The Evolution of Terrestrial Breeding in African Amphibians

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Introduction

Adaptation, life history and the comparative method

The study of adaptive traits – a trait or integrated suite of traits that increase the fitness of its possessor (Freeman and Herron 2007) – and the related process of adaptation has long been an important field of study for naturalists. However, it was not until Darwin and Wallace's theory on natural selection (Darwin & Wallace, 1858) that the concept of adaptive traits being the product of selection was understood and after which point the terms 'adaptation' and 'evolution' became almost interchangeable (but see e.g. Harvey and Pagel 1991; Stearns 1992 for discussion on different uses of the term). Adaptation as a response to environmental change is deeply embedded in biological theory (Dobzhansky 1950a; 1950b), but this interaction has historically been interpreted in a number of different ways. Lamarck for example, suggested that changes in an organism's immediate environment brought about 'adaptive traits' in the organism that better suit its environment, traits that are then passed on to the next generation (Futuyma 1998). In contrast, Darwin and Wallace proposed that the organism itself does not change in any significant (or heritable) way, but that population variation and changes in the environment (abiotic and biotic) shifts the probabilities for survival and reproductive success, thereby providing a mechanism for adaptive change over generations.

With the rediscovery of Mendel's law of inheritance in 1900 and developments in the field of genetics (Dobzhansky 1950c), the 'modern synthesis' of evolutionary theory could establish the relationship between two fundamental components of a trait: the genotype and the phenotype (Stearns 2000). The genotype (the inherited genetic information) allows for hereditable variability to persist and be passed on in a population, and the phenotype, the manifestation of the genotype in a given environment and developmental conditions, exhibits traits of different fitness upon which selection then acts. The study of the evolution of fitness components related to the life-cycle of an organism has forged the discipline of life history evolution (Stearns 1992).

One of the longstanding interests in life history evolution, in fact biology as a whole, has been to explain the remarkable diversity of reproductive strategies on earth. A reproductive strategy is a complex of interrelated life history components such as age at maturity, fecundity and length of life, and to understand the variation in these traits, studies have traditionally

adopted an optimality approach that has become known as the 'life history theory'. This theory predicts that natural selection acts to maximize an individual's inclusive fitness in a given environment, given underlying intrinsic (e.g. genetic) constraints (Stearns 2000). This foundation has lead to hallmark studies in ecology (e.g. Lack 1947; MacArthur and Wilson 1967) and has benefitted hugely from more recent inclusions of reaction norms and frequency and density dependent selection models (Stearns 2000). However, the optimality model is somewhat restricted to within-lineage variations and local adaptations, and less suited for studying how lineage-specific traits differ, at which taxonomic level differences occur and how they might have evolved (Stearns 1992). It is at this stage where life history evolution and comparative biology intersect.

Comparative biology uses comparisons of a variable (e.g. trait states, speciation rates, environmental conditions etc.) across a range of taxa to pose or test hypotheses on adaptation and other evolutionary processes (Futuyma 1998). For example, moving from marine to brackish and fresh water habitat has repeatedly resulted in increased egg size, decreased fecundity and abbreviated larval development in independent decapod lineages (Diesel et al. 2000), long-distance migration is likely to have played a key role in the origin of semelparity in various species of pacific salmon (Crespi and Teo 2002) and tropical birds have a slower pace of life than temperate birds (Wiersma et al. 2007). Although simple in its premise, some authors go so far as to say that 'comparative studies have taught us most of what we know about adaptation' (preface in Harvey and Pagel 1991). Before the popularization of integrating phylogenetic trees with comparative methods, comparative biology was largely restricted to non-directional studies where comparisons were made only across taxa at similar phylogenetic levels. Directional studies opened the door to estimating ancestral states and detecting correlated, parallel or convergent evolution (Harvey and Pagel 1991). Far more importantly, the inclusion of a phylogeny quantifies the degree of independence of an evolutionary occurrence, a fundamental assumption in comparative biology that was largely ignored for a long time (Felsenstein 1985). These advancements in comparative phylogenetic methods are making it increasingly possible to quantitatively study aspects of life history evolution, adaptation to changes in the environment and the implications these adaptations may have on the diversification and evolutionary success of lineages.

Using African amphibians as model taxa, this thesis investigates the evolution of life history strategies, how these may be evolutionarily correlated with the environment and

whether more terrestrial modes of reproduction may have favoured the diversification of lineages on a historically dry continent.

Amphibian life history and terrestrial breeding

Amphibians are tetrapod vertebrates that derived from osteolepiform fish in the Devonian, ca. 400 million years ago (Carroll 2001) and their life cycle are usually 'biphasic', consisting of an aquatic larval stage and a terrestrial adult stage. There are currently just over 7200 described, extant species of amphibians (Frost 2014) belonging to three orders: Anura (ca. 6350 species), Caudata (ca. 670 species) and Gymnophiona (ca. 200 species). Together, these make up the Lissamphibia (Figure 1). Anurans – frogs and toads – are the most wide spread group with a near global distribution, whereas caudates – salamanders and newts – are more or less restricted to the northern hemisphere (with recent immigration into northern South America; Elmer et al. 2013). Gymnophiona – the caecilians – are restricted to the tropics. How these three orders are related to each other and the monophyly of Lissamphibia has long been debated (summarized in Duellman and Trueb 1994), but there is a growing body of evidence in favour of the 'Batrachia hypothesis' (San Mauro et al. 2004; 2005; Roelants et al. 2007; San Mauro 2010) that places Gymnophiona as the sister lineage to Batrachia (Anuran + Caudata; Figure 1). Based on their distribution, it was traditionally thought that vicariance, caused by the breakup of Pangaea (Feller and Hedges 1998), was the likely process of cladogenesis among the main amphibian groups. However many of the amphibian lineages predate Pangaea fragmentation and so ecological specialization has been suggested as a plausible alternative (San Mauro et al. 2005).

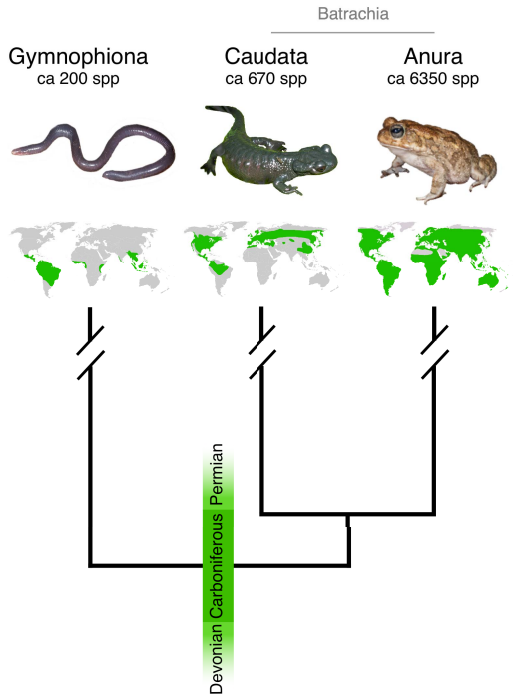

FIGURE 1. The phylogenetic relationship of lissamphibia based on the 'batrachian hypothesis' and their distributions.

The biphasic life history of many amphibians, particularly pronounced in anurans, is unique in vertebrates. In the plesiomorphic amphibian life cycle, aquatic larvae hatch from eggs placed in water and subsequently undergo a metamorphosis into a morphologically, physiologically, and ecologically distinct adult form. This 'double life' has interesting ecological and evolutionary consequences. For example, adults and larvae rarely compete for the same resources and a biphasic life cycle may allow for more effective exploitation of transient resources especially in seasonal environments (Moran 1994). Similarly, two species may have little niche overlap as adults but considerably more as larvae (Griffiths 1991) and independent adaptation can in cases lead to co-convergence of tadpole and adult phenotypes in unrelated lineages (Bossuyt and Milinkovitch 2000). Evolutionary conflicts are evident in toads, where adult of many species show highly adapted phenotypes for surviving in arid environments (Blair 1972; Van Bocxlaer et al. 2010), yet these species tend to have the most aquatic dependent larvae (Lutz 1948). Similarly, the Plethodontidae salamander species that have undergone an evolutionary loss of the larval stage show increased morphological innovation in adults, as if released from developmental constraints imposed by the larval stage in conspecifics (Wake and Roth 1989; but see Hanken 1992).

Amphibians are also unique because of the remarkable array of reproductive strategies that have evolved, ranging from extensive variations of the biphasic strategy to strategies where either the larval or adult stage is missing entirely (Duellman and Trueb 1994; Haddad and Prado 2005; Wells 2007; Vitt and Caldwell 2009). Attempts to classify these strategies tend to order modes from large, unprotected aquatic clutches with aquatic tadpoles to terrestrially laid eggs with larvae that drop, wriggle or are carried to water, on to modes with no larval stage or aquatic dependency at all such as direct development and viviparity (Duellman

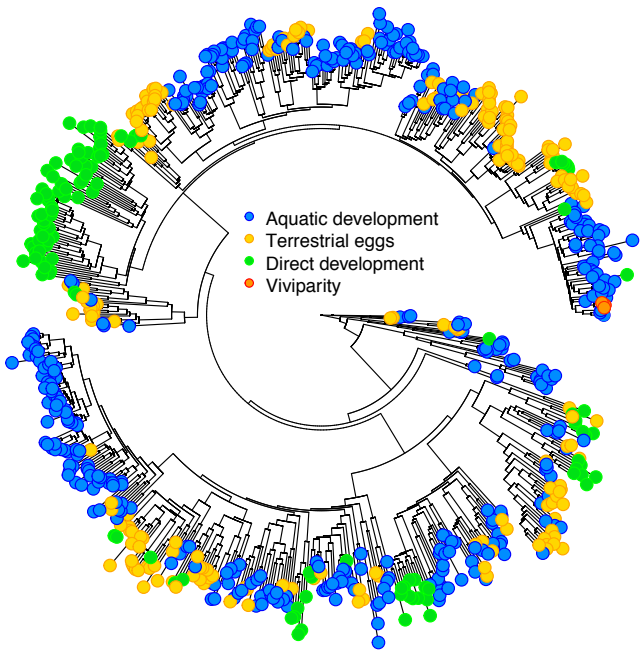

FIGURE 2: The phylogenetic distribution of reproductive modes in anurans indicates multiple independent origins of terrestrial breeding. Phylogeny from Pyron and Wiens (2011) and data adapted from Gomez-Mestre et al. (2012)

and Trueb 1994). Although an evolutionary sequence of adaptations to terrestrial reproduction is implied, a recent study on anurans has suggested that the evolution of terrestrial breeding has evolved multiple times independently (Figure 2) and not always requiring intermediate, semi-terrestrial steps (Gomez-Mestre et al. 2012). Nonetheless, there has been a historic interest in using extant amphibians as models for understanding the processes that may have lead to colonization of land by early amniotes (Romer 1957; Goin 1959; Tihen 1960a; Wilkinson and Nussbaum 1998; Laurin 2010). Laying eggs on land may have allowed for parents to better provision for young, reduce interspecific competition and avoid aquatic predators (Lutz 1948; Weygoldt 1980; Magnusson and Hero 1991). Although authors have speculated on a 'desiccation hypothesis' whereby terrestrial breeding has evolved to avoid aquatic eggs from drying out during periods of drought (Romer 1957), this is unlikely and it is now known that terrestrial breeding in amphibians and also in proto-amniotes must have evolved in very humid environments (Tihen 1960a; Poynton 1964; Gomez-Mestre et al. 2012). *Dendropsophus ebraccatus* for example usually lays eggs on leaves overhanging ponds, but deposits clutches in water if the banks of the pond are not sufficiently shaded (Touchon and Warkentin 2008). Similarly, anuran species with terrestrial oviparity occur most frequently in tropical climates characterized by high annual precipitation and temperature (Gomez-Mestre et al. 2012). Poynton (1964) reasoned that aquatic predation on eggs and larva or interspecific competition may indeed have imposed a selective pressure in favour of terrestrialization, but this transition must have occurred in moist forest to prevent desiccation of the eggs. Goin and Goin (1962) speculated that rugged, montane environments characterized by fast flowing streams pose a problem for biphasic breeders because eggs and larva are at risk of being washed downstream and so egg laying behaviour and tadpole morphology must either adapt to these torrential conditions (e.g. suckers in tadpoles to cling on to rocks in Atelopus Duellman and Lynch 1969) or alternatively, adopt a terrestrial strategy (Campbell and Duellman 2000). These alternative explanations for terrestrialization of development have remained generally poorly understood.

