African Journal of Herpetology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t917596259

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First published on: 04 January 2011

To cite this Article van der Meijden, Arie , Crottini, Angelica , Tarrant, Jeanne , Turner, Andrew and Vences, Miguel(2011) 'Multi-locus phylogeny and evolution of reproductive modes in the Pyxicephalidae, an African endemic clade of frogs', African Journal of Herpetology, 60: 1, 1 — 12, First published on: 04 January 2011 (iFirst)
To link to this Article: DOI: 10.1080/21564574.2010.523904
URL: http://dx.doi.org/10.1080/21564574.2010.523904

African Journal of Herpetology,
Vol. 60, No. 1, April 2011, 1–12

UNISA university of south africa PRESS | Taylor & Francis Taylor & Francis Group

Original article

Multi-locus phylogeny and evolution of reproductive modes in the Pyxicephalidae, an African endemic clade of frogs

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Abstract.—The ranoid family Pyxicephalidae is an endemic group of African frogs, with the majority of its diversity concentrated in South Africa. Here we present the first molecular phylogeny that includes as many as nine of the ten pyxicephalid genera, omitting only *Nothophryne*, based on four nuclear gene fragments and one mitochondrial gene fragment. This study corroborates the basal placement of *Pyxicephalus* and *Aubria*, and the sister group placement of *Natalobatrachus* and *Arthroleptella*, which were first signalled using smaller datasets. The evolution of endotrophic development has evolved twice within the Pyxicephalidae, once in the ancestor of *Anhydrophryne*, and independently in the ancestor of *Arthroleptella*. The implications for the evolution of body size, from the large species in *Pyxicephalus* and *Aubria* to the smallest pyxicephalid species in *Arthroleptella* and *Microbatrachella* are discussed.

Key words.—Amphibia, Anura, Ranoidea, Pyxicephalidae; *Amietia*, *Anhydrophryne*, *Arthroleptella*, *Cacosternum*, *Microbatrachella*, *Natalobatrachus*, *Poyntonia*, *Pyxicephalus*, *Strongylopus*, *Tomopterna*, molecular phylogeny

Molecular phylogenetics has in the past two decades revolutionised our understanding of the evolution and biogeography of many groups of organisms, especially where morphological homoplasy or evolutionary stasis has obscured the true relationships among taxa. Prominent among these groups are the amphibians for which molecular data have uncovered numerous previously unrecognised clades of taxa that often are diverse in morphology and ecology and therefore were previously thought to be unrelated (e.g. Vences *et al*. 2000; Frost *et al*. 2006; van der Meijden *et al*. 2007; Santos *et al*. 2009). A common pattern in these newly discovered clades is often that they show biogeographic coherence, i.e. they inhabit similar continents and regions in which they apparently diversified.

One such group is the family Pyxicephalidae, which occurs throughout Africa, but has its highest diversity of species and genera in southern Africa (van der Meijden *et al*. 2005). This highly diverse family contains frogs with body sizes from

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ISSN 2156-4574 print/ISSN 2153-3660 online

DOI: 10.1080/21564574.2010.523904
http://www.informaworld.com

about 15 mm (*Microbatrachella* and *Arthroleptella*) up to 245 mm (*Pyxicephalus*), terrestrial, and aquatic as well as semi-arboreal species such as *Natalobatrachus*. It contains species with explosive egg-laying of large numbers of small eggs into temporary ponds (e.g. *Tomopterna*), as well as species with nidiculous endotrophic reproduction in which a few large eggs develop in terrestrial nests into froglets, solely on the yolk reserve provided by the mother (e.g. *Anhydrophryne*, *Arthroleptella*).

