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Phylogenetic relationships of African microhylid frogs inferred from DNA sequences of mitochondrial 12S and 16S rRNA genes

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Abstract

The phylogenetic relationships of microhylid frogs are poorly understood. The first molecular phylogeny for continental African microhylids is presented, including representatives of all subfamilies, six of the eight genera, and the enigmatic hemisotid *Hemisus*. Mitochondrial 12S and 16S rRNA sequence data were analysed using parsimony, likelihood and Bayesian methods. Analyses of the data are consistent with the monophyly of all sampled subfamilies and genera. *Hemisus* does not nest within either brevicipitines or non-brevicipitines. It is possibly the sister group to brevicipitines, in which case brevicipitines might not be microhylids. *Phrynomantis* and *Hoplophryne* potentially group with non-African, non-brevicipitine microhylids, in agreement with recent morphological and molecular data. Within brevicipitines, *Breviceps* is recovered as the sister group to a clade of *Callulina* + *Spelaeophryne* + *Probreviceps*. The relationships among the genera within this latter clade are unclear, being sensitive to the method of analysis. Optimal trees suggest the *Probreviceps macrodactylus* subspecies complex might be paraphyletic with respect to *P. uluguruensis*, corroborating preliminary morphological studies indicating that *P. m. rungwensis* may be a distinct species. *P. m. loveridgei* may be paraphyletic with respect to *P. m. macrodactylus*, though this is not strongly supported. Some biogeographic hypotheses are examined in light of these findings.

Keywords: Microhylidae; Brevicipitinae; Melanobatrachinae; Phrynomerinae; *Hemisus*; Africa; Eastern Arc

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Introduction

Microhylids are a diverse group of subterranean, terrestrial and arboreal frogs occurring in northern Australasia, South and Southeast Asia, sub-Saharan Africa, Madagascar, and North and South America. The approximately 350 nominate species are classified in 64 genera and 10 subfamilies. This is the largest number of genera found in any amphibian family, comprising some 15% of all frog genera (Frost 2002). The status, composition, inter- and intrarelationships of Microhylidae have not been studied in detail, and the family remains in general poorly understood. Indeed, even the monophyly of Microhylidae is far from established (see below). In association with their ecological diversity, microhylids display great morphological variation, particularly in their cranial and pectoral girdle structure (Parker 1934; Carvalho 1954; Blommers-Schlösser 1993; Wu 1994). The inadequate state of microhylid systematics partly stems from the lack of comparative morphological studies. Blair (1962) suggested the use of non-traditional character systems for clarifying evolutionary relationships in frogs. More specifically, Largen and Drewes (1989) suggested molecular data would be useful for resolving relationships among African microhylids.

The suprageneric taxonomy of Microhylidae has barely changed since Parker's (1934) milestone monograph but, given the generally inadequate state of current knowledge, this is unlikely to prove stable. Currently, the eight African (excluding Madagascar) genera are divided into three subfamilies (Frost 2002). The African Brevicipitinae consists of twenty species in five genera. Three of these genera (*Probreviceps* Parker, *Callulina* Nieden, *Balebreviceps* Largen & Drewes) are found in evergreen forest, whereas the remainder (*Breviceps* Merrem, *Spelaeophryne* Ahl) are known to also inhabit some drier habitats. Among the moist forest genera, *Probreviceps* is the most speciose (3 species) and, except for the Zimbabwean *P. rhodesianus* Poynton & Broadley, is found principally in the mountain forests of Tanzania (Howell 1993). *P. macrodactylus* (Nieden) is subdivided into three subspecies (Parker 1934): *P. macrodactylus macrodactylus* (Nieden) from the Usambara, *P. macrodactylus loveridgei* Parker from the Uluguru and Udzungwa, and *P. macrodactlyus rungwensis* Loveridge from Rungwe and the Udzungwa. The latter two subspecies are sympatric in the Udzungwa Mountains, suggesting that they may be separate species. *Callulina* is also found throughout the Eastern Arc Mountains, and is known from *C. kreffti* Nieden and a new species from the West Usambaras (de Sá, Loader and Channing, unpublished). *Balebreviceps* is monotypic, with *B. hillmani* Largen & Drewes known from the Bale Mountains, Ethiopia (Largen and Drewes 1989). The only species of *Spelaeophryne*, *S. methneri* Ahl, is found in both low and highland areas of southeastern Tanzania, and *Breviceps* (15 species) is confined to eastern and southern Africa, being "concentrated in South Africa" (Poynton 1964; see also Channing 2001; Minter 2003). The Indo-African Melanobatrachinae comprises four species: *Melanobatrachus indicus* Beddome (Western Ghats, India), *Hoplophryne rogersi* Barbour & Loveridge (East Usambara, Tanzania), *H. uluguruensis* Barbour & Loveridge (Uluguru and Udzungwa, Tanzania), and *Parhoplophryne usambaricus* Barbour & Loveridge (East Usambara, Tanzania). These species all appear to be strictly confined to forests. The subfamily Phrynomerinae comprises five species of *Phrynomantis* Peters that have a wide distribution across savanna and woodland habitats in sub-Saharan Africa.

