Molecular phylogenetics of *Boulengerula* (Amphibia: Gymnophiona: Caeciliidae) and implications for taxonomy, biogeography and conservation

Simon P. Loader[1,2], Mark Wilkinson[2], James A. Cotton[3,4], G. John Measey[5,6], Michele Menegon[7], Kim M. Howell[8], Hendrik Müller[2,9] & David J. Gower[2]

[1]*Department of Environmental Sciences, Institute of Biogeography, University of Basel, Switzerland*
[2]*Department of Zoology, Natural History Museum, London, UK*
[3]*School of Biological and Chemical Sciences, Queen Mary University of London, UK*
[4]*Wellcome Trust Sanger Institute, Cambridge, UK*
[5]*Applied Biodiversity Research Division, South African National Biodiversity Institute, Cape Town, South Africa*
[6]*Department of Biodiversity and Conservation Biology, University of the Western Cape, Bellville, South Africa*
[7]*Sezione di Zoologia dei Vertebrati, Museo Tridentino di Scienze Naturali, Trento, Italy*
[8]*Department of Zoology and Marine Biology, University of Dar es Salaam, Tanzania*
[9]*Institut für Spezielle Zoologie und Evolutionsbiologie mit Phyletischem Museum, Universität Jena, Germany*

Phylogenetic relationships of the East African caeciliid *Boulengerula* were reconstructed using 12S, 16S and cytb mitochondrial gene sequences for 32 samples from Kenya and Tanzania. The generally well-supported and resolved phylogeny displayed the following relationships among the five nominate species sampled: (*B. boulengeri* ((*B. taitanus*, *B. niedeni*), (*B. changamwensis*, *B. uluguruensis*))). This resolution supports a formerly proposed bipartition of the genus, and differs significantly from previous, morphological phylogenies. Our analyses identified genetic differences between several mtDNA clades that potentially represent undescribed species. If substantiated, the necessary taxonomic revision will have implications for conservation assessments that depend to an important extent upon sizes of distributions. Overall, there is a positive correlation between genetic and geographic distance among and within the main clades. The two lowland, coastal individuals sampled are nested within primarily montane clades. Dating analyses suggest some temporally congruent divergences in *Boulengerula*, but other divergences happened at different times and over a long period, perhaps extending back to the Oligocene/Eocene. Our results for *Boulengerula* suggest a role for relative long-term environmental stability in the origins of the Eastern Arc Mountains biodiversity hotspot.

Key words: caecilians, mtDNA, Eastern Arc Mountains, Kenya, Tanzania

INTRODUCTION

The caecilian genus *Boulengerula* Tornier, 1896 is known from, and presumed to be restricted to, the Eastern Arc Mountains, lowland coastal forests, Albertine Rift and Malawi Shire Highlands of East Africa (Fig. 1). *Boulengerula* is the most speciose African caecilian genus, with the seven recognized species comprising about 39% and 4% of known extant African and global caecilian diversity respectively (Wilkinson et al., 2004; Müller et al., 2005; Wilkinson & Nussbaum, 2006; IUCN 2009). The centre of *Boulengerula* diversity is the Eastern Arc Mountains (EAM), a global biodiversity hotspot (Myers et al., 2000), from where four species have been described (Müller et al., 2005).

Immediately before Taylor's (1968) monographic treatment of caecilians, four species of *Boulengerula* were recognized: *B. boulengeri* Tornier, 1896, *B. uluguruensis* Barbour & Loveridge, 1928, *B. changamwensis* Loveridge, 1932, and *B. taitanus* Loveridge, 1935. Taylor (1968) partitioned the genus, retaining only the type species *B. boulengeri* in *Boulengerula* (Tornier, 1896) and erecting *Afrocaecilia* to receive the remaining species. Nussbaum & Hinkel (1994) described a new species, *B. fischeri*, carried out a morphological phylogenetic analysis of the genus, and placed the non-monophyletic (in their tree) *Afrocaecilia* in the synonymy of *Boulengerula*. Wilkinson et al. (2004) removed *B. denhardti* Nieden, 1912 from the synonymy of *Schistometopum* (*Dermophis*) *gregorii* (Boulenger, 1894), demonstrated that Nussbaum & Hinkel's (1994) phylogenetic results were not robust, and suggested that synonymy of *Afrocaecilia* with *Boulengerula* was premature. Most recently, Müller et al. (2005) have described an additional species, *B. niedeni*, which is currently the only IUCN "Critically Endangered" caecilian (IUCN et al., 2009).

Previous considerations of the molecular systematics of *Boulengerula* have included no more than three individuals (Wilkinson et al., 2003; Frost et al., 2006; Loader et al., 2007; Roelants et al., 2007; Wollenberg & Measey, 2009; Zhang & Wake, 2009). In addition to taxonomic significance, an expanded molecular perspective on *Boulengerula* systematics will contribute to a broader understanding of the biology of the group.

Correspondence: Simon P. Loader, Institute of Biogeography, Department of Environmental Sciences, Klingelbergstrasse, 27, University of Basel, Basel 4056, Switzerland. *E-mail:* Simon.Loader@unibas.ch