Recognition of the Pyxicephalidae as a monophyletic group began with the discovery (Bossuyt & Milinkovitch 2000; Vences *et al*. 2000) that the genus *Tomopterna* is an African endemic and species from Madagascar and India previously included in this genus belong to two different – Malagasy and Asian, respectively – clades, and that African *Tomopterna* might be related to the genera *Anhydrophryne*, *Cacosternum*, and *Pyxicephalus*. Van der Meijden *et al*. (2005) first provided strong evidence from multiple genetic markers that this African clade, with a clear centre of diversity and endemism in southern Africa, includes the genera *Afrana*, *Cacosternum*, *Natalobatrachus*, *Pyxicephalus*, *Strongylopus*, and *Tomopterna*. Scott (2005) combined mitochondrial DNA sequences and morphological data sets in a study of African ranoid frogs that also included numerous pyxicephalids. Bossuyt *et al*. (2006) included the genera *Arthroleptella* and *Poyntonia*, which were placed in the same monophyletic group, named Pyxicephalinae. Frost *et al*. (2006), with a dataset of largely different genes, confirmed this monophyletic group, too, including in it the additional genera *Amietia* and *Aubria*. These authors defined the clade as Pyxicephalidae and included in it, without molecular evidence, the genera *Microbatrachella* and *Nothophryne* (*Pyxicephalus* and *Aubria* were classified in the subfamily Pyxicephalinae, all other genera in the Cacosterninae). Tarrant *et al*. (2008) provided evidence for synonymy of the genus *Afrana* with *Amietia*. Dawood & Stam (2006) suggested that the Western Cape *Arthroleptella* species be maintained in the genus *Arthroleptella* and that the KwaZulu-Natal species be placed in *Anhydrophryne*, thus maintaining these two genera. Wiens *et al*. (2009) corroborated earlier findings on the placement of the subclades of the Pyxicephalidae in a re-analysis of available data on the Old World ranids. The temporal origin and initial diversification of pyxicephalids was placed in the Late Cretaceous to Early Paleocene (van der Meijden *et al*. 2005; Bossuyt *et al*. 2006; Roelants *et al*. 2007; Wiens *et al*. 2009).

Despite the obvious interest in the biogeography, conservation, and evolution of reproductive modes in this clade, no in-depth phylogenetic analysis of nuclear and mitochondrial gene sequences including all genera in the family has been conducted as yet. This study aims to contribute to a more complete understanding of the evolution of the Pyxicephalidae. We compiled multi-locus datasets for one mitochondrial and five nuclear gene fragments with complementing existing data from Genbank by new sequences. Our datasets include complete sequence sets for nine pyxicephalid genera plus the genus *Microbatrachella*, and a partial set of sequences for *Aubria*, with only one pyxicephalid genus (*Nothophryne*) missing.

Materials and Methods

Sampling Strategies and Molecular Methods

Tissue samples of five species of *Amietia*, two species of *Strongylopus* and one sample each of *Arthroleptella*, *Cacosternum*, *Microbatrachella*, *Pyxicephalus*, *Tomopterna*

and *Anhydrophryne* were available. *Rana temporaria* was used as the outgroup and *Petropedetes* and *Conraua* were added as additional hierarchical outgroups (details provided in Table 1). Leg muscle tissue and tadpole tail tissue (either frozen or preserved in 95% ethanol) were used for DNA extraction. Total genomic DNA was extracted from the tissue samples using proteinase K digestion (10 mg/ml concentration) followed by a standard salt extraction protocol (Bruford *et al*. 1992). One mitochondrial fragment (16S rRNA gene) plus four nuclear gene fragments were amplified: recombination activating gene 1 (RAG1), recombination activating gene 2 (RAG2), Rhodopsin (Rho) and Tyrosinase (Tyr).

Standard polymerase chain reactions were performed in a final volume of 11 µl and using 0.3 µl each of 10 pmol primer, 0.25 µl of total dNTP 10 mM (Promega), 0.08 µl of 5 U/ml GoTaq, and 2.5 µl 5X Green GoTaq Reaction Buffer (Promega). RAG2 fragments were amplified with a nested-PCR approach and a first amplification using external primers 31FN.Venk and Rag-2.Lung.460R, and a second amplification with internal primers Rag-2.Lung.35F and Lung.320R. Primers and detailed PCR conditions are provided in Table 2.

