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Molecular phylogeny of hyperoliid treefrogs: biogeographic origin of Malagasy and Seychellean taxa and re-analysis of familial paraphyly

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Abstract

Treefrogs of the family Hyperoliidae are distributed in Africa, Madagascar and the Seychelles. In this study, their phylogeny was studied using sequences of fragments of the mitochondrial 16S and 12S rRNA and cytochrome *b* genes. The molecular data strongly confirmed monophyly of the subfamily Hyperoliinae but indicated that the genus *Leptopelis* (subfamily Leptopelinae) is more closely related to species of the African family Astylosternidae. The Seychellean genus *Tachycnemis* was the sister group of the Malagasy *Heterixalus* in all molecular analyses; this clade was deeply nested within the Hyperoliinae. A re-evaluation of the morphological data did not contradict the sister group relationships of these two genera. The subfamily Tachycneminae is therefore considered as junior synonym of the Hyperoliinae. In addition, the molecular analysis did not reveal justification for a subfamily Kassininae. Biogeographically, the origin of Malagasy hyperoliids may not be well explained by Mesozoic vicariance in the context of Gondwana breakup, as indicated by the low differentiation of Malagasy hyperoliids to their African and Seychellean relatives and by analysis of current distribution patterns.

Key words: Amphibia – Anura – Hyperoliidae – *Heterixalus* – *Tachycnemis* – Madagascar – Seychelles – biogeography – phylogeny – 16S rRNA – 12S rRNA – cytochrome *b* – osteology

Introduction

The relationships and classification of the treefrogs of Africa, Asia and Madagascar have been controversial since the dawn of phylogenetic systematics. Old World treefrogs are defined by the presence of (cartilaginous or ossified) intercalary elements between ultimate and penultimate phalanges of fingers and toes. They belong to the superfamily Ranoidea based on their derived firmisternal shoulder girdle, as opposed to the superfamily Hyloidea, which has a plesiomorphic arciferal condition of the shoulder girdle, is mainly distributed in the New World, and also contains treefrog families that convergently evolved intercalary elements (Duellman and Trueb 1986).

For many years, all Old World treefrogs have been grouped in one family Rhacophoridae or Polypedatidae based on the presence of the intercalary element (e.g. Noble 1931). Laurent (1951) was the first to recognize that one large clade of Old World treefrogs was more closely related to a group of non-arboreal African frogs, today classified as family Astylosternidae. He considered this lineage as family Hyperoliidae. The separate status of the Hyperoliidae was later supported by the cladistic analyses of Liem (1970), Drewes (1984), Channing (1989) and Blommers-Schlösser (1993). The monophyly of the family, however, has recently been questioned by molecular data (Emerson et al. 2000).

The Hyperoliidae *sensu lato* include about 235 species (Glaw et al. 1998a) of mainly arboreal frogs which are characterized by combination of: (a) derived firmisternal condition of shoulder girdle; (b) derived presence of an intercalary element; and (c) plesiomorphic absence of a bony sternal style. These frogs are distributed in Africa, Madagascar and the Seychelles. The vast majority of genera and species occurs in Africa, whereas the monotypic *Tachycnemis* is endemic to the Seychelles, and *Heterixalus* (11 species; Vences et al. 2000b) is endemic to Madagascar. *Heterixalus* was placed by most workers close to African genera, mostly *Hyperolius* and *Afrixalus*, while *Tachycnemis* has often been considered as one of the most basal representative of the Hyperoliidae (Drewes 1984; Channing 1989). In contrast, the molecular study of Richards and Moore (1996), based on sequences of a fragment of the 12S rRNA gene in 14 hyperoliid taxa, arranged *Heterixalus* as the well-supported sister group of *Tachycnemis*. The *Tachycnemis–Heterixalus* clade was arranged as the sister group of *Afrixalus*, and *Leptopelis* was the sister group of all other included hyperoliid taxa. In a combined analysis of the available molecular and morphological data, Emerson et al. (2000) provided additional support for the *Tachycnemis–Heterixalus* relationships. By providing additional sequence data for a reduced set of taxa, these authors found indications of non-monophyly of hyperoliids, with *Leptopelis* being more closely related to the Arthroleptidae than to the other Hyperoliidae.

Because frogs are usually thought to be unable to disperse across the open sea (e.g. Bossuyt and Milinkovitch 2001), they appear to be a good model group to test plate tectonical events. The *Heterixalus–Tachycnemis* relationship appears to be congruent with the existence of a Madagascar–India continent in the Cretaceous, which also included the Seychelles (Richards and Moore 1996).

In the present study, our aim was to test the assumed relationships between Malagasy and Seychellean hyperoliids by analysis of additional DNA sequences and by a re-assessment of morphological characters. Additionally, we investigated the possible non-monophyly of the family by inclusion, for the first time, of crucial outgroup taxa such as scaphiophrynine microhylids and astylosternids.

Materials and methods

DNA was extracted using QIAmp extraction kits (QIAgen) from tissue samples (hindleg muscle, either fresh or preserved in 98% ethanol). Voucher specimens were deposited in the Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn (ZFMK; see Table 1). Other museum acronyms used are BMNH (The Natural History

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Table 1. List of specimens used for analysis, and of Genbank accession numbers for the sequenced fragments of the 16S and 12S rRNA genes and of the cytochrome *b* gene