True toads, anurans of the family Bufonidae, are interesting for studying the evolution of terrestriality in amphibians. The majority of species are habitat-generalists and very tolerant of arid, terrestrial environments. The generalized '*Bufo* phenotype' (sensu Van Bocxlaer et al. 2010) is well suited for water retention due to its large body size, thick glandular skin and inguinal fat-bodies. Interestingly, the thick skin, less suited for cutaneous gas exchange is

compensated for by well developed, vascularized lungs (Lutz 1948). Paradoxically, their life cycles have largely remained biphasic with no records of semi-terrestrial strategies (where eggs are laid on land, but tadpoles develop in water) and only very few cases of direct development. Yet, two out of the three known viviparous genera of anurans are bufonids, including the only known case of matrotrophic viviparity in anurans. How viviparity has evolved in bufonids and whether it is an adaptation to specific environments is not known and deserves more attention. Reconstructing a well-supported phylogeny of bufonidae has been elusive, with little consensus from morphology (e.g. Tihen 1960b; Martins 1972; Grandison 1981), karyology (Bogart 1972), albumin cross reactions (Maxson 1984) and molecular sequence data (Graybeal 1997). This has hindered our understanding of life history evolution in bufonids, especially for African taxa, a hurdle that this thesis aims to overcome.

Continental Africa

Continental Africa is the second biggest landmass on earth and is perhaps biologically most renowned for its megafauna, the rich cape flora and the origin of hominids (Kingdon 1990; Linder 2003; McCarthy et al. 2005). Although tectonic movements continued to rearrange most major landmasses long into the Cenozoic, the African continent has drifted a relatively small distance during this time and its current position is not far from the continent's location in the Cretaceous (Livingstone 1993). Regardless, Africa has experienced drastic climatic oscillations in the last 50-60 Myr as well as the reformation of major lakes and rivers, changing extent of the Sahara (e.g. Livingstone 1993) and shifts in vegetation patterns (e.g. Hamilton 1982). Perhaps most importantly for amphibians, the African tropics are, and most

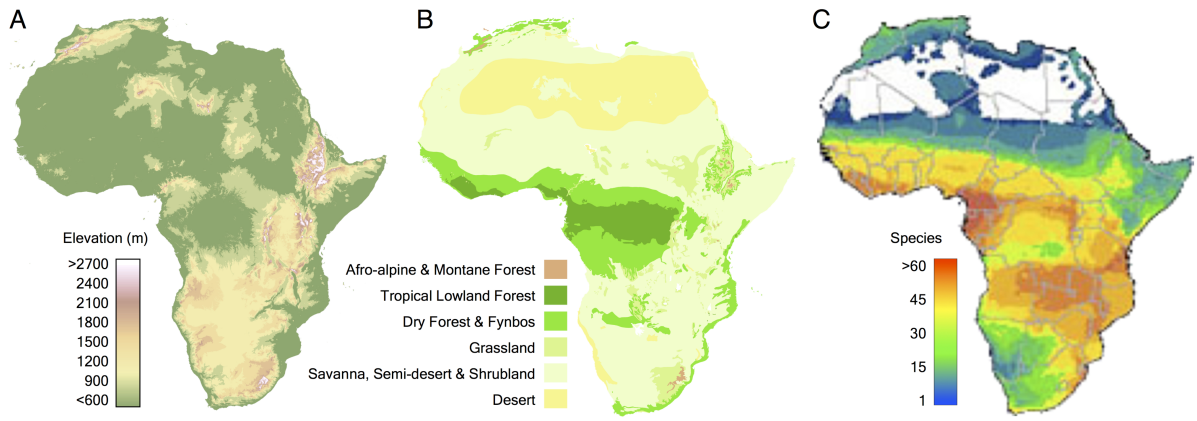

FIGURE 3a) Elevation map b) Vegetation map of Africa based on White (1983) and c) amphibian species diversity map from the Global Amphibian Assessment 2004.

likely always have been, much drier than other equatorial landmasses (Richards 1973; Livingstone 1993). Because the continent extends considerably farther north than South America for example and rainfall is governed by monsoonal winds from the Atlantic and Central Asia, both of which were weaker during ice ages, leading to severe droughts and the retraction of moist evergreen forests (Flenley 1979; Livingstone 1993). Most of sub-Saharan Africa lies above 900 m a.s.l. (Figure 3a) and the most prominent biome is savannah (Figure 3b). Humid lowland forest is almost entirely restricted to the Congo basin with a thin, continuous strip extending west to Sierra Leone, interrupted only by the 'Dahomey Gap' (Salzmann and Hoelzmann 2005). Montane forests are few and fragmented, with core areas being the Cameroonian highlands and the Eastern Afromontane Region, which includes the Ethiopian highlands, the Albertine Rift and the Eastern Arc Mountains.

Although there are notable diversity hotspots, Africa is amphibian species poor compared to other continents (Duellman 1999). South America has a species density upwards of 97.9 species/million km^2 compared to just 20.9 species/million km^2 in Africa and out of the three orders, Caudata is completely absent (in sub-Saharan Africa; Duellman 1999). Species richness is inversely correlated with aridity and core centres of richness and endemism include the Cameroonian highlands, the Eastern Arc mountains and adjacent coastal lowlands, the Albertine rift and southwestern Ivory Coast (Figure 3c; Buckley and Jetz 2007; Andreone et al. 2008). Approximately half of the amphibian species of Africa for which breeding biology is known, practice a terrestrial mode of reproduction (Figure 4; data from IUCN red list). These terrestrial forms include attaching eggs on leaves above water such as in many species of *Hyperolius,* where hatching lava drop into the water bodies below, laying eggs in terrestrial nests where larvae then also undergo metamorphosis such as in *Altiphrynoides malcolmi*, direct development as practiced by all *Arthroleptis* and viviparity, common among African caecilians, but restricted to two genera in anurans, *Nectophrynoides* and *Nimbaphrynoides*.

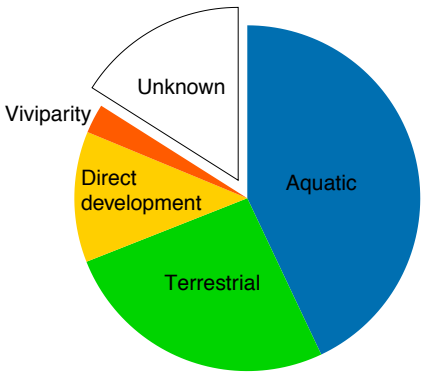

FIGURE 4: Proportion of breeding strategies of African amphibians (data from IUCN red list)

Objectives

The remarkable diversity of life history traits and behaviours of amphibians offers an overwhelming number of possibilities for testing ecological and evolutionary theories. With an ancestral dependency on aquatic habitats for reproduction (Vitt and Caldwell 2009), the majority of extant amphibians continue to have an aquatic larval stage. Yet, numerous adaptations in life history characters have allowed the colonization of terrestrial habitats or at least to become less dependent on open, standing bodies of water for egg deposition, larval development or both. This is particularly true for African amphibians. The selective pressures that favour such terrestrial breeding are not well understood and studies have broadly focused on two theories: predation on vulnerable larval stages and unsuitability of habitat. This thesis is focussed on understanding the latter; can geographic factors explain the evolution of terrestrial breeding in African amphibians?

A recent study has found correlations between terrestrial reproductive modes in anurans and increased precipitation and temperature on a global scale (Gomez-Mestre et al. 2012), but correlations with specific habitat types await empirical testing. Campbell and Duellman (2000) noted that in the Neotropics, montane forests are hazardous for biphasic breeding. In Africa too, terrestrial breeding strategies are frequent in montane environments (Goin and Goin 1962; Poynton 1964) and Goin and Goin (1962) proposed that there must be a causal relationship between terrestrial breeding and steep terrain. Fast flowing streams in montane environments pose problems for aquatic eggs and larvae that must avoid being washed downstream. To inhabit such environments, amphibians must evolve specialized tadpoles and egg laying behaviour (e.g. Inger 1960; McDiarmid and Altig 1999; Hirschfeld et al. 2012) or evolve terrestrial modes of reproduction. Poynton (1964) refuted this 'broken topography hypothesis', suggesting that the trend observed by Goin and Goin (1962) was misinterpreted and that the forest habitat was the true causal factor.

By studying the phylogenetic distribution of species with different life histories and correlating this with environmental parameters, we may better understand whether indeed forest or steep slopes, have provided the necessary conditions for terrestrial breeding to evolve. Furthermore, with Africa being a rather dry continent, one could speculate that terrestrial breeding strategies allow lineages to diversify at increased rates, taking advantage of terrestrial habitats that are unsuitable for biphasic breeders. This thesis aims to test such theories, first

by looking at a case study on the species-rich Eastern Arc Mountains, followed by three subsequent chapters focusing on the Bufonidae and aspects of their life history evolution, diversification and the evolution of viviparity.

Chapter overview

***Chapter 1**: Forests as promoters of terrestrial life-history strategies in East African amphibians*

Authors: Hendrik Müller*, **H. Christoph Liedtke***, Michele Menegon, Jan Beck, Liliana Ballesteros-Mejia, Peter Nagel, Simon P. Loader
**Authors contributed equally*

Status: Published (Biology Letters)

The Eastern Arc Mountains and adjacent lowlands of East Africa host a high number of diverse amphibian lineages, including viviparous anurans and caecilians. Here we test whether forest, specifically montane forest is associated with the distribution of terrestrial breeding species.

***Chapter 2**: Interspecific patterns for egg and clutch sizes of African Bufonidae (Amphibia: Anura)*

Authors: **H. Christoph Liedtke**, Hendrik Müller, Julian Hafner, Peter Nagel, Simon P. Loader

Status: Published (Zoologischer Anzeiger)

Bufonidae is one of the most globally successful amphibian families. It has been proposed that key to their success is laying large clutches. In Africa, bufonids are represented in almost all habitats, but information on two basic life history measures, fecundity and investment per egg

(egg size) are largely lacking or scattered in the literature. This study compiles all known information on these parameters from the literature and supplements this with new data from museum specimens to investigate how the clutch and egg size trade-off in African bufonids compares to that of other amphibian lineages and whether mixed data sources create artefacts that should be taken note of.

The published work of this chapter is supported by a subchapter where the phylogenetic non-independence of trait data is accounted for.

***Chapter 3**: No ecological opportunity on a continental Scale? Diversification and life-history evolution of African true toads (Bufonidae: Anura)*

Authors: **H. Christoph Liedtke**, Hendrik Müller, Mark-Oliver Rödel, Michele Menegon, LeGrand Nono Gonwouo, Michael F. Barej, Václav Gvoždík, Andreas Schmitz, Alan Channing, Peter Nagel, Simon P. Loader

Status: Manuscript under review

According to the Ecological Opportunity hypothesis, a colonization event of a competitor-free environment should lead to a bust in lineage diversification, taking advantage of the underutilised niche spaces. Subsequently, as niches become saturated, a density dependent slow-down of diversification should occur. Here we test whether the arrival of bufonids to Africa experienced such an opportunity and how aspects of life history, especially terrestrial breeding might have influenced diversification rates.

***Chapter 4:** The evolution of viviparity in African Anurans*

Authors: **H. Christoph Liedtke**, Hendrik Müller, Julian Hafner, Johannes Penner, Michele Menegon, David J. Gower, Mark-Oliver Rödel, Peter Nagel, Simon P. Loader

Status: Drafted manuscript

Viviparity is considered one of the most prominent examples of convergent evolution in vertebrate history. It is rare in amphibians however and even more so in anurans. Yet in bufonids, viviparity has evolved twice (out of three known instances in anurans), both times in Africa. How these lineages are related and what roles environmental factors and evolutionary precursors have played in driving the evolution of viviparity is investigated in this chapter.

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CHAPTER I

Forest as Promoters of Terrestrial Life-History Strategies in East African Amphibians

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Forests as promoters of terrestrial life-history strategies in East African amphibians

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Many amphibian lineages show terrestrialization of their reproductive strategy and breeding is partially or completely independent of water. A number of causal factors have been proposed for the evolution of terrestrialized breeding. While predation has received repeated attention as a potential factor, the influence of other factors such as habitat has never been tested using appropriate data or methods. Using a dataset that comprises 180 amphibian species from various East African habitats, we tested whether species occurring in different habitats show different patterns of terrestrialization in their breeding strategy. We recovered a significant association between terrestrialized breeding strategies and forest habitats. In general, forest seems to act as a facilitator, providing a permissive environment for the evolution of terrestrialized breeding strategies. However, while terrestrial oviposition is strongly correlated with lowland and montane forest habitat, complete terrestrial development is significantly correlated with montane forest only, indicating different selective pressures acting at different steps towards complete terrestrial development.

1. Introduction

Variations in life-history traits are known to be strongly associated with habitat [1–3]. This is evident from strategies adopted by individuals in a population along environmental gradients [4,5] and, on a broader scale, among taxa dispersed along altitudinal or latitudinal gradients or across habitats [6,7]. Investigating the ecological factors associated with the distribution of organisms with differing life-history strategies provides an opportunity to elucidate selective factors favouring particular life-history strategies in different environments.

Among major groups of vertebrates, amphibians exhibit by far the greatest diversity of reproductive strategies and have departed in many ways from the ancestral state of aquatic eggs and larvae that metamorphose into a more or less terrestrial adult [8]. For anurans alone, 39 reproductive modes have been described that have different combinations of traits, including oviposition site, developmental characters, larval habitat and the degree of parental care [8–10]. Thirty of the 39 described modes are characterized by some degree of terrestrial reproduction.