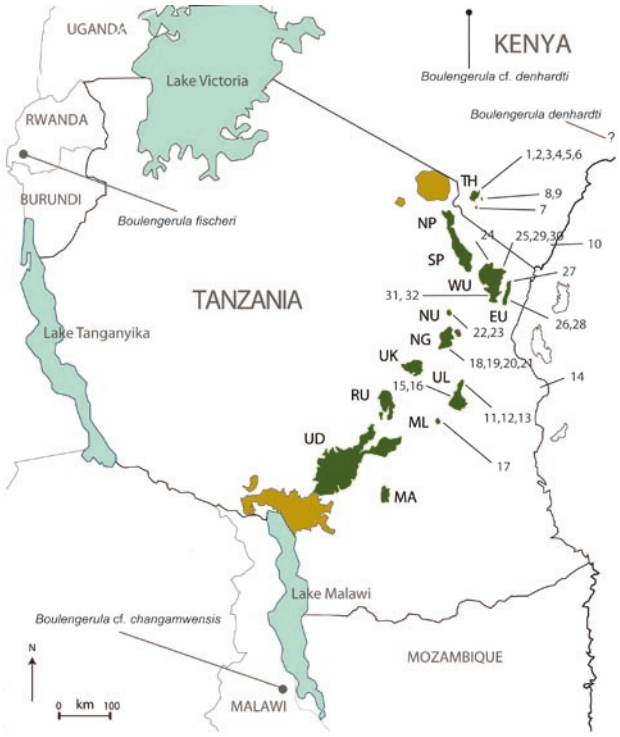

Fig. 1. Map of East Africa, with Eastern Arc mountain chain marked as dark areas. Collection localities of numbered samples are given in Table 1. This map covers the entire known range of *Boulengerula*. The locations of *B. changamwensis* (Malawi population), *B. denhardti*, *B.* cf. *denhardti* and *B. fischeri* not sampled in this study are also indicated. *Boulengerula denhardti* was described from an imprecise locality in the Tana River region. Abbreviations for montane blocks are: TH, Taita Hills; NP, North Pare; SP, South Pare; WU, West Usambara; EU, East Usambara; NU, Nguu; NG, Nguru; UK, Ukaguru; UL, Uluguru; ML, Malundwe; RU, Rubeho; UD, Udzungwa; and MA, Mahenge.

Although caecilian natural history is generally little studied and poorly understood (Gower & Wilkinson 2005), *Boulengerula* species have been the subject of pioneering quantitative field ecological studies (Garborieau & Measey, 2004; Gower et al., 2004; Malonza & Measey, 2005; Measey, 2006). Furthermore, *B. taitanus* is noteworthy for its remarkable reproductive biology including maternal dermatophagy and alloparental care (Kupfer et al., 2006, 2008). Finally, as a distinctive EAM lineage, *Boulengerula* has the potential to contribute to tests of hypotheses explaining the origins of the rich biodiversity of this region. Here we present a phylogenetic analysis of *Boulengerula* using a substantially expanded mtDNA dataset.

METHODS

Taxon and character sampling

Specimens of *Boulengerula* were obtained by targeted fieldwork (digging soil, pitfall trapping) in Kenya and Tanzania between 2000 and 2008 (Table 1). Tissues (generally liver and/or muscle) were preserved in 96% ethanol, with voucher specimens fixed in 4% formalin and stored in 70% ethanol. Samples were collected from all EAM localities where *Boulengerula* were previously recorded – East and West Usambara, Nguru, Taita Hills and Uluguru, (Nussbaum & Hinkel, 1994; Emmrich, 1994). Attempts were made to collect *Boulengerula* in places in the EAM where they potentially occur but have not previously been found, yielding the first specimens from Malundwe and Nguu. *Boulengerula* were not collected (and remain unreported) from Udzungwa, Mahenge, Rubeho, Ukaguru and North and South Pare. The coastal forests of Tanzania and Kenya were not surveyed as extensively, so that absence of *Boulengerula* throughout much of these areas is less certain. This study includes representatives of all nominate *Boulengerula* species except for *B. fischeri* and *B. denhardti*, non-EAM species (Fig. 1) known only from their holotypes. No attempt was made to sample a reported population of *B. changamwensis* from the Shire Highlands of Malawi (Nussbaum & Hinkel, 1994), or a population recently found in Ngaia, central Kenya by S. Spawls (pers. comm.) that most closely resembles *B. denhardti* among known species (see below). Based on the results of previous molecular analyses, the Central African *Herpele* is sister to *Boulengerula* (Wilkinson et al., 2003; Frost et al., 2006; Loader et al., 2007; Roelants et al., 2007), and so *H. squalostoma* was included as an outgroup. The rhinatrematid *Epicrionops marmoratus* was included as a more distant (e.g. Roelants et al., 2007), second outgroup.

Phylogenetics

An alignment of concatenated partial 12S, 16S and cytochrome b (*cytb*) sequences was assembled, based mostly on newly generated data. An outline of the methods for extraction, amplification and sequencing are given in Gower et al. (2002) and Wilkinson et al. (2003). Details of voucher specimens and GenBank accessions are given in Table 1.

Sequences were aligned initially in ClustalX using default parameters, were manually adjusted, and had any ambiguously aligned sites removed. The alignment is available from the senior author upon request. To investigate levels of saturation we plotted transition/transversion ratios against numbers of transversions for pairs of sequences. Parsimony analyses were performed with PAUP*4b6 (Swofford, 1998). Maximum likelihood (ML) analyses were performed with PAUP*4b6 and RAxML (Stamatakis, 2006), the latter using two partitions: rDNA (12S and 16S) and *cytb*. For ML analyses we used best-fit models as determined by Modeltest 3.04 (Posada & Crandall, 1998) using the Akaike Information Criterion (AIC). Empirical base frequencies were used in all analyses. PAUP* searches were heuristic, with 10 random addition sequence replicates and tree bisection recombination

Table 1. Details of *Boulengerula* and outgroup samples used in analyses plus GenBank accession codes. Collection abbreviations: BMNH (Natural History Museum, London, UK), KBM (Kate McQuaid Field Series), JM (John Measey Field Series), MTSN (Museo Tridentino di Scienze Naturali, Trento Italy), MW (field series to be deposited in BMNH), NMK (National Museum of Kenya), UMMZ (University of Michigan Museum, Ann Arbor, USA), and UTA (University of Texas, Arlington, USA). Vouchers were identified through comparisons with published descriptions and type material. FR = Forest Reserve, NR = Nature Reserve. GenBank accession codes in italics are from previous studies. Type localities (or likely within 20 km) for nominal species indicated by asterisks. (Taita Hills: Kenya; Uluguru, Malundwe, Nguru, Nguu, West and East Usambara: Tanzania.)

