to this Asian microhyline clade with low support, but this placement was consistent in all single gene analyses. *Asterophrys* and *Cophixalus*, represent the mainly New Guinean subfamily Asterophryinae are sister groups with high support. The scaphiophrynines *Scaphiophryne* and *Paradoxophyla* were resolved as sister taxa, forming a clade sister to the also Malagasy cophylines. Although this placement has only moderate support (83% ML bootstrap support, bayesian posterior probability 1.00), it was supported as well by the separate analyses of both *rag-1* and *rag-2*. The Asian genus *Calluella* is placed nested among the Microhylinae, and not with the Malagasy Dyscophinae. The positions of both *Otophryne* and *Hoplophryne* are basal within the Microhylidae. Although the placement of *Hoplophryne* is inconsistent between the different single gene analyses, *Otophryne* is placed consistently basal within the microhylid clade, and not with the remaining Gastrophryninae. The Phrynomerinae are also placed basally in the Microhylidae clade.

The molecular data from Frost et al. (2006) consisted of a total of 4015 basepairs after deletion of hypervariable parts of the rRNA genes. The 28*S* rRNA gene alignment consisted of 685 basepairs with 29 species represented. The histone *H3* sequence consisted of 328 basepairs, with 31 species represented. The *rhodopsin* alignment was 316 basepairs long, with only 14 species represented. The *SIA* alignment was 397 basepairs long with 29 species represented. The *tyrosinase* alignment, with a length of 532 basepairs, had 22 species represented. The mitochondrial section spanning 12*S*, the *Val tRNA* and 16*S* was 1786 basepairs in length and lacked only a complete sequence for *Scaphiophryne*, while some sequences were truncated.

Between the ML and MP phylogenies based on the molecular data from Frost et al. (2006), no well supported incongruencies were found. The genus *Ramanella*, represented by only a 16*S* sequence (which came from the same individual specimen as our sequences; Table 1), was excluded from the clade representing the Microhylidae in the ML tree (Fig. 2), whereas it was included as a basal microhylid taxon in the MP tree. The clade consisting of the three included taxa of the Brevicipitidae is highly supported. The clade representing all Microhylidae receives low bootstrap support in the ML analysis (30%) and moderate support in the MP analysis (71%, excluding *Ramanella*). Basal resolution within the microhylid clade is low, but several clades equivalent to those resolved using the dataset generated for this study are resolved with low support. Asterophryines were united in a single clade with low ML (9%) and moderate MP support (85%). The Cophylinae clade received moderate bootstrap support (ML 60%, MP 83%), and is resolved as sister to *Scaphiophryne* with low to moderate support (ML 32%, MP 72%). The Microhylinae form a clade with *Dyscophus* as its sister taxon, although support for this placement is vanishingly low. The Microhylines *Kalophrynus* and *Micryletta* were placed outside of the main Microhyline clade. Similar to the Microhylinae, the gastrophrynines form a poorly supported clade, with high support values only for the nodes uniting highly nested taxa such as the two representatives of the genus *Gastrophryne*, and *Nelsonophryne* and *Ctenophryne*. The gastrophrynine *Synapturanus* was placed outside the main Gastrophryninae clade, in low association with the Asterophryninae. Both *Hoplophryne* and *Phrynomantis* were placed basally in the microhylid clade.

3.1. Divergence time estimates

The estimated divergence times of the major groups included were based on the *rag-1*, *rag-2*, and *BDNF* datasets only (2919 characters). The results are shown in Table 2.

4. Discussion

4.1. Novel phylogenetic relationships among the Microhylidae

In addition to the comprehensive systematic studies of the Microhylidae of Parker (1934), Van Bocxlaer et al. (2006) and Frost et al. (2006) (but also see an unpublished