Globally, extant amphibian assemblages display differences in life-history strategies, possibly as an adaptive response to local conditions [11]. A number of hypotheses have been put forward to explain the various modes of terrestrial reproduction in amphibians in general and particularly in anurans. Lutz [12] and Tihen [13] suggested that the driving factor for the evolution of terrestrial egg deposition was predation on aquatic eggs and larvae, and plasticity in life-history traits as a response to predation is now well documented [5,14,15]. Others stressed the influence of the physical environment on the evolution of

terrestrial reproductive modes in amphibians (e.g. topography [16]; forest habitats [17]). Several recent studies have found a correlation between the diversity of reproductive modes in amphibians and the amount of rainfall, with more terrestrialized reproductive modes generally being present in more humid areas [18,19].

We analysed the distribution of amphibian species and their reproductive strategies across the lowland and highlands of East Africa, a region with a diverse array of habitats, including the Eastern Arc Mountains with montane grasslands and forests, and a broad range of different lowland habitats [20]. The high diversity of species, varying reproductive strategies, and different habitat types in East Africa makes it a suitable system for testing the influence of habitat on the evolution of terrestrialization of reproductive strategies. More specifically, we tested whether terrestrialized breeding strategies are evenly distributed or significantly associated with particular environments.

2. Material and methods

(a) Species sampling and breeding biology

We assembled a dataset of 166 anuran and 14 caecilian species of the East African coastal lowlands and the Eastern Arc Mountain chain, based on species lists and field survey data (see the electronic supplementary material). We assigned species to one of four habitat types—lowland forest, lowland non-forest, montane forest and montane grasslands—based on information from IUCN [21], Poynton *et al*. [22] and our own assessment of the taxa (see the electronic supplementary material).

Information on breeding biology was taken from the literature, particularly Channing & Howell [23] and the global amphibian assessment database [21], and references therein. We used a three state coding scheme to categorize breeding biology: 0—aquatic eggs and larvae, 1—terrestrial eggs and aquatic larvae, 2—complete terrestrial development.

Of the 180 amphibians included, 64 are predominantly non-forest coastal lowland species, 11 coastal lowland forest species, 90 montane forest species and 15 montane grassland species (see figure 1 and electronic supplementary material). Sixty species were categorized as aquatic, 42 as semi-terrestrial and 71 as completely terrestrial breeders. The breeding biology of seven species was unknown (see figure 1 and electronic supplementary material).

(b) Comparative analysis of breeding biology

We assembled a phylogeny for all East African taxa (see the electronic supplementary material for details). Correlates of breeding strategy and habitat types were identified using a phylogenetic generalized least-squares approach [25] using the package APE [26] in R v. 2.13.0 [27]. The regression models correct for phylogenetic non-independence by implementing a Brownian motion (BM), a Pagel's lambda (λ) or an Ornstein–Uhlenbeck (OU) error structure. Akaike Information Criterion (AIC) scores of each regression were compared (models with $\Delta\text{AIC} > 2$ were deemed as acceptable alternative models). A number of different analyses were performed to explore potential bias in the data (see the electronic supplementary material).

Our coding system for the breeding biology of amphibians is based on two traits: place of egg deposition and larval habitat. To test whether the evolution of these two traits is correlated with a particular environment, any habitat recovered as having a significant correlation with breeding strategy was carried forward, and correlated evolution was tested using the DISCRETE module in BayesTraits [28]. Both likelihood and Bayesian approaches were implemented, and likelihood ratio (LR) and Bayes factor (BF) scores of models where habitat and life-history traits evolve dependently or independently of each other were compared. LR scores follow a χ^2 distribution with 4 d.f., and a difference in BF scores greater than 10 was considered as strong evidence in favour of one model over the other (see the electronic supplementary material for model settings).

The sequence alignment, phylogeny and all comparative analysis datasets were deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.8f74d [24].

3. Results

Habitat type and breeding biology contain a phylogenetic signal ($\lambda = 0.635$ and $\lambda = 0.985$, respectively). Regression models that incorporate a λ error structure outperformed the BM and OU models, with an AIC score of 46.735 over the BM and OU scores of 93.847 and 51.005, respectively. The λ model shows that, against non-forest lowland habitats, lowland and montane forests have a significant, positive effect on the terrestrialization of breeding biology. Montane grasslands have no effect on terrestrialized breeding, indicating that altitude as such does not appear to be associated with terrestrialized reproduction (table 1).

Because both types of forest have a positive effect on terrestrialization of breeding strategy, both were carried forward to the BayesTraits analysis to test for correlated evolution of habitat and either terrestrial oviposition or terrestrial larval development (including direct development, ovoviviparity and viviparity). LR and log-BF tests demonstrate significant correlations between terrestrial egg-laying and both montane and lowland forest habitat ($\text{LR} = 36.221$, $p < 0.001$, $\text{BF} = 22.454$ and $\text{LR} = 10.922$, $p < 0.05$, $\text{BF} = 11.696$, respectively; table 2). Furthermore, the likelihood analyses reveal that montane forest is also significantly correlated with terrestrial larval development ($\text{LR} = 12.512$, $p < 0.05$, although this conclusion is not supported by the Bayesian analysis, $\text{BF} = -1.776$; table 2), whereas both likelihood and Bayesian analyses indicate no correlation between terrestrial larval development and lowland forest ($\text{LR} = 0.154$, $p = 0.997$, $\text{BF} = 4.125$). The BayesTraits analyses robustly indicate that forest in general is linked to the evolution of terrestrial egg deposition. Additional, somewhat more equivocal evidence suggests that the evolution of terrestrial larval development is associated specifically with montane, but not with lowland forest. These results remain robust even when excluding newly discovered species and also when excluding viviparous and ovoviviparous species, all of which are predominately found in montane forest areas (see the electronic supplementary material).

4. Discussion

Many amphibian species worldwide show partly or fully terrestrialized modes of reproduction. However, until now the link between habitat and terrestrialization of amphibian life history had not been assessed quantitatively within a comparative phylogenetic and geographical framework. Our analysis recovered forest as the best predictor of the distribution of amphibians with terrestrialized reproductive modes in East Africa. This suggests that forest may play a role in the evolution and maintenance of terrestrialized reproductive modes, assuming a stable association between

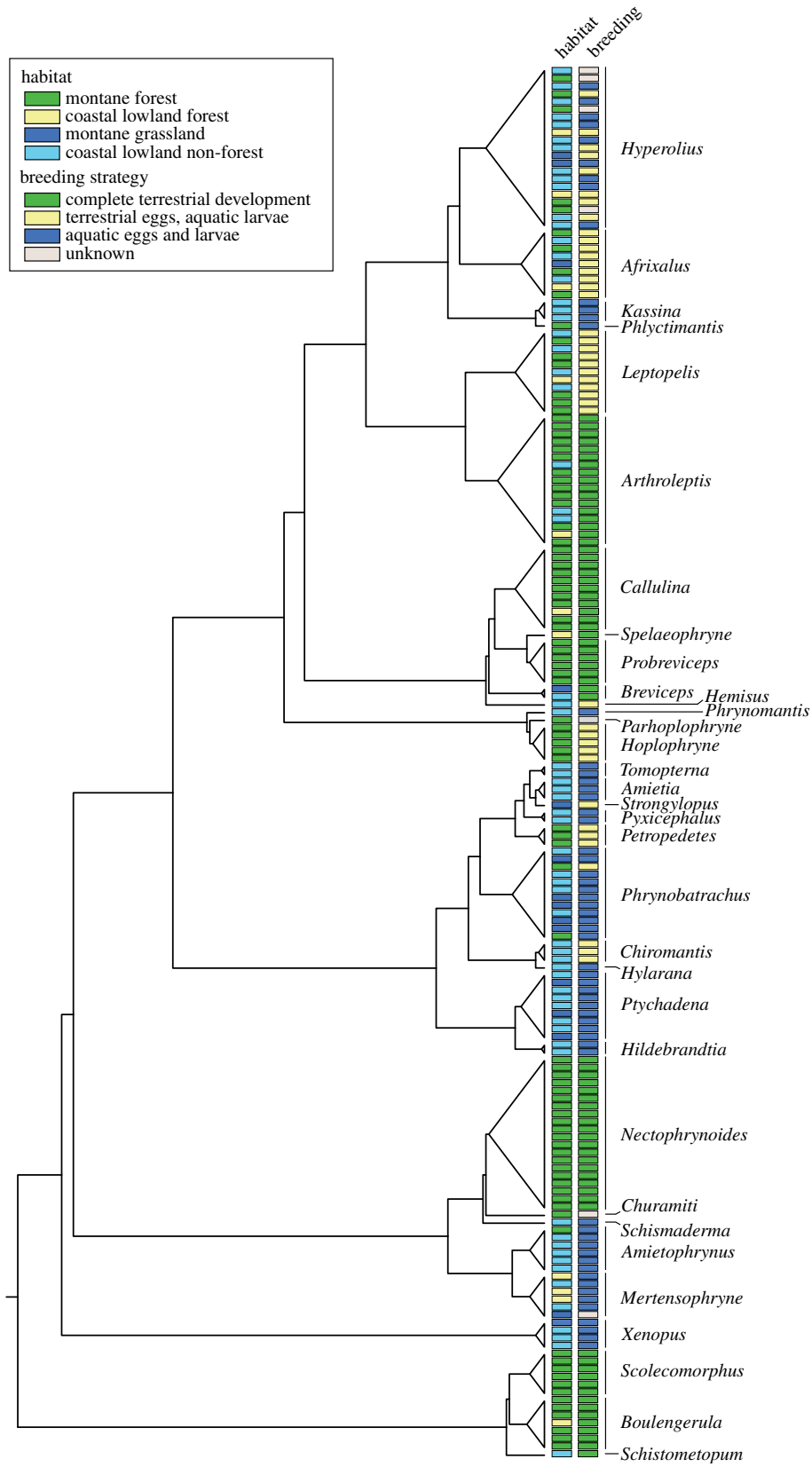

Figure 1. Phylogeny and phylogenetic distribution of habitat preference and breeding biology of East African amphibians. (Online version in colour.)

Table 1. Phylogenetic generalized least-squares regression implementing a Pagel's lambda model of evolution to test the effect of habitat on breeding biology.

	coefficient ± s.e.	*t*-value	*p*-value
Pagel's lambda model; $\lambda = 0.635$, AIC = 46.735			
intercept	1.204 ± 0.773	1.557	0.121
coastal lowland forest	0.256 ± 0.071	3.582	<0.001
montane forest	0.230 ± 0.052	4.429	<0.001
montane grassland	0.030 ± 0.061	0.489	0.625

Table 2. Correlated evolution of breeding strategy and habitat in BayesTraits-DISCRETE showing log likelihood scores and harmonic means for independent and dependent evolution of traits.

	log likelihood				MCMC harmonic mean		
	independent	dependent	likelihood ratio	*p*-value	independent	dependent	Bayes factor
terrestrial egg—montane forest	−140.556	−122.445	36.221	<0.001	−145.416	−134.189	22.454
terrestrial egg—coastal lowland forest	−92.491	−87.029	10.922	<0.05	−104.587	−98.739	11.696
terrestrial larva—montane forest	−100.574	−94.318	12.512	<0.05	−107.237	−108.125	−1.776
terrestrial larva—coastal lowland forest	−52.509	−52.432	0.154	0.997	−71.978	−69.916	4.125

species and their habitat throughout their evolutionary history. This study does not support or reject hypotheses on the precise causal factors that drive the evolution of different breeding strategies, but it is the first study to quantify the trend observed in previous studies that terrestrial forms of breeding are associated with particular environments [16,17].

Terrestrial egg-laying in East Africa is strongly correlated with forest habitat of any kind, which suggests that common biotic and/or abiotic factors of low- and highland forests promote terrestrial egg-laying. Humidity has recently been shown to influence the occurrence of terrestrial breeders [5,18,19]. Forest may be instrumental in providing humidity levels permissive for the evolution of terrestrial oviposition, e.g. owing to a lower risk of egg desiccation. At the same time complete terrestrial development is associated with montane forest only, suggesting selective factors that are unique to that environment. Topographic complexity and the availability of aquatic breeding sites are different in lowland and montane forests, and might explain the observed differences in developmental habitat. Montane forest habitats are generally characterized by a paucity of standing bodies of water and, at least at times, by swift-flowing streams, both of which might exert strong selective pressures against aquatic larvae and thus promote complete terrestrial development (including viviparity and ovoviviparity; [29]). Interestingly, dragonflies, damselflies and water beetles (whose larvae are important predators of amphibian larvae) show similar patterns of terrestrial breeding specialization in relation to montane forest habitats [30–32]. We conclude that terrestrially breeding East African amphibians have strong affinities with forests, particularly montane forests, and we predict that analyses in other regions will produce broadly similar results.