	Voucher	Species	Locality	Forest reserve	12S	16S	*cytb*
	UMMZ 190478	*Epicrionops marmoratus*	Ecuador	Cotopaxi	*AY101206*	*AY101226*	*AY101246*
	UTA 38889	*Herpele squalostoma*	Cameroon	Mundemba	FN652665	FN652697	FN652729
1	NMK A/4008	*B. taitanus*	Taita Hills	Ngangao FR	FN652685	FN652717	FN652749
2	NMK A/3112	*B. taitanus*	Taita Hills	Wundanyi*	AY450614	*AY450621*	*EU200986*
3	NMK A/3111/3	*B. taitanus*	Taita Hills	Wundanyi*	FN652667	FN652699	FN652731
4	NMK A/3112/1	*B. taitanus*	Taita Hills	Wundanyi*	FN652666	FN652698	FN652730
5	NMK A/3111/1	*B. taitanus*	Taita Hills	Wundanyi*	FN652669	FN652701	FN652733
6	JM 228	*B. taitanus*	Taita Hills	Chawia FR	*FN652689*	*FN652721*	*FN652753*
7	JM 849	*B. taitanus*	Taita Hills	Kasigau	FN652687	FN652719	FN652751
8	NMK A/4294	*B. niedeni*	Taita Hills	Sagala*	FN652691	FN652723	FN652755
9	NMK A/4295	*B. niedeni*	Taita Hills	Sagala*	FN652692	FN652724	FN652756
10	NMK A/4129	*B. changamwensis*	Coastal Kenya	Changamwe*	FN652690	FN652722	FN652754
11	BMNH 2005.216	*B. uluguruensis*	Uluguru	Mkungwe FR, 580 m asl	FN652670	FN652702	FN652734
12	BMNH 2005.215	*B. uluguruensis*	Uluguru	Mkungwe FR, 800 m asl	FN652672	FN652704	FN652736
13	BMNH 2005.214	*B. uluguruensis*	Uluguru	Mkungwe FR, 650 m asl	FN652671	FN652703	FN652735
14	BMNH 2005.996	*B. uluguruensis*	Coastal forest, Tanzania	Kazizumbwi FR, 180 m asl	FN652674	FN652706	FN652738
15	BMNH 2005.187	*B. uluguruensis*	Uluguru	Tegetero, 1000 m asl*	FN652684	FN652716	FN652748
16	JM 966	*B. uluguruensis*	Uluguru,	Tandai Village	FN652688	FN652720	FN652752
17	KBM 003	*B.* cf. *uluguruensis*	Malundwe		FN652694	FN652726	FN652758
18	BMNH 2002.959	*B.* cf. *uluguruensis*	Nguru	Komboro, Nguru South FR	FN652676	FN652708	FN652740
19	MTSN 8292	*B.* cf. *uluguruensis*	Nguru	Pemba, Nguru South FR	FN652693	FN652725	FN652757
20	BMNH 2002.928	*B.* cf. *uluguruensis*	Nguru	Komboro, Nguru South FR	FN652675	FN652707	FN652739
21	BMNH 2002.932	*B.* cf. *uluguruensis*	Nguru	Komboro, Nguru South FR	FN652679	FN652711	FN652743
22	MW 7291	*B.* cf. *uluguruensis*	Nguu	Nguu North FR	FN652695	FN652727	FN652759
23	MW 6638	*B.* cf. *uluguruensis*	Nguu	Nguu North FR	FN652696	FN652728	FN652760
24	BMNH 2005.1343	*B.* cf. *boulengeri*	West Usambara	Lushoto	FN652681	FN652713	FN652745
25	BMNH 2005.1358	*B.* cf. *boulengeri*	West Usambara	Mazumbai FR	FN652678	FN652710	FN652742
26	JM 150	*B. boulengeri*	East Usambara	Shambangeda, Amani NR*	FN652686	FN652718	FN652750
27	BMNH 2002.776	*B. boulengeri*	East Usambara	Nilo FR	FN652673	FN652705	FN652737
28	BMNH 2002.95	*B. boulengeri*	East Usambara	Amani-Kwamkoro FR*	*AY450613*	*AY450620*	*EU200987*
29	BMNH 2005.1359	*B.* cf. *boulengeri*	West Usambara	Mazumbai FR	FN652680	FN652712	FN652744
30	BMNH 2005.1357	*B.* cf. *boulengeri*	West Usambara	Mazumbai FR	FN652677	FN652709	FN652741
31	BMNH 2005.1349	*B.* cf. *boulengeri*	West Usambara	Ambangula FR	FN652682	FN652714	FN652746
32	BMNH 2005.1352	*B.* cf. *boulengeri*	West Usambara	Ambangula FR	FN652683	FN652715	FN652747

branch swapping. RAxML searches employed 200 runs on distinct random starting trees. Bayesian analysis was performed using MrBayes 3.1 (Huelsenbeck & Ronquist, 2001) both with and without partition using parameters estimated by Modeltest. Data were analysed in runs with 2,000,000 generations, trees sampled every 1000 generations. We used Tracer 1.2.1 (Rambaut & Drummond, 2005) to check that MCMC runs had reached stationarity and the first 1000 trees were discarded as "burn-in".

To test whether the data have significantly more hierarchical structure than expected by chance alone we used a permutation tail probability (PTP), employing 100 ran-

domizations of the data (Faith & Cranston, 1991; Archie, 1989). The partition homogeneity/incongruence-length difference test implemented in PAUP* was used to determine if different partitions of the data have significantly different signals. Support for clades was quantified with Bayesian posterior probabilities, bootstrap proportions (Felsenstein, 1985) based on 1000 pseudoreplicates, and decay indices (Bremer, 1988) determined by enforcing converse topological constraints. PAUP* was used to implement *a priori* Kishino–Hasegawa (Kishino & Hasegawa, 1989) and Templeton (Templeton, 1983) tests (under ML and parsimony, respectively) of null hypothesis of non-significant differences between optimal and selected suboptimal trees reflecting prior taxonomic, biogeographic and phylogenetic hypotheses.

Biogeography and dating

Roelants et al. (2007) estimated that the split between *Herpele* and *Boulengerula* occurred 96.7 Ma (95% confidence intervals 71.8–119.6 Ma) based on a broad sampling of amphibians, multiple genetic markers (including nuclear genes) and 22 calibration points. In the absence of any primary calibration points, we used this estimate as a single secondary point calibration for a dating analysis of the unpartitioned ingroup data using BEAST 1.4.6 (Drummond & Rambaut, 2007) with default settings. Two independent chains each of 10 million generations were run under the exponential uncorrelated clock models of Drummond et al. (2006), using Yule process (Yule, 1924), proportional-to-distinguishable arrangement (PDA) and coalescent priors on the tree, under a GTR+I+Gamma model of sequence evolution (the model selected by Modeltest 3.04). Although the Yule prior is the most natural null model for the speciation and extinction of lineages, it is well known that this simple model does not match the pattern of cladogenesis in real phylogenies (Mooers & Heard, 1997; Aldous, 2001), so we explored the effect of this prior assumption using the two additional priors. Generations prior to convergence, evaluated by visual inspection that each pair of independent chains sampled the same posterior distribution, were deleted for all subsequent analyses. The initial tree topology for all analyses was the ML tree estimated using PAUP*, and chains were run both allowing the tree topology to vary during the MCMC process and keeping the tree topology fixed, with all other MCMC operators run as default. All date estimates are given as means and 95% highest posterior density (HPD) confidence intervals are based on pooled results: from three chains for each (PDA and Yule) of the exponential uncorrelated relaxed clock. We used a point estimate (96.7 Ma) rather than the 95% confidence interval (71.8–119.6) for our calibration because it enables stronger tests of hypotheses of temporal congruence (which can be rejected if 95% confidence intervals for divergence dates are non-overlapping) as in relative dating (Loader et al., 2007). To accommodate uncertainty in the inference of absolute divergences we simply rescaled the inferred divergence dates and confidence intervals to those implied by the upper and lower bounds (71.8 and 119.6 Ma; Roelants et al., 2007) of the 95% confidence interval of the calibration point. Mantel tests of correlation between geographic distances (m) and genetic (uncorrected p) distances were conducted using MANTEL (Cavalcanti, 2005) with 100,000 permutations.

