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A previously unrecognized radiation of ranid frogs in Southern Africa revealed by nuclear and mitochondrial DNA sequences

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Abstract

In sub-Saharan Africa, amphibians are represented by a large number of endemic frog genera and species of incompletely clarified phylogenetic relationships. This applies especially to African frogs of the family Ranidae. We provide a molecular phylogenetic hypothesis for ranids, including 11 of the 12 African endemic genera. Analysis of nuclear (*rag-1*, *rag-2*, and *rhodopsin* genes) and mitochondrial markers (12S and 16S ribosomal RNA genes) provide evidence for an endemic clade of African genera of high morphological and ecological diversity thus far assigned to up to five different subfamilies: *Afrana, Cacosternum, Natalobatrachus, Pyxicephalus, Strongylopus*, and *Tomopterna*. This clade has its highest species diversity in southern Africa, suggesting a possible biogeographic connection with the Cape Floral Region. Bayesian estimates of divergence times place the initial diversification of the southern African ranid clade at ~62–85 million years ago, concurrent with the onset of the radiation of Afrotherian mammals. These and other African ranids (*Conraua, Petropedetes, Phrynobatrachus, and Ptychadena*) are placed basally within the Ranoidae with respect to the Eurasian groups, which suggests an African origin for this whole epifamily.

Keywords: Amphibia; Ranidae; Cacosterninae; Petropedetinae; Pyxicephalinae; Raninae; Tomopterninae; Molecular phylogeny; Conservation priorities; Biodiversity hotspot; Africa

1. Introduction

The recent report of the Global Amphibian Assessment project (Stuart et al., 2004) shows that at least a disturbing 42% of amphibian species are experiencing declines, in large part due to still unknown processes. In some cases entire diverse clades of frogs are heavily declining (Lötters et al., 2004). Such non-random extinctions can lead to a severe loss of evolutionary history (Purvis et al., 2000) and a reliable phylogeny of all amphibians is needed to identify them. In several very species-rich cosmopolitan groups of frogs the phylogenetic relationships are still insufficiently known. This lack of a robust phylogenetic hypothesis is especially true for the family Ranidae or True Frogs that contains over 700 species, which are distributed throughout the world. A single genus (*Rana*) is thought to occur on all continents except Antarctica. Yet the phylogenetic relationships among *Rana*, and ranids in general, are largely uncharted (Emerson et al., 2000b). Recent molecular studies have provided important progress in the understanding of ranids and their related groups (Bossuyt and Milinkovitch, 2000; Hoegg et al., 2004; Van der Meijden et al., 2004; Vences et al., 2003b). Some studies have identified India as a reservoir of ancient ranid lineages, and proposed these animals as a model for "Out of India" dispersal of vertebrates (Bossuyt and Milinkovitch, 2001; Roelants et al., 2004). These works demonstrated

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the potential of ranids to decipher general patterns of biogeography and diversification although only a part of the currently recognized ranid diversity has been studied so far. Because most of the endemic African ranid genera are still unstudied from a molecular perspective the biogeographical insights remain incomplete.

Despite recent compelling evidence for the ability for transoceanic dispersal in amphibians (Hedges et al., 1992; Vences et al., 2003b, 2004), there is little doubt that continental drift has had a major influence in shaping their current distribution and phylogeny. The close relationships of the recently discovered *Nasikabatrachus* from India with *Nesomantis* from the Seychelles strikingly demonstrated the importance of the Gondwanan breakup for the vicariance biogeography and hence phylogeny of these basal Neobatrachian frogs (Ranoidei sensu Sokol, 1977). Africa is generally seen as the place of origin for the current distribution of frogs in the superfamily Ranoidea (Biju and Bossuyt, 2003; Feller and Hedges, 1998; Savage, 1973), and one of its subclades, the Arthroleptoidae (Fig. 1), is endemic to this continent (with a few species in Madagascar and on the Seychelles).

Africa is renowned for several endemic radiations such as the Afrotherian mammals (Springer et al., 1997) and the haplochromine cichlid fishes (Verheyen et al., 2003). Africa was united with South America, Australia, Antarctica, India, and Madagascar in the supercontinent Gondwanaland until the end of the late Jurassic. After the breakup of Gondwanaland, Africa remained isolated until it connected with Eurasia. The India–Seychelles–Madagascar plate broke off from Africa 158 to 160 million years ago[1] (mya), and Greater India started to drift northwards across the Indian Ocean about 96–84 mya (Briggs, 2003). The India–Madagascar plate has been suggested as possible biogeographic origin of Asian ranoid subclades (Duellman and Trueb, 1986; Bossuyt and Milinkovitch, 2001), i.e., the Rhacophoridae and at least part of the Ranidae.

Land bridges that connected Africa with Eurasia after its long isolation from other continents allowed Eurasian faunal elements to disperse into Africa, including several ranoid representatives. Species (1) of the dicroglossine genus *Hoplobatrachus*, (2) of the ranine lineage containing the genera/subgenera *Rana* and *Amnirana*, and (3) the rhacophorid genus *Chiromantis* have dispersed into Africa from Eurasia (Kosuch et al., 2001; Vences et al., 2003b). Currently, 21 ranid genera are restricted in their distribution to Africa, most of which are limited to sub-Saharan Africa.

By analyzing nuclear and mitochondrial DNA sequences of representatives of all but one subfamily of ranids we here provide the first inclusive molecular phylogeny of ranid relationships. Our data provide compelling evidence for a deep evolutionary history of many African endemic ranid groups and, unexpectedly, uncover an endemic radiation that includes taxa that had so far been classified into up to five different subfamilies.