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Chapter II

Interspecific Patterns for Egg and Clutch Sizes of African Bufonidae (Amphibia: Anura)

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Interspeci c patterns for egg and clutch sizes of African Bufonidae (Amphibia: Anura)

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ABSTRACT

Little is known about reproductive trade offs in African amphibians, but such data, particularly in the form of quantitative measurements, are a key for investigating life history evolution. Here we compile and analyze known data on African bufonids from published material and new data from preserved museum specimens, to investigate interspeci c patterns of egg and clutch sizes variation. Our data is a composite of mixed sources, including ova data from dissected females and laid clutches from observations in the eld. Our study shows that, as body size increases, clutch size increases but egg size decreases, and when correcting for body size, egg size is inversely correlated with clutch size. These parameter interactions however, are different for different reproductive modes. In free swimming larval developing species, the same trends are recovered, but for lecithotrophic viviparous species no signi cant correlations could be recovered for clutch size and body size nor for the trade off between clutch size and egg size, and egg size is positively related to body size. The egg size of *Nimbaphrynoides occidentalis* (Angel, 1943) is a clear outlier, which may be due to its matrotrophic viviparous reproduction. In addition, we observed no statistical difference between ova data collected from dissections and laid clutch data from eld observations, which suggests that such a mixed dataset has utility in comparative analyses.

1. Introduction

Life history theory predicts that key ontogenetic processes such as the timing and allocation of reproductive efforts are strongly subjected to natural selection in favour of maximizing an individ ual s inclusive tness. The study of life history theory is therefore largely concerned with understanding why such an immense vari ation in reproductive strategies exists in nature and whether an optimization hypothesis can always be recovered as the underly ing explanation (Stearns, 2000). The trade off between the number of offspring and parental investment per offspring for example has been the focus of many fundamental concepts in ecology and evolution (e.g. Lack, 1947; MacArthur and Wilson, 1967; Van Noordwijk and de Jong, 1986) and the size and number of eggs per clutch is known to vary strongly both within (Cummins, 1986; Williamson and Bull, 1995; Christians, 2002; Berven, 2008) and between (Kuramoto, 1978; Blackburn, 1991; Figuerola and Green, 2005; Martin et al., 2006) species.

For amphibians, relationships between egg diameter and the number of eggs per clutch are central measures used to characterize reproductive modes, along with oviposition site, rate and duration of development, size of hatchling and type of parental care (Salthe and Duellman, 1973). Already in 1886, Boulenger noted that ter restrially breeding amphibians generally have larger eggs, but lay fewer than their aquatic breeding counterparts (Boulenger, 1886). Since then, numerous other studies have investigated the interspe ci c relationship of egg and clutch size (e.g. Wake, 1978; Barbault, 1984; H dl, 1990; Pupin et al., 2010); reviewed in (Duellman and Trueb, 1994; Wells, 2007), but African taxa tend to be underrepre sented in broad scale comparative analyses (e.g. Summers et al., 2006; Wells, 2007; Gomez Mestre et al., 2012), or are only the subject of studies that focus on a single taxon (Barbault, 1984; *Phrynobatrachus* R del and Ernst, 2002; *Boulengerula* Malonza and Measey, 2005). Here, we investigate interspeci c patterns in clutch and egg size in relation to body size of true toads of Africa (Fam ily Bufonidae) to test whether a trade off exists between the two. Bufonids are interesting for this kind of study given the starkly con trasting breeding strategies they exhibit (e.g. Van Bocxlaer et al., 2010) and African bufonids speci cally cover a particularly broad range of life history strategies, from large bodied, temporary pond breeders such as *Amietophrynus gutturalis* (Power, 1927) depositing

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tens of thousands of eggs (Channing and Howell, 2006) to the small, habitat specialist, matrotrophic viviparous toad *Nimbaphrynoides occidentalis* with extremely reduced clutches of no more than 17 eggs (Angel and Lamotte, 1944). Wells (2007) has shown that egg and clutch size relationships vary between different reproductive modes, but restricted his sampling to New World amphibian lin eages. To test whether the same is true for African bufonids, we compare trends in life history parameters in species with free swimming larva and species that give birth to live young in the form of lecithotrophic viviparity (formerly referred to as ovoviviparity; Blackburn, 1999) and matrotrophic viviparity.

Researchers working on Afro tropical systems are often faced with problems of data availability and compatibility. Even when data are available, observations stem from varying types of quan titative and qualitative measures and composites of this nature are often needed to compile a suitable number of data points for mean ingful analyses. We therefore also investigated whether egg counts and measurements taken from dissections of preserved, gravid females are comparable to data collected from eld observations of laid clutches by testing whether trends observed for ovarian clutches are signi cantly different than those for laid clutches .

2. Methods

2.1. Data collection

An exhaustive literature search for data on egg diameter and the number of eggs per clutch for African bufonid species was carried out. Information was compiled from primary literature indexed and searchable via Google Scholar (Google Inc., CA, USA) and webof knowledge.com (Thomson Reuters, Zurich, Switzerland) and from library searches for un indexed journals, books and eld guides in personal literature collections and the library of the University of Basel as well as the Natural History Museum (NHM), London. In cases where the literature source did not explicitly state or other wise infer how counts or measurements were obtained, data was assumed to refer to laid clutches, not dissections. Information on reproductive modes was obtained from the IUCN Red List online database (www.iucnredlist.org).

The literature dataset was complemented with new data col lected for this study. The collections of the NHM and the Museum f r Naturkunde, Berlin were visited and gravid females with visi bly distended abdomens were dissected to retrieve the ovarian egg mass. Investigators were careful not to cause excessive damage to specimen, by either using pre existing incisions (likely made by collectors to allow for preservatives to enter the body cavity) or by making incisions on only one side of the specimen, by cutting a crescent shape from just below the armpit along the ank towards the inguinal region. Eggs were gently lifted out of the body cavity with forceps and placed onto a glass plate and kept moist with 70% methylated spirit.

Information on the clutch and egg size for *Barbarophryne brongersmai* (Hoogmoed, 1972) was also generated de novo for this study, but refers to a laid clutch from a breeding program, not from a dissected female.

Clutch sizes below 500 eggs were counted exactly and clutches larger than this were divided into smaller, equal sized portions, one of which was counted and this number was then multiplied by the number of egg portions to get an estimate total clutch size. Egg diameter was measured to the nearest 0.1 mm using dial callipers. Where possible, multiple individuals of each species were dissected to obtain repeated measures per species.

2.2. Statistical analyses

The ideal dataset for this kind of study would consist of egg, clutch and body size measurements of the same female. However, this information is rarely published and so maximum records per species were used, which produces the most extensive dataset. Snout vent length, the measurement from the tip of the snout to the cloaca, was used as a body size measurement, egg diameter without a gelatinous layer was used as an egg size measure ment and counts of the number of eggs in one clutch determined clutch size. All measures were natural log transformed, and cor relations of egg and clutch size with body size were explored with linear regressions. Separate regression slopes were calculated for species with different reproductive modes and clutch types (ovarian and laid clutches). Reproductive mode categories were de ned as development as free swimming tadpoles (including *Altiphryniodes malcolmi* [Grandison, 1978], which is arguably not strictly free swimming, but see discussion), lecithotrophic vivipar ity and matrotrophic viviparity (as de ned by Wourms, 1981), however the last was excluded from statistical analyses due to having a sample size of one (*Nimbaphrynoides occidentalis*). All cod ing is listed in Table 1. To test whether the regression slopes were signi cantly different for each of the groupings, Analyses of Cova riances (ANCOVAs) with type III sum of squares were carried out using the Anova function in the *car* package (Fox and Weisberg, 2011) in R v.3.0.0 (R core team, 2013). In cases where the assump tions for parametric testing were not met, signi cance was tested using a permutation test implementing the *aovp* function in the R package *lmPerm* (Wheeler, 2010). The residuals for egg and clutch size on body size of a reduced dataset with species containing miss ing data removed were then used to plot egg size residuals against clutch size residuals. Although the variables at hand show linear relationships (after natural log transformations), using residuals to partial out the effect of a third variable is still considered bad practice (Garcia Berthou, 2001) and this was therefore only done to graphically explore the relationship between these two traits. To statistically test whether a signi cant correlation exists and whether this is affected by either reproductive modes or clutch types, ANCOVAs with female body size as a covariate were carried out. For all tests, non signi cant interaction terms were removed and if the reduced model was not a signi cantly worse t (tested using the anova function in the basic *stat* package in R), this model was preferred.

3. Results

Egg and clutch size data was collected from dissections of 35 females covering 19 species (Table 1S). The total dataset comprises 60 species (of just over 100 described species of African bufonids; AmphibiaWeb, 2013), clutch size data for 56, and egg size data for 54 of these species are included, with 50 species having information for both (Table 1; literature sources in Table S2).

3.1. Clutch size

The frequency distribution of clutch sizes is heavily skewed with the majority of African bufonid species laying less than 2000 eggs per clutch (mean = 3597; Fig. 1A). For the complete data set, clutch size is strongly, positively related to female body size (= 3.552, adjusted $R^2 = 0.818$, $p < 0.001$). When taking account of the differ ent clutch types and reproductive modes, individual regression slopes continue to show a positive relationship of ovarian and laid clutch size with body size (Fig. 1B and C), however this relationship is not statistically supported for lecithotrophic viviparous species (adjusted $R^2 = 0.306$, $p = 0.071$).

The ANCOVA on clutch size and body size with clutch type as a treatment effect shows that there is no signi cant interaction between body size and clutch type suggesting that the two clutch type slopes are similar and the interaction term can be removed

Table 1
Maximum female body size (measured as snout vent length in mm), clutch size and egg size (diameter in mm) for all species included in this study and coding for the two treatment classes clutch type (whether data originated from eld observations of laid clutches [laid], or dissected gravid females from museum collections [ovarian]) and reproductive mode (whether species undergo larval development as free swimming tadpoles [FST] or give birth to live young in the form of either lecithotrophic [LV] or matrotrophic viviparity [MV]).

Species	Max. female body size (in mm)	Max. clutch size	Max. egg size (in mm)	Clutch type	Reproductive mode
Altiphrynoides malcolmi (Grandison, 1978)	31	31	3.9	laid	FST
Altiphrynoides osgoodi (Loveridge, 1932)	62	307	3	laid	FST
Amietophrynus brauni (Nieden, 1911)	110	9000	1	ovarian	FST
Amietophrynus camerunensis (Parker, 1936)	91	2100	1.7	ovarian	FST
Amietophrynus channingi Barej, Schmitz, Menegon, Hillers, Hinkel, B hme and R dl, 2011	143	4500	2	laid	FST
Amietophrynus funereus (Bocage, 1866)	66	unknown	1.4	ovarian	FST
Amietophrynus garmani (Meek, 1897)	115	20,000	1.2	laid	FST
Amietophrynus gracilipes (Boulenger, 1899)	41	unknown	1.5	laid	FST
Amietophrynus gutturalis (Power, 1927)	120	23,000	1.45	laid	FST
Amietophrynus kisoloensis (Loveridge, 1932)	87	2400	1.9	ovarian	FST
Amietophrynus lemairii (Boulenger, 1901)	70	2500	1.5	ovarian	FST[(a)]
Amietophrynus maculatus (Hallowell, 1854)	80	8000	1.5	laid	FST
Amietophrynus mauritanicus (Schlegel, 1841)	150	10,000	1.5	laid	FST
Amietophrynus pantherinus (Smith, 1828)	140	24,476	unknown	laid	FST
Amietophrynus pardalis (Hewitt, 1935)	147	14,000	1.5	ovarian	FST
Amietophrynus poweri (Hewitt, 1935)	100	23,000	unknown	laid	FST
Amietophrynus rangeri (Hewitt, 1935)	115	10,760	1.3	laid	FST
Amietophrynus regularis (Reuss, 1833)	130	11,000	1.3	laid	FST
Amietophrynus superciliaris (Boulenger, 1888)	163	4000	2	laid	FST
Amietophrynus tuberosus (G nther, 1858)	74	4200	1.5	ovarian	FST
Amietophrynus xeros (Tandy, Tandy, Keith, and Duff MacKay, 1976)	92.7	5000	1	laid	FST
Barbarophryne brongersmai (Hoogmoed, 1972)	51	690	1.7	laid	FST
Bufo pentoni Anderson, 1893	95	2600	2	laid	FST
Capensibufo rosei (Hewitt, 1926)	39	90	2.5	laid	FST
Capensibufo tradouwi (Hewitt, 1926)	48	60	2	laid	FST
Didynamipus sjostedti Andersson, 1903	19	18	2.3	ovarian	FST[(a)]
Duttaphrynus dodsoni (Boulenger, 1895)	64	470	1.5	ovarian	FST
Laurentophryne parkeri (Laurent, 1950)	27.1	30	2.0	ovarian	unknown
Mertensophryne anotis (Boulenger, 1907)	46	105	2.5	laid	FST
Mertensophryne howelli (Poynton and Clarke, 1999)	45	60	2.5	ovarian	FST[(a)]
Mertensophryne lindneri (Mertens, 1955)	34	81	2.1	ovarian	FST[(a)]
Mertensophryne lonnbergi (Andersson, 1911)	44	125	2.5	laid	FST
Mertensophryne loveridgei (Poynton, 1991)	38	131	2.1	ovarian	FST[(a)]
Mertensophryne melanopleura (Schmidt and Inger, 1959)	27	35	2	laid	FST
Mertensophryne micranotis (Loveridge, 1925)	24	70	1.8	ovarian	FST
Mertensophryne taitana (Peters, 1878)	33	350	2	laid	FST
Mertensophryne usambarae (Poynton and Clarke, 1999)	45	60	2.4	ovarian	FST[(a)]
Mertensophryne uzunguensis (Loveridge, 1932)	30	188	2	ovarian	FST
Nectophryne afra Buchholz and Peters, 1875	25	40	2.5	ovarian	FST
Nectophryne batesii Boulenger, 1913	25	45	2.5	ovarian	FST
Nectophrynoides asperginis Poynton, Howell, Clarke and Lovett, 1999	29	16	2.4	laid	LV
Nectophrynoides cryptus Perret, 1971	34	25	2.2	ovarian	LV
Nectophrynoides laticeps (Channing, Menegon, Salvidio and Akker, 2005)	24	60	1.8	ovarian	LV[(a)]
Nectophrynoides minutus Perret, 1972	22	31	2	ovarian	LV
Nectophrynoides paulae Menegon, Salvidio, Ngalason and Loader, 2007	24	20	unknown	ovarian	LV[(a)]
Nectophrynoides poyntoni Menegon, Salvidio and Loader, 2004	24	10	unknown	ovarian	LV[(a)]
Nectophrynoides tornieri (Roux, 1906)	34	37	2	laid	LV
Nectophrynoides vestergaardi Menegon, Salvidio and Loader, 2004	24	46	unknown	ovarian	LV[(a)]
Nectophrynoides viviparus (Tornier, 1905)	60	160	2.9	ovarian	LV
Nimbaphrynoides occidentalis (Angel, 1943)	32.5	17	0.6	ovarian	MV
Poyntonophrynus dombensis (Bocage, 1895)	40	900	1.8	laid	FST
Poyntonophrynus fenoulheti (Hewitt and Methuen, 1912)	43	2000	1.8	laid	FST
Schismaderma carens (Smith, 1848)	92	2500	2.5	laid	FST
Vandijkophrynus amatolicus (Hewitt, 1925)	37	unknown	2	laid	FST
Vandijkophrynus angusticeps (Smith, 1848)	58	3000	2	laid	FST
Vandijkophrynus gariepensis (Smith, 1848)	95	unknown	1.5	laid	FST
Vandijkophrynus robinsoni (Branch and Braack, 1996)	57	2000	unknown	laid	FST
Werneria bambutensis (Amiet, 1972)	38	483	2	ovarian	FST
Werneria tandyi (Amiet, 1972)	41.2	629	1.5	ovarian	FST
Wolterstorf na parvipalmata (Werner, 1898)	35	2.5	219	laid	FST