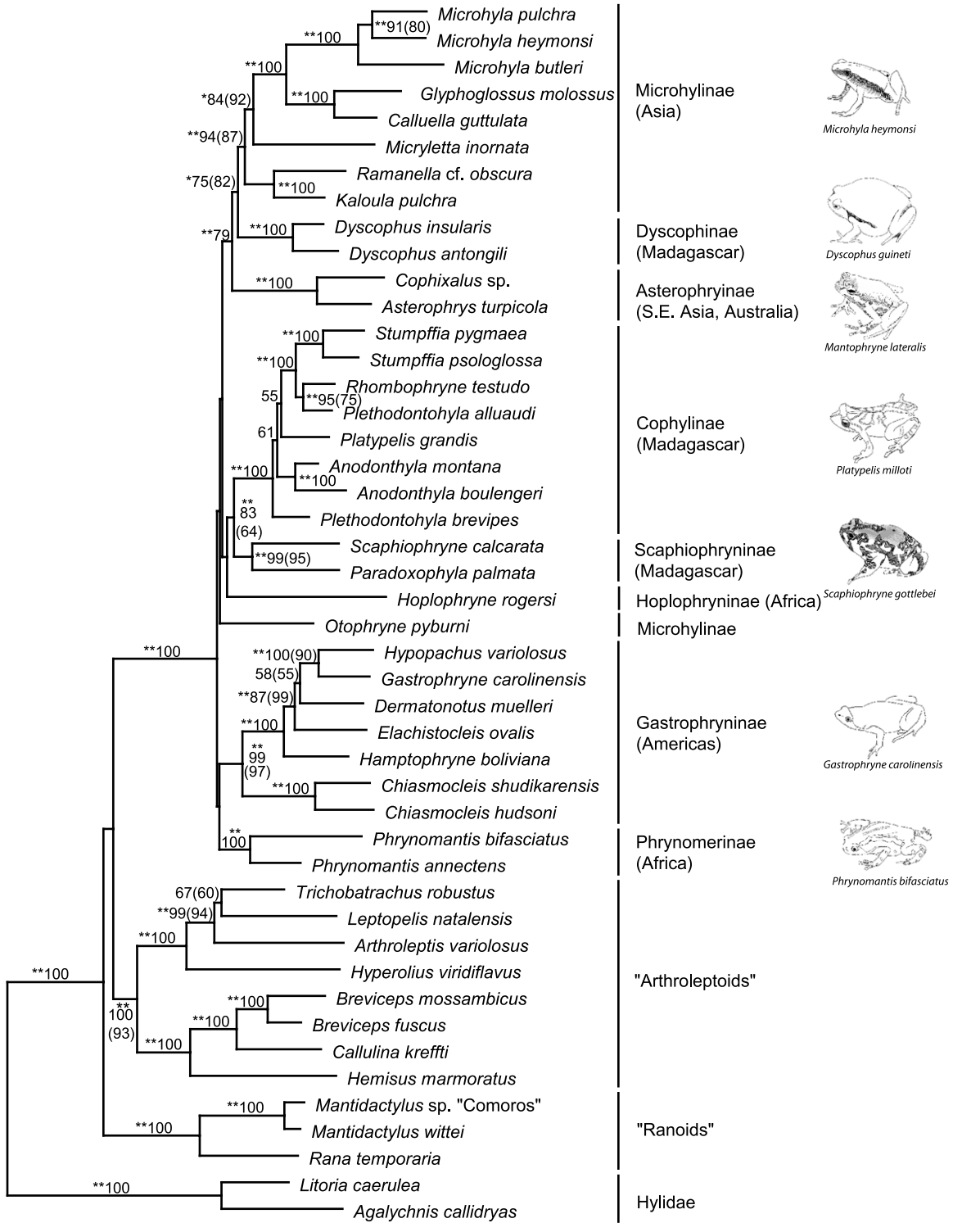

Fig. 1. ML tree, hierarchical outgroups omitted. Values are ML bootstrap support percentages of 500 replicates. Values under 50% not shown. MP bootstrap support percentages of 500 replicates given in parentheses where less than 100%. A single asterisk indicates a bayesian posterior probability of over 0.95, two asterisks indicate a bayesian posterior probability of 1.00. Drawings show representative species of major clades.

Ph.D. thesis by S.-H. Wu from the University of Michigan, 1994), our study provides strong support for a number of clades within this family. Many of these agree with those resolved in previous studies, but several novel phylogenetic relationships were discovered.

One of the more surprising results is the moderately supported placement of the Scaphiophryninae as sister to the Cophylinae. Such a sister group relationship of these two endemic Malagasy subfamilies has, to our knowledge, hitherto not been proposed. The placement of the Scaphiophryninae

Table 2
Divergence time estimates (Age), 95% Confidence interval (CI), and corresponding standard deviation (SD)

Clade	Age (Mya) ± SD (My)	95% CI (My)
Lissamphibia—Amniota (min. 338 Mya)	**356 ± 46**	**281–446**
Anura—Caudata (min. 230 Mya)	**326 ± 40**	**255–412**
Diapsids—Synapsids (338–288 Mya)	**305 ± 41**	**232–392**
Anura	215 ± 28	164–276
Ranoidea—Hyloidea	151 ± 32	116–197
Xenopus—Pipa (min. 110 Mya)	**142 ± 23**	**102–193**
Ranoidea	119 ± 17	89–157
Microhylidae—"Arthroleptoids"	116 ± 17	87–153
"Arthroleptoids"	103 ± 16	76–137
Hyperolius—(*Arthroleptis—*(*Trichobatrachus—Leptopelis*))	77 ± 13	55–106
Brevicipitidae—*Hemisus*	75 ± 13	53–104
Mantellidae—Ranidae	61 ± 11	43–84
Litoria—Agalychnis (min. 42 Mya)	**53 ± 9**	**42–75**
Mantidactylus wittei—M. sp. "Comoros" (max. 15 Mya)	**11 ± 2**	**6.7–15**
Alytes muletensis—A. dickhilleni (min. 5 Mya)	**10 ± 5**	**2.3–23**
Microhylidae		
Microhylidae	66 ± 11	47–90
Microhylinae—Asterophryinae	57 ± 10	40–79
Microhylinae—Dyscophinae	55 ± 10	39–76
Scaphiophryninae—Cophylinae	53 ± 9	38–74
Gastrophryninae	53 ± 10	37–74
Microhylinae	52 ± 9	37–72
Phrynomerinae	47 ± 9	32–66
Scaphiophryninae	45 ± 8	31–64
Cophylinae	32 ± 7	22–47
Cophixalus—Asterophrys	20 ± 5	12–30
Dyscophinae	19 ± 5	11–29
Hypopachus—Gastrophryne	17 ± 4	10–27

Ages of higher taxa are the divergence times of the most basal branches in that clade, based on the taxa included in this study.
Constraints imposed by calibration points are given in the first column, and the posterior age estimates recovered for these nodes are indicated in bold face.

as sister to the Cophylinae is rather well supported by our data and biogeographically more parsimonious, and the relationships among these taxa therefore demand closer scrutiny. The study of Frost et al. (2006) however, placed the Scaphiophryne basal to the Asian Microhylines, far removed from the Cophylinae. Our reanalyses of these data excluding morphological characters concur with the placement of the Scaphiophryninae with the cophylines (Fig. 2), be it with a low bootstrap support value of 32% (MP 72%). The often proposed position of the Scaphiophryninae as sister to the remaining Microhylidae (Scoptanura) (Wassersug, 1984; Duellman and Trueb, 1986; Wassersug, 1989; Ford and Cannatella, 1993; Haas, 2003) cannot be unambiguously ruled out by our data.