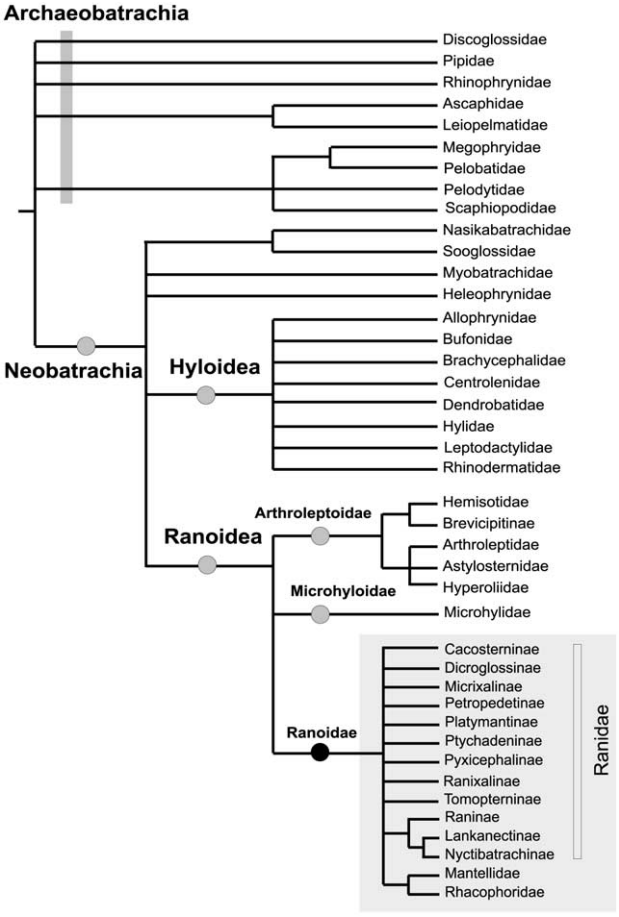

Fig. 1. Schematic representation of the classification of ranids and their phylogenetic position among frogs following Vences and Glaw (2001), with some additions from Dubois (1992) and Blommers-Schlösser (1993), and with modifications from the trees of Biju and Bossuyt (2003), Dubois (2003), Hoegg et al. (2004), Roelants et al. (2004), Van der Meijden et al. (2004), and own unpublished data: the family Ranidae is a paraphyletic assemblage that together with the Mantellidae and Rhacophoridae forms the epifamily Ranoidae. Together with two other epifamilies (the Arthroleptoidae and Microhyloidae) they form the superfamily Ranoidea in the Neobatrachia. The familial scheme used here includes Bombinatoridae in Discoglossidae, and Limnonastidae and Rheobatrachidae in Myobatrachidae.

2. Materials and methods

2.1. Taxonomy and selection of taxa

Duellman and Trueb's (1986) characterization of ranid systematics being 'in a state of chaos' has been heavily quoted but the situation has not much improved

[1] *Abbreviations used:* mya, million years ago; MP, maximum parsimony; ML, maximum likelihood; BI, Bayesian Inference; NJ, neighbour joining.

since. As a convention we here follow the taxonomic scheme of Vences and Glaw (2001), with some modifications from more recent research as outlined in Fig. 1. At present, accepting the proposals of subfamilial arrangement by Dubois and Ohler (2001) and Roelants et al. (2004) to the classifications of Dubois (1992) and Blommers-Schlösser (1993), the Ranidae consists of about 12 subfamilies (Fig. 1), of which five are endemic to Africa. Ranids are a paraphyletic group that, together with the Mantellidae and Rhacophoridae, forms the epifamily Ranoidae. These are hierarchically a fraction of the superfamily Ranoidea and the suborder Neobatrachia which both probably are monophyletic (Hoegg et al., 2004).

Sequences were obtained from taxa representing all ranid subfamilies except the Micrixalinae (Table 1), as well as from the families Mantellidae and Rhacophoridae. We furthermore included taxa belonging to the Arthroleptoidae and Microhyloidae. *Latimeria, Homo*, *Gallus*, the salamander *Lyciasalamandra*, two archaeobatrachians of the genus *Alytes,* and two hylid neobatrachians, genera *Agalychnis* and *Litoria*, as hierarchical outgroups (not shown in figures).

2.2. DNA sequencing

DNA was extracted from muscle or skin tissue fixed in 99% ethanol. Tissue samples were digested using proteinase K (final concentration 1 mg/mL), homogenized and subsequently purified following a standard salt extraction protocol. Primers for *rag-1* and *rag-2* were from Hoegg et al. (2004) as reported in Chiari et al. (2004). Primers for one fragment of the 12S rRNA gene and one fragment of the 16S rRNA gene were 12SA-L and 12SB-H and 16SA-L and 16SB-H of Palumbi et al. (1991), respectively (see Vences et al., 2003a). Primers for a fragment of *rhodopsin* exon (Rhod1A and Rhod1D) were from Bossuyt and Milinkovitch (2000). PCR was performed in 25 µl reactions containing 0.5–1.0 U of REDTaq DNA Polymerase (Sigma, Taufkirchen, Germany), 0.01 U of *Pwo* DNA polymerase (Roche, Mannheim, Germany), 50 ng genomic DNA, 10 pmol of each primer, 15 nmol of each dNTP, 50 nmol additional $MgCl_2$, and the REDTaq PCR buffer (in final reaction solution: 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, and 0.01% gelatine). 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 10 cycles with 94 °C for 30 s, annealing temperatures increasing by 0.5 °C per cycle from 52 to 57 °C and extending for 3 min at 68 °C. 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. For 12S and 16S the denaturation step was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 90 s.

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) on an ABI 3100 capillary sequencer using the manufacturer's instructions. New sequences for 37 species were combined with existing sequences taken from GenBank in the final dataset. These sequences were deposited in GenBank (for accession numbers see Table 1).

2.3. Data analysis

DNA sequences were aligned using ClustalW (Thompson et al., 1994). Gapped and hypervariable sites, totalling 729 characters, were excluded from the analyses. A homogeneity partition test (Farris et al., 1994) as implemented in PAUP* (Swofford, 2002) rejected homogeneity of the different markers. Besides a combined analysis of the combined dataset we therefore also performed separate analyses of the various genes.

The combined dataset was used to calculate neighbor- joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) phylogenies using PAUP* (Swofford, 2002). Heuristic searches were performed using 10 replicates of a stepwise addition of taxa. The best fitting models of sequence evolution for ML analyses (Table 2) were determined by hierarchical likelihood ratio tests and by the AIC criterion in Modeltest 3.06 (Posada and Crandall, 1998). Bootstrap branch support values were calculated with 500 MP replicates and 100 ML replicates.