Based on morphology and behaviour, Blommers-Schlösser (1993) argued that brevicipitines are not microhylids, but actually belong with the enigmatic African taxon *Hemisus* Günther in the Hemisotidae. Wu's (1994) phylogenetic analysis of morphology also found support for brevicipitines being more closely related to *Hemisus* than to non-brevicipitine microhylids. The currently more orthodox view that brevicipitines are microhylids and only distantly related to *Hemisus* was summarised by Ford and Cannatella (1993). Recent studies of larval morphology (Haas 2003) and DNA sequence data (Biju and Bossuyt 2003; Vences et al. 2003) have reinforced the view that *Hemisus* is only distantly related to a monophyletic Microhylidae, but none of these studies sampled any brevicipitine taxa.

The limited ability of most amphibians to disperse across biogeographical barriers (e.g. the sea or arid habitats) has led some workers (e.g. Savage 1973; Duellman and Trueb 1994; Bossuyt and Milinkovitch 2001) to argue that the distribution of amphibians reflects changes in geology and geography at various scales, such as continental drift and orogenesis. The current distribution of microhylids has been interpreted as reflecting the break-up of Gondwana (Savage 1973). At a finer scale, the high species diversity and strong patterns of endemism in amphibians (including microhylids) of the Eastern Arc are thought to be intimately related to more recent geographic events (Fjeldå and Lovett 1993; Howell 1993).

In this paper, we present the first phylogenetic analysis of mitochondrial DNA sequence data for African microhylids, sampling all subfamilies and six of the eight genera found in continental Africa. We focus especially on brevicipitines. *Hemisus* is also included, in order to explore the relationship of this genus with microhylids. The results of phylogenetic analyses are compared briefly with some existing biogeographic hypotheses.

Material and methods

Samples

A total of 27 terminal taxa were used in this study (Table 1). Sequences for 23 terminal taxa were generated from newly collected material from Tanzania and Ivory Coast. These were supplemented by sequences for 4 species obtained from GenBank (Benson et al. 1998). Although microhylids are also distributed elsewhere in sub-Saharan Africa, collecting was concentrated in Tanzania because all but one genus (*Balebreviceps* from the Bale Mts, Ethiopia; Largen and Drewes 1989) of African microhylids occur there. All species known to occur in Tanzania are represented in this study by at least one specimen, except for *Parhoplophryne usambaricus* which is known from the single type specimen only (Barbour and Loveridge 1928). Beyond Tanzania, this study lacks intensive sampling of *Breviceps*, with only one of 15 species included. The sub-Saharan *Phrynomantis* is represented by two of the five known species. The only species of *Probreviceps* not included in this study is the Zimbabwean *P. rhodesianus*.

Four non-African microhylids were included, including representatives of at least two major lineages within the family, the exclusively Madagascan Scaphiophryninae (*Scaphiophryne* Boulenger) and the more cosmopolitan Microhylinae (*Microhyla* Tschudi, *Kaloula* Gray). All microhylid taxa for which 12S and 16S data are currently deposited in GenBank were included, with the exception of the Madagascan dyscophine *Dyscophus guineti* (Grandidier), for which the available data do not match the regions sequenced here and contain several ambiguities. In addition to microhylids, we included the East African *Hemisus marmoratus* Steindachner and West African *H. sudanensis* (Steindachner).

DNA extraction, amplification and sequencing

DNA was extracted from liver and/or thigh muscle preserved in aqueous 95% ethanol, and purified using phenol/chloroform extractions. The primers used in amplification and sequencing were 12Sa and 12Sb for the 12S rRNA gene (Kocher et al. 1989), and 16Sa and 16Sb for the 16S rRNA gene (Palumbi 1996). Successful polymerase chain reaction (PCR) gel bands were removed and purified. PCR products were sequenced using an ABI 377 automated sequencer (PE Biosystems, Warrington, UK), following the manufacturer's protocols. Each published sequence represents a consensus of both strands. GenBank accession numbers for sequences are given in Table 1.

Phylogenetic analysis

Sequences were aligned manually. Length differences were resolved by inserting alignment gaps, and positions that could not be aligned unambiguously were excluded. Parsimony and maximum likelihood (ML) analyses were performed with PAUP*4b6 (Swofford 1998); ML analyses used models recommended by Modeltest 3.04 (Posada and Crandall 1998), with empirical base frequencies. All analyses were heuristic, with 10 random addition sequence replicates and tree bisection recombination branch swapping. Zero length branches were suppressed. Bayesian analysis was performed using MrBayes (Huelsenbeck and Ronquist 2001) with a six substitution category model and empirical base frequencies. The Markov chain Monte Carlo search was run with four chains for 1,000,000 generations. The first 1000 generations were discarded as 'burn-in', and subsequent trees were sampled every 1000 generations.

Faith and Cranston's (1991) permutation tail probability (PTP) was determined with parsimony analyses of 99 randomisations of the data. Support for clades was measured with bootstrap proportions (Felsenstein 1985; 1000 pseudoreplicates), and decay indices (Bremer 1988) determined by enforcing converse topological constraints. The significance of length differences between most parsimonious and suboptimal trees found in constrained analyses was assessed using a non-parametric test (Templeton 1983). This test is only unbiased when comparing trees chosen a priori, i.e. not on the basis of their fit to the data. When trees are selected because of their maximal fit to the data, the tests are too liberal. Thus, we here accept the failure to reject the null hypothesis at face value, while rejection of the null hypothesis is interpreted more cautiously (see Wilkinson et al. 2003). Rate heterogeneity among taxa was investigated by performing relative rates tests using RRTree (Robinson-Rechavi and Huchon 2000).