Cases where reproductive mode is assumed are indicated with the annotation [(a)].

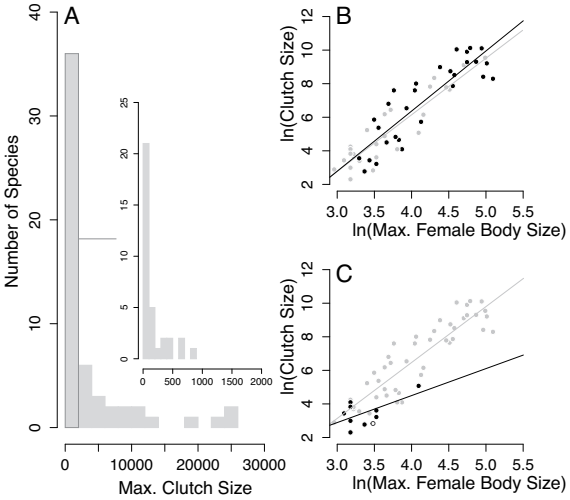

Fig. 1. Clutch sizes of African bufonids. (A) Frequency histogram of clutch sizes per species with a magni cation of clutch sizes below 2000. (B) Clutch size in relation to female body size with different regression slopes for laid clutches (black; = 3.583, adjusted $R^2 = 0.757$, $p < 0.001$) compared to ovarian clutches (grey; = 3.371, adjusted $R^2 = 0.817$, $p < 0.001$). (C) Regression slopes for lecithotrophic viviparous species (black; = 1.607, adjusted $R^2 = 0.306$, $p = 0.071$) compared to lar val developing species (grey; = 3.331, adjusted $R^2 = 0.794$, $p < 0.001$). The hollow point represents the matrotrophic viviparous *Nimbaphrynoides occidentalis*.

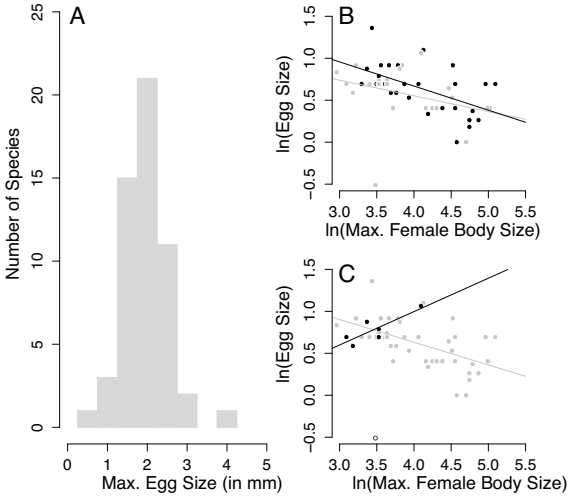

Fig. 2. Egg sizes of African bufonids. (A) Frequency histogram of egg size of African bufonids. (B) Egg size in relation to female body size with different regression slopes for laid clutches (black; = −0.288, adjusted $R^2 = 0.274$, $p = 0.002$) com pared to ovarian clutches (grey; = −0.133, adjusted $R^2 = 0.058$, $p = 0.134$). (C) Regression slopes for lecithotrophic viviparous species (black; = 0.398, adjusted $R^2 = 0.634$, $p = 0.036$) compared to larval developing species (grey; = −0.270, adjusted $R^2 = 0.298$, $p < 0.001$). The hollow point represents the matrotrophic viviparous *Nimbaphrynoides occidentalis*.

Table 2a
ANOVA table for effect of body size on clutch size with clutch type as the treatment variable (interaction terms were not signi cant).

	Sum of Sq.	Df	*F*	*p*
Intercept	54.750	1	51.673	<0.001
Female Body Size	210.568	1	198.736	<0.001
Clutch Type	0.487	1	0.460	0.501
Residuals	56.156	53		

from the model. The reduced model is not a signi cantly worse t ($F = 0.181$, $p = 0.672$) and is therefore preferred over one includ ing the interaction term. In this model, body size shows a strong, positive effect on clutch size ($F = 198.736$, $p < 0.001$; Table 2a), with no signi cant treatment effect of clutch type ($F = 0.460$, $p = 0.501$; Table 2a).

The homogeneity of variance assumption of an ANCOVA when using reproductive mode as a treatment effect was not met (Lev ene s test; $F = 18.817$, $p < 0.001$) and therefore a permutation test was used instead (Table 2b). The interaction term for body size and reproductive mode was not signi cant and was therefore removed. The reduced model is not a signi cantly worse t ($F = 0.2447$, $p = 0.124$) and is therefore preferred over one including the inter action term. For the reduced model, both female body size and reproductive mode were recovered as having a signi cant effect on clutch size ($F = 179.674$, $p < 0.001$ and $F = 5.676$, $p < 0.05$ respec tively; Table 2b), which indicates that although clutch size varies with body size, there is also a difference in pattern between

Table 2b
Permutation ANOVA table for effect of body size on clutch size with reproductive mode as the treatment variable.

	Df	R Sum of Sq.	R Mean Sq.	*F*	*p*
Female Body Size	1	170.387	170.387	179.674	<0.001
Reproductive mode	1	5.383	5.383	5.676	0.021
Residuals	51	48.364	0.948		

lecithotrophic viviparous and free swimming larva species. The regression slopes depicted in Fig. 1C suggest that for both repro ductive modes, a positive relationship of clutch size and female body size can be observed, with the effect being strong in free swimming larva species, but not statistically different from zero for lecithotrophic viviparous species.

3.2. Egg size

Egg size shows a slight log normal distribution with a mean diameter of 1.936 mm (Fig. 2A). Without sub setting the data, egg size is inversely correlated to female body size (= −0.209, adjusted $R^2 = 0.140$, $p < 0.05$). *Nimbaphrynoides occidentalis*, the only matrotrophic viviparous anuran, is a clear outlier, with an egg size well below what is expected for its body size (represented by a hollow point in Fig. 2C).

The inverse relationship is maintained when sub setting the data into ovarian and laid clutches, although the slope for ovar ian data is not statistically different from zero (adjusted $R^2 = 0.058$, $p = 0.135$; Fig. 2B). If *N. occidentalis* is treated as an outlier and removed, a signi cant negative relationship is recovered (= −0.234, adjusted $R^2 = 0.280$, $p = 0.005$; slope not shown). For reproductive mode as a treatment effect, the regression slope for species with larval development indicates a negative relationship for egg size and body size (adjusted $R^2 = 0.298$, $p < 0.001$; Fig. 2C), but for lecithotrophic viviparous species, this relationship is posi tive (adjusted $R^2 = 0.634$, $p < 0.05$; Figure 2C).

When comparing the two clutch types, the interaction term for the ANCOVA of egg size and body size is not signi cant, sug gesting that the two slopes are similar and as for clutch size, the reduced model is not a signi cantly worse t ($F = 0.507$, $p = 0.480$). Body size has a signi cant effect on clutch size ($F = 12.027$, $p < 0.05$; Table 3a), with no signi cant treatment effect of clutch type ($F = 2.347$, $p = 0.132$, Table 3a).

When looking at reproductive mode as the grouping variable, the interaction term was signi cant ($F = 5.399$, $p < 0.05$; Table 3b),

Table 3a
ANCOVA table for effect of body size on clutch size with clutch type as the treatment variable (interaction terms were not signi cant).

	Sum of Sq.	Df	*F*	*p*
Intercept	2.479	1	31.024	<0.001
Female body size	0.961	1	12.027	0.001
Clutch type	0.188	1	2.347	0.132
Residuals	4.075	51		

Table 3b
ANCOVA table for effect of body size on clutch size with reproductive mode as the treatment variable.

	Sum of Sq.	Df	*F*	*p*
Intercept	2.616	1	51.912	<0.001
Female body size (FBS)	1.089	1	21.623	<0.001
Reproductive mode (RM)	0.264	1	5.238	0.027
FBS × RM	0.272	1	5.399	0.024
Residuals	2.418	48		

meaning the slopes of the two regression lines (Fig. 2C) are signi cantly different from one another. Body size is inversely correlated with egg size in larval developing species but the reverse is true for lecithotrophic viviparous species.

3.3. Egg vs. clutch size

Clutch size and egg size regressions on body size are more or less linear (see Figs. 1 and 2) and therefore the residuals of each regression could be used as a means of removing the effect of body size. When doing so, there is a negative overall relationship between the residuals of egg size and clutch size (= −0.079, adjusted $R^2 = 0.064$, $p = 0.045$). This relationship is intensi ed when *Nimbaphrynoides occidentalis* is removed (= −0.127, adjusted $R^2 = 0.308$, $p < 0.001$). For both laid and ovarian clutches, the negative relationship is maintained (Fig. 3A), but only if *N. occidentalis* is removed, is the slope for the ovarian clutch dataset signi cantly different from zero (= −0.166, adjusted $R^2 = 0.459$, $p < 0.001$; regression line not shown). For the regression slopes representing the different reproductive modes, both larval developing and lecithotrophic viviparous species show a negative relationship (Fig. 3B) although the relationship for the latter is not statistically different from zero (= −0.180, adjusted $R^2 = 0.230$, $p = 0.189$).

When comparing the two clutch types (not including *N. occidentalis*), none of the interaction terms for the ANCOVA are signi cant suggesting the slopes are similar and the reduced model is not a signi cantly worse t ($F = 0.940$, $p = 0.451$). In the reduced model, clutch size has the strongest effect on egg size ($F = 21.303$, $p < 0.001$; Table 4a) with female body size and clutch type having no signi cant effect ($F = 2.148$, $p = 0.150$ and $F = 3.864$, $p = 0.056$ respectively; Table 4a).

When comparing the two reproductive modes, again, none of the interaction terms for the ANCOVA are signi cant and similarly, the reduced model is not a signi cantly worse t ($F = 1.219$, $p = 0.318$). In the reduced model, reproductive mode has no signi cant effect on the model ($F = 2.057$, $p = 0.159$; Table 4b) and the main driver is clutch size ($F = 22.454$, $p < 0.001$; Table 4b) with female body size not contributing signi cantly ($F = 3.537$, $p = 0.067$; Table 4b).

Table 4a
ANCOVA table for effect of clutch size on egg size with female body size and clutch type as covariates (interaction terms were not signi cant).

	Sum of Sq.	Df	*F*	*p*
Intercept	0.356	1	9.734	0.004
Clutch size	0.778	1	21.303	<0.001
Female body size	0.078	1	2.148	0.150
Clutch type	0.141	1	3.864	0.056
Residuals	1.607	44		

Table 4b
ANCOVA table for effect of clutch size on egg size with female body size and reproductive modes as covariates (interaction terms were not signi cant).