Bayesian inference (BI) of the combined and of separate datasets was conducted with MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001), using models (Table 2) estimated with Modeltest under the AIC criterion, with 250,000 generations, sampling trees every 10th generation (and calculating a consensus tree after omitting the first 3000 trees). For the combined dataset, 1,000,000 generations were computed, with a burn-in of 10,000. These BI phylogeny reconstructions were repeated five times each, resulting in only very minor differences in the resulting trees, all referring to unsupported branches without relevance for the present study.

2.4. Divergence time estimation

We used the MultiDivTime package (Thorne and Kishino, 2002; Thorne et al., 1998) to estimate the divergence times, based on nuclear sequences only. Calibration

Table 1
Voucher specimens and GenBank accession numbers of taxa studied

Species	Family	Locality and voucher specimen	Accession Nos.				
			12S	16S	*Rag-1*	*Rag-2*	Rhodopsin
Homo sapiens	*Hominidae*	GenBank	NC_001807	NC_001807	NM_000448	BC022397	NM_000539
Latimeria sp.	*Coelacanthidae*	GenBank	Z21921	Z21921	AY442925	AF369087	AF131253
Gallus gallus	*Phasianidae*	GenBank	AY235571	AY235571	AF143730	M58531	D00702
Lyciasalamandra luschani	*Salamandridae*	GenBank	AF154053	AF154053	AY323753	AY323797	U36574*
			—	—	—	—	—
Alytes dickhilleni	*Discoglossidae*	Parejo, Spain (no voucher)	AY333672	AY333710	DQ019494	DQ019517	AY341817
Alytes muletensis	*Discoglossidae*	Mallorca, Spain (no voucher)	AY333671	AF224729	AY323755	AY323781	AY323731
Agalychnis callidryas	*Hylidae*	Pet trade (no voucher)	AY330898	AY330890	AY323765	AY323780	AY323750
Litoria caerulea	*Hylidae*	Pet trade (no voucher)	AY330903	AY330894	AY323767	AY323793	AY323751
Heterixalus tricolor	*Hyperoliidae*	Madagascar, ZSM 700/2001	AF215434	AF215220	AY323768	AY323787	AY323741
Hyperolius viridiflavus	*Hyperoliidae*	Barberton, South Africa, ZFMK 66726	AY330901	AY323789	AY323740	AF215440	AF215223
Aglyptodactylus madagascariensis	*Mantellidae*	Madagascar, ZSM 183/2002	AF215179	AY341678	AY571640	DQ019516	DQ019552
Boophis doulioti	*Mantellidae*	Madagascar, ZSM 185/2002	AY341608	AY341663	AY571643	DQ019519	AY341792
Laliostoma labrosum	*Mantellidae*	Madagascar, UADBA-MV 2001.1466	AF215178	AY341679	AY571652	DQ019530	AF249106
Mantella madagascariensis	*Mantellidae*	Pet trade (no voucher)	AF124101	AF124131	DQ019500	DQ019532	AY263284
Mantidactylus sp.	*Mantellidae*	Mayotte, ZSM 652/2000	AY330906	AY330888	AY323775	AY323794	AY323742
Mantidactylus wittei	*Mantellidae*	Madagascar, ZSM 405/2000	AY330904	AF317691	AY323774	AY323795	AY323743
Breviceps fuscus	*Microhylidae*	Big Tree, South Africa, ZFMK 66716	DQ019578	AF215366	AY571644	DQ019520	DQ019553
Dyscophus antongilii	*Microhylidae*	Maroantsetra, Madagascar (no voucher)	DQ019581	DQ019601	AY571648	DQ019525	DQ019558
Kaloula pulchra	*Microhylidae*	Pet trade (no voucher)	AY330902	AY330893	AY323772	AY323790	AF249100*
Plethodontohyla alluaudi	*Microhylidae*	Madagascar, ZSM 3/2002	DQ019589	DQ019606	AY571661	DQ019541	DQ019568
Scaphiophryne calcarata	*Microhylidae*	Madagascar, ZSM 115/2002	DQ019593	AJ314811	AY571660	DQ019548	DQ019573
Afrana angolensis	*Ranidae*	Barberton, South Africa (no voucher)	DQ019576	DQ019596	DQ019493	DQ019515	DQ019551
Amnirana (Hylarana) lepus	*Ranidae*	Cameroon, pet trade, ZFMK 64831	DQ019584	AY014377	AY571641	DQ019529	DQ019561
Amolops hainanensis	*Ranidae*	Hainan island, China, MVZ 230383	DQ019577	DQ019597	DQ019495	DQ019518	AY322231*
Cacosternum boettgeri	*Ranidae*	Hardap, Namibia, ZFMK 66727	AF124096	AF215414	AY571645	DQ019521	DQ019554
Ceratobatrachus guentheri	*Ranidae*	Pet trade (no voucher)	DQ019579	DQ019598	DQ019496	DQ019522	DQ019555
Conraua crassipes	*Ranidae*	Nlonako, Cameroon, ZFMK 75446	DQ019580	DQ019600	DQ019498	DQ019524	DQ019557
Fejervarya sp.	*Ranidae*	ZFMK uncatalogued (MV-PBl1)	DQ019582	DQ019602	AY571649	DQ019526	DQ019559
Hoplobatrachus occipitalis	*Ranidae*	Voucher not collected	AJ564734	AY341689	AY571650	DQ019527	AJ564730
Hylarana (Rana) gracilis	*Ranidae*	Belihuloya, Sri Lanka, ZFMK (MNHN 2000.614)	DQ019583	AY014376	DQ019499	DQ019528	DQ019560
Indirana sp.	*Ranidae*	*Indirana* sp., several different specimens	AF215194	AF215392	AF249122	AF215194	AF215391
Lankanectes corrugatus	*Ranidae*	Kandy, Sri Lanka, MNHN 2000.616	DQ019586	DQ019603	AY571653	DQ019531	DQ019562

(*continued on next page*)

Table 1 (*continued*)