Family	Species	Origin	Collection number	16S (1/2)	12S	Cyt *b*
Bufonidae	*Bufo asper*	Tanak Masa island, West Sumatra (16S)	–	AF124109/AJ437011	U52733*	–
Bufonidae	*Bufo melanostictus*	Hong Kong	MVZ 217645	–	–	L10977*
Arthroleptidae	*Arthroleptis variabilis*	Cameroon	ZFMK 68794	AF124107	AF215140	–
Arthroleptidae	*Arthroleptis* sp.	Nlonako, Cameroon	Not collected	–	AF215139	–
Arthroleptidae	*Cardioglossa* cf. *leucomystax*	Comoe National Park, Ivory Coast	Not collected	AF124110	–	–
Astylosternidae	*Trichobatrachus robustus*	Nkongsamba, Cameroon	ZFMK 66453	AF124136	AF124104	–
Astylosternidae	*Astylosternus schiotzi*	Edib, Cameroon	ZFMK 67733	AF124108/AJ437008	AF215142	AF215490
Astylosternidae	*Scotobleps gabonicus*	Nlonako, Cameroon	ZFMK 69155	AF215341	AF215141	–
Hemisotidae	*Hemisus marmoratus*	Comoe National Park, Ivory Coast	ZFMK 68418	AF215342	AF215143	–
Hyperoliidae	*Acanthixalus spinosus*	Nyasoso, Cameroon	ZFMK 72000	AF215427/AJ437002	AF215214	AF215488
Hyperoliidae	*Afrixalus delicatus*	Kwambonambi, South Africa	ZFMK 68792	AF215428/AJ437005	AF215215	AF215489
Hyperoliidae	*Afrixalus knysnae*	South Africa	Not preserved	AF215429	AF215216	–
Hyperoliidae	*Afrixalus laevis*	Edib, Cameroon	ZFMK 67423	AF215430	AF215217	–
Hyperoliidae	*Afrixalus* sp.	Pet trade (no locality)	ZFMK 68790	AF215431	AF215218	–
Hyperoliidae	*Cryptothylax gresshoffi*	Pet trade (Ghana?)	Not preserved	AF215432	–	–
Hyperoliidae	*Heterixalus alboguttatus*	Ranomafana, Madagascar	Not preserved	AF215433/AJ437003	AF215219	AF215491
Hyperoliidae	*Heterixalus boettgeri*	Near Tolagnaro, Madagascar	Not preserved	AF215435	AF215221	–
Hyperoliidae	*Heterixalus madagascariensis*	Masoala, Madagascar	Not preserved	AF215437	–	–
Hyperoliidae	*Heterixalus tricolor*	Kirindy, Madagascar	ZFMK 66689	AF215434/AJ437004	AF215220	AF215492
Hyperoliidae	*Heterixalus luteostriatus*	Kirindy, Madagascar	ZFMK 72027	AF215436	–	–
Hyperoliidae	*Hyperolius* sp.	Cameroon	Not preserved	AF215438	–	AF215493
Hyperoliidae	*Hyperolius tuberilinguis*	Mtunzini, South Africa	ZFMK 66725	AF215443/AJ437007	AF215226	–
Hyperoliidae	*Hyperolius argus*	Mtunzini, South Africa	ZFMK 68780	AF215439	AF215222	–
Hyperoliidae	*Hyperolius viridiflavus*	Barberton, South Africa	ZFMK 66726	AF215440	AF215223	–
Hyperoliidae	*Hyperolius viridiflavus*	Little Brak, South Africa	ZFMK 66718	AF215441	AF215227	–
Hyperoliidae	*Hyperolius nasutus*	Rundu, Namibia	ZFMK 66764	AF215442	AF215224	–
Hyperoliidae	*Hyperolius semidiscus*	Silaka, South Africa	ZFMK 68779	–	AF215225	AF215494
Hyperoliidae	*Kassina senegalensis*	St. Lucia, South Africa	Not preserved	AF215445	AF215228	AF215495
Hyperoliidae	*Kassina maculata*	Kwambonambi, South Africa	ZFMK 66445	AF215444	AF215229	–
Hyperoliidae	*Opisthothylax immaculatus*	Cameroon	ZFMK uncatalogued	AJ437012	–	–
Hyperoliidae	*Semnodactylus weali*	Kokstadt, South Africa	Not preserved	AF215450/AJ437006	AF215232	AF215499
Hyperoliidae	*Tachycnemis seychellensis*	Mahé, Seychelles	ZFMK 62856	AF215451	–	–
Hyperoliidae	*Tachycnemis seychellensis*	Praslin, Seychelles	ZFMK 62879	AF215452	AF215233	AF215500
Hyperoliidae	*Leptopelis mossambicus*	Sta. Lucia, South Africa	Not preserved	AF215446	–	–
Hyperoliidae	*Leptopelis brevirostris*	Cameroon	ZFMK 72065	AF215447	–	–
Hyperoliidae	*Leptopelis modestus*	Cameroon	ZFMK 67976	AJ437013/AJ437009	AF215230	AF215497
Hyperoliidae	*Leptopelis natalensis*	Mtunzini, South Africa	ZFMK 68785	AF215448/AJ437010	AF215231	AF215498
Hyperoliidae	*Leptopelis natalensis*	Silaka, South Africa	ZFMK 68783	AF215449	–	–
Microhylidae (Scaphiophryninae)	*Scaphiophryne brevis*	Kirindy, Madagascar	Not preserved	AF215384	–	–
Microhylidae (Scaphiophryninae)	*Scaphiophryne gottlebei*	Pet trade (no locality)	Not preserved	AF215385	AF215144	–
Mantellidae	*Aglyptodactylus madagascariensis*	Andasibe, Madagascar	ZFMK 64137	AF215330	AF215179	–
Mantellidae	*Boophis doulioti*	Isalo, Madagascar	ZFMK 70495	AF215332	AF215163	AF215487
Mantellidae	*Mantella madagascariensis*	Ranomafana, Madagascar	ZFMK 64138	AF215301	AF215177	–
Mantellidae	*Mantidactylus grandisonae*	Ambato, Madagascar	ZFMK 66669	AF215315	AF215149	AF215485
Ranidae (Cacosterninae)	*Cacosternum boettgeri*	Bredell, South Africa (12S); Hardap, Namibia (16S)	ZFMK 66727	AF215414	AF215208	–
Ranidae (Raninae)	*Rana temporaria*	Koblenz, Germany	ZFMK 69883	AF124135	AF124103	–
Rhacophoridae	*Chiromatis rufescens*	Cameroon	ZFMK 72067	AF215347	–	–
Rhacophoridae	*Chiromantis xerampelina*	Pet trade (no locality)	ZFMK 72070	AF215348	–	–
Rhacophorinae	*Rhacophorus nigropalmatus*	Laos	MNHN 1997.4092	AF215359	AF215188	–

Sequences marked with an asterisk were obtained from Genbank and refer to Graybeal (1997).

Museum, London), MRSN (Museo Regionale di Scienze Naturali, Torino), MVZ (Museum of Vertebrate Zoology, Berkeley; FC, frozen tissue collection), UMMZ (University of Michigan Museum of Zoology), ZMA (Zoölogisch Museum Amsterdam), ZMB (Zoologisches Museum der Universität, Berlin). Four pairs of primers were used to amplify sections of the mitochondrial 16S rRNA, 12S rRNA and cytochrome *b* genes. Primer sequences and PCR conditions are summarized in Table 2. Of these fragments, the 12S rRNA portion was homologous to the sequences of Richards and Moore (1996) and Emerson et al. (2000), and could therefore be combined with these previous data sets. Our 16S rRNA sequences, in contrast, correspond to sections that had not been sequenced by these authors, and could therefore not be submitted to a combined analysis.

PCR products were purified using QIAquick purification kits (QIAagen). Single-stranded fragments were sequenced using an automatic sequencer (ABI 377). Sequences were aligned using the CLUSTAL option of the computer program SEQUENCE NAVIGATOR (Applied Biosystems); alignments were subsequently adjusted manually. Sequences were submitted to Genbank (see Table 1 for accession numbers). Highly variable regions of the 16S and 12S fragments were excluded from the analysis.

To assess whether the different gene fragments of the reduced taxa set could be submitted to combined analysis, partition homogeneity tests as implemented in PAUP*, version 4b8 (Swofford 2001) were used. Prior to phylogenetic reconstruction, we explored which substitution model fits our sequence data the best. Hierarchical likelihood ratio tests were applied for testing the goodness-of-fit of nested substitution models using the program MODELTEST (Posada and Crandall 1998).