PCR products were purified through QIAquick purification kit (Quiagen) according to the manufacturer's instructions. Purified PCR templates were sequenced using dye-labelled dideoxy terminator cycle sequencing on an ABI 3130 automated DNA sequencer or on an ABI 3730xl at Macrogen Inc. Chromatographs were checked and sequences were edited using BioEdit, ver. 7.0.9 (Hall 1999) and MEGA 4.1 (Kumar *et al.* 2008) and aligned using Muscle (Edgar 2004). All newly determined sequences have been deposited in GenBank (HQ014417-HQ014446, details in Table 1).

Phylogenetic Analyses

Three datasets were analysed for a combination of mitochondrial (16S) and nuclear (RAG1, RAG2, tyrosinase and rhodopsin) genes: (1) an alignment containing 20 taxa for which sequences for the 16S rRNA gene, RAG1 and RAG2 were all available, encompassing a total of 2 805 bp; (2) an alignment containing 25 taxa for which any of the genes of 16S rRNA, RAG1 and RAG2 were available (2 805 bp); and (3) an alignment containing 13 taxa for which any of the genes 16S, RAG1, RAG2, tyrosinase and rhodopsin were available (3 669 bp). In addition to these concatenated datasets, datasets for each single gene were also analysed except for RAG1 and RAG2, which were combined due to their close functional association.

For each of the datasets, two methods of phylogenetic analysis, namely maximum likelihood (ML) and Bayesian Inference (BI) searches were conducted using PhyML, version 2.4.4 (Guindon & Gascuel 2003) and MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, 2004), respectively. The best fit models of nucleotide evolution were determined under the Akaike information criterion in Modeltest 3.7 (Posada & Crandall 1998) and MrModeltest (Nylander 2002). Nodal support for the topologies recovered in the ML analyses were obtained with 1 000 bootstrap replicates. The BI analyses were run with 5 000 000 generations, sampling trees every 10th generation (and calculating a consensus tree after omitting the first 125 000 trees). Log likelihood scores for the remaining trees were graphed in Tracer 1.5 (http://beast.bio.ed.ac.uk/Tracer) and checked for appropriateness of the burnin-period.

Table 1. List of taxa included in the present study, with their respective localities, collection or field numbers of voucher specimens (where available) and GenBank accession numbers (16S, RAG1, RAG2, Rhodopsin and Tyrosinase). Newly determined sequences are in bold. Other sequences are taken from the works of 1, Bossuyt *et al.* 2006; 2, Tarrant *et al.* 2008; 3, Frost *et al.* 2006; 4, Turner *et al.* 2004; 5, Vences *et al.* 2000; 6, van der Meijden *et al.* 2007; 7, van der Meijden *et al.* 2005; 8, Scott 2005; 9, Chen *et al.* 2005; 10, Dawood & Uqubay 2004; 11, Dawood & Stam 2006; 12, Vences *et al.* 2003; 13, Hoegg *et al.* 2004; 14, Bossuyt & Milinkovitch 2000. *Dataset 1; †dataset 2.