Species	Family	Locality and voucher specimen	Accession Nos.				
			12S	16S	*Rag-1*	*Rag-2*	Rhodopsin
Nanorana parkeri	*Ranidae*	Sichuan Prov., China, MVZ 231206	AF206110	AY322283	DQ019501	DQ019533	AY322219
Natalobatrachus bonebergi	*Ranidae*	The Haven, South Africa, ZFMK 66443	AF215198	AF215396	DQ019502	DQ019534	DQ019563
Nyctibatrachus major	*Ranidae*	Ooty, India, ZFMK 74837	AF249017	AY341687	AY571655	DQ019535	AF249113
Occidozyga lima	*Ranidae*	China, Hainan Prov., MVZ 236659	AF161027	AF285213	DQ019503	DQ019536	DQ019564
Paa verrucospinosa	*Ranidae*	Vietnam, Vinh Phu Prov., MVZ 223858	AF205552	AY322284	DQ019504	DQ019537	AY322234*
Petropedetes parkeri	*Ranidae*	Cameroon, Voucher not collected	AY341628	AF124132	DQ019505	DQ019538	AY341813
Phrynobatrachus natalensis	*Ranidae*	Mtunzini, South Africa, ZFMK 73452	DQ019588	DQ019605	DQ019507	DQ019540	DQ019567
Ptychadena mascareniensis	*Ranidae*	Madagascar, ZSM 190/2002	AY341624	AY341690	AY571658	DQ019542	AY341809
Pyxicephalus adspersus	*Ranidae*	Rundu, Namibia (no voucher)	AF206091	AF215505	DQ019508	DQ019543	DQ019569
Rana aurora	*Ranidae*	Del Norte co., CA, USA, MVZ 188965	DQ019590	DQ019607	DQ019509	DQ019544	DQ019570
Rana berlandieri	*Ranidae*	Mexico, Coahuila, MVZ 145474	AY115111	DQ019608	DQ019510	DQ019545	DQ019571
Rana sylvatica	*Ranidae*	Tompkins co., NY, USA, MVZ 137426	DQ019591	AF175977	DQ019511	DQ019546	DQ019572
Rana temporaria	*Ranidae*	voucher not collected	AF124103	AF124135	AY323776	AY323803	AF249119
Strongylopus fasciatus	*Ranidae*	Little Brak, South Africa, ZFMK 66444	DQ019594	AF215412	DQ019513	DQ019549	DQ019574
Tomopterna sp. "Khorixas"	*Ranidae*	Khorixas, Namibia, ZFMK 66403	DQ019595	DQ019610	DQ019514	DQ019550	DQ019575
Chirixalus cf. *Vittatus*	*Rhacophoridae*	Myanmar, pet trade, ZFMK 65463	AF458131	AF215346	AY571646	DQ019523	DQ019556
Philautus cf. *Macropus*	*Rhacophoridae*	Belihuloya, Sri Lanka (no voucher)	DQ019587	DQ019604	DQ019506	DQ019539	DQ019566
Polypedates maculatus	*Rhacophoridae*	voucher not collected	AF215184	AF215358	AY323777	AY323802	AF249124*
Rhacophorus dennysii	*Rhacophoridae*	Pet trade, ZFMK 65461	DQ019592	DQ019609	AY571659	DQ019547	AF249125*

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, France; MVZ—Museum of Vertebrate Zoology, University of California at Berkeley, USA; UADBA—Université d'Antananarivo, Département de Biologie Animale, Madagascar, numbers being field numbers 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 *rhodopsin* sequence of a different salamander genus, *Ambystoma*.

points were applied as follows: (1) minimum age of the frogs-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 dickhillenii* split at 5 mya (Mediterranean salinity crisis; Fromhage et al., 2004); and (5) age interval of the split between diapsids and synapsids at 338–288 mya (Graur and Martin, 2004).

3. Results

After exclusion of highly variable regions of 12S and 16S rRNA, the concatenated dataset consisted of 2995 nucleotides from nuclear genes (*rag-1, rag-2*, and *rhodopsin*) and mitochondrial genes (12S and 16S). Of these, 318 nucleotides were uninformative and 1212 base pairs were parsimony informative. For *rag-1* 606 sites were parsimony informative and 589 were constant of a total of 1330 sequenced nucleotides. For *rag-2* 755 base pairs were sequenced and contained 472 informative and 219 constant sites. *Rhodopsin* had 127 informative and 134 constant of a total of 289 characters. The fragments of the mitochondrial genes 12S and 16S had 119 and 141

Table 2
Models and parameter values used in the maximum likelihood (ML) analysis estimated with Modeltest (Posada and Crandall, 1998), and in the Bayesian (BI) analyses estimated with MrModeltest (Nylander, 2002)

Gene and analysis	Model	Base frequencies				Rate parameters						Shape parameter	Proportion invariable sites
		A	C	G	T	A–C	A–G	A–T	C–G	C–T	G–T		
Combined (ML-HLRT)	TrN + I + G	0.3160	0.2403	0.1927	0.2510	1.0000	3.8766	1.0000	1.0000	5.1032	1.0000	0.9517	0.3287
Combined (BI-HLRT)	GTR + I + G	0.3278	0.2251	0.1952	0.2520	1.7919	4.4416	1.4886	1.2223	7.3845	1.0000	0.7962	0.2860
Combined (ML and BI-AIC)	GTR + I + G	0.3021	0.2286	0.2079	0.2614	1.7245	4.9953	1.3206	1.2157	6.9370	1.0000	0.9443	0.3254
Rag-1 (BI-AIC)	GTR + I + G	0.3023	0.2279	0.2140	0.2557	1.4603	4.3679	1.0199	1.0124	5.7222	1.0000	1.0672	0.3688
Rag-2 (BI-AIC)	GTR + I + G	0.2915	0.2299	0.2025	0.2760	1.3504	4.7911	0.9256	1.3892	4.8758	1.0000	1.2424	0.2409
Rhodopsin (BI-AIC)	HKY + I + G	0.2261	0.3095	0.1804	0.2841	—	—	—	—	—	—	1.2011	0.3778
12S + 16S (BI-AIC)	GTR + I + G	0.3527	0.2287	0.2056	0.2130	3.5116	8.4158	5.1886	0.4111	28.2360	1.0000	0.5347	0.2952

The upper two models were estimated using hierarchical likelihood ratio tests (HLRT), the lower ones are according to the AIC criterion.

informative, and 82 and 188 constant of 253 and 368 characters, respectively. None of the nuclear gene fragments showed saturation when transitions and transversions were plotted against sequence divergence. All the phylogenies based on the combined dataset resolved the hierarchical outgroups in the expected relationships.