Phylogenetic analyses were carried out using PAUP*. We calculated maximum parsimony (MP) trees with gaps treated as a fifth character, and neighbour-joining (NJ) and maximum likelihood (ML) trees with gaps treated as missing data. In the MP analyses, we conducted heuristic searches with initial trees obtained by simple stepwise addition, followed by branch swapping using the TBR (tree bisection-reconnection) routine implemented in PAUP*. Only minimal length trees were saved and zero length branches were collapsed. As homogeneity of base frequencies was rejected in the combined analysis, we chose the LogDet model for NJ analysis, which is robust against possible variation of sequence evolution among lineages (Lockhart et al. 1994). The ML trees were obtained using heuristic searches with settings as in MP, using the substitution model proposed by MODELTEST for each data subset, respectively.

Following Hedges (1992), 2000 bootstrap replicates were run (Felsenstein 1985) in all analyses except ML, where only 50–100 replicates were run because of computational constraints. Additionally, the robustness of nodes was tested by Shimodaira–Hasegawa tests as implemented in PAUP* (RELL bootstrap, 1000 replicates, one-tailed test).

Own data on osteology refer to the following cleared and stained (Dingerkus and Uhler 1977; Plösch 1991) specimens: *Afrixalus delicatus* (ZFMK 68792); *Afrixalus fornasinii* (ZFMK 68789); *Afrixalus fulvovittatus* (ZFMK 62576);*Afrixalus* sp. (ZFMK 68790, 68791); *Heterixalus alboguttatus* (ZFMK 68793); *Heterixalus andrakata* (ZFMK 52561, 52564); *Heterixalus betsileo* [ZMA 6724, ZMA 6756, ZMA uncatalogued (field number 995), MRSN A399.4]; *Heterixalus luteostriatus* (MRSN A393.7); *Heterixalus madagascariensis* (ZFMK 52574, 52647);*Heterixalus punctatus* (ZFMK 60018); *Heterixalus rutenbergi* (ZFMK 59844); *Heterixalus tricolor* (ZFMK 52583); *Heterixalus* '*variabilis*' (ZFMK 52578, 53606); *Hyperolius argus* (ZFMK 68780); *Hyperolius nasutus* (ZFMK 68782); *Hyperolius pusillus* (ZFMK 68781); *Hyperolius semidiscus* (ZFMK 68779); *Hyperolius tuberilinguis* (ZFMK 68778); *Hyperolius viridiflavus* (ZFMK 68773–68777); *Kassina decorata* (ZFMK 67841); *Leptopelis* cf. *bocagei* (ZFMK 68787, 68788); *Leptopelis natalensis* (ZFMK 68783–68786); *Leptopelis modestus* (ZFMK 67412); *Leptopelis mossambicus* (ZFMK 29444); *Leptopelis rufus* (ZFMK 67992); *Phlyctimantis verrucosus* (ZFMK 58824); *Tachycnemis seychellensis* (ZFMK 62859, 62879, BMNH 1976.1958).

Familial arrangement throughout follows Vences and Glaw (2001). Considering the phylogenies of Emerson et al. (2000) and anticipating the results obtained herein, the following names are used when referring to hyperoliid treefrogs: (1) Hyperoliidae *sensu lato*, to refer to

Table 2. Primers and PCR conditions (Vences et al. 2000c; Mausfeld et al. 2000) used for amplifications of four gene fragments (16S, 12S rRNA; cytochrome *b*)

	16S rRNA fragment 1	16S rRNA fragment 2	12S rRNA	Cytochrome *b*
L-Primer	16SAL: 5′ – CGC CTG TTT ATC AAA AAC AT– 3′ (Palumbi et al. 1991)	16SL3: 5′ – AGC AAA GAH YWW ACC TCG TAC CTT TTG CAT– 3′ (Hedges 1994: modified)	12SAL: 5′ – AAA CTG GGA TTA GAT ACC CCA CTA T– 3′ (Palumbi et al. 1991)	L14841: 5′ – CTC CCA GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC TTC G– 3′ (Kocher et al. 1989: modified)
H-Primer	16SBH: 5′ – CCG GTC TGA ACT CAG ATC ACG T – 3′ (Palumbi et al. 1991)	16SAH: 5′ – ATG TTT TTG ATA AAC AGG CG– 3′ (reverse of 16SAL)	12SBH: 5′ – GAG GGT GAC GGG CGG TGT GT– 3′ (Palumbi et al. 1991)	CB3H: 5′– GGC AAA TAG GAA GTA TCA TTC TG– 3′ (Palumbi et al. 1991)
Length of analysed DNA fragments	ca. 530 bp	470 bp	ca. 400 bp	415 bp
PCR conditions				
Initial denaturation	90 s: 94°C	90 s: 94°C	–	–
Denaturation	45 s: 94°C	45 s: 94°C	45 s: 94°C	90 s: 95°C
Annealing	45 s: 55°C	45 s: 55°C	60 s: 50°C	60 s: 50°C
Extension	90 s: 72°C	90 s: 72°C	120 s: 74°C	90 s: 72°C
Cycles (2– 4)	33	33	35	34

For each primer, the original reference is given. The lengths of the analysed fragments are only approximate in the rRNA genes, which contain a large number indels among taxa. The data refer to the analysed number of base pairs, not to the lengths of the original gene sections as amplified by the primers given; for instance, the portion of the 16S rRNA gene amplified by the primers 16SL3 and 16SAH (fragment 2) has a length of more than 630 bp.

the family as currently understood (e.g. Drewes 1984; Duellman and Trueb 1986), this entity is probably paraphyletic; (2) Hyperoliinae to refer to all Hyperoliidae *sensu lato* except for *Leptopelis*; (3) Leptopelinae to refer to species of the genus *Leptopelis*. For a phylogenetic definition of the latter two taxa, see the section Discussion.

Results

Analysis of 16S rRNA sequences

A chi-square test did not reject the hypothesis of homogeneity of base frequencies across taxa ($p = 1$). MODELTEST selected a general time-reversible substitution model with a proportion of invariable sites of 0.287, a gamma distribution shape parameter of 0.534, empirical base frequencies (freqA = 0.3458; freqC = 0.2338; freqG = 0.1701; freqT = 0.2504) and substitution rates (A–C = 3.9941; A–G = 11.0765; A–T = 7.3495; C–G = 2.0263; C–T = 36.6485; G–T = 1) as best fitting the combined data set. After exclusion of hypervariable sites, 471 characters were available for analysis. Of these, 225 were constant, 59 were variable but parsimony-uninformative and 187 were parsimony-informative.

Figure 1 shows the results of an ML analysis carried out under the substitution model suggested by MODELTEST. The topologies of MP and NJ trees largely agreed with this tree and are not shown. Three main lineages were distinguished and supported by relevant bootstrap values in ML, MP and NJ analyses: (1) a lineage containing all included Hyperoliinae (bootstrap support 95–98%); (2) a lineage containing all Ranidae, Rhacophoridae and Mantellidae (81–95%); (3) a clade containing the Leptopelinae (*Leptopelis*) (100%). The latter was grouped with arthroleptids, astylosternids and *Hemisus*, but no relevant bootstrap support was found for these relationships.