We chose not to include a range of putative outgroups (e.g. ranids, hyperoliids, artholeptids, rhacophorids) for three main reasons. First, the monophyly of, and interrelationships among, many major groups of neobatrachian frogs are not well established (e.g. Ford and Cannatella 1993; Hay et al. 1995; Haas 2003) so that selection of specific outgroups would be somewhat arbitrary. Second, countering this by including a broad range of outgroups was resisted because, based on preliminary analyses, it increases ambiguity in the alignment and the potential for long-branch attraction. Third, previous studies (e.g. Hay et al. 1995; Wilkinson et al. 2003; Hertwig et al. 2004) suggest that 12S and 16S mitochondrial data alone are unlikely to provide a robust, well-resolved picture of higher relationships across such a wide range of amphibian families. Thus, we use unrooted trees to test previous hypotheses of

Table 1. Details of *Hemisus* and microhylid samples used in analyses

	Species	Voucher	Locality	GenBank accession no.
1	*Hemisus marmoratus* Steindachner	MW 1856	Sali FR, Mahenge Mts., Tanzania	AY531831, AY531854
2	*Hemisus sudanensis* (Steindachner)	MOR C00.1	Comoé National Park, Ivory Coast	AY531830, AY531853
3	*Phrynomantis microps* Peters	MOR C97.1	Comoé National Park, Ivory Coast	AY531832, AY531855
4	*Phrynomantis bifasciatus* (Smith)	MW 3842	Mkomazi Game Reserve, Tanzania	AY531833, AY531856
5	*Scaphiophryne brevis* (Boulenger)			AF 026357, AF 215384
6	*Scaphiophryne gottlebei* Busse & Böhme			AF 215144, AF 215385
7	*Hoplophryne uluguruensis* Barbour & Loveridge	KMH 22723	West Kilombero Scarp FR, Uzungwa Mts, Tanzania	AY531835, AY531858
8	*Hoplophryne rogersi* Barbour & Loveridge	KMH 23364	Nilo FR, East Usambara Mts. Tanzania	AY531834, AY531857
9	*Microhyla* cf. *ornate* (Duméril & Bibron)			AF 249003, AF 215371
10	*Kaloula taprobanica* Parker			AF 249004, AF 249057
11	*Breviceps mossambicus* Peters	MW 1826	Sali FR, Mahenge Mts., Tanzania	AY531836, AY531859
12	*Breviceps mossambicus* Peters	MW 1848	Sali FR, Mahenge Mts., Tanzania	AY531837, AY531860
13	*Spelaeophryne methneri* Ahl	KMH 21547	Uluguru Mountains, Milawilila FR, Tanzania	AY531838, AY531861
14	*Spelaeophryne methneri* Ahl	MW 1850	Sali FR, Mahenge Mts., Tanzania	AY531839, AY531862
15	*Callulina* n. sp.	MW 3215	Ambangula FR, West Usambara Mts, Tanzania	AY531841, AY531864
16	*Callulina* n. sp.	MW 1968	Mazumbai FR, West Usambara Mts, Tanzania	AY531840, AY531863
17	*Callulina kreffti* Nieden	KMH 23534	Nilo FR, East Usambara Mts., Tanzania	AY531842, AY531865
18	*Probreviceps m. rungwensis* Loveridge	KMH 19141	West Kilombero Scarp FR, Uzungwa Mts, Tanzania	AY531843, AY531866
19	*Probreviceps m. rungwensis* Loveridge	KMH 18974	Ndundulu FR, Uzungwa Mts., Tanzania	AY531844, AY531867
20	*Probreviceps uluguruensis* (Loveridge)	KMH 21570	Uluguru South FR, Uluguru Mts., Tanzania	AY531845, AY531868
21	*Probreviceps uluguruensis* (Loveridge)	KMH 21577	Uluguru South FR, Uluguru Mts., Tanzania	AY531846, AY531869
22	*Probreviceps m. loveridgei* Parker	KMH 21461	Mkungwe FR, Uluguru Mts., Tanzania	AY531847, AY531870
23	*Probreviceps m. loveridgei* Parker	KMH 21532	Kasanga FR, Uluguru, Tanzania	AY531848, AY531871
24	*Probreviceps m. loveridgei* Parker	KMH 22702	West Kilombero Scarp FR, Uzungwa Mts., Tanzania	AY531849, AY531872
25	*Probreviceps m. loveridgei Parker*	KMH 22067	West Kilombero Scarp FR, Uzungwa Mts., Tanzania	AY531850, AY531873
26	*Probreviceps m. macrodactylus* (Nieden)	KMH 16360	Amani NR, East Usambara Mts., Tanzania	AY531851, AY531874
27	*Probreviceps m. macrodactylus* (Nieden)	KMH 21399	Nilo FR, East Usambara Mts., Tanzania	AY531852, AY531875

Vouchers were identified through comparisons with published descriptions (Barbour and Loveridge 1928; Parker 1934; Laurent 1972; Poynton and Broadley 1985; Rödel 2000) and paratype material held in the Natural History Museum, London. Voucher specimens are stored in the Zoology department of the Natural History Museum, London (KMH and MW field series) and M.-O. Rödel's research collection (MOR) deposited in the Staatliches Museum für Naturkunde Stuttgart and the Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn. FR = Forest Reserve, NR = Nature Reserve, m = macrodactylus.

monophyly and sister-group relationships, and we explore the implications of alternative rootings.