The phylogenies based on the combined dataset obtained through MP, NJ, and ML consistently show the superfamily Ranoidea as a clade with high bootstrap support (Fig. 2), containing the included representatives of Ranoidae, Microhyloidae, and Arthroleptoidae (support values from MP bootstrap and ML bootstrap; 100% and BI analysis 98%). The clustering of *Breviceps* with the hyperoliids (82, 62, and 100) provides further support for the exclusion of this genus from the Microhylidae, as already indicated in an earlier study using only *rag-1* (Van der Meijden et al., 2004). A more inclusive arthroleptoid sampling including the Hemisotidae is necessary to determine the position of *Breviceps* and related genera, since the Hemisotidae were found to be closely related to the hyperoliids (Biju and Bossuyt, 2003).

The clade Ranoidae receives high support (100, 97, and 100), as do several subclades within this epifamily that agree with current classification. This pertains to the Rhacophoridae, Mantellidae, Dicroglossinae, and Raninae. Basal resolution within the Ranoidae epifamily is low, however. The nested position of the Rhacophoridae and Mantellidae renders the Ranidae paraphyletic. The position of the platymantine *Ceratobatrachus* is inconsistent between the different methods of analysis and remains only weakly resolved, indicating that the subfamily Platymantinae is distinct from other ranoids. The relationships of the African genera *Phrynobatrachus* and *Ptychadena* to the remaining Ranoidae could not be resolved unambiguously. Excluding these two genera and a further clade of African species (see below), the remaining Eurasian and American Ranoidae form a monophyletic clade with some, albeit low, support (<50, 60, and 100). The African species *Hoplobatrachus occipitalis* and *Amnirana lepus* are nested within the largely Asian Dicroglossinae and Raninae, supporting the hypothesis of their Asian origin (Kosuch et al., 2001).

Most remarkable is the presence of a highly supported clade (100, 94, and 100) containing representatives of six sub-Saharan genera, most of which have so far not been considered to be related (Fig. 2): *Afrana, Cacosternum, Natalobatrachus*, *Pyxicephalus, Strongylopus* and *Tomopterna*. Biogeographically, the center of diversity and endemism of this divergent set of taxa is in southernmost Africa (Fig. 4). This endemic southern African clade is highly distinct and supported irrespective of the type of phylogenetic analysis. Further African genera such as *Petropedetes* and *Conraua* may be among its basal representatives (Fig. 2) but support for this placement is weak and they are thus not considered further here. The clade is resolved, at least partially, also in separate Bayesian analyses of the gene fragments used (Fig. 3). *rag-1* and *rag-2* were congruent in strongly supporting a