Within the Hyperoliinae, *Afrixalus* was not monophyletic. The *Afrixalus–Opisthothylax* lineage was monophyletic in the MP and NJ analyses (not shown) but not in the ML analysis (Fig. 1). *Kassina* was paraphyletic and included *Semnodactylus*. *Heterixalus* was paraphyletic and included *Tachycnemis*. *Hyperolius* was monophyletic. However, no convincing bootstrap support was found for any of these topologies. In contrast, the monophylum containing *Tachycnemis* and *Heterixalus* received high support (91–100%).

Analysis of 12S rRNA sequences

For the data set including the sequences from Richards and Moore (1996) and Emerson et al. (2000) and those obtained by us, MODELTEST selected a general time-reversible substitution model with a proportion of invariable sites of 0.268, a gamma distribution shape parameter of 0.738, empirical base frequencies (freqA = 0.3386; freqC = 0.2066; freqG = 0.1715; freqT = 0.2833) and substitution rates (A–C = 1.9913; A–G = 4.8308; A–T = 3.1322; C–G = 0.2361; C–T = 13.7671; G–T = 1). After exclusion of hypervariable sites, 321 characters were available for analysis. Of these, 148 were constant, 35 were variable but parsimony-uninformative and 138 were parsimony-informative.

The ML tree obtained using this substitution model (Fig. 2) largely agreed with the one based on the 16S sequences, although bootstrap support for most aspects of the topology was lower. Sequences from Genbank clustered with conspecific sequences obtained by us in four cases (*Heterixalus boettgeri*, *T. seychellensis*, *Hyperolius viridiflavus*, *Hyperolius argus*) corroborating the validity of combining the two data sets. In three cases the conspecific sequences did not result to be sister groups: the two included *Kassina maculata* sequences were very similar to each other but were arranged paraphyletically with respect to *Kassina senegalensis*. A similar situation was found in *Heterixalus tricolor*, but in neither case was the topology supported by relevant bootstrap values. In contrast, the three representatives of the Arthroleptidae studied by Emerson et al. (2000) did not form a lineage with the *Arthroleptis* sequences obtained by us, possibly because of a relatively large number of uncertainties in our sequences.

Analysis of combined 16S rRNA, 12S rRNA and cytochrome *b* sequences

In the analysis of the combined data in a reduced set of taxa, chi-square tests contradicted homogeneity of base frequencies in the combined data set ($p < 0.05$) but not in any of the separate fragments in the reduced set of taxa ($p > 0.5$). A partition homogeneity test did not reject the null hypothesis of congruence of the included gene fragments (1000 replicates; $p = 0.53$), thereby not contradicting their suitability for combination in phylogenetic analysis. MODELTEST selected a Tamura–Nei substitution model with a proportion of invariable sites of 0.276, a gamma distribution shape parameter of 0.521, empirical base frequencies (freqA = 0.3714; freqC = 0.2278; freqG = 0.1268; freqT = 0.2740) and substitution rates (A–G = 2.2692; C–T = 6.7289; all other rates = 1) as best fitting the combined data set. This model was used in ML analyses (Fig. 3). Of the total of 1821 included characters, 976 were constant, 279 were variable but parsimony-uninformative and 566 were parsimony-informative.

Astylosternus was the sister group of *Leptopelis* in MP, NJ and ML analyses (bootstrap support 84–99%) and the Hyperoliinae were a monophylum (100%). *Tachycnemis* was the sister group of *Heterixalus* (100%), and *Acanthixalus* was the sister group of the *Semnodactylus* lineage (59–85%). The two included species of *Heterixalus* were sister groups (79–99%), rejecting the hypothesis of paraphyly of *Heterixalus* versus *Tachycnemis* as suggested by the topology of the 12S and 16S ML trees. Other splits were not sufficiently resolved. Shimodaira–Hasegawa tests excluded significantly ($p < 0.05$) all alternative topologies in which *Tachycnemis* and *Heterixalus* were not sister groups, and all topologies in which *Leptopelis* was nested within the Hyperoliinae; they did not reject significantly, however, the alternative topology with *Leptopelis* being the sister group of the Hyperoliinae.

In analyses of each of the separate gene fragments (16S rRNA, 12S rRNA, cytochrome *b*), the *Tachycnemis–Heterixalus* and the *Astylosternus–Leptopelis* lineage were monophyletic under ML, MP and NJ models (not shown).

Reassessment of the relationships between *Heterixalus* and *Tachycnemis*

Our molecular results corroborated those of Richards and Moore (1996) regarding the close relationships between *Heterixalus* (Madagascar) and *Tachycnemis* (Seychelles). The sequences of the *Tachycnemis* specimens from the islands of Praslin and Mahé were almost identical (a single substitution in the 16S fragment), confirming the view of Nussbaum and Wu (1995) that the *Tachycnemis* populations from the different