Taxon	Locality	Voucher	16S	RAG1	RAG2	Rhodopsin	Tyrosinase
Amietia angolensis			DQ347318[1*]	DQ347257[1*]	DQ019515[7*]		
Amietia dracomontana	Tsatsane, Lesotho	QQ0009	FJ411440[2*]				
Amietia dracomontana	Sani Top, Lesotho	SP0007		**HQ014422**[2*]	**HQ014430**[2*]		
Amietia fuscigula	Cape of Good Hope		**HQ014417**[4*†]			DQ283794[3†]	DQ282909[3†]
Amietia fuscigula	Freestate, South Africa	QQ0139		**HQ014423**[2*†]	**HQ014431**[2*†]		
Amietia umbraculata	Makhaleng, Lesotho	DT5	FJ411427[2*]	FJ411443[2*]	**HQ014432**[*†]		
Amietia vandijki	Swartberg, South Africa	MH0107	**HQ014418**[2*†]	**HQ014424**[2*†]			
Amietia vertebralis	Vemvane, South Africa	MH1211	FJ411432[2*]	FJ411448[2*]	**HQ014433**[*]		
Arthroleptella lightfooti	Silvermine reserve, South Africa		AY205282[4*†]	**HQ014425**[*†]	**HQ014434** [*†]	**HQ014442**[†]	
Aubria subsigillata	Cameroon		DQ283352[3]			DQ283977[3]	DQ282975[3]
Cacosternum boettgeri			AF215414[5*†]	AY571645[6*†]	DQ019521[7*†]	DQ019554[7†]	DQ347141[1†]
Cacosternum namaquense			**HQ014419**[*]	**HQ014426**[*]	**HQ014435**[*]		
Microbatrachella capensis	Betty's bay		DQ022357[8*†]	**HQ014427**[*†]	**HQ014436**[*†]	**HQ014443**[†]	
Natalobatrachus bonebergi			AF215396[5*†]	DQ019502[7*†]	DQ019534[7*†]	DQ019563[7†]	DQ347144[1†]
Poyntonia paludicola	Groot Drakenstein (RAG2)		DQ347341[1*†]	DQ347283[1*†]	**HQ014437**	DQ347402[1†]	
Pyxicephalus adspersus			AF206472[9*†]	DQ019508[7*†]	DQ019543[7*†]	DQ019569[7†]	
Pyxicephalus edulis							**HQ014445**
Strongylopus bonaespei			DQ347345[1]	DQ347288[1]			DQ347196[1†]
Strongylopus fasciatus			AF215412[5*†]	DQ019513[7*†]	DQ019549[7*†]	DQ019574[7†]	
Strongylopus grayii	Polka Draai, South Africa		**HQ014420**[2*]	**HQ014428**[*]	**HQ014438**[*]		
Strongylopus wageri	Sani Pass, South Africa	SP0002	FJ411441[2*]	FJ411457[2*]	**HQ014439**[2*]		
Tomopterna cryptotis	Freestate, South Africa	QQ0133	AY255099[10]		**HQ014440**[2*†]		
Tomopterna delalandii			**HQ014421**[†]				DQ283005[3†]

Table 1 (*Continued*)

Taxon	Locality	Voucher	16S	RAG1	RAG2	Rhodopsin	Tyrosinase
Tomopterna damarensis			DQ019610[7*]	DQ019514[7*†]	DQ019550[7*†]	DQ019575[7†]	
Tomopterna tandyi			AY838891[11]				
Anhydrophryne rattrayi	Hogsback		AF215413[5*†]	**HQ014429**[*†]	**HQ014441**[*†]	**HQ014444**[†]	**HQ014446**
Conraua crassipes			DQ019600[7*†]	DQ019498[7*†]	DQ019524[7*†]		
Petropedetes parkeri			AY341724[12*†]	DQ019505[7*†]	DQ019538[7*†]		
Rana temporaria			DQ283128[3*†]	AY323776[13*†]	AY323803[13*†]	DQ283914[3†]	AF249182[14†]

Table 2. List of primers and PCR conditions used for molecular analyses. PCR conditions start with temperature (°C) of each step followed by the time in seconds. Sources are as follows: 1, Palumbi *et al.* 1991; 2, Hoegg *et al*. 2004; 3, Venkatesh *et al.* 2001; 4, Brinkmann *et al*. 2004; 5, Bossuyt & Milinkovitch 2000; 6, San Mauro *et al.* 2004; 7, Chiari *et al.* 2004 and unpublished.