Based on the great differences between the tadpole of *Scaphiophryne*, which possess a horny beak and oral denticles and resembles a ranoid tadpole, and *Paradoxophyla*, which lacks all keratinized oral structures and which is a typical microhylid tadpole, it was suggested that the scaphiophrynines might not be monophyletic (Ford and Cannatella, 1993). Haas (2003) suggested based on larval characters that *Paradoxophyla* is more closely related to the Phrynomerinae. Our molecular data provide strong evidence for a sister-group relationship of *Paradoxophyla* and *Scaphiophryne*. Parsimony arguments therefore suggest that the morphology of the *Scaphiophryne* tadpole, intermediate between a ranoid and microhylid larval morphology, should be interpreted to be a reversal from the microhylid type. The alternative, considering the *Scaphiophryne* tadpole as the ancestral form for microhylids, would instead imply the unlikely hypothesis that *Scaphiophryne* has evolved its filter-feeding tadpole morphology fully in parallel to other microhylids.

The isolated position of *Otophryne* corroborates the suggestion of Wassersug and Pyburn (1987) that this species is not a microhyline. They suggested its unique characters might merit the erection of a new subfamily. The phylogenetic hypothesis by Wild (1995), based on the combined morphological data from Zweifel (1986) and Donnelly et al. (1990), instead indicated that *Otophryne* is nested in the American Gastrophryninae. Our molecular data resolve *Otophryne* as a distinct taxon in the microhylid clade, not closely associated with any other microhylid subclade, based both on the combined as well as the single gene analyses. Although basal resolution in the current phylogeny is too low to interpret the position of *Otophryne* as being basal, the possibility of such a placement allows us to consider some of the unique morphological traits of the psammonic larva, especially the keratinized mouthparts, as a possible plesiomorphic character rather than an adaptation related to its burrowing lifestyle. Excluding *Otophryne*, the remaining Gastrophryninae form one of the most distinct