	Sum of Sq.	Df	*F*	*p*
Intercept	0.274	1	7.207	0.010
Clutch size	0.852	1	22.454	<0.001
Female body size	0.134	1	3.537	0.067
Reproductive mode	0.078	1	2.057	0.159
Residuals	1.670	44		

4. Discussion

In African bufonids, both egg number per clutch and egg size are correlated with body size. As body size increases, clutch size increases, but egg size decreases, and when correcting for body size, a strong negative correlation is evident for egg size on clutch size. Whether data originated from laid clutches or from dissected females had no effect on any general patterns and thus, we propose that data from both sources could be combined for broad scale comparative studies in the future. However, reproductive mode had a signi cant effect on how egg size and clutch size are correlated with body size (though not on how these two parameters are correlated with each other after correcting for body size), in line with what Wells (2007) observed for Neotropical species. Our dissections of *Mertensophryne micranotis* (Loveridge, 1925) and *M. uzunguensis* (Loveridge, 1932) also provide new record number of eggs for these species, with egg counts for both exceeding any previous records by a factor of two or more (Grandison and Ashe, 1983; Poynton et al., 2005).

Larval developing species retain a signi cant, positive correlation of clutch size with body size as well as inverse correlations of egg size with body size, and of egg size with clutch size (after correcting for body size). For lecithotrophic viviparous species, the slopes of the regression lines for clutch size on body size and for body size corrected egg size on clutch size showed the same trends as for larval developing species, however they were not significantly different from zero, suggesting weak correlations. The regression slope for egg size on body size was signi cantly different from zero and supported a positive correlation of egg size with body size, the reverse for what was recovered for larval developing species.

The positive relationship between clutch size and body size is one that has been recovered in previous studies on amphibians (Kuramoto, 1978; Barbault, 1984; Duellman and Trueb, 1994; Prado and Haddad, 2005; Wells, 2007) and the most straight forward explanation for this is that larger bodied females can carry larger numbers of eggs (Roff, 2002). This however assumes that egg size is relatively constant and one cannot rule out that both body size and fecundity respond to external factors in a collinear fashion and thus there may not be a direct causal link between the two. For example, L ddecke (2002) found that within a single species, body size increased with altitude as did clutch size, even after the effect of increasing body size was removed.

Salthe and Duellman (1973) note that New World anurans practicing the same reproductive mode show a positive interspeci c correlation between egg size and female body size, but when investigating this relationship across multiple reproductive modes, the correlation is inverted. Egg size and body size of African Bufonids appears to behave similarly, showing an overall inverse correlation, but as the dataset is subdivided into distinct reproductive modes, lecithortophic viviparous species show a positive correlation. Larval developing species continue to show a negative correlation,

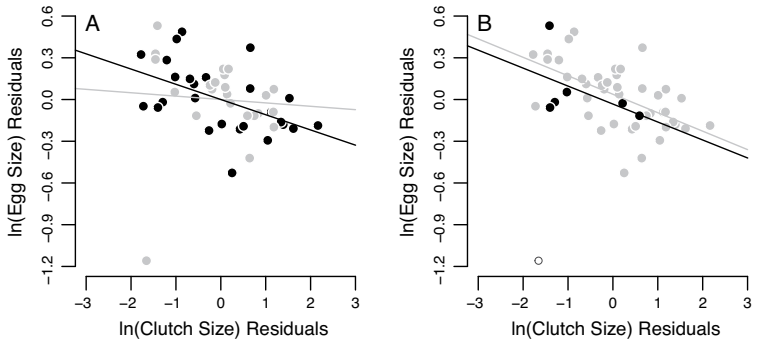

Fig. 3. Residuals for clutch size and egg size on body size, showing different regression slopes for (A) laid clutches (black; = −0.109, adjusted R^2 = 0.224, p = 0.009) and ovarian clutches (grey; = −0.024, adjusted R^2 = −0.043, p = 0.757) and for (B) lecithotrophic viviparous species (black; = −0.180, adjusted R^2 = 0.230, p = 0.189) and larval developing species (grey; = −0.134 adjusted R^2 = 0.327, p < 0.001). The hollow point represents the matrotrophic viviparous *Nimbaphrynoides occidentalis*.

which may be an indication that this category is too heterogeneous, pooling explosive pond breeders such as *Amietophrynus gutturalis* with terrestrial nest breeders such as *Altiphrynoides malcolmi*. Finer categorization of reproductive modes of African bufonids is ham pered by the lack of detailed knowledge of the life history of many taxa and highly specialized reproductive modes such as the breed ing in tree cavities and provisioning of post hatching parental care as practiced by *Nectophryne* spp Bucholz and Peters, 1875 are usu ally represented by too low numbers of species to obtain suf cient sample sizes for statistical testing. A statistically viable re ne ment of the free swimming larval developing category would be to rstly remove species with highly specialized modes such as those involving terrestrial nest building (e.g. *Altiphrynoides mal colmi*) or internal fertilization (e.g. *Mertensophryne micranotis*) and then separating species with larva developing in permanent water bodies from those with larva developing in temporary water bod ies. The recovered regression slopes for these two groups continue to show negative correlations of egg size and body size (= −0.178 and −0.315 respectively), but both slopes are no longer signi cantly different from zero (p = 0.063 and 0.057 respectively).

The viviparous *Nimbaphrynoides occidentalis* produces consid erably smaller eggs than is expected for its body size and Angel and Lamotte (1944) comment that the eggs are hugely deprived of yolk. The toad is the only known matrotrophic viviparous anuran and embryos undergo complete development in the uterus of the mother over a period of nine months (Gallien, 1959; Castanet et al., 2000). A similar egg size reduction associated with matrotrophic viviparity has been observed for reptiles (Blackburn et al., 1984) and mammals (Dunbrack and Ramsay, 1989), as developmental energy is no longer provided by yolk stores in the egg, but directly from the mother.

After correcting for body size, bufonids of both reproduc tive modes (free swimming larva versus live bearing) exhibit an inverse correlation between egg size and clutch size, corre sponding to previous ndings (Duellman and Trueb, 1994; Wells, 2007; Vitt and Caldwell, 2009) as well as the general principle of MacArthur and Wilson s theory of r versus K selection in popu lations (MacArthur and Wilson, 1967; Pianka, 1970). This theory predicts that if there are no density effects or competition, the optimum strategy for an organism would be to maximize fecun dity, with minimal investment into each individual (r selection). If an environment is saturated, the optimum shifts to the other extreme, wherein it is more bene cial for an organism to reduce the number of offspring produced, but to increase the invest ment per offspring (K selection). Dobzhansky (1950) reasoned that K selection should be favoured in climatically stable environments such as the tropics, whereas in temperate or high altitude regions, r selection strategies would be more successful. The clear trade off seen in African bufonids may therefore re ect the environments to which individual species are adapted and therefore offers an interesting system for investigating the relationship of life history parameters and habitat.

The collection of life history data in the eld is often dif cult. Direct observations of species are often frustrated by the geograph ical location of species and/or the frequency and rarity of some species. In addition, species with more derived life histories often breed in cryptic or dif cult to observe locations, making it chal lenging to obtain quantitative and qualitative life history data. This is particularly true for Africa where basic data on the ecology and breeding biology of many species are still lacking. In our study, we compared the utility of data obtained from eld observations and museum specimens and proved that there is no signi cant differ ence between both data sources, meaning that both sets of data can be combined in more comprehensive analyses. Preserved material from natural history collections is therefore an important resource for signi cantly adding to our knowledge on amphibian life history.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcz.2014.02.003.

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CHAPTER II – SUPPLEMENT

Phylogenetic Non-Independence of Trait Data

Introduction and Methods

The independence of data is an important assumption of general linear models, but is one that is frequently violated in comparative studies due to the underlying phylogenetic relationships of species (Felsenstein, 1985). The primary intention of the preceding publication was to accumulate as much quantitative information on egg, clutch and body size of African Bufonidae as possible to ensure that this group can be better represented in future studies (currently largely absent in e.g. Wells, 2007). The dataset also provided the opportunity to carry out basic correlation studies of these traits to allow for discussion on trade-offs as has been previously documented for other groups (summarized in Duellman & Trueb, 1994; Wells, 2007; Vitt & Caldwell, 2009). Not correcting for phylogenetic non-independence inflates type I error (false positive) rates and such regression results must therefore be treated with caution. As a supplement to this chapter, the important analyses are repeated here with corrections for non-independence of data points using the phylogeny reconstructed for chapter 4 (that was not available at the time of publication). We test for phylogenetic signal in the data using two measures; Pagel's λ (Pagel, 1997) and Blomberg et al.'s K (Blomberg, Garland & Ives, 2003) using the phytools package v0.4-05 (Revell, 2012) in R. To test overall relationships we use a phylogenetic generalized least squares approach (pGLS; Martins & Hansen, 1997), which includes a patristic distance matrix as an error structure in the models, using the R package ape v3.1-1 (Paradis, Claude & Strimmer, 2004) and nlme v3.1-117 (Pinheiro *et al.*, 2014). Three models of trait evolution were implemented for the correlation structure, a Brownian motion, a Pagel's λ and an Ornstein-Uhlenbeck model. Akaike Information Criterion (AIC) scores of each regression were compared and models with $\Delta AIC>2$ were deemed as acceptable alternative models. In cases where $\Delta AIC<2$, but the effect size and significance levels were similar in both models, only the results of the model with the lowest AIC score are printed.

Results and Discussion

Tables

Phylogenetic signal

TABLE 1. Phylogenetic signal in trait data. All traits were natural log transformed.

Trait	Number of species	Pagel's λ	Blomberg et al.'s K
Body size	70	λ= 0.809, p<0.001	K=1.029, p<0.001
Clutch size	51	λ= 0.972, p<0.001	K=1.029, p<0.001
Egg size	48	λ= 0.907, p<0.001	K=0.788, p<0.001

Effect of body size on clutch size

TABLE 2. pGLS results for best fitting model for natural log transformed body size on transformed clutch size.

	Coefficient	Std. Error	t-value	p-value
(Intercept)	-3.911	1.381	-2.832	0.007
Body size	2.559	0.347	7.367	<0.001
		Model: Pagel's λ, λ =0.921, AIC=137.702 (ΔAIC=9.403)		

TABLE 3. pGLS results for best fitting model for natural log transformed body size and reproductive mode (live bearing) on transformed clutch size for breeding biology.

	Coefficient	Std. Error	t-value	p-value
(Intercept)	-3.377	1.354	-2.495	0.016
Body size	2.473	0.337	7.335	<0.001
Live-bearing	-1.529	0.695	-2.201	<0.001
		Model: Pagel's λ, λ=0.910, AIC=133.9132 (ΔAIC=10.400)		

TABLE 4. pGLS results for best fitting model for natural log transformed body size on transformed clutch size for a) larval and b) live-bearing species (excluding *N. occidentalis*) separately.

Table 4a)	Coefficient	Std. Error	t-value	p-value
(Intercept)	-4.775	1.532	-3.117	0.003
Body size	2.828	0.384	7.375	<0.001
		Model: Pagel's λ, λ=0.910, AIC=111.643 (ΔAIC=10.356)		
Table 4b)	Coefficient	Std. Error	t-value	p-value
(Intercept)	-0.811	2.169	-0.374	0.721
Body size	1.310	0.634	2.068	0.084
		Model: Brownian Motion, AIC=1.725 (ΔAIC=1.438)		

Effect of body size on egg size

TABLE 5. pGLS results for best fitting model for natural log transformed body size on transformed egg size. The analysis was carried out on a) the full dataset and b) repeated with *Nimbaphrynoides occidentalis* removed.

Table 5a)	Coefficient	Std. Error	t-value	p-value
(Intercept)	1.042	0.340	3.069	0.004
Body size	-0.098	0.083	-1.192	0.239
		Model: Brownian Motion, AIC=21.288 (ΔAIC=1.438)		
Table 5b)	Coefficient	Std. Error	t-value	p-value
(Intercept)	1.074	0.268	4.010	<0.001
Body size	-0.094	0.065	-1.439	0.157
		Model: Brownian Motion, AIC=-0.653 (ΔAIC=3.621)		

TABLE 6. pGLS results for best fitting model for natural log transformed body size and reproductive mode (live bearing) on transformed egg size for breeding biology. The analysis was carried out on a) the full dataset and b) repeated with *Nimbaphrynoides occidentalis* removed.

Table 6a)	Coefficient	Std. Error	t-value	p-value
(Intercept)	1.201	0.311	3.861	<0.001
Body size	-0.115	0.075	-1.541	0.130
Live-bearing	-0.721	0.216	-3.341	0.002
		Model: Brownian Motion, AIC= 14.334 (ΔAIC=1.344)		
Table 6b)	Coefficient	Std. Error	t-value	p-value
(Intercept)	1.080	0.276	3.915	<0.001
Body size	-0.094	0.066	-1.427	0.161
Live-bearing	-0.030	0.265	-0.113	0.910
		Model: Brownian Motion, AIC=2.164 (ΔAIC=1.823)		

TABLE 7. pGLS results for best fitting model for natural log transformed body size on transformed egg size for a) larval and b) live-bearing species (excluding *N. occidentalis*) separately.