Gene	Primer name	Sequence (5′→3′)	Source	PCR conditions
16S rRNA	16S AL	CGC CTG TTT ATC AAA AAC AT	1	94(90), [94(45), 55(45), 72(90)x33], 72(300)
16S rRNA	16S BH	CCG GTC TGA ACT CAG ATC ACG T	1	
RAG1	AmpF1	ACA GGA TAT GAT GAR AAG CTT GT	7	94(140), [50(50), 72(300), 72(600)x39], 94(45), 72(300)
RAG1	AmpR1	AAC TCC GCT GCA TTK CCA ATR TCA CA	7	
RAG1	AmpR2	GGT GYT TYA ACA CAT CTT CCA TYT CRT A	7	
RAG1	Amp-Rag1F	AGC TGC AGY CAR TAC CAY AAR ATG TA	7	94(120), [94(30), 48(45), 68(90)x35], 68(420)
RAG1	Amp-Rag1R	TTR GAT GTG TAG AGC CAG TGG TGY TT-	7	94(120), [94(30), 48(45), 68(90)x35], 68(420)
RAG2	31FN. Venk (external primer)	TTY GGI CAR AAR GGI TGG CC	3	94(120), [94(20), 50(40), 68(180)x35], 68(300)
RAG2	Lung.460R (external primer)	GCA TYG RGC ATG GAC CCA RTG ICC	4	
RAG2	Rag-2Lung.35F (internal primer)	GGC CAA AGA GRT CYT GTC CIA CTG G	2, 7	94(120), [94(20), 50(40), 68(180)x35], 68(300)
RAG2	Lung.320R (internal primer)	AYC ACC CAT ATY RCT ACC AAA CC	2, 7	
Rhodopsin	Rhod-ma	AAC GGA ACA GAA GGY CC	5	94(90), [94(30), 57(45), 72(30)x35], 72(240)
Rhodopsin	Rhos-md	GTA GCG AAG AAR CCT TC	5	
Tyrosinase	Tyr-1a	AGG TCC TCT TRA GCA AGG AAT G	5	94(120), [94(30), 57(50), 72(120)x36], 72(600)
Tyrosinase	Tyr-1g	TGC TGG CRT CTC TCC ART CCC A	5	

RESULTS

The trees obtained by analysing datasets 2 and 3 are shown in Figs. 1 and 2, respectively. The analysis of dataset 1 yielded a congruent topology to that of dataset 2, of which it is a subset. The results of dataset 1 are therefore not shown in the figures. The single gene datasets did not resolve any highly supported topological differences at the genus level to the topology shown in Fig. 1. In the analyses of datasets 1 and 2, where more than one species per genus was included, genera were always recovered as monophyletic with high support values (Fig. 1). Several nodes were strongly recovered by all analyses: (1) the monophyly of pyxicephalids; (2) the placement of *Pyxicephalus* (plus *Aubria* in dataset 3) as the basal-most clade; (3) the placement of *Arthroleptella* as sister to *Natalobatrachus*. Datasets 1 and 2 also provided strong support for a clade including *Cacosternum*, *Microbatrachella*, and *Poyntonia*, but surprisingly dataset 3 (with two additional genes but fewer taxa) found only weak ML bootstrap support for this group, although partitioned Bayesian analyses confirmed the monophyly of this group. Moderate support from all three datasets was furthermore found for a clade including *Amietia*, *Arthroleptella*, *Cacosternum*, *Microbatrachella*, *Natalobatrachus*, *Poyntonia* and *Strongylopus*, to the exclusion of *Anhydrophryne* and *Tomopterna*. None of the trees recovered the taxa with endotrophic development (*Anhydrophryne* and *Arthroleptella*) in one monophyletic group.

DISCUSSION

Several aspects of our analyses are congruent with previous studies, which partly used different gene fragments and analysis methods. The basal position of *Pyxicephalus* in the Pyxicephalidae has been a common finding in all studies so far (van der Meijden *et al*. 2005; Bossuyt *et al*. 2006; Frost *et al*. 2006; Roelants *et al*. 2007; Wiens *et al*. 2009). The same is true for the placement of *Tomopterna*, splitting off from the tree after the *Pyxicephalus-Aubria* clade (van der Meijden *et al*. 2005; Bossuyt *et al*. 2006; Frost *et al*. 2006) and the placement of *Arthroleptella* sister to *Natalobatrachus* (Bossuyt *et al*. 2006; Frost *et al*. 2006).