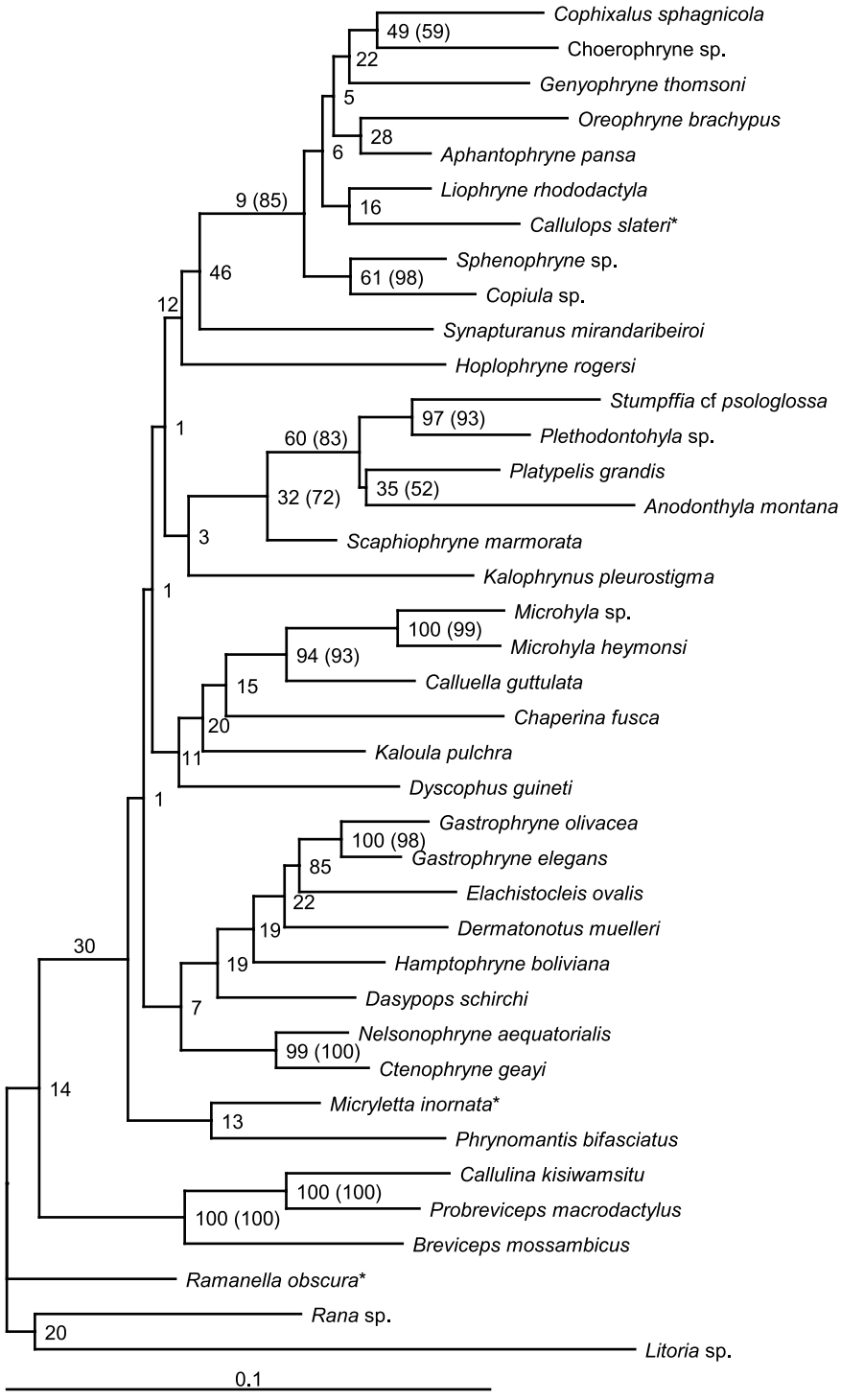

Fig. 2. ML tree of reanalyzed molecular data from Frost et al. (2006). Values are ML (MP) bootstrap support percentages of 300 replicates and 500 replicates, respectively. MP bootstrap values lower than 50% not shown. Taxa for which only 12S or 16S sequences are available are marked with an asterisk.

and well supported groups within the microhylid clade, clearly unrelated to the Asian Microhylinae. This is congruent with the findings of Haas (2003) using larval morphological characters, and those of Frost et al. (2006) based on both morphological and molecular data. Resolution within this group is high and the two North American taxa, *Hypopachus* and *Gastrophryne*, are firmly nested within the remaining South American clade.

The Microhylinae are here resolved as a highly supported clade. Basal to this clade are the Malagasy dyscophines and the representatives of the mainly New Guinean subfamily Asterophryinae (*Asterophrys* and *Cophixalus*).

The topology resulting from our reanalysis of the molecular data from Frost et al. (2006) differed markedly from that presented in the Frost et al. (2006) paper in a

number of points. Their placement of *Kalophrynus* as the sister taxon to the remaining Microhylidae was not recovered by our reanalysis which rather placed this taxon at an unresolved basal position within the Microhylidae. Also the position of *Synapturanus* basal to all microhylids save *Kalophrynus* was not supported by our reanalysis. However, the exclusion of *Synapturanus* from the Gastrophryninae clade is corroborated, placing this South American species basal in the Microhylidae clade outside of the Gastrophryninae, similar to *Otophryne*. The possible basal position places more emphasis of the enigmatic character of these two taxa. Our reanalysis also differed in the placement of the Malagasy taxa *Scaphiophryne* and *Dyscophus*. The former was resolved by Frost et al. (2006) as the sister taxon to the Microhylinae, whereas our reanalysis found this taxon to be the sister taxon of the cophylines, a placement in agreement with our own data. *Dyscophus* was placed sister to the Asterophryinae in the study of Frost et al. (2006), whereas our data placed this taxon sister to the Microhylinae, similar to the findings of Van Bocxlaer et al. (2006). Our reanalysis of the molecular data from Frost et al. (2006) corroborated the placement of *Dyscophus* with the Microhylinae, be it with little support for this placement. The similarity of the phylogeny based on the molecular data of Frost et al. (2006) and that based on our own data transfers high credibility to our topology. The alternative topology recovered by Frost et al. (2006) can probably be attributed to the different methods of alignment and analysis used by these authors (optimization parsimony) but may also be due to the inclusion of morphological characters and hypervariable regions of the rRNA genes in their dataset. Further work will be required to resolve this point.

Despite the inclusion of over 4 kbp of sequence data in our analysis, the basal relationships among the well resolved microhylid clades remain somewhat unresolved. The inclusion of more sequence data, and a more comprehensive taxon sampling might improve the resolution in this part of the tree. Lack of resolution among the basal nodes of the Microhylidae was also evident in the study of Frost et al. (2006) and Van Bocxlaer et al. (2006). The contrast between the lack of resolution basally within the Microhylidae with the otherwise well resolved clades in three separate studies could point to a "biological" polytomy, due to a fast initial radiation. This pattern is similar to that found basally in the Ranoidae (Bossuyt et al., 2006; Van der Meijden et al., 2005), and similarly complicates the selection among alternative biogeographic scenarios.

4.2. Relationships among "Arthroleptoids"

The position of *Leptopelis* as sister to the arthroleptid *Trichobatrachus*, corroborates the findings of Emerson et al. (2000), that *Leptopelis* is distinct from the hyperoliids, and those of Vences et al. (2003a) and Frost et al. (2006), that the leptopelines are closely related to the astylosternids. Our study supplies additional evidence of the close relation of *Hemisus* to the brevicipitids, which has previously been shown by Frost et al. (2006). This relationship had previously been hypothesized by Blommers-Schlosser (1993) based on morphological arguments, but Channing (1995) showed that only a single morphological synapomorphy (a single median thyroid gland) supported the sister relationship between the Brevicipitinae and *Hemisus*.