Table 7a)	Coefficient	Std. Error	t-value	p-value
(Intercept)	1.417	0.263	5.397	<0.001
Body size	-0.181	0.064	-2.851	0.007
		Model: Brownian Motion, AIC= 7.725 (ΔAIC=0.789)		
Table 7b)	Coefficient	Std. Error	t-value	p-value
(Intercept)	-0.738	0.453	-1.630	0.202
Body size	0.442	0.130	3.404	0.042
		Model: Brownian Motion, AIC=3.272 (ΔAIC=0.653)		

Effect of clutch size on egg size with body size as a covariate

TABLE 8. pGLS results for best fitting model for natural log transformed clutch size on transformed egg size with body size as a covariate. The analysis was carried out on a) the full dataset and b) repeated with *Nimbaphrynoides occidentalis* removed.

Table 8a)	Coefficient	Std. Error	t-value	p-value
(Intercept)	0.559	0.453	1.234	0.224
Body size	0.134	0.156	0.860	0.395
Clutch size	-0.069	0.047	-1.470	0.149
		Model: Brownian Motion, AIC=26.366 (ΔAIC=1.921)		
Table 8b)	Coefficient	Std. Error	t-value	p-value
(Intercept)	0.414	0.307	1.345	0.186
Body size	0.267	0.109	2.454	0.018
Clutch size	-0.121	0.032	-3.822	<0.001
		Model: Pagel's λ, λ=0.838, AIC=-6.627 (ΔAIC=0.596)		

TABLE 9. pGLS results for best fitting model for natural log transformed clutch size on transformed egg size and reproductive mode (live-bearing) with body size as a covariate. The analysis was carried out on a) the full dataset and b) repeated with *Nimbaphrynoides occidentalis* removed.

Table 9a)	Coefficient	Std. Error	t-value	p-value
(Intercept)	0.618	0.378	1.633	0.110
Body size	0.235	0.132	1.780	0.083
Clutch size	-0.124	0.041	-3.033	0.004
Live-bearing	-0.906	0.206	-4.403	<0.001
		Model: Brownian Motion, AIC= 13.439 (ΔAIC=1.675)		
Table 9b)	Coefficient	Std. Error	t-value	p-value
(Intercept)	0.400	0.308	1.300	0.201
Body size	0.295	0.107	2.749	0.009
Clutch size	-0.133	0.033	-4.031	<0.001
Live-bearing	-0.177	0.224	-0.788	0.435
		Model: Brownian Motion, AIC=-4.692 (ΔAIC=0.801)		

TABLE 10. pGLS results for best fitting model for natural log transformed clutch size on transformed egg size with body size as a covariate for a) larval and b) live-bearing species (excluding *N. occidentalis*) separately.

Table 10a)	Coefficient	Std. Error	t-value	p-value
(Intercept)	0.888	0.346	2.567	0.015
Body size	0.128	0.122	1.046	0.303
Clutch size	-0.107	0.034	-3.158	0.003
		Model: Brownian Motion, AIC=-8.686 (ΔAIC=1.740)		
Table 10b)	Coefficient	Std. Error	t-value	p-value
(Intercept)	-0.769	0.501	-1.534	0.265
Body size	0.543	0.207	2.629	0.119
Clutch size	-0.085	0.124	-0.682	0.566

Model: Brownian Motion, AIC= 7.195 (ΔAIC=1.487)

Summary

All three traits show significant phylogenetic signal (Table 1) indicating that these characters are phylogenetically conserved.

Clutch size – Body size is significantly, positively correlated with clutch size (Table 2) a relationship that persists even when including reproductive modes in the model (Table 3) and clutch sizes are significantly smaller in live-bearing species compared to species with aquatic larval development (Table 3). When looking at the body size/clutch size relationship for each reproductive strategy separately, positive correlations are found in both, but only for aquatic larval species is this relationship significant (Table 4).

Egg size – No significant effect of body size on egg size was recovered (Table 5a), even when removing *Nimbaphryniodes occidentalis* (Table 5b). When including reproductive modes in the model, body size continues to have no significant effect on egg size (Table 6a and b), but reproductive mode has a significant effect (Table 6a). This significance is removed however when *N. occidentalis* is removed (Table 6b). Within aquatic larval species, there is a significant inverse correlation of body size with egg size (Table 7a) and in live bearing species (excluding *N. occidentalis*) this relationship is significantly positive.

Egg size/clutch size trade off –When comparing clutch size to egg size with body size as a covariate, a significant inverse relationship is recovered for the dataset without *N. occidentalis* both without (Table 8b) and with (Table 9b) reproductive mode included in the model. Within each reproductive mode, there is a negative correlation of egg size and clutch size, but this is only significantly different from no correlation for species with aquatic larva (Table 10a and b)

In summary, in species with aquatic modes of reproduction, clutch size increases with body size and egg size decreases. Similarly, when correcting for body size, a trade off exists where egg size decreases with increasing clutch size. In live bearing species (excluding *N. occidentalis*), no significant relationship between body size and clutch size exists (although a positive trend is evident) and egg size increases with body size. Furthermore, no significant trade off is evident for egg size and clutch size. Clutch sizes of larval developers are bigger than those of live bearing species, but egg sizes do not significantly differ, nor is there a significant difference in the clutch size/egg size trade-off between larval and live bearing

species. When comparing these results to the non-phylogenetic autocorrelation corrected results presented in the manuscript, the recovered patterns remain largely the same, with the exception that significance for the egg size/body size relationship and when comparing relationships for species with different breeding strategies is lost.

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Chapter III

No Ecological Opportunity on a Continental Scale? Diversification and Life-History Evolution of African True Toads (Bufonidae: Anura)

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Status: Under Review

No Ecological Opportunity on a Continental Scale? Diversification and Life-History Evolution of African True Toads (Bufonidae: Anura)

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Abstract

True toads (Bufonidae) have rapidly diversified across most of the terrestrial world, adapting to a range of habitats. In Africa too, bufonids are represented in all major, terrestrial biomes and have evolved a wide spectrum of life history strategies. Here we investigate whether the first bufonid radiation to colonize Africa shows signs of density dependent lineage accumulation as predicted by the Ecological Opportunity hypothesis and whether there is heterogeneity in rates across subclades or reproductive strategies. Furthermore, we investigate whether lineage diversification patterns coincide with body, clutch and egg size disparity patterns through time. By reconstructing the most complete, multi-locus molecular phylogeny for this group to date (comprising ca. 70% of all described species and uncovering an unexpectedly high number of cryptic taxa) and fitting a number of diversification rate models to this reconstruction, we find that the diversification of lineages on the African continent has been relatively constant throughout time, across clades and reproductive modes, with no evidence for an early burst or a density dependent slow down. In contrast to the constant rate of lineage diversification, we find that life history traits were partitioned early on, which is indicative of rapid change, potentially fitting an EO model, and therefore might suggest that the diversification rate models may be underestimating extinction rates. We conclude that a number of potential, non-mutually exclusive, explanations might account for bufonid diversification patterns. These include ecological competitors, relative homogeneity in topography, or the erosion of signals over time. Overall, compared to more insular systems, the diversification of lineages on a continental scale appears to be characterised by more gradual, slower diversification rates.

Keywords

Lineages through time, disparity through time, MuSSE, BAMM, GMYC, egg size, clutch size, reproductive modes

Introduction

How species and species assemblages respond to a release from ecological competition is a fundamental question in evolutionary biology (Simpson 1953; Schluter 2000). The colonization of islands (Robichaux et al. 1990; Grant 1999; Whittaker and Fernandez-Palacios 2007) or the survival of mass extinction events (Sepkoski 1998) are classic examples of where the sudden availability of empty or underutilized adaptive zones has presented organisms with an 'Ecological Opportunity' (EO; Simpson 1953) to rapidly diversify, unimpeded by competition. In support of the EO theory, signatures of an 'early-burst' followed by density dependent declining rates of diversification as competition increases have been detected mostly in insular systems (Grant 1999; Harmon et al. 2008a; Jønsson et al. 2012), but also in localized mainland systems (Hughes and Eastwood 2006; Kozak and Wiens 2006; Rabosky and Lovette 2008a; Pinto et al. 2008; Slingsby et al. 2014). Yet, whether this same pattern can also be detected for lineages that have colonized entire continents, has only been addressed relatively recently (Derryberry et al. 2011; Day et al. 2013; Barker et al. 2013; Schenk et al. 2013; McGuire et al. 2014) and needs to be investigated in more detail. Large, continental systems provide an interesting test of how land areas, buffer zones, and historical and recent landscape heterogeneity might impact diversification patterns.

An interesting system for investigating EO and diversification rates on a continental scale is the colonization of Africa by true toads (family Bufonidae) ca. 30 Ma (Van Bocxlaer et al. 2010), which also adapted to vastly differing habitats in the process. With 585 currently described species worldwide, Bufonidae is the third most species-rich family of amphibians (Frost 2014). Both fossil and molecular evidence point to a Neotropical origin of this group (Tihen 1962; Blair 1972; Pramuk et al. 2008) at around 60-70 Ma (Pramuk et al. 2008; Van Bocxlaer et al. 2010) followed by a rapid global diversification which occurred around the mid Eocene (Pramuk et al. 2008). By the mid Oligocene (Van Bocxlaer et al. 2010), bufonids were established on all continents except Australasia and Antarctica, neither of which host endemic bufonids lineages. Van Bocxlaer et al. (2010) proposed that the evolution of an 'optimal range-expansion phenotype' was crucial for their success, a phenotype that was also characteristic of the first lineage to colonize Africa.

Adapting to new habitats when presented with EO should not only be evident in the pattern of lineage accumulation through time, but it should also be reflected in the early

disparity of characters (Schluter 2000; Harmon et al. 2003; Slater et al. 2010; Jønsson et al. 2012). An indication that such a partitioning may have occurred in African bufonids, is the remarkable versatility in breeding strategies, which includes specialized tadpole habitats including discarded snail shells (*Mertensophryne micranotis*) or terrestrial nests (*Altiphrynoides malcolmi*) and the only known case of matrotrophic viviparity for anurans (*Nimbaphrynoides occidentalis*). It is known that specific reproductive modes are associated with specific habitats in African amphibians (Goin and Goin 1962; Poynton 1964; Müller et al. 2013) making it a useful aspect of life history to investigate. Similarly the partitioning of reproductive investment into laying a large number of small eggs versus laying a small number of large eggs again is influenced in part by extrinsic conditions (Duellman and Trueb 1994; Roff 2002; Räsänen et al. 2008) and a broad spectrum of this trade-off is represented in African bufonids (Liedtke et al. 2014). How the disparity of these strategies has been structured over time may therefore give further clues as to how bufonids diversified across the continent.

Here we test whether the colonization of Africa by toads shows signs of an early-burst of lineage accumulation with a subsequent slowdown in diversification rates and whether these rates are homogenous across all subclades. With life-history evolution as our focus for elucidating the occurrence of an early and rapid adaptation phase to new habitats, we also investigate whether the evolution of any of five broad reproductive modes (free-swimming larva, free-swimming larva in micro water body, larva in terrestrial nest, lecithotrophic viviparity, and matrotrophic viviparity) is associated with different rates of diversification and whether the trade-off between clutch versus egg size occurred early in the history of African toads.

Methods

Taxon Sampling

The task of reconstructing a reliable phylogeny for African bufonids requires that several obstacles be overcome. Firstly, the current number of described species is unlikely to be close to the true number of species. Frost (2014) lists 103 species for African genera of bufonids, but this includes *Amietophrynus chudeaui* and *A. cristiglans*, two species which are no longer valid taxa (Rödel 2000). The taxonomic validity of others is questionable (e.g. *Amietophrynus buchneri, A. djohongensis*, *Mertensophryne mocquardi* and *M. nairobensis*), others have not been collected in recent history and their population status is unknown (e.g. *Amietophrynus perreti*,

A. danielae, Altiphrynoides osgoodi and *Laurentophryne parkeri*) and a large number of candidate species have been collected in recent years, but have not yet been formally described (M.O. Rödel, M. Menegon, S.P. Loader unpubl. data). Secondly, the socio-political instability of certain regions of Africa throughout recent history poses logistical problems for sampling. As examples, *A. fuliginatus, A. funereus, M. schmidti, L. parkeri* all occur in the Congo basin, and *Poyntonophrynus grandisonae* and *P. dombensis* are endemic to Angola, localities that have been unsafe for field work in recent decades. Thirdly, all previous phylogenies (Frost et al. 2006; Pramuk et al. 2008; Van Bocxlaer et al. 2009; 2010; Pyron and Wiens 2011; Beukema et al. 2013) suggest a geographic paraphyly of African bufonids although with a degree of uncertainty, and good coverage of Eurasian lineages must therefore also be included in any reconstructions.