Results

A total of 760 aligned sites were analysed, of which 479 were constant, 44 variable but parsimony uninformative, and 237 parsimony informative. The data have a parsimony PTP of 0.01, allowing rejection of the null hypothesis that they contain no more hierarchical structure than expected by chance alone. Relative rates tests indicated that *Spelaeophryne methneri*, *Hemisus marmoratus*, and *Breviceps mossambicus* evolved more rapidly than the other taxa ($p = 0.04$). There is no significant base composition bias for any taxon, whether or not uninformative sites are considered. Plots of transitions vs. transversions (not shown) suggest that saturation is not a problem with these data.

Parsimony analysis yielded three most parsimonious trees (MPTs), which differed only in the position of the two Uluguru samples of *Probreviceps macrodactylus loveridgei* (Fig. 1). The ML analysis used the GTR + I + G model (as recommended by both criteria used in Modeltest). The optimal ML tree (Fig. 2) is similar to the MPTs. Most relationships common to parsimony and ML trees are well supported as judged by bootstrap proportions and decay indices (Fig. 1). Bayesian posterior probabilities are high (>0.87), perhaps unreasonably so, for all splits in the optimal ML tree (Fig. 2), including for relationships not found in the MPTs. A minority of the investigated splits were not significantly better supported than alternatives, as judged by Templeton tests (Fig. 1).

Discussion

The unrooted optimal trees recovered by parsimony and ML (Figs. 1 and 2) are consistent with the monophyly of all previously recognised genera, subfamilies (except Microhylinae) and families, in that the trees can be rooted such that all these taxa are clades. The ML tree can be rooted such that Microhylinae (*Microhyla* + *Kaloula*) is a clade, but the corresponding split has a low posterior probability and is not recovered in the MPTs, which allow for this clade only as one of the possible resolutions of a polytomy. With the exception of the Brevicipitinae, bootstrap proportions for the splits corresponding to the other supraspecific taxa are high (>95%).

Higher relationships

Despite uncertainty over the position of the root, we are able to examine relationships among four main groups: Brevicipitinae (B), *Hemisus* (H), Scaphiophryninae (S), and the remaining, paraphyletic non-brevicipitine, non-scaphiophrynine microhylids (N). Ford and Cannatella (1993) defined Scoptanura as non-scaphiophrynine microhylids, including brevicipitines. Our optimal trees are inconsistent with the Scoptanura hypothesis (H, S (B, N)). Templeton tests ($p < 0.031$) do not require us to attribute the difference (16 steps) between our MPTs and the best trees consistent with Scoptanura monophyly to random sampling error. The same is also true ($p < 0.02$) for the alternative hypothesis (H, N (B, S)). Assuming that brevicipitines are monophyletic (see below) and that *Hemisus* is monophyletic, our data suggest that the Brevicipitinae is the sister group to *Hemisus*, to a clade containing all non-brevicipitine microhylids sampled here, or to a clade including both these groups.

Given that *Hemisus* is only distantly related to non-brevicipitine microhylids (Biju and Bossuyt 2003; Haas 2003; Vences et al. 2003), the implication is that if brevicipitines are the sister group to *Hemisus*, then they are not microhylids. Support for the resolution ((S, N)(H, B)) comes from Blommers-Schlösser's (1993) and Wu's (1994) phylogenetic analyses of morphology. These tentative insights point to a need for a major revision of microhylid classification. Additional taxon sampling and data from other (probably nuclear) genes and/or from more morphological systems will be needed to further resolve phylogenetic relationships before this can be undertaken with confidence.

Non-brevicipitine microhylids

The non-brevicipitine microhylids sampled here were recovered as a putative clade in all analyses. The bootstrap proportion, decay index, and posterior probability for this group are high, and Templeton tests ($p > 0.0339$) do not compel us to attribute this support to sampling error (Figs. 1 and 2). The position of *Hoplophryne* Barbour & Loveridge within a putative clade comprising a mixture of widely geographically distributed, non-brevicipitine microhylids is uncontroversial. The similar nesting of *Phrynomantis* is supported by detailed studies of morphology (Laurent 1941; Haas 2003). Noble (1931) placed *Phrynomantis* in its own subfamily, not closely allied to any other microhylids. Parker (1934) excluded *Phrynomantis* from Microhylidae based on the presence of intercalary cartilages, a character now known to be present in other microhylids as well (Wu 1994). Data from larval morphology strongly support the nesting of *Phrynomantis* within a clade of non-scaphiophrynine microhylids (Haas 2003).