4.3. Alternate biogeographic scenarios for the Microhylidae

Divergence time estimates place the initial divergence within the Microhylidae at 66 Mya (with a 95% confidence interval of 47–90 Mya), but the last common ancestor of the Microhylidae and their closest ranoid relatives at 116 Mya (87–153 Mya). This dating largely agrees with the recent analysis of Bossuyt et al. (2006) who found an initial split within microhylids immediately after the Cretaceous-Paleocene boundary, and with Van Bocxlaer et al. (2006) who recovered a divergence time of about 88 Mya for the initial microhylid divergence and 127 Mya for their split from their nearest relatives. The early microhylids found themselves thus on Gondwana, around or after the disconnection of Madagascar and India from the mainland 121 Mya (Sanmartin and Ronquist, 2004). Several scenarios have been proposed to explain their current distribution.

4.3.1. Ancient vicariance

Savage (1973) proposed a scenario based almost entirely on vicariance due to the fragmentation of Gondwana. Savage suggested that vicariance events led to the presence of microhylids in South America, Africa, Madagascar, India, and Australia. The origins of the Microhylinae in Asia have been proposed to be due to their presence in Madagascar-India (Duellman and Trueb, 1986), where they originated after an ancient vicariant split from Africa. After the separation of Madagascar and India by the Mascarene basin, 88–84 Mya, India would have acted as "biotic ferry" and transported several ranoid frogs (Natatanura sensu Frost et al., 2006) as well as microhylids to Asia, where they underwent extensive radiations (Duellman and Trueb, 1986; Bossuyt and Milinkovitch, 2001). These frog lineages previously would have been present on the Madagascar-India continent since its disconnection from Africa. Madagascar and India, however, had broken off from Africa (121 Mya) before the initial divergence of microhylids at 66 Mya (47–90 Mya). Assuming that our age estimates are correct, the initial splits among microhylids would therefore be distinctly younger than this continental separation, making it unlikely that the presence on Madagascar of cophylines and scaphiophrynines, which originated 53 Mya (38–74 Mya), is due to vicariance.

The basal position of both the African taxa *Hoplophryne* and *Phrynomantis*, and the South American taxa *Otophryne* and *Synapturanus* suggest a possible vicariant split between African and South American taxa. Africa

and South America had separated at 110 Mya, before the initial radiation of the extant Microhylidae, and therefore vicariance is unlikely in light of this divergence time estimate. South America remained indirectly connected to Australia and New Guinea, but not to Africa, through Antarctica until 35 Mya. A hypothesis of ancestors from South America colonizing Asia via Antarctica and Australia is in accordance with the basal position of the Asterophryinae and Genyophryninae to the Microhylinae, and the divergence between asterophryines from microhylines at 57 Mya (40–79 Mya). The initial divergence between the Australo-New Guinean taxa is estimated at 20 Mya, but only two possibly closely related taxa (Sumida et al., 2000) are included in our study. The fossil asterophryine *Australobatrachus*, from the Oligocene to Miocene of Australia provides further evidence for this possibility. Counter to this scenario is the low species diversity presently found in Australia compared to that on New Guinea.

4.3.2. Recent dispersal

Feller and Hedges (1998) proposed that the origin of the Microhylidae lies in Africa, and subsequent dispersal to Asia, and from there to South America via North America led to the current distribution. Although appealing, this scenario is at odds with our phylogenetic reconstructions and divergence time estimates. The dispersal of the Microhylidae to Asia after the reconnection of Africa to Eurasia through the Arabian Peninsula, latest 10 Mya (McQuarrie et al., 2003) as proposed by Feller and Hedges, is in disagreement with our divergence time estimates. The initial radiation of the Asian Microhylinae is estimated at 52 Mya (37–72 Mya), well before the reconnection of Africa to Eurasia. Our data also show the North American microhylines to be nested within the South American clade. This contradicts the hypothesis of the colonization of South America from North America, unless one postulates extinction and recolonization for North America. On the contrary, North America was probably colonized by dispersal from South America. Feller and Hedges (1998) propose, for hyloid frogs, a dispersal route to North America across the proto-Antilles in the late Cretaceous. It is unlikely that the microhylids also used this route because the split between North and South American taxa is younger: divergence time estimates place the most recent common ancestor of the North American taxa *Hypopachus* and *Gastrophryne* at 17 Mya (10–27 Mya), closer to the connection of North and South America through the Panama isthmus approximately 5–3.5 Mya. The North American microhyline taxa might therefore have dispersed from South America across the Panama isthmus or across the sea prior to the great faunal interchange. Since the monophyly of the North American taxa is well corroborated and was also found by Frost et al. (2006), to explain the colonization of North America along with the great faunal interchange would require to assume the extinction of the putative closest relatives of *Gastrophryne*, and of the ones of *Hypopachus*, in southern America.