As a major difference from our trees, Frost *et al*. (2006) found *Anhydrophryne* sister to *Cacosternum*. This placement was based partly on rhodopsin and tyrosinase sequences that we also included in our dataset 3, which may explain why the support values are lower in the trees based on this dataset. It will be necessary to verify this placement with an independent set of sequences.

Considering the available evidence for pyxicephalid relationships, some conclusions can be drawn on the evolution of this family. Most basal in the family are frogs of large (*Pyxicephalus*, *Aubria*) or medium size (*Tomopterna*), which live in lowland rainforests or savannas and reproduce explosively in temporary ponds (*Pyxicephalus*, *Tomopterna*). *Pyxicephalus* occurs in southern Africa, and *Aubria* does not, neither of these two basal-most genera are endemic to the region. *Tomopterna* ranges widely over most of Africa, but according to current knowledge has its centre of diversity in southern Africa. Various other clades sequentially splitting off the pyxicephalid tree (*Anhydrophryne*, *Arthroleptella-Natalobatrachus*) are endemic to southern Africa and partly (*Anhydrophryne*, *Arthroleptella*) contain numerous microendemic taxa in the

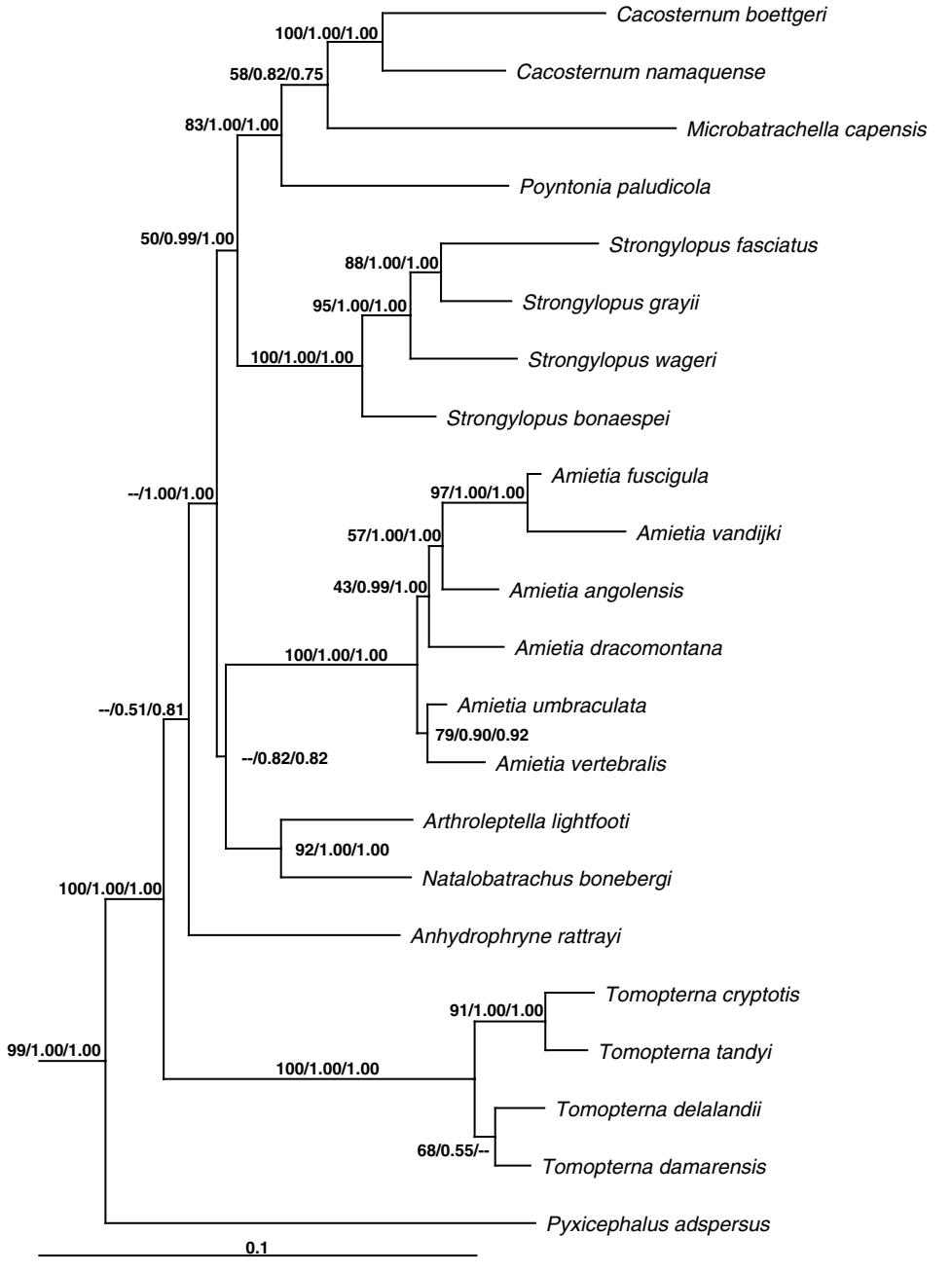

Figure 1. Maximum likelihood tree of the Pyxicephalidae based on dataset 2 (2 805 bp of the 16S rRNA, Rag1 and Rag2 genes, including all taxa for which at least one of these genes was available). Numbers at nodes are support values from a ML bootstrap analysis (bootstrap proportions in per cent), and posterior probabilities from unpartitioned and a partitioned BI analyses. *Rana temporaria*, *Conraua crassipes* and *Petropedetes parkeri* were used as outgroups (not shown).