Savage (1973) speculated that the three extant African microhylid subfamilies (Brevicipitinae, Melanobatrachinae, Phrynomerinae) diversified prior to Gondwana

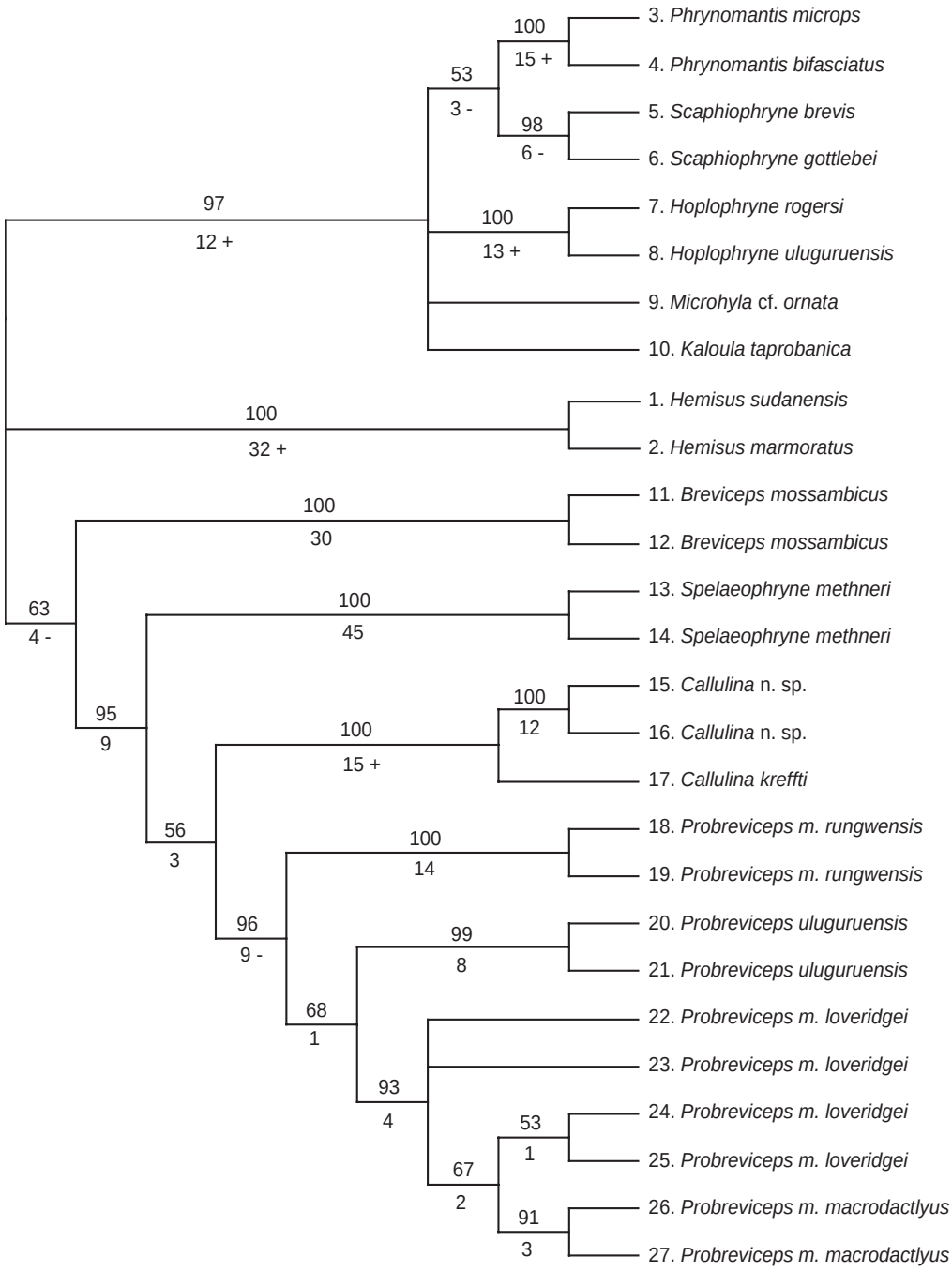

Fig. 1. Strict consensus of three unrooted most parsimonous trees (MPTs). Descriptive statistics (with all characters/without uninformative characters): tree length = 677/629 steps, CI = 0.5746/0.5421, RI = 0.7732/0.7732. Numbers above branches are bootstrap proportions. Numbers below internal branches are decay indices; symbols following the decay index values show the results of Templeton tests for differences in length between the MPTs and the best suboptimal trees obtained from converse topological constraints: presence (+) or lack (−) of support at the $p \leqslant 0.05$ level is indicated for previously hypothesised supraspecific taxa. m = *macrodactylus*.

fragmentation. In contrast, Duellman and Trueb (1994, p. 489) argued that a brevicipitine–phrynomerine lineage diversified only after Gondwana fragmentation. We reject Duellman and Trueb's hypothesis, because there is no rooting of our optimal trees in which *Phrynomantis* and brevicipitines form a clade. We are not compelled to attribute the difference (16 steps) between our MPTs and the best trees in which *Phrynomantis* and brevicipitines are a potential clade to sampling error (Templeton test, $p < 0.02$).