Table 2. Support for nodes a–w labelled in Fig. 2 (B = bootstrap, MP= maximum parsimony, ML = maximum likelihood, BPP = Bayesian posterior probability, DI = decay index).

	Unpartitioned			Partitioned		
	B MP	B ML	BPP	BPP	B ML	DI
a	100	98	100	100	97	5
b	100	100	100	100	100	31
c	100	100	100	100	100	41
d	96	92	100	100	99	10
e	63	78	91	91	85	0
f	86	89	100	100	91	0
g	86	87	99	99	91	0
h	100	96	100	100	98	0
i	98	96	100	100	100	0
j	99	98	85	83	100	0
k	100	100	100	100	100	8
l	99	99	100	100	100	0
m	96	98	100	100	100	8
n	99	91	100	100	100	11
o	100	100	100	100	100	30
p	56	72	75	79	78	0
q	99	94	100	100	96	7
r	90	83	100	100	100	6
s	70	81	97	96	91	7
t	100	100	100	100	100	7
u	95	83	89	87	87	7
v	100	100	100	100	100	59
w	68	62	85	89	70	4

RESULTS

The concatenated alignment comprises 1360 sites: 304 12S, 423 16S and 633 *cytb*. Of these, 823 are constant, 156 variable but parsimony uninformative, and 381 parsimony informative. The data appear non-random (PTP = 0.01), and not significantly heterogeneous (ILD test; P= 0.55). Plots reveal little evidence of saturation within any partition, including 3rd position sites in *cytb*.

The optimal unpartitioned ML phylogeny is shown in Fig. 2, with clade support values for different analyses reported in Table 2. Analyses using various methods and parameters yielded trees with almost identical and generally well-supported relationships. The optimal ML and Bayesian trees (for unpartitioned and partitioned data) have topologies that are identical to each other and to one of the five most parsimonious trees (MPTs). The remaining MPTs differ only in the relationships among the three *B. boulengeri* samples in clade q (Fig. 2), and in whether

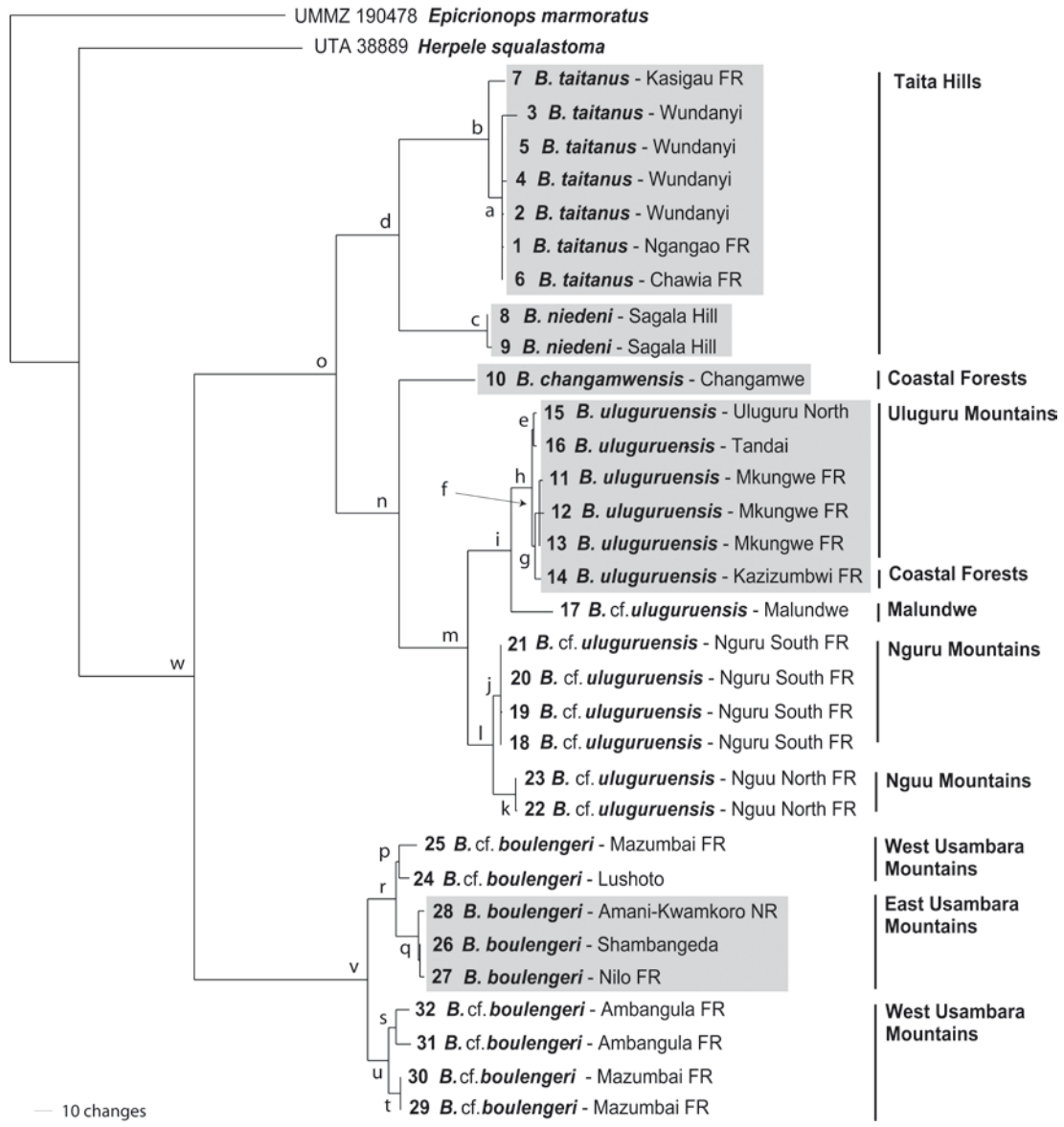

Fig. 2. Maximum likelihood tree from unpartitioned data. (–ln likelihood = 6666.26813). Nucleotide frequencies: A = 0.34310, C = 0.2508, G = 0.1447, T = 0.26140. Number of substitution parameters = 6; rate matrix = 1.00, 3.2018, 1.000, 1.000 and 8.6411; gamma shape parameter = 0.8971; proportion of invariant sites = 0.3935). Letters a–w label nodes referred to in Tables 2–4. Quantitative support values for nodes are given in Table 2. Shaded boxes indicate bounds of five nominate species.