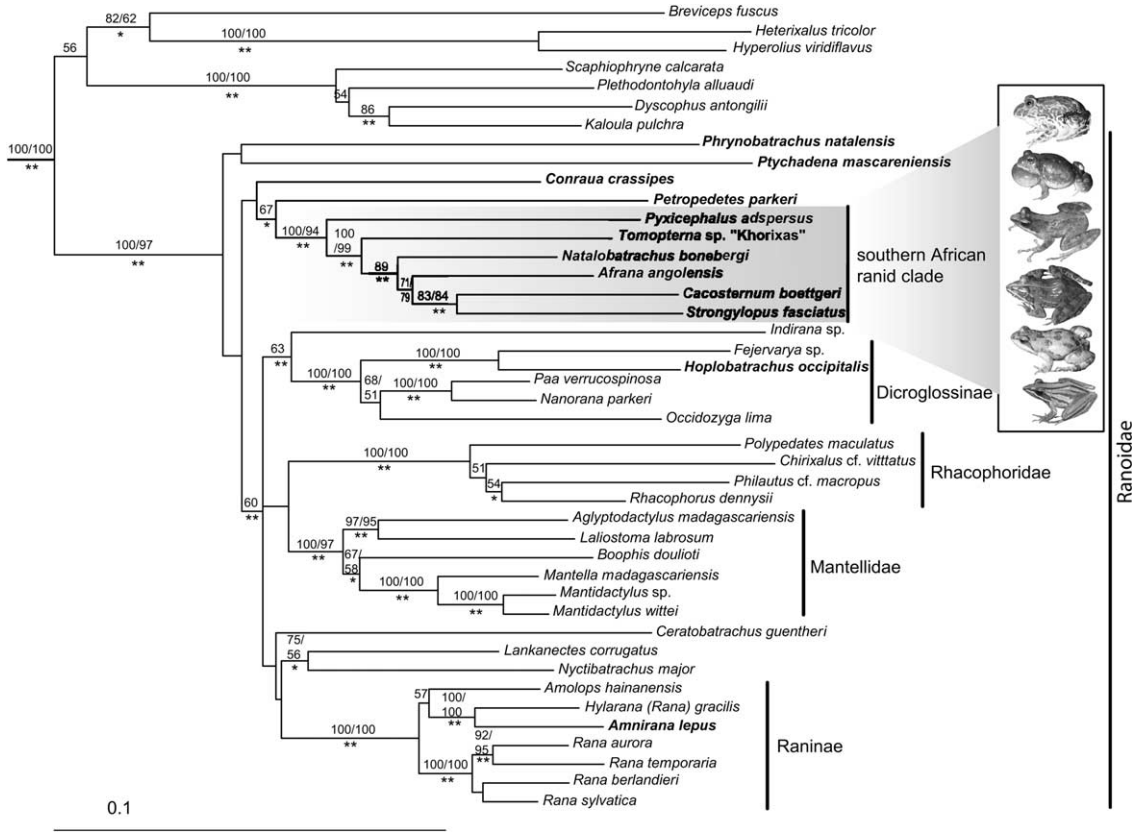

Fig. 2. Maximum likelihood phylogram of the superfamily Ranoidea, rooted with hierarchical outgroups *Latimeria*, *Homo*, *Gallus*, *Lyciasalamandra*, *Alytes*, *Agalychnis*, and *Litoria* (not shown), based on the combined dataset of nuclear and mitochondrial sequences. Support values above branches are ML/MP bootstrap values, a single asterisk below a branch indicates a Bayesian posterior probability above 95%, and two asterisks indicate a Bayesian posterior probability of 100%. When only one value is shown it refers to the ML support (the MP support in these cases was below 50%). This tree was obtained using a substitution model suggested by hierarchical likelihood ratio tests; a tree calculated under the substitution model suggested by the AIC criterion in Modeltest (Posada and Crandall, 1998) was identical except in placing *Ceratobatrachus* sister to rhacophorids. African Ranoidae are marked in bold. Inset shows the diversity of the representatives of the endemic African clade, in order from top to bottom *Pyxicephalus adspersus*, *Tomopterna* sp. (Khorixas), *Natalobatrachus bonebergi*, *Afrana angolensis*, *Cacosternum boettgeri*, and *Strongylopus fasciatus*.

monophyletic group of all six taxa, whereas *rhodopsin* supported a group of only five of them (excluding *Afrana*), and 12S+16S included, in addition, *Petropedetes* and *Phrynobatrachus* in this clade (although with conspicuously long branches, indicative of a possibly spurious placement).

Bayesian analysis of divergence times (Table 3) provided a posterior age estimate of the epifamily Ranoidae of 91.9 mya (95% confidence intervals 65.9–124.4 mya), of the endemic ranid clade with *Pyxicephalus* as the most basal taxon of 69.9 (48.9–96.5) mya and of the more inclusive clade with also *Petropedetes* and *Conraua* of 85.6 (61.1–116.4) mya.

4. Discussion

4.1. Endemic ranids from Southern Africa form an unexpected novel and divergent clade

With over 200 species of ranids found only in Africa, it is, after Asia, the continent with the second highest species diversity of this family. Most of these species have never been included in global phylogenetic studies. Several thorough osteological studies on African taxa (e.g., Clarke, 1981; Deckert, 1938) included no or very few Asian taxa whereas most of the recent molecular studies on ranids (Bossuyt and Milinkovitch, 2000; Emerson et al., 2000a; Marmayou et al., 2000; Roelants et al., 2004) focused on Asian taxa.

Despite this relative lack of knowledge, the finding of a highly supported Southern African ranid clade in our analysis was still most surprising. The phylogenetic relationships implied by Fig. 2 have not been previously hypothesized based on morphological data (e.g., Clarke, 1981). This clade, with *Pyxicephalus* basal to the other five genera, is strongly supported by the combined analysis and by separate analyses of *rag-1* and *rag-2*. Phylogenies based on *rhodopsin*, and on the mitochondrial 12S and 16S rRNA, provide some additional support for close relationships of taxa in this clade in the combined analyses but are less unequivocal when analysed individually (Fig. 3). However, the low

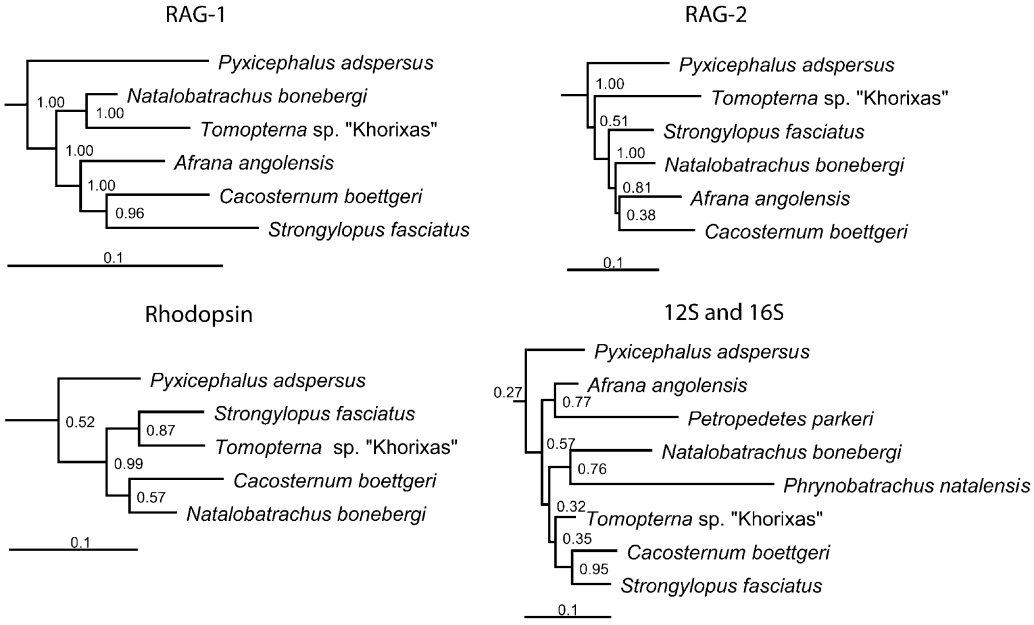

Fig. 3. Separate Bayesian trees of the gene fragments. Mitochondrial markers (12S and 16S rRNA fragments) were combined in a single dataset. Numbers are posterior probabilities.