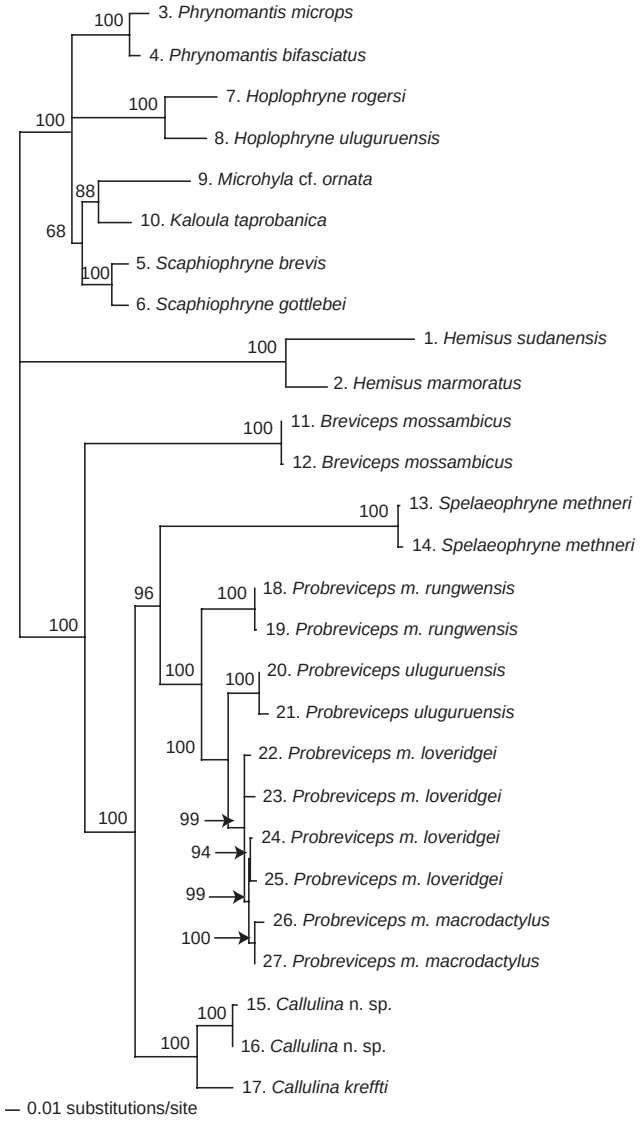

Fig. 2. Maximum likelihood phylogram (unrooted), showing branch lengths (–ln likelihood = 4275.79863, proportion of invariant sites = 0.3491, gamma shape parameter = 0.4952); support values above nodes are Bayesian posterior probabilities.

Brevicipitines

Whether trees are rooted with *Hemisus* or any of the non-brevicipitine microhylids sampled, the data presented in this paper support the monophyly of Brevicipitinae. Quantitative support for this node is not compelling (Figs. 1 and 2), although it is further corroborated by morphological evidence (Parker 1934; Blommers-Schlösser 1993; Wu 1994) and is accepted here. Parker (1934) commented on the special nature of the brevicipitine vomer (prevomer in Parker's usage) which is reduced posteriorly (post-choanally) but bearing a large anterior and medial expansion. Parker also noted other characters (e.g. retention of a complete shoulder girdle) that readily distinguished brevicipitines from all other microhylids, but further work is required to determine derived and plesiomorphic conditions.

Phylogenetic relationships of the genera within Brevicipitinae have been briefly explored by Poynton (1964, 1999), Poynton and Pritchard (1976), Largen and Drewes (1989), and Wu (1994). As their genus names suggest, *Probreviceps* and *Breviceps* have been thought to be closely related, and Poynton (1999, p. 515) proposed that *Breviceps* "can be derived from sylvicolous East African *Probreviceps*". This was based on the observation of clinal variation in the lengths of limbs and digits along the continuous North to South distribution of the two genera (Poynton and Pritchard 1976). *Probreviceps* from Tanzania have the longest limbs and toes, followed by *P. rhodesianus* (further South, in Zimbabwe), then *Breviceps* (which occurs further southwards) with the shortest. In contrast, Wu (1994) hypothesised that *Callulina* and *Probreviceps* comprise a clade, with successive sister groups formed by a paraphyletic *Breviceps*, and *Spelaeophryne*. Focussing on pectoral girdle morphology, Largen and Drewes (1989) questioned the monophyly of *Probreviceps* + *Breviceps* by suggesting that *Probreviceps* is more closely related to *Balebreviceps* (not included in our analyses). Our analyses strongly exclude *Breviceps* from a clade comprising *Probreviceps*, *Callulina* and *Spelaeophryne*. Judged by the Templeton test ($p < 0.03$), it is unnecessary to attribute the difference (14 steps) between our MPTs and the best trees containing a *Probreviceps* + *Breviceps* clade to random sampling error. Despite this, the optimal trees recovered in our analyses (Figs. 1 and 2) do not preclude the possibility that *Breviceps* evolved from a *Probreviceps*-like ancestor, as in Poynton's hypothesis.