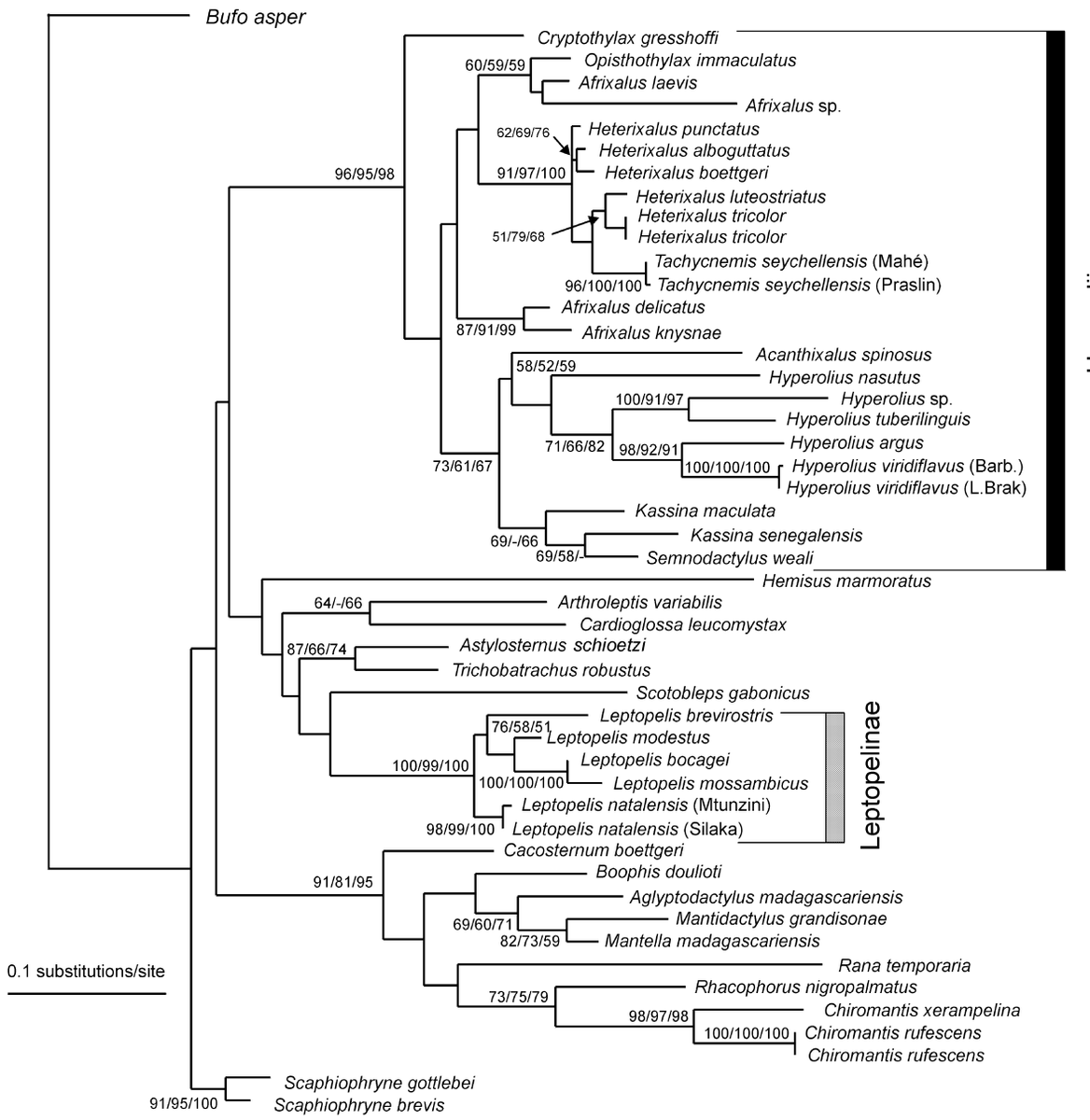

Fig. 1. Maximum likelihood phylogram based on analysis of 471 bp of mitochondrial 16S ribosomal RNA gene sequences (16S fragment 1) of frogs of the family Hyperoliidae *sensu lato* and other ranoid taxa. Vertical bars mark the two hyperoliid subfamilies recognized herein and mark the apparent paraphyly of the family. Numbers above or below branches are bootstrap values in percentage (ML: 50 replicates; MP and NJ: 2000 replicates; values below 50% not given). *Bufo asper* was used as the outgroup

Seychellean islands are conspecific. All three analysed gene fragments unequivocally suggested a sister group relationship of *Tachycnemis* and *Heterixalus*. This stands in conflict with the morphological and osteological phylogeny as available from the literature (Drewes 1984). We therefore undertook a re-evaluation of the osteology and morphology of these two genera. According to Drewes (1984: Fig. 25), *Tachycnemis* is the sister group of a clade containing *Callixalus*, *Acanthixalus, Chrysobatrachus*, *Opisthothylax*, *Hyperolius*, *Afrixalus* and *Heterixalus*, and is phylogenetically characterized by the autapomorphies 1(2), 24(1) and the reversal (relative to the state considered as plesiomorphic in the Hyperoliidae *sensu lato*) 9(1). The remaining lineage is characterized by the synapomorphies 1(0), 20(2). The *Heterixalus–Hyperolius–Afrixalus* lineage is characterized by the synapomorphy 18(5). *Heterixalus* is characterized by the autapomorphy 9(0) and the reversal 14(1) (see below for an explanation of these characters and character states).

Comparing the character states of *Tachycnemis* and *Heterixalus* in appendix B of Drewes (1984), phylogenetically relevant differences between *Heterixalus* and *Tachycnemis* regard his characters 1, 14, 18, 20, 23, and 24. Additionally, we here reviewed character 9, although the mentioning of a difference in this character probably is a typing error in the legend to Fig. 25 of Drewes (1984). Drawings and figures of the different character states are included in Drewes (1984). Four of the distinctive states do not refer to reliable characters with a constant distribution of states among hyperoliid genera according to the data presented in Drewes (1984). The sphenethmoid (character 1) is either present or absent (states 0 and 1) in different species of *Kassina*; the terminal phalanx of the third finger (character 14) can be either claw-shaped

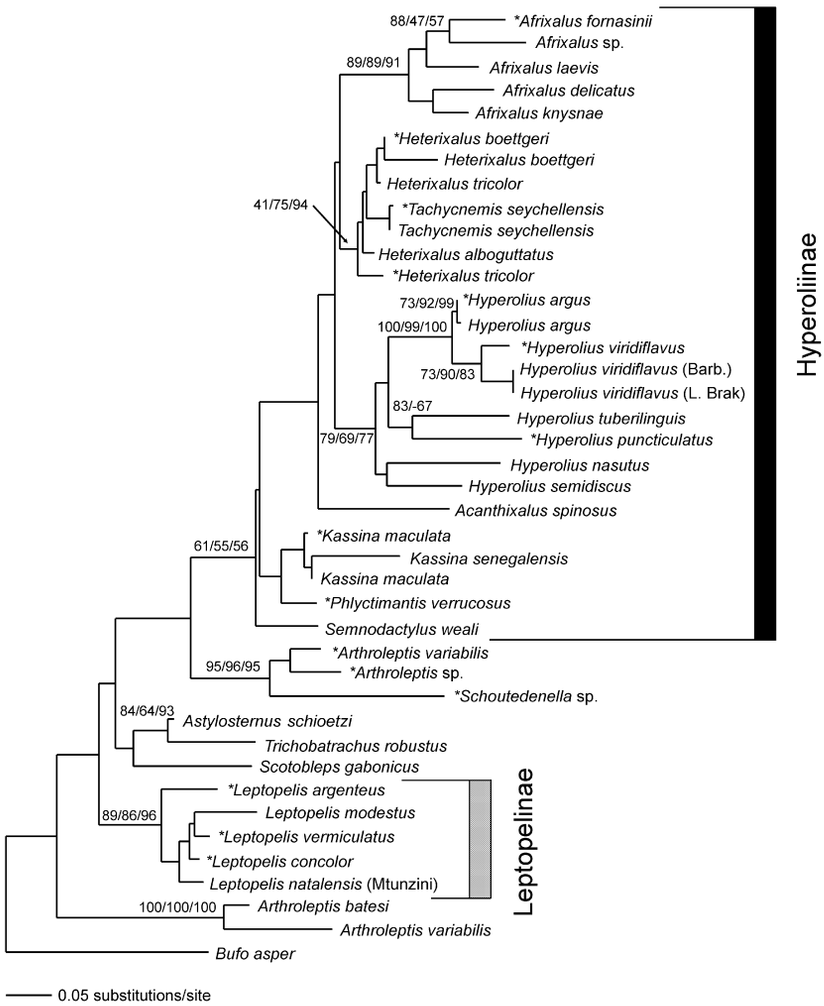

Fig. 2. Maximum likelihood phylogram based on analysis of 321 bp of 12S ribosomal RNA gene sequences of frogs of the families Hyperoliidae, Arthroleptidae and Astylosternidae. Numbers are bootstrap values in percentage (ML: 60 replicates; MP and NJ: 2000 replicates; values below 50% not given). *Bufo asper* was used as the outgroup. Taxa marked with asterisks correspond to sequences from the work of Richards and Moore (1996) as obtained from Genbank