Table 3
Posterior time estimates of most relevant splits within the Ranoidea from a Bayesian analysis using the MultiDivTime program (Thorne and Kishino, 2002) with calibrations and settings given in Section 2

Clade	Age (MY)	Standard deviation	95% confidence interval
Ranoidea	133.6	19.8	99.2–176.7
Arthroleptoidae–Microhylidae	127.1	19.4	93.2–169.4
Ranoidae	91.9	14.9	65.9–124.4
African endemic clade + *Conraua* + *Petropedetes*	85.6	14.1	61.1–116.4
Non-African Ranoidae including *Amnirana*, *Hoplobatrachus*, and *Chirixalus*	83.3	13.7	59.4–113.2
African endemic clade + *Petropedetes*	81.7	13.7	58.0–111.6
Mantellidae + Rhacophoridae	73.1	12.4	51.6–100.1
African endemic clade	69.9	12.3	48.9–96.5
African endemic clade excluding *Pyxicephalus*	61.7	11.3	42.7–86.0
Mantellidae	58.2	10.2	40.7–80.5
Natalobatrachus, *Afrana*, *Cacosternum*, *Strongylopus*	50.4	9.7	34.1–71.8
Afrana, *Cacosternum*, *Strongylopus*	47.7	9.3	31.9–68.0
Cacosternum, *Strongylopus*	40.2	8.4	25.9–58.9
Rhacophoridae	36.0	7.5	23.3–52.8
Raninae (excluding *Afrana*)	33.9	7.5	21.4–50.6

phylogenetic resolution of the latter three genes is not surprising because (a) for *rhodopsin*, we only included a short fragment (289 bp), and (b) and for mitochondrial genes such as 12S and 16S it is known that they are less informative in resolving deep phylogenetic relationships compared to single-copy protein coding nuclear genes (e.g., Springer et al., 2001).

One of the six genera unambiguously included in the endemic southern African clade, *Natalobatrachus*, is classified in the Petropedetinae (Blommers-Schlösser, 1993; considered as the family Phrynobatrachidae by Dubois, 1992) mainly based on osteological and dental characters (Laurent, 1986). *Cacosternum* has been placed in the Cacosterninae (Blommers-Schlösser, 1993). *Afrana* and *Strongylopus* have been classified in the Raninae, *Tomopterna* and *Pyxicephalus* in the Tomopterninae and Pyxicephalinae, respectively (Blommers-Schlösser, 1993; Dubois, 1992). While the Cacosterninae, Pyxicephalinae and Tomopterninae may belong to the endemic African clade in their totality (except for the Asian *Nannophrys* that was placed in the Cacosterninae by Blommers-Schlösser, 1993), this is not the case for the Petropedetinae and Raninae. Indeed, the Raninae included here form a well-defined clade when *Strongylopus* and *Afrana*, considered subgenera of *Rana* by Dubois (1992), are excluded (Fig. 2).

The elusion of this molecularly well-distinguishable endemic southern African clade of ranids to morphological analyses suggests a high incidence of homoplasy in morphological characters used for their classification. Other regional radiations, such as the Madagascan and Indian tree frogs (Bossuyt and Milinkovitch, 2000), or Indian and African burrowing frogs (Biju and Bossuyt, 2003) show a similar pattern of morphological homoplasy. In other ranoid frogs such as microhylids (Wild, 1995) and brevicipitines (Blommers-Schlösser, 1993; van der Meijden et al., 2004) homoplasy occurs in morphological characters as well. Alternatively, the placement of these taxa into separate subfamilies could have been be due to the lesser amount of attention that this large and highly diverse African ranid fauna has received relative to the other ranids, and therefore an artefact of observation. The genera in the endemic southern African clade were not only considered to belong to five different subfamilies or families, they also are morphologically and ecologically most distinct. *Cacosternum* are small frogs of generalized ecology and reproductive biology, many *Tomopterna* are burrowing savanna-dwelling frogs, *Afrana* are generalized semi-aquatic frogs, *Natalobatrachus bonebergi* is a semi-arboreal species living along rainforest streams, and *Pyxicephalus* are giant bullfrogs possessing fang-like projections of the lower jaw and a complex parental care behaviour. The genus *Anhydrophryne* is also likely to belong to this clade based on previously published mitochondrial data (Vences et al., 2000). These hogsback frogs live in humid South African forests and have direct development (Channing, 2001). Other South African genera of the Cacosterninae probably belong to the endemic southern African clade as well, although molecular data are lacking so far (see caption to Fig. 4) which would further increase the ecological diversity in this lineage.

The trend of species-richness of the southern African clade (Fig. 4) does not match the distribution of the total amphibian diversity, which tends to be highest in the humid region around Cameroon and an area covering Zambia, Mozambique, Tanzania and the Southeast of the Democratic Republic of the Congo (Stuart et al., 2004). Some species and genera of the African endemic clade (especially *Pyxicephalus* and *Tomopterna*) have succeeded in colonizing vast savanna areas of Africa, but other genera in this clade are restricted to Southern Africa, such as *Natalobatrachus* and *Anhydrophryne*. On the contrary, there is no genus in the clade restricted to any other region of Africa. This suggests that these frogs originated in this region and some lineages subsequently radiated across sub-Saharan