Bootstrap support for the *Spelaeophryne* + *Callulina* + *Probreviceps* clade, and for the monophyly of the constituent genera, is high in all analyses, although the best trees in which *Probreviceps* is constrained to be non-monophyletic do not have a significantly worse fit to the data (Fig. 1). The relationships among these three genera are not clearly resolved by our data, although no analyses recovered one of the three possible resolutions, i.e. the pairing of *Callulina* + *Spelaeophryne*. Currently, morphological data that might provide decisive support for one of the two competing hypotheses (in the optimal parsimony and ML trees) are lacking. The conflict and lack of resolution might be caused by heterogeneous rates of molecular evolution (i.e. *Spelaeophryne* relative to other brevicipitines), inadequate taxon sampling (*Balebreviceps hillmani*; additional species of *Breviceps*), or simply too few sequence data.

The referral of a new species to *Callulina* based on morphology (de Sá, Loader & Channing, unpublished) is strongly supported by our molecular analyses. The

status of the *Probreviceps macrodactylus* complex has not been investigated previously in a phylogenetic context. Our analyses suggest (Figs. 1 and 2) that *P. macrodactylus* is paraphyletic with respect to *P. uluguruensis*, but this is poorly supported as judged by the Templeton test ($p > 0.21$), bootstrap proportion and decay index values (Fig. 1). *Probreviceps macrodactylus rungwensis* can be distinguished from other *Probreviceps* by its large tympanum and notably pointed snout (J. C. Poynton, pers. comm.), and it perhaps represents a distinct species. We sampled *P. m. rungwensis* from the Udzungwa only, so that future sampling of this taxon from its type locality of Rungwe (part of the Southern Highlands rather than the Eastern Arc) is recommended, particularly in light of the apparently significant biogeographical barrier (the 'Makambo Gap', e.g. Keilland 1990; Lovett 1990; Gravlund 2002) between these regions. Limited morphological studies on *P. m. macrodactylus* and *P. m. loveridgei* (Parker 1934; Poynton, unpublished) and our molecular data suggest that there are very few differences between these subspecies, and the molecular data suggest that the latter may be paraphyletic with respect to the former (Figs. 1 and 2).

Tanzanian *Probreviceps* are confined to upland evergreen forest of the isolated constituent blocks of the Eastern Arc Mountains and Southern Highlands (e.g. Howell 1993). Taken at face value, the optimal phylogenies recovered in our analyses (Figs. 1 and 2) suggest that divergence of lineages giving rise to extant Udzungwa and Uluguru *Probreviceps* has occurred at least twice. The combined distributional and phylogenetic evidence does not fit with a simple, single vicariance/dispersal event, but is seemingly in accordance with the hypothesis that climatic fluctuations have repeatedly isolated (and reconnected) Eastern Arc montane forests over the last 2.8 Myr and driven speciation (e.g. see Roy 1997, and references therein). However, we stress that the relationships on which this is based are not well supported.

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References

Barbour, T., Loveridge, A., 1928. A comparative study of the herpetological faunae of the Uluguru and Usambara mountains, Tanganyika Territory, with descriptions of new species. Mem. Mus. Comp. Zool. 50, 87–265.

Benson, D.A., Boguski, M.S., Lipman, D.J., Ostell, J., Ouellette, B.F.F., 1998. GenBank. Nucleic Acids Res. 26, 1–7.

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

Blair, W.F., 1962. Non-morphological data in Anuran classification. Syst. Zool. 11, 72–84.

Blommers-Schlösser, R.M.A., 1993. Systematic relationships of the Mantellinae Laurent, 1946 (Anura: Ranoidea). Ethol. Ecol. Evol. 5, 199–218.

Bossuyt, F., Milinkovitch, M.C., 2001. Amphibians as indicators of early Tertiary 'out-of-India' dispersal of vertebrates. Science 292, 93–95.

Bremer, K., 1988. The limits of amino acid sequence data in Angiosperm phylogenetic reconstruction. Evolution 42, 795–803.

Carvalho, A.L.de., 1954. A preliminary synopsis of the genera of American microhylid frogs. Occas. Pap. Mus. Zool. Univ. Mich. 555, 1–19.

Channing, A., 2001. Amphibians of Central and Southern Africa. Cornell University Press, New York.

Duellman, W.E., Trueb, L., 1994. Biology of Amphibians. Johns Hopkins University Press, Baltimore, London.

Faith, D.P., Cranston, P.S., 1991. Could a cladogram this short have arisen by chance alone? On permutation tests for cladistic structure. Cladistics 7, 1–28.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Fjeldå, J., Lovett, J.C., 1993. Geographical patterns of old and young species in African forest biota: the significance of specific montane areas as evolutionary centres. Biodivers. Conserv. 6, 325–346.