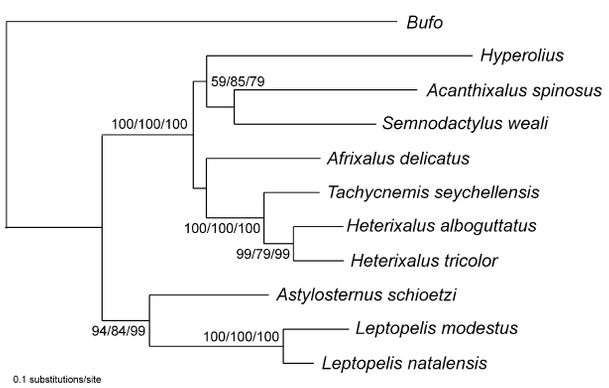

Fig. 3. Maximum likelihood phylogram based on a combined analysis of 415 bp of the cytochrome *b* gene, as well as 409 bp of the 12S and 997 bp (two fragments) of the 16S ribosomal RNA gene sequences of frogs of the families Hyperoliidae and Astylosternidae. Numbers are bootstrap values in percentage (ML: 500 replicates; MP and NJ: 2000 replicates; values below 50% not given)

(state 0) or peniform (state 1) in *Hyperolius*; the vocal sac openings (character 20) are lateral slits in most *Kassina* (state 1) but anterior slits (state 2) in *Kassina lamottei*; the tympanum (character 23) has even an own state (state 1) according to Drewes (1984), which codes ambiguity, i.e. 'tympanum visible or hidden'.

Drewes (1984) had one skeleton of *Heterixalus madagascariensis*, one X-ray figure of *Heterixalus rutenbergi*, and three skeletons of *Tachycnemis* available for comparison. In the course of the present study, 15 skeletons of nine *Heterixalus* species and three skeletons of *Tachycnemis* were examined. External morphology was examined in a larger number of specimens of all known *Heterixalus* species and in *Tachycnemis* specimens from different islands.

Character 1 [sphenethmoid: invisible or barely visible dorsally in *Heterixalus* (state 0), 0.3–0.5 the length of frontoparietals in *Tachycnemis* (state 2); Drewes 1984]. In the ZFMK material available to us, the sphenethmoid was barely visible dorsally, as a partly divided element just anterior to the frontoparietals. BMNH 1976.1958 had a wider sphenethmoid exposure, but only if referring to cartilaginous elements; the mineralized structures did not reach anteriorly the level of the nasals. Drewes (1984) emphasized the sphenethmoid extension in the largest hyperoliids, *Leptopelis* and *Tachycnemis*, as invading the planum anteorbitale and consisting of a completely ossified space between nasals. We assume that in *Tachycnemis* this is the case only in very large (female) specimens, as the sphenethmoids of the smaller males we examined were by no means comparable with that of similarly sized *Leptopelis* (e.g. *L. natalensis*, ZFMK 68784 and 68786).

These actually had a very distinct mineralized frontoparietal extending anteriorly into the region between the nasals.

Character 9 [posterolateral process of hyoid: present in *Heterixalus* (state 0), absent in *Tachycnemis* (state 1) (Drewes 1984: Fig. 25), although present in both taxa according to Drewes (1984: appendix B, and corresponding genus diagnoses)]. Such processes are typical for most neobatrachian anurans (Trewavas 1933), and are generally well developed and easily recognizable. However, in *Tachycnemis* the process is extremely small and barely visible (Drewes 1984: Fig. 5, and own observations on BMNH 1976.1958). Own examinations showed that the process was present in *Heterixalus alboguttatus*, *Heterixalus madagascariensis*, and as very small rudiment in *Heterixalus betsileo*, but was absent in *Heterixalus luteostriatus, Heterixalus rutenbergi* and *Heterixalus* '*variabilis*'.

Character 14 [terminal phalanx of third finger: claw-shaped in *Heterixalus* (state 0), peniform with a notable constriction near tip, less curved in *Tachycnemis* (state 1); Drewes 1984]. The shape of the terminal phalanx of the third finger clearly is not a valid character to distinguish between *Heterixalus* and *Tachycnemis* specimens according to our observations. Many *Heterixalus* do have rather claw-shaped terminal phalanges (e.g. *Heterixalus madagascariensis*, ZFMK 52574), but other specimens (e.g. *Heterixalus luteostriatus*, MRSN A393.7) have a very distinct terminal constriction. On the other hand, in one examined *Tachycnemis* (ZFMK 62879) this constriction was very indistinct, and the phalanx was not distinguishable from the more claw-shaped elements of most *Heterixalus*.

Character 18 [gular gland: a median disc with free lateral and posterior margins, overlying the vocal pouch which consists of extensive folds of thin, non-pleated skin in *Heterixalus* (state 5), a medial disc or oval surrounded by loose, thin, unfolded, non-distensible skin in *Tachycnemis* (state 3); Drewes 1984]. The difference in structure and arrangement of gular gland and vocal pouch was not a consistent difference between *Heterixalus* and *Tachycnemis* in the material examined. All *Tachycnemis* actually had a gular gland which, as described by Drewes (1984), was a median disk surrounded by unfolded and non-distensible skin. In *Heterixalus*, several specimens had the state described by Drewes (1984), i.e. the gland disc was free posteriorly, and overlying a vocal pouch of extensive folds of thin skin (e.g. in ZFMK 57410, *Heterixalus luteostriatus*). However, other specimens clearly lacked the vocal pouch as in one *Heterixalus betsileo* specimen from Montagne d'Ambre (ZFMK 57411).

Character 20 [vocal sac openings: lateral slits in *Heterixalus* (state 1), anterior slits in *Tachycnemis* (state 2); Drewes 1984]. We have not been able to determine reliably the position of the vocal slits that requires a careful dissection (Drewes 1984).

Character 23 [tympanum: always obscured by epidermis or absent in *Heterixalus* (state 2), externally visible in *Tachycnemis* (state 0); Drewes 1984]. The tympanum in *Heterixalus* is generally largely concealed (Blommers-Schlösser and Blanc 1991), but in most preserved specimens it is possible to recognize and measure it (e.g. Glaw and Vences 1993). On the other hand, although most large *Tachycnemis* specimens have generally a rather well visible tympanum margin, smaller specimens approach the state of *Heterixalus*. So, in ZFMK 33303, the tympanum is almost invisible. The visibility of the silhouette of the tympanum may also be related to the state of fixation and preservation of the examined specimens. Those that have been fixed using 96% ethanol during an extended period of time, or being partly desiccated, have a less concealed tympanum as their skin fits tighter around the ear region. This observation holds true both for *Tachycnemis* and *Heterixalus*.