sample 24 or 25 (W Usambara *B.* cf. *boulengeri*) alone is sister to clade q. Monophyly of *Boulengerula* is not overwhelmingly supported (Table 2), but we do not doubt it on the basis of more extensive molecular phylogenetic analyses of caecilian mt and nuclear DNA not reported here. Each of the four nominate species represented by multiple individuals (*B. boulengeri*, *B. niedeni*, *B. taitanus* and *B. uluguruensis*) were recovered as strongly supported monophyla. The basal split in the ingroup is between *B. boulengeri* and all other lineages, consistent with Taylor's (1968) partitioning of *Boulengerula*. The recently described *B. niedeni* is sister to its geographically and phenotypically closest neighbour *B. taitanus,* and these are sister to a clade comprising *B. changamwensis* and *B. uluguruensis* plus Malundwe, Nguu and Nguru populations of *B.* cf. *uluguruensis.*

Pairwise uncorrected distances were calculated for all terminals (see Supporting Online Material). Differences between all currently recognized species exceed 7%. Pairwise distances between *B. boulengeri* and other *Boulengerula* (>19%) are similar to those between *Boulengerula* and *Herpele* (>22%). Uluguru *B. uluguruensis* are 4% and 2% different from *B.* cf. *uluguruensis* from Nguu + Nguru and from Malundwe, respectively. Less substantial differences (1–3%) exist among *B. boulengeri* from various Usambara localities.

Table 3. Results of topological tests of *a priori* phylogenetic hypotheses (T = Templeton Test, KH = Kishino–Hasegawa Test). * = significantly better than the alternative (<0.05), w = significantly worse than the alternative. Nodes are labelled in Fig. 2.

Hypothesis	Node	T	KH
Monophyletic *Boulengerula taitanus*	b	$P<0.001$*	$P<0.01$*
Monophyletic *Boulengerula niedeni*	c	$P<0.0001$*	$P<0.0001$*
Monophyly of Taita Hills *Boulengerula*	d	$P<0.05$*	$P=0.124$
Monophyletic *Boulengerula uluguruensis*	h	$P=0.103$	$P=0.053$
Monophyletic *Boulengerula boulengeri*	q	$P<0.05$*	$P=0.176$
Monophyletic Usambara *Boulengerula*	v	$P<0.0001$*	$P<0.0001$*
Nussbaum & Hinkel's morphological phylogeny		$P<0.0001^{w}$	$P<0.0001^{w}$
Monophyletic coastal *Boulengerula*		$P<0.0003^{w}$	$P<0.0001^{w}$
Monophyletic EAM montane *Boulengerula*		$P<0.0001^{w}$	$P<0.0001^{w}$

Judged by the KH and Templeton tests, the best trees constrained to be consistent with Nussbaum & Hinkel's (1994) and Wilkinson et al.'s (2004) morphological phylogenies of *Boulengerula* are significantly suboptimal for both ML and parsimony ($P<0.0001$) (see Table 3). Other significantly suboptimal resolutions are those best trees including a non-monophyletic "*Afrocaecilia*" (non-*boulengeri Boulengerula)* ($P<0.001$), paraphyletic Usambara *B. boulengeri* ($P<0.0001$), monophyletic coastal ($P<0.0003$) and monophyletic EAM samples ($P<0.0001$). Monophyly of West Usambara *B. boulengeri* cannot be rejected ($P>0.1$).

Table 4. Bayesian estimates of divergence dates (Ma) with different priors assuming divergence of *Herpele* and *Boulengerula* at 96.7 Ma. Letters in first column refer to nodes on Fig. 2 and Fig. 3. 95% = confidence/credibility interval; Absolute = interval rescaled to take into account the confidence interval (71.8–119.6 Ma) of the single secondary calibration point (Roelants et al., 2007).

	PDA			Yule		Coalescent	
	Mean	95%	Absolute	Mean	95%	Mean	95%
a	7.68	5.69–9.45	4.22–11.78	4.64	3.44–5.71	2.70	2.00–3.32
b	15.18	11.24–18.68	8.35–23.28	9.65	7.15–11.88	6.37	4.72–7.84
c	3.23	2.39–3.97	1.77–4.96	2.30	1.70–2.83	1.40	1.04–1.73
d	41.28	30.57–50.80	22.7–63.31	27.87	20.64–34.30	20.49	15.17–25.22
e	4.39	3.25–5.40	2.41–6.73	2.91	2.16–3.58	1.68	1.25–2.07
f	2.15	1.59–2.64	1.18–3.3	1.43	1.06–1.76	0.81	0.60–0.99
g	6.17	4.57–7.59	3.39–9.46	3.97	2.94–4.89	2.37	1.75–2.92
h	9.94	7.36–12.23	5.46–15.24	6.37	4.72–7.84	3.96	2.94–4.88
i	18.27	13.53–22.48	10.05–28.02	11.76	8.71–14.47	7.77	5.76–9.57
j	1.50	1.11–1.84	0.82–2.3	1.06	0.79–1.31	0.58	0.43–0.72
k	1.77	1.31–2.18	0.97–2.71	1.20	0.89–1.48	0.76	0.56–0.94
l	12.92	9.57–15.90	7.16–19.81	8.23	6.09–10.13	5.32	3.94–6.55
m	30.42	22.53–37.44	16.73–46.65	20.48	15.16–25.20	14.62	10.83–17.99
n	44.73	33.12–55.05	24.59–68.6	31.65	23.43–38.95	24.45	18.10–30.08
o	62.21	46.06–76.55	34.2–95.41	47.48	35.16–58.43	39.89	29.54–49.09
p	10.56	7.82–12.99	5.81–16. 2	6.63	4.91–8.16	3.92	2.90–4.82
q	5.37	3.97–6.60	2.95–8.24	3.54	2.62–4.36	2.12	1.57–2.61
r	18.35	13.59–22.59	10.09–28.14	11.70	8.66–14.40	7.48	5.54–9.21
s	10.09	7.47–12.41	5.55–15.47	6.08	4.50–7.49	3.53	2.61–4.34
t	1.59	1.17–1.95	0.87–2.44	1.03	0.77–1.27	0.56	0.42–0.69
u	17.89	13.25–22.02	9.84–27.44	11.04	8.18–13.59	7.00	5.18–8.62
v	34.25	25.36–42.15	18.83–52.523	23.85	17.66–29.35	17.36	12.85–21.36
w	90.41	66.95–111.27	49.71–138.66	76.34	56.53–93.95	68.88	51.00–84.76