4.3.3. Ancient dispersal

As summarized above, the original "biotic ferry" hypothesis strictly implied vicariance to explain the distribution of microhylids and natatanurans in Africa, Madagascar and India/Asia, but the molecular age estimates indicate that microhylids and natatanurans are younger than the generally accepted age of geological separation among these land masses. Van Bocxlaer et al. (2006) alternatively proposed that land connections among Gondwanan landmasses may have persisted longer, contrary to the traditional paleogeographical reconstructions. In fact, the Kerguelen plateau provided a connection between Madagascar-India and Antarctica (and thereby with Australia and South America) in the Late Cretaceous which led to faunal interchanges (Krause et al., 1999; Noonan and Chippindale, 2006), and the drifting Indian continent may have been simultaneously close to Madagascar, Africa, and Asia (Patriat and Segoufin, 1988; Briggs, 2003). The hypothesis of Van Bocxlaer et al. (2006) largely relies on a phylogenetic reconstruction of microhylids, based on a dataset of 2865 nucleotides, which similar to our tree has almost no support for basal relationships among major clades. It therefore is too early to fully evaluate this scenario that places two African taxa (*Phrynomantis* and *Hoplophryne*) as most basal microhylids. However, it is obvious that the hypothesis of Van Bocxlaer et al. (2006), in contradiction to the title of their paper, is strongly dispersal-oriented. There is no doubt that Gondwana was highly fragmented already in the Early Cretaceous, and according to the available divergence time estimates microhylids and natatanurans had not yet diverged at this time. An initial widespread Gondwanan distribution of these frogs can therefore not be invoked, and they must instead have originated on one of the Gondwana fragments. At least for the Natatanura this was most likely Africa since the basal lineages of ranoids and natatanurans are endemic to that continent (Van der Meijden et al., 2005). After a presumed secondary contact between India and Africa (Briggs, 2003), a subset of these frogs could have dispersed to India, radiated there, and several subgroups then again dispersed to Madagascar (and possibly from there, once more, over the Kerguelen plateau and Antarctica to South America). Hence, the initial phylogenetic splits explaining the current transcontinental distribution of these frogs would have been two or more dispersals (over land or crossing narrow sea straits). Only subsequently would the connections between Africa–India and India–Madagascar have been severed (causing definitive isolation that could be interpreted as vicariance), and the "biotic ferry" India completed its journey towards Asia. In fact, Van Bocxlaer et al. (2006) themselves use the term "mass coherent dispersal" to explain the encountered patterns. This variant of the scenario is even more appealing because it easily explains, without assuming multiple extinctions, why the Malagasy taxa (especially the natatanuran family Mantellidae) are deeply nested within the Indo-Asian taxa.

Again assuming accretions of landmasses, a further alternative not mentioned by Van Bocxlaer et al. (2006) and equally explaining the initial splits in microhylids and natatanurans, would be a trans-Tethyan connection leading to a colonization of Eurasia via Africa, and subsequently a dispersal route via India to Madagascar, and then via Antarctica to Australia and South America. We do not favor this scenario which is unlikely despite previous suggestions of Cretaceous-Paleocene connections across the Tethys (e.g., Gheerbrant, 1990). It exemplifies, however, that a plethora of alternative explanations for the current distribution patterns are available, and that the biotic data so far do not unequivocally contradict any particular aspect of the prevalent geological reconstructions.

4.3.4. Biogeographic conclusions

From the discussions above it becomes clear that a more comprehensive taxon sampling as well as a better phylogenetic resolution will be necessary to unambiguously resolve the biogeographic history of the Microhylidae. The inclusion of more New Guinean asterophrynines, as well as inclusion of Australian taxa might possibly resolve the order of colonization of these two areas. Increased taxon sampling, as well as a larger dataset might better resolve the relationships between the Microhylinae, the Dyscophinae and the Asterophryninae and thus help understanding the direction of colonization in this case. Considering the very low support for the basal relationships among microhylids, and the short time interval in which it occurred (66–53 Mya), it seems likely that these frogs arose by a radiation which may have been too fast to be fully reconstructed. Some overseas dispersal probably occurred (e.g., from southern to northern America) as well as some dispersal across landmasses and subsequent vicariance. Eventually, it is not unlikely that the biogeographic history of this and other frog families will turn out to be highly complex, shaped by a mixture of vicariance and dispersal events rendering unidimensional hypotheses as too simplistic and optimistic.

Acknowledgments

We thank Jens V. Vindum at the California Academy of Sciences, San Francisco, Steve Richards at the James Cook University, Frank Glaw at the Zoologische Staatssammlung München, Alexander Kupfer at the Natural History Museum, London, and Carla Cicero at the Museum for Vertebrate Zoology, Berkeley (Loan No. 6205), for generously providing tissue samples crucial to this work. Thanks to Dirk Steinke and Frank Glaw for valuable discussions, and to Franky Bossuyt and an anonymous reviewer for their helpful comments. We thank our fellow microhylophilic colleagues with whom we have enjoyed constructive correspondence under the auspice of AmphibiaTree. This work was funded by the Deutsche Forschungsgemeinschaft (Grant ME1725/10-1).

References

Amphibiaweb, Information on amphibian biology and conservation. [web application]. 2006. Berkeley, California: AmphibiaWeb. Available: http://amphibiaweb.org/.

Andreone, F., Vences, M., Vieites, D.R., Glaw, F., Meyer, A., 2005. Recurrent ecological adaptations revealed through a molecular analysis of the secretive cophyline frogs of Madagascar. Mol. Phylogenet. Evol. 34, 315–322.

Barnes, W.M., 1994. PCR amplification of up to 35 kb DNA with high fidelity and high yield from lambda 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, 711–713.

Blommers-Schlosser, R.M.A., 1993. Systematic relationships of the Mantellinae Laurent 1946 (Anura Ranoidea). Ethol. Ecol. Evol. 2, 199–218.

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.

Bossuyt, F., Milinkovitch, M.C., 2001. Amphibians as indicators of early tertiary "Out-of-India" dispersal of vertebrates. Science 292, 93–95.

Bossuyt, F., Brown, R.M., Hillis, D.M., Cannatella, D.C., Milinkovitch, M.C., 2006. Phylogeny and biogeography of a cosmopolitan frog radiation: Late Cretaceous diversification resulted in continent-scale endemism in the family Ranidae. Syst. Biol. 55, 579–594.