Character 24 [intercalary element: peripherally mineralized with cartilaginous central part in *Heterixalus* (state 2), cartilaginous, unmineralized in *Tachycnemis* (state 1); Drewes 1984]. Regarding the degree of mineralization of the intercalary elements, our observations do not agree with those of Drewes (1984). Although one specimen of *Tachycnemis* (ZFMK 62859) had only slightly mineralized intercalary elements (with a distinct peripheral calcification, however), in the second examined specimen (ZFMK 62879) the intercalaries were strongly stained by alizarin red, and thus largely calcified. No difference was noted between that specimen and most *Heterixalus*. On the other hand, intercalary elements in several *Heterixalus* showed very little calcification and were largely stained only by alcian blue (e.g. *Heterixalus luteostriatus*, MRSN A393.7). We also observed calcification in the intercalaries of some *Leptopelis* (e.g. *L. natalensis* ZFMK 68784), but the mineralized part was located internally in the centre of the element, not externally as in other hyperoliids. This confirms the observation of Drewes (1984) that the intercalaries of *Leptopelis* are different from those of other hyperoliids, and may be composed of juvenile cartilage. The fact that the mineralization of the intercalary elements both in *Tachycnemis* and *Heterixalus* starts peripherally emphasizes the distinction of *Tachycnemis* from *Leptopelis*, and indicates that the slight differences between the elements of *Tachycnemis* and *Heterixalus* are only gradual modifications of a basically identical structure.

Discussion

Non-monophyly of the Hyperoliidae *sensu lato*

One conspicuous result of the 16S tree (Fig. 1) was the lack of support of monophyly of the Hyperoliidae *sensu lato*. Instead, two main clusters were distinguished, the Leptopelinae (containing the genus *Leptopelis* only) and the Hyperoliinae (containing all other genera). This topology was corroborated by the combined sequence analysis, in which species of *Leptopelis* were placed with *Astylosternus* rather than with the Hyperoliinae (Fig. 3). *Leptopelis* lack a number of important apomorphies common to the Hyperoliinae (Drewes 1984), especially the gular gland of males. Its classification within the Hyperoliidae *sensu lato* was largely based on the presence of an intercalary element, which however is known to be of little phylogenetic value (Glaw et al. 1998b). In the analyses of Liem (1970), Drewes (1984) and Channing (1989), as well as in the molecular study of Richards and Moore (1996), the Hyperoliidae *sensu lato* resulted as monophyletic group, but the taxa which most likely are their closest relatives (the Astylosternidae and Arthroleptidae) were not or not adequately sampled and included. In contrast, Emerson et al. (2000) also found evidence for paraphyly of the Hyperoliidae *sensu lato*. The molecular trees and total evidence trees (combining the available molecular and morphological data) of these authors were not congruent regarding the phylogenetic position of *Leptopelis*, but the genus was never placed in a monophyletic group with the Hyperoliinae. Despite the relatively high bootstrap values indicating non-monophyly of the Hyperoliidae *sensu lato*, the null hypothesis of their monophyly could not be significantly rejected by the Shimodaira–Hasegawa tests performed herein. We suppose that future work will identify the actual sister group of

Leptopelis and thereby contribute to a better understanding of the basal ranoid radiation in Africa.

Intergeneric relationships

Summarizing the data presented herein, none of the morphological and osteological characters used by Drewes (1984) to distinguish phylogenetically *Tachycnemis* from *Heterixalus* can be used convincingly as argument against a close relatedness of both genera. Some of the differences (especially the sphenethmoid extension) are perhaps explained by retention of paedomorphic characters in the (smaller) *Heterixalus*. Thus, the monophyletic group containing these two genera was supported by analysis of 16S, 12S and cytochrome *b* sequences and not convincingly contradicted by morphology, and it should therefore be considered as one of the best-evidenced clades in the Hyperoliidae. Their close relationships are also emphasized by the fact that the 16S and the 12S Maximum Likelihood trees suggested a paraphyly of *Heterixalus* with respect to *Tachycnemis* (Figs 1 and 2). However, the combined analysis (Fig. 3), which included two of the most divergent *Heterixalus* species (*H. boettgeri* and *H. tricolor*) clearly supported the monophyletic group containing these two species, as opposed to their sister group *Tachycnemis*. This indicates that the paraphyletic arrangements of *Heterixalus* in the other trees, which in no case was supported by relevant bootstrap values, most likely are artefacts. They might have been caused by a relatively low number of informative sites with respect to the differentiation within the *Heterixalus–Tachycnemis* lineage.

Afrixalus has been related to *Kassina* by Laurent (1944) but clustered close to *Hyperolius* and *Heterixalus* in the studies of Liem (1970) and Drewes (1984). Laurent (1972) explained this with the presumably paedomorphic character states in *Afrixalus*, which often are very small frogs. The available molecular data are equivocal regarding the position of *Afrixalus*, but the genus never was placed as a direct sister group to *Kassina* (and related genera). This seems to be better in accordance with the points of view of Liem (1970) and Drewes (1984) than with the opinion of Laurent (1944, 1972). On the other hand, *Opisthothylax immaculatus*, a representative of a monotypic genus from western Africa, was placed close to species of *Afrixalus* in the 16S analysis. The morphological analysis of Drewes (1984) did not detect close phylogenetic relationships between these two genera, but they share the behaviour of depositing their clutches into folded leafs (*Opisthothylax* further producing a foam nest; Schiøtz 1999).

Subfamilial classification

Several proposals of subfamilial classification of the Hyperoliidae *sensu lato* have so far been published. Dubois (1981) proposed to divide the family into three subfamilies, the Leptopelinae, Kassininae and Hyperoliinae. Channing (1989) introduced a subfamily Tachycneminae, assuming that *Tachycnemis* was the most basal hyperoliid after *Leptopelis*. Blommers-Schlösser (1993) accepted explicitly the Leptopelinae beside 'other hyperoliids'. Considering the results presented herein, we propose to accept only two subfamilies, the Leptopelinae (*Leptopelis*) and the Hyperoliinae (all remaining genera). The names Tachycneminae and Kassininae are considered as junior synonyms of Hyperoliinae. The sister groups of both the Leptopelinae and the Hyperoliinae have not been reliably identified by the available phylogenetic analyses, and therefore any stem-based definition of these taxa seems difficult at present. As all genera included in the Hyperoliinae share one conspicuous and derived character (the gular gland of males) we consider it as most appropriate to define the subfamily as apomorphy-based taxon that includes all ranoid frogs characterized by this synapomorphy. In contrast, the Leptopelinae are not known to share any unique derived character. They could be defined phenetically as firmisternal frogs with distinctly vertical pupil and with an intercalary element between ultimate and penultimate phalanges of fingers and toes. By combination of these three characters, a species can be easily recognized as representative of the genus *Leptopelis*. We therefore define the Leptopelinae as node-based taxon containing all members of the genus *Leptopelis*.