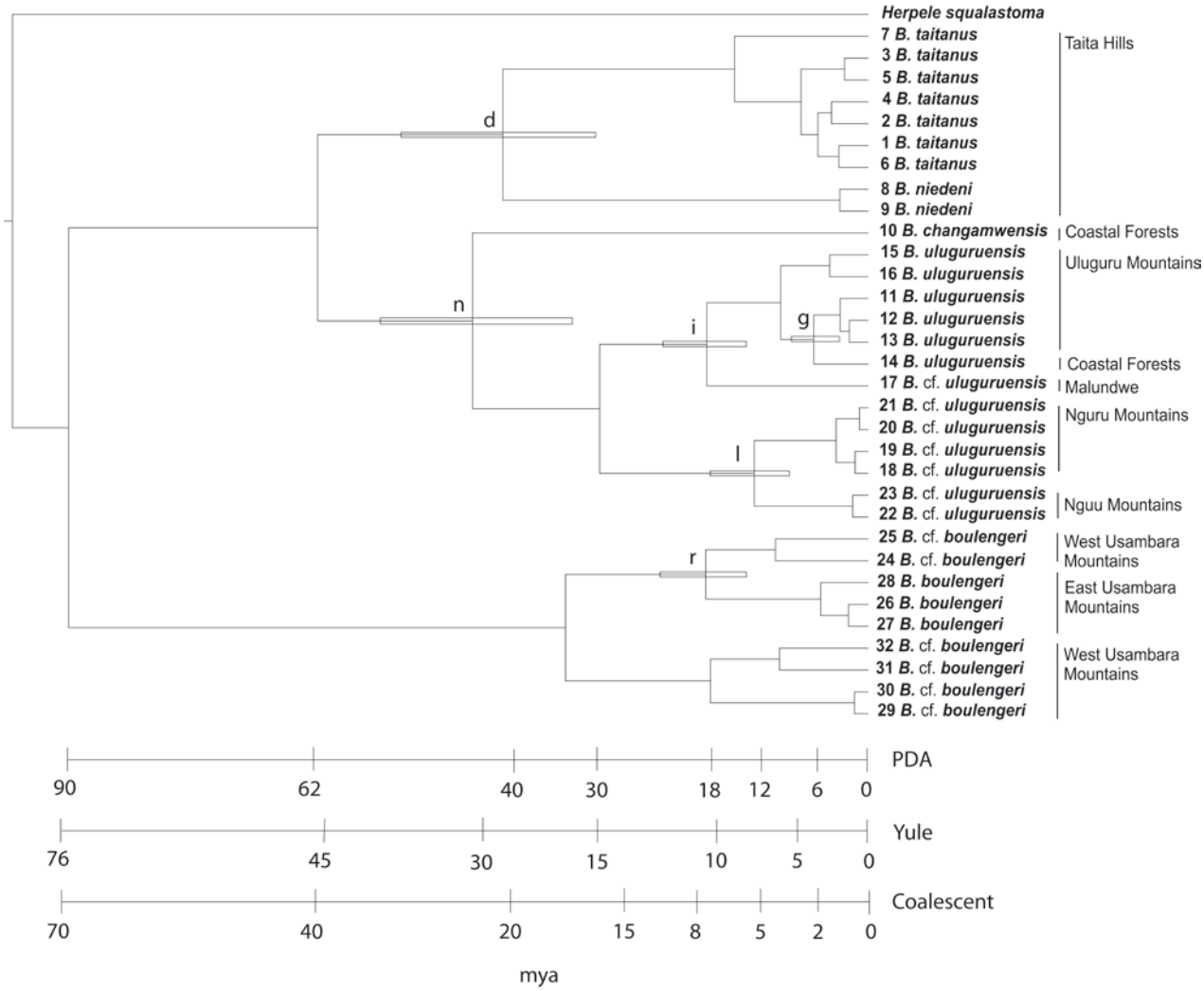

Fig. 3. Ultrametric tree from Bayesian relaxed clock analysis using exponential uncorrelated clock PDA priors. PDA, Yule and coalescent prior scales based on point calibration of *Herpele–Boulengerula* split at 96.7 Ma. Letters above branches identify nodes referred to in Tables 2–4 and Fig. 2. Bars indicate 95% confidence intervals (see Table 4).

In unconstrained optimal trees, sister lineages are often those that would be predicted from geographical proximity, including East + West Usambara, Nguru + Nguu, Malundwe + Uluguru. Mantel tests indicate significant positive correlation between genetic and geographic distance: for the complete alignment (P=0.0001), for "*Afrocaecilia*" only and for *B. boulengeri* only (P<0.05).

Dating results from the fixed tree topology analyses (Fig. 3, Table 4) are very similar to those allowing topology to vary (not shown). The PDA prior resulted in consistently oldest estimates of divergence, with estimates based on coalescent and Yule priors about 20% and 10% younger, respectively. No prior allows us to reject the null hypothesis of temporal congruence of 1) three geographically adjacent splits, Nguru–Nguu (node l), East-West Usambara (node r), Uluguru–Malundwe (node i), and 2) the deeper divergence of *B. changamwensis* (node n) and the split between the Taita Hills *B. niedeni* and *B. taitanus* (node d), but each prior rejects the hypothesis that the latter are contemporaneous with the former. The topology of the tree (Fig. 2) implies that the two divergences between coastal forest and EAM *Boulengerula*, the Kazizumbwi–Uluguru *B. uluguruensis* split (node g) and the *B. changamwensis–B. uluguruensis* split (node n), were asynchronous, and the dating suggests the former occurred substantially more recently than the latter (Table 4).