Briggs, J.C., 2003. The biogeographic and tectonic history of India. J. Biogeogr. 30, 338–381.

Bruford, M.W., Hanotte, O., Brookfield, J.F.Y., Burke, T., 1992. Single-locus and multilocus DNA fingerprinting. In: Hoezel, A.R. (Ed.), The South American Herpetofauna: Its Origin, Evolution, and Dispersal. Molecular Genetic Analysis in Conservation. IRL Press, Oxford.

Channing, A., 1995. The relationship between *Breviceps* (Anura: Microhylidae) and *Hemisus* (Hemisotidae) remains equivocal. J. Herpet. Ass. Afr. 44, 55–57.

Darst, C.R., Cannatella, D.C., 2004. Novel relationships among hyloid frogs inferred from 12S and 16S mitochondrial DNA sequences. Mol. Phylogenet. Evol. 31, 462–475.

Donnelly, M.A., De Sá, R.O., Guyer, C., 1990. Description of the tadpoles of *Gastrophryne pictiventris* and *Nelsonophryne aterrima* (Anura: Microhylidae), with a review of morphological variation in free-swimming microhylid larvae. Am. Mus. Novit. 2976, 1–19.

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

Dubois, A., 2005. Amphibia Mundi. 1.1.; An ergotaxonomy of recent amphibians. Alytes 23, 1–24.

Duellman, W.E., 1979. The South American Herpetofauna: Its Origin, Evolution, and Dispersal. Museum of Natural History. The University of Kansas, Lawrence, Kansas.

Duellman, W.E., Trueb, L., 1986. Biology of Amphibians. McGraw Hilll, New York.

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.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1994. Testing significance of incongruence. Cladistics 10, 315–319.

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

Felsenstein, J., 1984. Distance methods for inferring phylogenies: A justification. Evolution 38, 16–24.

Ford, L.S., Cannatella, D.C., 1993. The major clades of frogs. Herpetol. Monogr. 7, 94–117.

Fromhage, L., Vences, M., Veith, M., 2004. Testing alternative vicariance scenarios in Western Mediterranean discoglossid frogs. Mol. Phylogenet. Evol. 31, 308–322.

Frost, D.R., Grant, T., Faivovich, J., Baina, R.H., Haas, A., Haddad, C.F.B., Sá, R.O.D., Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C.J., Campbell, J.A., Blotto, B.L., Moler, P., Drewes, R.C., Nussbaum, R.A., Lynch, J.D., Green, D.M., Wheeler, W.C., 2006. The amphibian tree of life. Bull. Amer. Mus. Nat. Hist. 297, 1–291.

Gheerbrant, E., 1990. On the early biogeographical history of the African placentals. Historical Biol. 4, 107–116.

Graur, D., Martin, W., 2004. Reading the entrails of chickens: Molecular timescales of evolution and the illusion of precision. Trends Genet. 20, 80–86.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

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

Hanken, J., Wake, D.B., 1993. Miniaturization of body size: Organismal consequences and evolutionary significance. Annu. Rev. Ecol. Syst. 24, 501–519.

Hebert, P., Cywinska, A., Ball, S., Dewaard, J., 2003. Biological identification through DNA barcodes. Proc. R. Soc. B. 270, 313–321.

Hebert, P., Stoeckle, M., Zemlak, T., Francis, C., 2004. Identification of birds through COI DNA barcodes. PLoS Biol. 2, e312.

Hedges, S.B., Hass, C.A., Maxson, L.R., 1992. Caribbean biogeography: Molecular evidence for dispersal in West Indian terrestrial vertebrates. Proc. Natl. Acad. Sci. USA 89, 1909–1913.

Hoegg, S., Vences, M., Brinkmann, H., Meyer, A., 2004. Phylogeny and comparative substitution rates of frogs inferred from sequences of three nuclear genes. Mol. Biol. Evol. 21, 1188–1200.

Hoskin, C.J., 2004. Australian microhylid frogs (*Cophixalus* and *Austrochaperina*): Phylogeny, taxonomy, calls, distributions and breeding biology. Aust. J. Zool. 52, 237–269.

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

Köhler, J., Vieites, D.R., Bonett, R.M., Garcı´a, F.H., Glaw, F., Steinke, D., Vences, M., 2005. New amphibians and global conservation: A boost in species discoveries in a highly endangered vertebrate group. BioScience 55, 693–696.

Krause, D.W., Rogers, R.R., Forster, C.A., Hartman, J.H., Buckley, G.A., Sampson, S.D., 1999. The late cretaceous vertebrate fauna of Madagascar: Implications for Gondwanan paleobiogeography. GSA Today (Publ. Geol. Soc. Am.) 9, 1–7.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5, 150–163.

Loader, S.P., Gower, D.J., Howell, K.M.N.D., Rödel, M.-O., Clarke, B.T., De Sá, R., Cohen, B.L., Wilkinson, M., 2004. Phylogenetic relationships of African microhylid frogs inferred from DNA sequences of mitochondrial 12S and 16S rRNA genes. Org. Divers Evol. 4, 227–235.

McDiarmid, R.W., Altig, R., 1999. Tadpoles: The Biology of Anuran Larvae. xvi, 444. University of Chicago Press, Chicago and London.

McQuarrie, N., Stock, J.M., Verdel, C., Wernicke, B.P., 2003. Cenozoic evolution of Neotethys and implications for the causes of plate motions. Geophys. Res. Lett. 30, 6.1–6.4.