Biogeography

The sister group relationship of the Malagasy *Heterixalus* to the Seychellean *Tachycnemis*, and the placement of this lineage among the African hyperoliids is well corroborated. This topology was explained by Richards and Moore (1996) as result of continental drift events. According to geological data (Barron et al. 1981; Rabinovitz et al. 1983; Pitman et al. 1993; Storey 1995; Storey et al. 1995), Madagascar separated from Africa at 165–121 million years before present (myr) but remained attached to India and the Seychelles. The separation between Africa and South America has been dated at 101–86 myr, and the separation between Madagascar and India at 88–63 myr.

It is generally assumed that the ancestors of the endemic Malagasy frog taxa, including the Mantellidae and Hyperoliinae, were present on the Madagascar–India continent and evolved in isolation after the separation of India and the Seychelles from Madagascar (Duellman and Trueb 1986; Richards and Moore 1996; but see Vences et al. 2000a). It is further hypothesized that rhacophorids and ranids reached Asia on the drifting Indian subcontinent (Bossuyt and Milinkovitch 2001).

Presence of the Hyperoliinae on the Madagascar–India continent implies a very early age of their evolution. Such an assumption, however, meets with several contradicting facts. The first problem is the absence of these frogs in South America which separated from Africa at least 20 myr later than Madagascar–India (Barron et al. 1981; Rabinovitz et al. 1983; Pitman et al. 1993). Furthermore, Madagascar was apparently connected to South America via the Kerguelen plateau and Antarctica in the Late Cretaceous (Sampson et al. 1998; Krause et al. 1999). Many representatives of the Hyperoliinae are very adaptive savanna species. Some are widespread, such as the forms of the *Hyperolius viridiflavus* complex, which occur in most of subsaharan Africa (Schiøtz 1999). Only few derived hyperoliid lineages can be considered as strict inhabitants of rain forests or of refugial mountain habitats (e.g. Wieczorek et al. 2000). This ecological adaptability is shared by the Malagasy *Heterixalus* (which largely breed in rice fields and are most common outside forested areas; Blommers-Schlösser 1982; Glaw and Vences 1993, 1994; Vences et al. 2000b) and by African genera such as *Hyperolius*, *Afrixalus* and *Kassina*. It may therefore be speculated that their common ancestor was characterized by similar features. It is difficult to understand why such a vagile group would not have been able to colonize the presumably vast savannahs of

South America before its separation from Africa (considering the rather seasonal mesozoic climates in the interior of the large continental landmasses; e.g. Spicer et al. 1994), and why South America should not at least harbour some relicts of these early invaders. Furthermore, if presence of the Hyperoliinae on the Madagascar–India–Seychelles continent is assumed, it has to be explained why they went extinct in India but not on the very small Seychelles Islands.

A second more relevant problem for the vicariance scenario of hyperoliid origins is the low genetic differentiation between the taxa involved. Molecular clock rates have been calibrated for the 16S rRNA gene in many different animal groups (see Avise 2000), and usually range between 0.2 and 1% pairwise divergence per million year. Mean 16S divergence between *Tachycnemis* and *Heterixalus* was 7.5%, mean divergence between the *Tachycnemis*/*Heterixalus* lineage and the African *Afrixalus* (its sister group in our analyses) was 13%. Molecular clock estimates are often of limited value due to statistical qualifications (e.g. Hillis et al. 1996), and no calibrations are so far available for the Hyperoliinae. However, it can be stated that vicariance scenarios for their divergences (130 myr for the split between Africa and Madagascar/Seychelles, and 63 myr for the split between Madagascar, India and the Seychelles) would require the assumption of unprecedentedly low substitution rates of about 0.1% pairwise divergence per myr in the 16S rRNA gene.

Anurans are unlikely to cross saltwater barriers as indicated by their absence on oceanic island (Darwin 1859). However, the origin of anurans on Caribbean islands is best explained by oversea dispersal (Hedges et al. 1992). Hyperoliids may have reached Madagascar from Africa during the Tertiary using temporarily existing islands and land bridges as stepping stones (McCall 1997; see also Rage 1996). An origin by dispersal is probable for many extant Malagasy reptile and mammal groups (e.g. Yoder et al. 1996; Caccone et al. 1999; Mausfeld et al. 2000; Vences et al. 2001a,b). Frogs adapted to arid conditions and resting on sun-exposed leafs or in leaf axils, as many representatives of the Hyperoliinae, are certainly among the amphibians most likely to disperse by rafting.

Palaeontological data indicate an important faunal turnover in Madagascar after the Cretaceous (Krause et al. 1996, 1997, 1999). Feller and Hedges (1998) have recently pointed out that the almost complete absence of ranoid frogs in South America, and the lack of several major groups of hyloids in Africa and Asia (e.g. leptodactylids, dendrobatids, centrolenids), may indicate that the main split between these two groups of neobatrachian anurans was caused by vicariance in the context of the Africa–South America separation. This implies an origin and dispersal of Old World treefrogs after the separation of Africa and Madagascar. Although vicariance often offers more appealing explanations, dispersal hypotheses should not be disregarded when investigating into the origin of the extant Malagasy frog radiations.

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Zusammenfassung

Molekulare Phylogenie der Riedfrösche (Hyperoliidae): Biogeographischer Ursprung der auf Madagaskar und den Seychellen verbreiteten Taxa mit einer Neuanalyse der Paraphylie der Familie

Die Familie der Riedfrösche (Hyperoliidae) ist in Afrika, Madagaskar und den Seychellen verbreitet. In dieser Arbeit untersuchten wir die Phylogenie dieser Gruppe auf der Basis von partiellen Sequenzen der mitochondrialen 16S und 12S rRNA und Cytochrom *b* Gene. Die molekularen Daten unterstützten die Monophylie der Unterfamilie Hyperoliinae, aber wiesen darauf hin, dass die Gattung *Leptopelis* (Unterfamilie Leptopelinae) näher mit Vertretern der afrikanischen Familie Astylosternidae verwandt ist. Die endemische Seychellen-Gattung *Tachycnemis* war die Schwestergruppe der madagassischen *Heterixalus*; die Linie aus diesen beiden Gattungen stand innerhalb der übrigen Hyperoliinae. Eine genauere Neuanalyse morphologischer Merkmale lieferte keine überzeugenden Argumente, die dieser Schwestergruppenbeziehung widersprechen. Die Unterfamilie Tachycneminae wird daher als Synonym der Hyperoliinae aufgefasst. Zudem lieferten die molekularen Daten auch keine überzeugende phylogenetische Begründung für die Aufrechterhaltung einer eigenen Unterfamilie Kassininae. Die schwache molekul molekulare are Differenzierung der madagassischen Hyperoliiden zu ihren nächsten Verwandten auf den Seychellen und in Afrika, sowie das Gesamtareal der Hyperoliidae, weisen darauf hin, dass ihr biogeographischer Ursprung nicht oder nicht vollständig durch mesozoische Vikarianzereignisse im Zusammenhang mit dem Auseinanderbrechen von Gondwana erklärt werden kann.

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