Ford, L., Cannatella, D.C., 1993. The major clades of frogs. Herpetol. Monogr. 7, 94–117.
Frost, D.R., 2002. Amphibian Species of the World: an Online Reference. V2.21 (15 July 2002). http://research.amnh.org/herpetology/amphibia/index.html
Gravlund, P., 2002. Molecular phylogeny of Tornier's cat snake (*Crotaphopeltis tornieri*), endemic to East African mountain forests: biogeography, vicariance events and problematic species boundaries. J. Zool. Syst. Evol. Res. 40, 46–56.
Haas, A., 2003. Phylogeny of frogs inferred from primarily larval characters (Amphibia: Anura). Cladistics 19, 23–89.
Hay, J.M., Ruvinsky, I., Hedges, S.B., Maxson, L.R., 1995. Phylogenetic relationships of Amphibian families from DNA sequences of mitochondrial 12S and 16S ribosomal RNA genes. Mol. Biol. Evol. 12, 928–937.
Hertwig, S., Sá, R.O.de., Haas, A., 2004. Phylogenetic signal and the utility of 12S and 16S mtDNA in frog phylogeny. J. Zool. Syst. Evol. Res. 42, 2–18.
Howell, K.M., 1993. Herpetofauna of the Eastern African forests. In: Lovett, J.C., Wasser, S.K. (Eds.), Biogeography and Ecology of the Rain Forests of Eastern Africa. Cambridge University Press, Cambridge, pp. 173–203.
Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.
Keilland, J., 1990. Butterflies of Tanzania. Hill House, Melbourne, London.
Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 89, 6196–6200.
Largen, M.J., Drewes, R.C., 1989. A new genus and species of brevicipitine frog (Amphibia Anura Microhylidae) from high altitude in the mountains of Ethiopia. Trop. Zool. 2, 13–30.
Laurent, R.F., 1941. Note sur l'osteologie des genres *Breviceps* et *Phrynomerus* (Batraciens). Rev. Zool. Bot. Afr. 35, 417–418.
Laurent, R.F., 1972. Tentative revision of the genus *Hemisus* Günther. Ann. Mus. R. Afr. Cent. Sci. Zool. 194, 1–67.
Lovett, J.C., 1990. Classification and status of the moist forests of Tanzania. Mitt. Inst. Allg. Bot. Hamb. 23c, 287–300.
Minter, L.R., 2003. Two new cryptic species of *Breviceps* (Anura: Microhylidae) from southern Africa. Afr. J. Herpetol. 52, 9–21.
Noble, G.K., 1931. The Biology of the Amphibia. Dover Publications, New York.
Palumbi, S.R., 1996. The polymerase chain reaction. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), Molecular Systematics, second ed. Sinauer Associates, Sunderland, MA, pp. 205–247.
Parker, H.W., 1934. A Monograph of the Frogs of the Family Microhylidae. Trustees of the British Museum (Natural History), London.
Posada, D., Crandall, K., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.
Poynton, J.C., 1964. The Amphibia of southern Africa. Ann. Natal Mus. 17, 1–334.
Poynton, J.C., 1999. Distribution of amphibians in sub-Saharan Africa, Madagascar, and Seychelles. In: Duellman, W.D. (Ed.), Patterns of Distribution of Amphibians, a Global Perspective. Johns Hopkins University Press, Baltimore, pp. 483–539.
Poynton, J.C., Broadley, D.G., 1985. Amphibia Zambesiaca 1. Scolecomorphidae, Pipidae, Microhylidae, Hemisidae, Arthroleptidae. Ann. Natal Mus. 26, 503–553.
Poynton, J.C., Pritchard, S., 1976. Notes on the biology of *Breviceps* (Anura: Microhylidae). Zool. Afr. 11, 313–318.
Robinson-Rechavi, M., Huchon, D., 2000. RRTree: relative-rate tests between groups of sequences on a phylogenetic tree. Bioinformatics 16, 296–297.
Rödel, M.-O., 2000. Herpetofauna of West Africa, Vol. I: amphibians of the West African savanna. Edition Chimaira, Frankfurt/M.
Roy, M.S., 1997. Recent diversification of African Greenbuls (Pycnonotidae: *Andropadus*) supports a montane speciation model. Proc. R. Soc. London B 264, 1337–1344.
Savage, J.M., 1973. The geographic distribution of frogs: patterns and predictions. In: Vial, J.L. (Ed.), Evolutionary Biology of the Anurans. University of Missouri Press, Columbia, pp. 351–445.
Swofford, D.L., 1998. PAUP*, Phylogenetic Analysis Using Parsimony (*and Other Methods). Test version 4b6. Sinauer Associates, Sunderland, MA, USA.
Templeton, A.R., 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution 37, 221–244.
Vences, M., Kosuch, J., Glaw, F., Böhme, W., Veith, M., 2003. 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.
Wilkinson, M., Loader, S.P., Gower, D.J., Sheps, J.A., Cohen, B.L., 2003. Phylogenetic relationships of African caecilians (Amphibia: Gymnophiona): insights from mitochondrial rDNA gene sequences. Afr. J. Herpetol. 52, 83–92.
Wu, S.-H., 1994. Phylogenetic relationships, higher classification and historical biogeography of the Microhyloid frogs (Lissamphibia: Anura: Brevicipitidae and Microhylidae). Ph.D. Thesis, University of Michigan.

Note added in proof

Since this paper was accepted, two publications have appeared that provide evidence that brevicipitines (Darst and Cannatella 2003; Van der Meijden et al. 2004) and *Hemisus* (Darst and Cannatella 2003) are more closely related to hyperoliids and arthroleptids than to non-brevicipitine microhylids. Each study included a single brevicipitine.

Darst, C.R., Cannatella, D.C., 2003. Novel relationships among hyloid frogs inferred from 12S and 16S mitochondrial DNA sequences. Mol. Phyl. Evol. 31, 462–475.
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. Roy. Soc. Lond. B (Suppl.) 271, S378–S381.