Measey, G.J., Vences, M., Drewes, R.C., Chiari, Y., Melo, M., Bourles, B., 2007. Freshwater paths across the ocean: Molecular phylogeny of the frog *Ptychadena newtoni* gives insights into amphibian colonization of oceanic islands. J. Biogeogr. 34, 7–20.

Noonan, B.P., Chippindale, P.T., 2006. Vicariant origin of malagasy reptiles supports late cretaceous antarctic land bridge. Amer. Nat. 168, 730–741.

Parker, B.A., 1934. A Monograph of the Frogs of the Family Microhylidae. The British Museum, London.

Patriat, P., Segoufin, J., 1988. Reconstruction of the central indian ocean. In: Scotese, C.R., Sager, W.W. (Eds.), Mesozoic and Cenozoic Plate Reconstructions. Tectonophysics, 155, pp. 211–234.

Posada, D., Crandall, K.A., 1998. Modeltest: Testing the model of DNA substitution. Bioinformatics 14, 817–818.

Ruta, M., Coates, M.I., Quicke, D.L., 2003. Early tetrapod relationships revisited. Biol. Rev. Camb. Philos. Soc. (London) 78, 251–345.

San Mauro, D., Vences, M., Alcobendas, M., Zardoya, R., Meyer, A., 2005. Initial diversification of living amphibians predated the breakup of Pangaea. Am. Nat. 165, 590–599.

Sanchiz, B., 1998. Encyclopedia of Palaeoherpetology Part 4. Salienta, Pfeil, München.

Sanmartin, I., Ronquist, F., 2004. Southern hemisphere biogeography inferred by event-based models: Plant versus animal patterns. Syst. Biol. 2, 216–243.

Savage, J.M., 1973. The geographic distribution of frogs: Patterns and predictions. In: Vial, J.L. (Ed.), Evolutionary Biology of Anurans: Contemporary Research on Major Problems. University of Missouri Press, Colombia, Missouri.

Scott, E., 2005. A phylogeny of ranid frogs (Anura: Ranoidea: Ranidae), based on a simultaneous analysis of morphological and molecular data. Cladistics 21, 507–574.

Seddon, J.M., Baverstock, P.R., Georges, A., 1998. The rate of mitochondrial 12S rRNA gene evolution is similar in freshwater turtles and marsupials. J. Mol. Evol. 4, 460–464.

Sumida, M., Allison, A., Nishioka, M., 2000. Evolutionary relationships among 12 species belonging to three genera of the family Microhylidae in Papua New Guinea revealed by allozyme analysis. Biochem. Syst. Ecol. 8, 721–736.

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

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: Improving the sensitivity of the progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Thorne, J.L., Kishino, H., 2002. Divergence time and evolutionary rate estimation with multilocus data. Syst. Biol. 5, 689–702.

Van Bocxlaer, I., Roelants, K., Biju, S.D., Nagaraju, J., Bossuyt, F., 2006. Late Cretaceous Vicariance in Gondwanan Amphibians. PLoS ONE 1, e74.

Van der Meijden, A., Vences, M., Meyer, A., 2004. Novel phylogenetic relationships of the enigmatic brevicipitine and scaphiophrynine toads as revealed by sequences from the nuclear Rag-1 gene. Proc. R. Soc. B 271, S378–S381.

Van der Meijden, A., Vences, M., Hoegg, S.I., Meyer, A., 2005. A previously unrecognized radiation of ranid frogs in Southern Africa revealed by nuclear and mitochondrial DNA sequences. Mol. Phylogenet. Evol. 37, 674–685.

Vences, M., Kosuch, J., Glaw, F., Böhme, W., Veith, M., 2003a. Molecular phylogeny of hyperoliid treefrogs: Biogeographic origin of Malagasy and Seychellean taxa and re-analysis of familial paraphyly. J. Zool. Syst. Evol. Res. 41, 205–215.

Vences, M., Vieites, D.R., Glaw, F., Brinkmann, H., Kosuch, J., Veith, M., Meyer, A., 2003b. Multiple overseas dispersal in amphibians. Proc. R. Soc. B. 1532, 2435–2442.

Vences, M., Thomas, M., Van der Meijden, A., Chiari, Y., Vieites, D.R., 2005. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. Front Zool. 2, 5.

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

Wassersug, R., 1989. What, if anything is a microhylid (Orton type II) tadpole? In: Splechtna, H. (Ed.), Trends in Vertebrate Morphology. G. Fischer, Stuttgart, New York.

Wassersug, R.J., Pyburn, W.F., 1987. The biology of the Pe-ret' toad, *Otophryne robusta* (Microhylidae), with special consideration of its fossorial larva and systematic relationships. Zool. J. Linn. Soc. 91, 137–169.

Wiens, J.J., Fetzner, J.W., Parkinson, C.L., Reeder, T.W., 2005. Hylid frog phylogeny and sampling strategies for speciose clades. Syst. Biol. 5, 719–748.

Wild, E.R., 1995. New genus and species of Amazonian microhylid frog with a phylogenetic analysis of New World genera. Copeia 4, 837–849.

Yang, Z., 1997. PAML: A program package for phylogenetic analysis by maximum likelihood. Computer Applications in the BioSciences 13, 555–556.

Zweifel, R.G., 1986. A new genus and species of microhylid frog from the Cerro de la Neblina region of Venezuela and a discussion of relationships among New World microhylid genera. Am. Mus. Novit. 2863, 1–24.