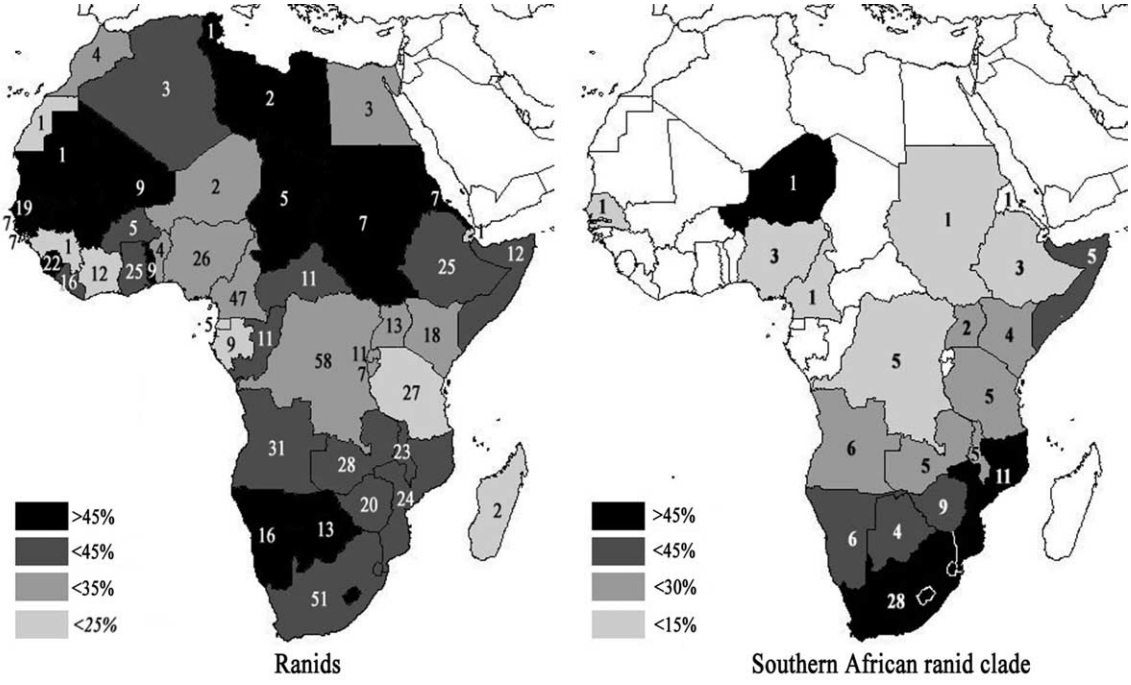

Fig. 4. Left map shows percentages of all ranid species as part of the total frog species numbers per country, with absolute number of ranids in each country (data from AmphibiaWeb.org).[2] Percentage of the ranid species belonging to the endemic Southern African clade of the total ranid species count is shown on the right. Numbers represent absolute species numbers of frogs from the endemic Southern African clade. Frogs occurring in Lesotho and Swaziland were included in the South African species counts. The analysis considered all representatives demonstrated to belong to the Southern African clade herein (Fig. 2) and the genus *Anhydrophryne* that was closely related to *Cacosternum* in the molecular study of Vences et al. (2000). Inclusion of the other African cacosternine genera (*Arthroleptella, Microbatrachella*, *Poyntonia*, and *Nothophryne* (Blommers-Schlösser, 1993; Dubois, 2003)) would lead to an even stronger diversity hotspot in South Africa.

Africa. The large range in biology and ecology suggest an adaptive radiation in these frogs similar to that of the Leptodactylidae in South America.

South Africa has a high endemism for flora, as it has the entire Cape Floristic Region within its borders (Mittermeier et al., 1998). The degree of endemism for amphibians is also spectacularly high; 54% of the 118 frogs that occur in South Africa are only found there. Of the 51 ranids that occur there, 27 (53%) are endemic (calculated using data from AmphibiaWeb.org).[2] Conservation International has marked the Cape Floristic Region as one of the world's biodiversity hotspots with 5682 endemic plant species and 53 endemic vertebrates (Myers et al., 2000). Future data will allow testing possible biogeographic correlates between the high floral endemism of the Cape Floristic Region and the southern African diversity hotspot of the endemic ranid clade identified herein. An entire radiation at the family level associated to some degree with the Cape Floral region will further strengthen its status as a biodiversity hotspot, and can possibly serve as a flagship example of endemic biodiversity.

4.2. Endemic African ranids are phylogenetically basal

Despite the inclusion of almost 3 kbp sequence data into our analysis, basal relationships among major ranoid clades remained largely unsolved. The lack of resolution basal within the Ranoidae, in contrast to the good resolution at levels below and above, could be a 'hard' polytomy caused by a relatively rapid radiation of the Ranidae. Alternatively, this pattern could be alleviated by the inclusion of more species. Independent from the uncertainty of their precise phylogenetic position, various species had high genetic divergences from all other taxa included. This applies to the African *Petropedetes*, *Ptychadena, Phrynobatrachus*, and *Conraua*, but also to the Asian *Ceratobatrachus*, a Solomon and Bourgainville island representative of platymantines that are endemic to the Philippines, Papua New Guinea, the Moluccas, New Britain, Admiralty, Solomon, and Fiji islands. The long branches of these taxa (Fig. 2) are indicative of long independent evolutionary histories. The age of their splits from their closest relatives were estimated between 92 and 86 mya (Table 3). As predicted by Roelants et al. (2004) the inclusion of these taxa leads to the identification of areas of endemism for deep evolutionary ranid lineages in addition to South Asia, namely the Philippine and Pacific region, and southern and central Africa.

The long isolation of Africa subsequent to Gondwana fragmentation has, similar to the faunal histories of South America and Australia, allowed for the development of a unique endemic mammalian radiation: the Afrotheria (Murphy et al., 2001). This radiation includes a range of animals as dissimilar as elephants, aardvarks and golden moles. The time of the onset of the Afrotheria radiation has been estimated to be 79.9 mya, with 95% confidence intervals of 73.0–85.8 mya, by Springer et al. (2003). Bayesian analysis of the divergence time of the endemic ranid clade with *Pyxicephalus* as the most basal taxon provided an estimate of 69.9 (48.9–96.5) mya. Divergence time of the clade including *Petropedetes* and *Conraua* is 85.6 (61.1–116.4) mya (Table 3). This indicates that the radiation of the endemic African ranid clade may have occurred roughly in the same period as the Afrotherian radiation, although the large confidence intervals of our estimates inhibit more precise interpretations. This endemic clade and the other deep African lineages identified in our study, therefore contain a large amount of evolutionary history. This should be taken into consideration when outlining conservation strategies. In fact, in South Africa, Lesotho and Swaziland (Minter et al., 2004), 30% (11 out of 37) species belonging to the endemic African clade (if cacosternines are considered as belonging to it entirely) are in a threatened red list category, while this applies to only 18% (14 out of 78) of the remaining frog species. This might be seen as indication for non-random extinction processes.

5. Conclusion

The amphibian decline problem and the persistent elusiveness of its causes highlight the limits of our knowledge of amphibian biodiversity and emphasize the urgency of the need for further studies to design informed conservation measures. The discovery of a clade that is supported by several independent nuclear as well as mitochondrial markers, but which has eluded workers using morphological characters, is indicative of the need of a well-resolved molecular phylogeny of amphibians. Possibly, high levels of phenotypic homoplasy so far hindered the discovery of reliable morphology-based phylogenetic relationships, especially of the ranoids. Clearly, further studies are necessary to investigate the characters that are particularly homoplasious. The uncovered endemic clade may be affected more strongly than others by declines, thereby stressing the importance of a phylogenetic framework for effective conservation priority assessment.

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[2] Although the data in the AmphibiaWeb.org database is incomplete regarding African ranids awaiting an update with findings from the Global Amphibian Assessment, the general picture and the identified hotspots for the endemic clade are unlikely to change.

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