DISCUSSION

Taxonomy

Previous molecular phylogenetic analyses of *Boulengerula* have not included more than one specimen per species and no more than three nominate species. Our phylogenetic results are entirely consistent with previous findings of a deep divergence between *B. boulengeri* and *B. taita-*

nus or *B. uluguruensis* (Wilkinson et al., 2004; Frost et al., 2006; Loader et al., 2007; Roelants et al., 2007; Zhang & Wake, 2009) and the position of *B. boulengeri* outside *B. taitanus* + *B. uluguruensis* (Wollenberg & Measey, 2009; Zhang & Wake, 2009). New findings, including the sister-group relationships between *B. taitanus* + *B. niedeni* and between *B. changamwensis* + *B. uluguruensis* are also well supported. Our other main new findings are divergent mitochondrial lineages representing potentially undescribed species of *B*. cf. *boulengeri* in West Usambara, and *B*. cf. *uluguruensis* in Malundwe, Nguu and Nguru.

Our results are consistent with Taylor's (1968) bipartition of the genus into *Boulengerula* (*boulengeri*) and *Afrocaecilia* (*changamwensis*, *taitanus*, *uluguruensis*). Nussbaum & Hinkel (1994) were unimpressed by the two characters used to diagnose these two groups (presence/absence of inner mandibular teeth with anterior tongue attachment; presence/absence of dorsal exposure of mesethmoid; Taylor, 1968; Nussbaum & Wilkinson, 1989), and provided morphological phylogenetic evidence of the non-monophyly of *Afrocaecilia* as additional support for their placement of this genus into the synonymy of *Boulengerula*. Our new molecular phylogenetic data build on Wilkinson et al.'s (2004) reanalysis of Nussbaum & Hinkel's (1994) morphological data in demonstrating that the synonymy of *Afrocaecilia* with *Boulengerula* is not supported on phylogenetic grounds. However, we refrain from resurrecting *Afrocaecilia*, not least because of incomplete molecular sampling. In particular, the position of the as yet unsampled *B. denhardti* will have important implications for the diagnosis of *Boulengerula* and *Afrocaecilia* (should the latter be reinstated) given that, like *B. boulengeri*, this species lacks inner mandibular teeth and has an anteriorly attached tongue.

The close relationship between *B. uluguruensis* and *B. changamwensis* is unsurprising given that these are morphologically similar species differentiated from each other mainly in the arrangement of the palatal dentition and numbers of annuli (e.g. Taylor, 1968; Nussbaum & Hinkel, 1994). These two species also include the only lowland *Boulengerula* sampled in our study. *Boulengerula changamwensis* was described originally from coastal southern Kenya (see Malonza & Müller, 2004), but has also been reported from the Shire Highlands of Malawi, some 1400 km to the south, based on a single specimen collected in the 1800s (Nussbaum & Hinkel, 1994). Given the great distance and lack of intervening records, we doubt that the Malawi and Kenya samples represent the same species. However, although montane East African caecilians appear in general to have small ranges, we know much less about range size of their lowland counterparts.

The only other lowland *Boulengerula* we sampled, from coastal forest at Kazizumbwi, is a *B. uluguruensis* showing little genetic distinction from its Uluguru montane conspecifics. This Kazizumbwi specimen is the only extra-Uluguru record for the species. The lack of *B. uluguruensis* records between Uluguru and the coast can be explained by local extinction and/or lack of sampling; the latter cannot be rejected because little dedicated caecilian field effort in Tanzania to date has focused on the lowlands. Within the *B. uluguruensis* clade, all analyses retrieve the same relationships that show some sign of geographical structuring: a clade comprising samples from two localities (Tandai; Tegetero) that are separated by about 6 km, is sister to a clade that includes the three samples from Mkungwe (about 25 km W of Tandai) with the coastal Kazizumbwi sample from a further 100 km west.

The sister pairing of Taita Hills *B. taitanus* and *B. niedeni* is unsurprising given their geographic proximity and morphological similarity (Müller et al., 2005). The single *B. taitanus* sampled from Kasigau is a well supported, but somewhat genetically distant (1.8%) sister group of its conspecifics from the main Taita montane block. Kasigau is some 60 km to the south of the main Taita block, much further than the type locality of *B. niedeni*, Sagalla (Müller et al., 2005: fig. 1). Individuals from the Kasigau population are notably darker than *B. taitanus* from the main block (H. Müller, pers. obs.), and this population merits more detailed study.

Our phylogenetic analyses recovered three mtDNA clades among the Usambara populations. *Boulengerula boulengeri* was originally described from material from East Usambara, and our East Usambara samples form a clade that can be readily referred to this species. The existence of an undescribed new species from West Usambara has been suggested based on morphological data (Vestergaard, 1994; Channing & Howell, 2005), and at least some West Usambara populations include specimens with more annuli and vertebrae than those from East Usambara. Genetic (uncorrected p-) distances between our West and East Usambara samples (up to 3.6%; see Supporting Online Material) provide some support for this suggestion, but the paraphyly of our West Usambara samples (including the paraphyly of the three samples from Mazumbai) and the wide range of genetic distances (1.6–3.6%; see Supporting Online Material) indicate a complexity that will require more detailed investigation before any taxonomic action.

Other potential new species indicated by our results are *B*. cf. *uluguruensis* from Malundwe, Nguru and Nguu. Both the phylogeny (reciprocal monophyly of individuals from these three separate montane areas) and genetic distances (>2.9% from Uluguru *B. uluguruensis*) suggest that each area might harbour its own endemic species of *Boulengerula*, and preliminary observations suggest differences also in colour and overall body proportions. Although the known amphibian diversity of the EAM is already high, there are molecular and traditional data that provide strong evidence that the numbers of species and levels of endemism remain substantially underestimated (e.g. Menegon et al., 2008; Blackburn, 2009; Loader et al., 2010), particularly in the Nguru Mountains (Menegon et al., 2008).