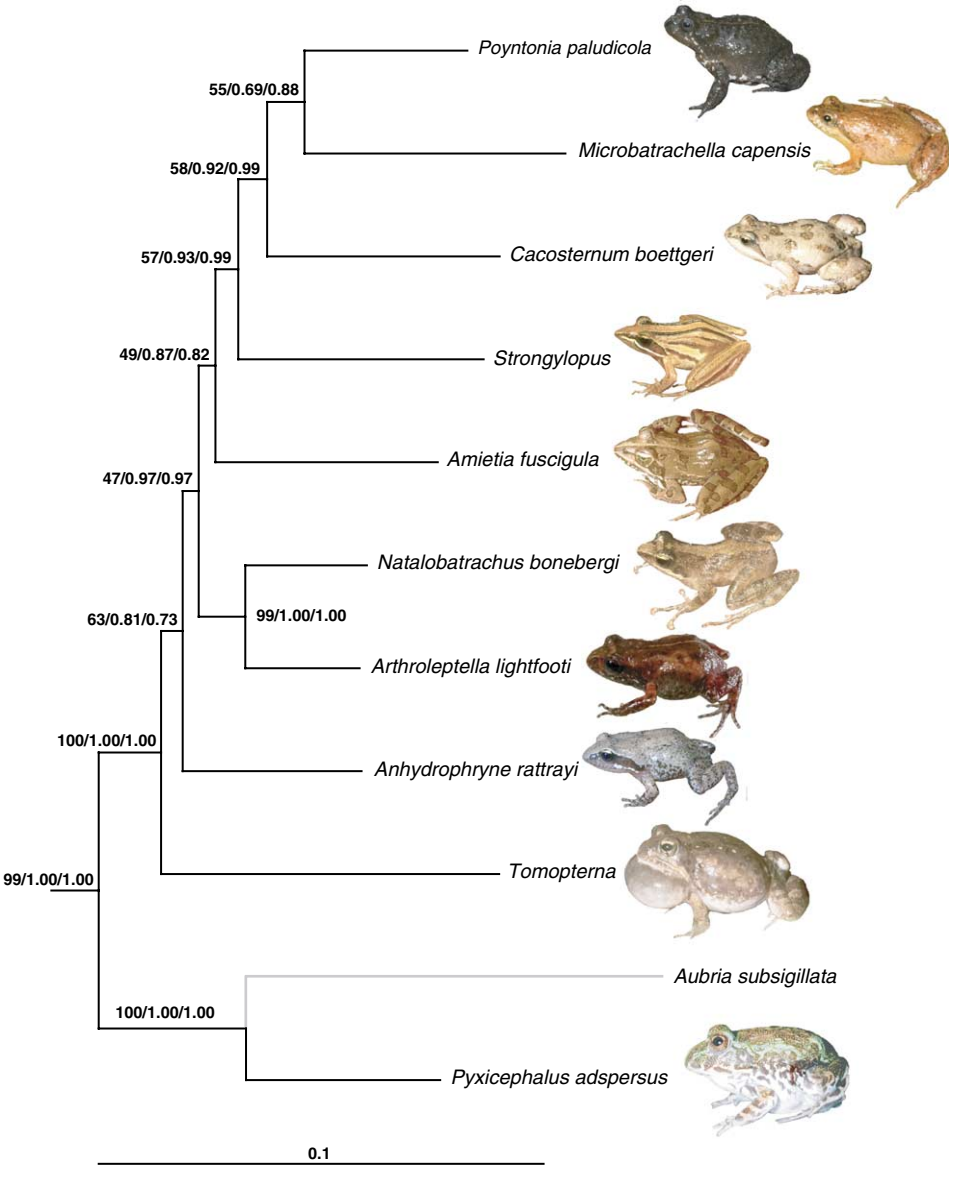

Figure 2. Maximum likelihood tree of the Pyxicephalidae based on dataset 3 (3 669 bp of the 16S, Rag1, Rag2, rhodopsin, and tyrosinase genes). Numbers at nodes are support values from an ML bootstrap analysis (bootstrap proportions in per cent), and posterior probabilities from unpartitioned and partitioned BI analyses. *Rana temporaria*, *Conraua crassipes* and *Petropedetes parkeri* were used as outgroups (not shown). The branch leading to *Aubria* has been coloured grey to indicate its placement is based on only 16S, tyrosinase, and rhodopsin sequences.

region. The remaining genera are either microendemic in southern Africa as well (*Microbatrachella*, *Poyntonia*) or have representatives ranging more widely into Africa (*Amietia*, *Strongylopus*, partly *Cacosternum*). In summary, this pattern

suggests an origin of the Pyxicephalidae from wide-ranging and medium to large-sized African frogs that inhabited savanna and lowland forest, similar to the extant genera *Pyxicephalus*, *Aubria* and *Tomopterna*. In southern Africa, and several times independently, some ancestral pyxicephalids adapted to special habitats by reducing their body size (e.g. *Anhydrophryne*, *Arthroleptella*, Cacosternum, *Microbatrachella*, *Natalobatrachus*, *Poyntonia*) and specialising in semi-arboreal habits (*Natalobatrachus*) or endotrophic nidiculous reproduction (*Anhydrophryne* and *Arthroleptella*). Our data are unambiguous in suggesting that endotrophic development evolved independently twice, once in *Arthroleptella* and once in *Anhydrophryne.* None of our trees placed these genera as sister genera. The fact that *Arthroleptella* is the sister group of *Natalobatrachus*, a semi-arboreal species that deposits its eggs outside of the water, on branches overhanging the water where the exotrophic tadpoles develop, might indicate that such a non-aquatic egg deposition (otherwise not found among pyxicephalids with exotrophic development) might have characterised also the ancestor of the *Natalobatrachus-Arthroleptella* lineage and might have represented a predisposition facilitating the evolution of fully terrestrial, endotrophic development in *Arthroleptella*.

ACKNOWLEDGEMENTS

We are grateful to numerous friends and colleagues who helped with collecting or provided samples: Marius Burger, Alan Channing, Ylenia Chiari, Michael Cunningham, Louis du Preez and SAIAB (South African Institute of Aquatic Biodiversity). Meike Kondermann, Gabi Keunecke and Eva Saxinger helped in the lab. Thanks to Stefan Lötters, David Blackburn, Eli Greenbaum and one anonymous reviewer for their insightful comments on the manuscript. AvdM was supported by FCT post-doctoral grant SFRH/BPD/48042/2008.

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Received: 27 May 2010; Final acceptance: 9 September 2010