A photograph of a caecilian from Ngaia Forest, Kenya (not sampled in our study) provided by S. Spawls appears to represent a *Boulengerula*, based on body and head shape, lack of externally visible eyes, unsegmented terminal shield and lack of secondary annular grooves. If confirmed, this would be the most northerly record for the genus, and the only one north of the equator. The individual has about 153 annuli (more than are known for

any species except the 161 of *B. denhardti* and 186 of *B. fischeri*), and its purplish colour (darker dorsally) with whitish annular grooves readily distinguishes it from *B. changamwensis*, *B. fischeri*, *B. niedeni*, *B. taitanus* and *B. uluguruensis*. More detailed study will be required to determine whether this form is distinct also from *B. denhardti*.

Biogeography

The two main (and not mutually exclusive) hypotheses put forward to explain the high diversity and local endemism of the EAM biota are long-term environmental stability and habitat fragmentation across an island-like chain of mountains (Burgess et al., 2007; Lovett et al. 2005). Testing these and other possible explanations depends ultimately on empirical data for different lineages occurring in the region. In particular, robust phylogenies and relative and/or absolute estimates of divergence dates should promote the required multi-taxon analyses. Molecular phylogenetic studies of EAM organisms to date (e.g. Roy, 1997; Gravlund, 2002; Möller et al., 1997; Lindqqvist & Albert, 2001; Bowie et al., 2004a,b, 2005; Beresford et al., 2004; Blackburn & Measey, 2009) have drawn attention to the presumed importance of Pleistocene and Pliocene climate fluctuations in shaping diversification here, but few general patterns have yet emerged.

Excluding the position of our two coastal samples and the relationship between *B. boulengeri* and other lineages, geographical proximity is a good predictor of sister-group relationships in our phylogeny, and this is underlined by our Mantel test results, which corroborate significant positive correlation between geographic and genetic distance within and among clades. This is consistent with short-range dispersal and/or vicariance being a more important historical driver of diversification than long-range dispersal.

Failure to reject the hypothesis that three of the main divergences in the *Boulengerula* tree occurred contemporaneously is consistent with a possibly regional scale abiotic event that promoted the expansion/fragmentation of moist forest habitat and dispersal/fragmentation of *Boulengerula* populations (see Fig. 3 and Table 4). The divergence between the Taita Hills *B. neideni* and *B. taitanus* was not contemporaneous with this putative burst of speciation but occurred substantially earlier, providing evidence of a more complex history than a single cycle of EAM habitat expansion/fragmentation. Given the lack of robust primary calibration points, inferred absolute dates must be viewed with caution. With the PDA prior, and taking uncertainty in the calibration into account, the basal split within the sampled *Boulengerula* (node w) occurred anytime between 49 and 139 Ma, the divergences of nodes i, r and l, if contemporaneous, occurred between 10 and 20 Ma, and the split between the Taita Hills *B. neideni* and *B. taitanus* occurred between 22 and 64 Ma. With the other priors the estimated divergences are younger. These timings can be interpreted tentatively as evidence for important diversification during the Miocene (5.3 to 23 Ma), with the primary split within *Boulengerula* (*Boulengerula*–"*Afrocaecilia*") possibly stretching back to the Palaeocene. These divergence estimates are substantially older than the Pleistocene (0.01 to 1.8 Ma) and Pliocene (1.8 to 5.3 Ma) – the two climatically volatile epochs generally cited as most important for the origins of extant EAM diversity (e.g. Beresford et al., 2004; Bowie et al., 2005; Kahindo et al., 2007; Fjeldså & Bowie, 2008; Blackburn & Measey, 2009). Substantial environmental change also occurred in Africa during the Miocene (Trauth et al., 2005), and other studies have indicated that at least some other EAM herpetofaunal lineages are "old" (e.g. *Rhampholeon*, Mathee et al., 2004; *Hoplophryne*, Van Bocxlaer et al., 2006; brevicipitines, Roelants et al., 2007; *Nectophrynoides–Churamiti*, Van Bocxlaer et al., 2009). These findings suggest that a broader historical perspective will be required to formulate appropriate hypotheses of EAM biotic diversification. Evidence of important pre-Pliocene EAM diversification comes also from traditional distributional data (Jetz et al., 2004) and very high levels of endemism (e.g. Basilewsky, 1962, 1976; Scharff, 1992; Johanson & Willassen, 1997; Warui & Jocqué, 2002; Vandenspiegel, 2001; Stanley et al., 2005; Mathee et al., 2004; Tilbury et al., 2006; Davenport et al., 2006; Mariaux et al., 2008), further substantiating prolonged history in explanations of the origins and maintenance of the EAM biodiversity hotspot (see also Burgess et al., 2007).

High species diversity in the EAM has been contrasted with substantially lower diversity in East African lowland forests (Burgess et al., 1998), but molecular phylogenetic assessments of lowland genetic diversity are very rare to date. For *Boulengerula*, our tree is consistent with EAM origins of lowland populations, with the two lowland individuals nested within different, primarily montane lineages. Similar patterns have been recovered for East African lizards (Matthee et al., 2004) and angiosperm plants (Möller et al., 1997). The commonality of this pattern across other East African lineages is another important hypothesis to test in the effort to better understand the EAM hotspot.

Conservation

Boulengerula niedeni is ranked number three in the EDGE of Existence amphibian conservation programme (www.edgeofexistence.org). This species' status as currently the only IUCN "Critically Endangered" caecilian is because "…it has an extent of occurrence of less than 100 km^2, is restricted to one location, and its habitat is undergoing a continuing decline in quality" (IUCN, 2009). Approximately two thirds of caecilian species are "Data Deficient" for the IUCN Red List, largely because of inadequate taxonomy and particularly scant data on distribution and ecology (Gower & Wilkinson, 2005). For *Boulengerula*, three of the seven species are currently Data Deficient, and whether *B. changamwensis*, *B. denhardti* and *B. fischeri* qualify for Least Concern or a threatened category will probably most rapidly be resolved by greatly improving data on their distribution. The current IUCN assessments for *B. boulengeri* and *B. uluguruensis* (Least Concern) include maps of populations (West Usambara and Nguu + Nguru, respectively) that might represent undescribed species. As well as reducing the range size of the nominate species, formal description of these potential new

species would establish taxa with small ranges that might require a "threatened" conservation assessment. This is particularly the case for the Malundwe population which, if endemic to the montane forest there, might exist in only a very small (perhaps less than 6 km^2) fragment of habitat. Building upon previous work with further surveying and morphological taxonomic reassessment is going to be crucial for *Boulengerula* conservation biology as well as underpinning studies of East African caecilian evolution.

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Gower et al. (2011) report new material of *Boulengerula fischeri* and add it to the molecular phylogenetic data set analysed here.

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