Taxon sampling has been extensive to try to minimize the impact of the above listed caveats. At least one representative of every African genus was included, with the exception of *Laurentophryne*, a monotypic genus from eastern Democratic Republic of the Congo that has not been sighted since its original collection and description (Laurent 1950), despite recent efforts (Greenbaum and Kusamba 2012; IUCN SSC Amphibian Specialist Group 2013). We also sampled as many geographic localities as possible per species to try to uncover additional cryptic or undescribed taxa. Tissues were accumulated through the authors' own field collections and through tissue loans from museum repositories. In total, 1676 sequences from 432 individuals were generated *de novo* for this study, and in combination with sequence data from GenBank, the complete dataset includes 591 individuals of at least 112 species including outgroups. This covers almost 70% of all described African species (69 out of 101), 14 out of 18 Eurasian genera and a selection of New World bufonids to allow for the inclusion of more fossil calibration points.

Generating Molecular Sequence Data

DNA was extracted from either leg muscle or liver tissue stored in >96% ethanol or RNAlater, using a Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., CA, USA) and the default protocol. A total of ~3439 base pairs comprising five markers including partial sequences of two ribosomal RNA genes; 12S and 16S rRNA (~380 and ~575 bp), and three coding regions: cytochrome-oxidase subunit 1 (COI; mitochondrial, ~840 bp), C-X-C chemokine receptor type 4 (CXCR4; nuclear, 711 bp), and recombination activating gene-1

(RAG1; nuclear, ~933 bp) were amplified via Polymerase Chain Reaction (PCR) using Illustra puReTaq Ready-To-Go PCR beads (GE healthcare, Buckinghamshire, UK; see primers and cycling profiles in online Appendix 1). Q-solution (by Qiagen) was added to the PCR reaction to improve amplification of CXCR4, RAG1 and COI. PCR products were visualized on 1% agarose gels and successful amplifications were sent to Microsynth AG (Balgris, CH) for purification and sequencing. Complementary strands were sequenced and subsequently proofread using Codoncode Aligner v4.4.1 (Codoncode Cooperation, MA, USA). All sequences were deposited on GenBank and assigned accession numbers (online Appendix 2).

Aligning and Concatenating Sequences

Sequences generated *de novo* in this study were supplemented with existing relevant bufonid sequences available on GenBank and processed using the bioinformatics platform Geneious Pro v5.6.7 (created by Biomatters, available from http://www.geneious.com). Sequence lists were created for each gene fragment separately and concatenated GenBank sequences spanning over multiple genes were split appropriately. Each sequence list was then aligned with MAFFT v7.017 (Katoh and Standley 2013) using the auto setting for all coding genes and the E-INS-i algorithm for 12S and 16S. The alignments and where available the sequence chromatograms were manually checked. GBlocks (Castresana 2000) was used to remove poorly aligned, ambiguous nucleotide and gap positions in the 12S and 16S alignments caused by low conservatism of loop regions, to standardize alignment manipulations, with the options set to allow for smaller final blocks and less strict flanking positions, but no gap positions. The coding genes were realigned and translated using TranslatorX (Abascal et al. 2010) to find the open reading frame. All five genes were concatenated and an optimal partitioning scheme and nucleotide substitution models were determined using partitionfinder v1.1.1 (Lanfear et al. 2012) based on Akaike Information Criterion scores (AIC) implementing the greedy search algorithm and unlinked branch lengths. Non-coding genes and each codon position for coding genes were treated as individual partitions (totalling to 11 potential partitions).

To qualitatively evaluate the degree of saturation in each partition, a Maximum Likelihood (ML) search was carried out using the HPC-MPI version of RAxML v7.2.8 (Stamatakis 2006) using a GTR+Γ model of substitution and 1000 nonparametric bootstrap

replicates. Pairwise transitions and transversions were then plotted against the patristic distances of the GTR model (Online Appendix 3) using the ape package (Paradis et al. 2004) in R (R core team 2013). The transitions in the 3rd codon position of COI showed a high degree of saturation, indicated by the flattening out of points, and this partition was therefore removed for the phylogenetic reconstruction.

Phylogenetic Inferences

Two DNA alignments and subsequent phylogenetic inferences to investigate African bufonid phylogeny were utilized. How these two alignments and all resulting trees have been derived is graphically outlined in Online Appendix 4. The first, 'full tree' inference (tree A in Online Appendix 4) favoured gene over taxon coverage to establish a well-resolved backbone phylogeny, allow for geological time calibration and to investigate paraphyly of African taxa. Only samples for which sequence data of all five gene-regions was available were included in this alignment (with the exception of *Incilius* spp. and *Bufotes surdus* that were included for calibration purposes). All African genera (except for *Laurentophryne*; see taxon sampling) are represented in this tree, but only 60 of the 101 described species are covered. For the purpose of getting a more complete understanding of the diversity of African lineages, the second alignment and phylogenetic reconstruction was carried out using sequence data for as many individuals as possible, even if not all five genes were available (tree B in Online Appendix 4). This second alignment was restricted to include only members of the first African radiation (FAR; this excludes *Werneria, Wolterstorffina, Nectophryne* and *Laurentophryne*; see results for details on paraphyly) because an EO driven signal in diversification is unlikely to be relevant for subsequent colonization events (Schenk et al. 2013). The resulting nucleotide matrix for this second inference favours taxon sampling (covering 60 of the 89 described species), but at the cost of missing sequence data, fossil calibration points and species not belonging to the FAR clade.

Joint posterior distribution of all model parameters for both trees were estimated using Bayesian MCMC searches in BEAST v1.7.5 (Drummond et al. 2012). For the full tree, a three-partition scheme was recovered as optimal with the following substitution models GTR+Γ+I (12S, 16S and COI-cp1), GTR+Γ+I (COI-cp2, CXCR4-cp1, CXCR4-cp2, RAG1-cp1 and RAG1-cp2) and GTR+Γ (CXCR4-cp3 and RAG1-cp3). For the first two partitions, GTR+Γ was implemented instead of GTR+Γ+I to avoid over-parameterization due

to non-independence of estimates for the proportion of invariable sites and among-site rate variations (Yang 2006). For the FAR tree, a partitioning scheme treating all partitions as one, with a GTR+Γ substitution model had the lowest AIC score. Molecular clock models were estimated for a linked set of mitochondrial markers (12S, 16S and COI) and for CXCR4 and RAG1 separately using uncorrelated lognormal relaxed clock (ucld) priors (Drummond et al. 2006). Speciation tree priors were chosen over coalescent priors because although the dataset is heterogeneous (in cases containing multiple individuals per species), the former is more appropriate given that taxon sampling comprises distantly related genera. Alternatively, *BEAST (Heled and Drummond 2010) designed for multispecies coalescent processes requires a prior knowledge of species delimitations, a condition that is problematic with the current dataset. Both birth-death (Gernhard 2008) and pure-birth (Yule 1925; Gernhard 2008) speciation tree priors were tested however, and model selection was based on log 10 Bayes Factors calculated from the harmonic means of marginal log likelihood scores (ln P(model|data)) from the resulting combined BEAST log files with 1000 bootstrap replicates using Tracer v1.5 (Rambaut and Drummond 2007). A ratio greater than 2 was taken as decisive evidence for favouring one model over the other (Kass and Raftery 1995). The full tree was calibrated to recover a geological time scale by including four fossil node constraints: the origin of the *Rhinella marina* species-group (11.8 Ma), the most recent common ancestor of *Anaxyrus* and *Incilius* (20 Ma), the oldest unambiguously identified *Bufo bufo* (9.6 Ma) and the age of the *Bufotes viridis* lineage (18 Ma). Details on prior settings and justification of dates are provided in Online Appendix 5. As these fossils are not contained within the FAR clade, the crown age of the FAR tree ingroup was calibrated using the age of the most recent common ancestor of the FAR clade in the full tree. No other constraints were implemented for either reconstruction.

A total of three MCMC searches with 100 million generations and three with 50 million generations, sampling every 2000th iterations were conducted to assess convergence and stability of parameters. An additional MCMC search on priors only (i.e. with an empty alignment) was also executed to assess whether the signal in the data for estimating parameters is overwhelmed by the prior settings. Convergence and effective sample sizes (EES) of parameters in the log files were visually inspected using Tracer, and AWTY (Wilgenbusch et al. 2004) was used to assess whether the MCMC analyses were run long

enough to allow the tree topologies to be adequately sampled in proportion to their true posterior probability distribution.

Multiple tree files from the independent searches were combined using LogCombiner v1.7.5 (Rambaut and Drummond 2012a). Appropriate burn-in thresholds were set for each run based on the inspection of the chain in Tracer and states were resampled at a lower frequency to obtain ca. 20,000 posterior trees. These trees were then summarized on a maximum clade credibility tree (MCC tree) using TreeAnnotator v1.7.5 (Rambaut and Drummond 2012b) using median node heights and no limit on the posterior probability. Trees have been submitted to TreeBase (submission ID: 15589).

Species Delimitation

Extensive field and lab work by the authors and collaborators has revealed a large number of undescribed species of African bufonids. Investigating diversification rates using only described species is therefore not a true representation of the phylogenetic diversity of African bufonids. To objectively obtain a tree that includes undescribed, but distinct taxa, the General Mixed Yule-Coalescent model (GMYC; Pons et al. 2006) implemented in the R package splits v1.0-19 (Ezard et al. 2009) was used to identify suitable delimitation points on the chromatogram generated for the densely sampled first radiation (FAR tree). This delimitation method was chosen over others that are more accommodating to multi-locus datasets, such as BPP (Yang and Rannala 2010) for example, because the GMYC method requires no prior taxonomic assumptions to be made. The guide tree necessary for BPP can strongly influence the resulting delimitations (Leaché and Fujita 2010) and given the uncertainty and the large-scale nature of our dataset, this seemed inappropriate.

The GMYC method uses a ML approach to find break points where diversification rates shift from lineage branching pattern that resembles a Yule speciation model to a pattern that better fits to a neutral coalescent model. The single-threshold method was chosen due to its higher delimitation accuracy (Fujisawa and Barraclough 2013) and the lower sensitivity to user-settings (as recommended by the package authors), but we relaxed the scaling parameters (intervals=c(0,10)) to relax the assumptions of the rate models (Pons et al. 2006). This method does not take phylogenetic uncertainty into consideration. In order to allow some uncertainty to still be represented in downstream analyses, the MCC tree was used to calculate delimitation points, pruned to contain only one representative per delimited element

(tree D in Online Appendix 4) and a random subset of 1000 posterior trees was then also pruned to include only these terminals.

A number of diversification rate estimation methods allow the incorporation of biased undersampling information in the models. Although taxon sampling is incomplete in the GMYC-pruned FAR tree (from here on 'GMYC tree'), the documented species numbers are not a reliable measure to scale our analyses due to the questionable taxonomic validity of some taxa and the large number of cryptic species in a number of clades (see introduction). The analyses carried out with the GMYC tree were therefore not corrected to account for missing taxa as this would be trivial at best, given the current state of taxonomic knowledge of this group. As a comparison, the same analyses were repeated using the FAR phylogeny pruned to include only a single representative per formally described species (from here on 'DS tree'; tree C in Online Appendix 4) and incorporating bias information for incomplete sampling whenever methods allowed.

Lineage Diversification

Three aspects of lineage diversification and rate shifts in the FAR clade (using both the GMYC and the DS tree) were modelled to try to estimate likely speciation and extinction patterns for African bufonids: a) net diversification rates and temporal patterns under different models were estimated for the entire phylogeny, b) traces of lineage-specific rate shifts were investigated and c) whether or not rate shifts in concordance with life history trait changes are evident.

Detecting rate shifts through time.—Net diversification rates (*r*; speciation minus extinction) were calculated for models assuming no extinction (ε=0, where "ε" is the extinction fraction: extinction/speciation) and high extinction rates (ε=0.9) using the R package geiger v.1.99-3.1 (Harmon et al. 2008b), to obtain a lower and upper range estimate (Magallón and Sanderson 2001). The γ statistic (Pybus and Harvey 2000) was calculated to test whether the net diversification of a given phylogeny departs from an exponential, pure-birth-like accumulation of lineages. A significantly negative γ would indicate a deceleration in lineage accumulation, where branching events are more concentrated near the root of the tree as would be expected under an early burst scenario. To account for missing taxa in the DS tree, we employed a Monte Carlo Constant Rate (MCCR) test, which calculates a γ for a simulated set of 5000 complete (i.e. including all 89 described species belonging to the FAR

clade) random trees under a constant rate pure-birth model and then randomly prunes tips to simulate incomplete sampling (Pybus and Harvey 2000). The accumulation of lineages through time for the GMYC and the DS tree were plotting and compared to a plot of the median of 1000 simulated lineages generated under a pure-birth process limited to 89 species, the described number of species of the FAR based on traditional taxonomy (Frost 2014).

To further investigate whether diversification rates have changed over time, we compared two rate-constant models; a pure-birth and birth-death model, to three rate-variable models; a two-rate Yule model (Y2R), a density dependent exponential model (DDX) and a density dependent linear model (DDL), using the fitAICrc function in the R package laser (Rabosky and Shliep 2013) and adjusting the number of intervals to 100 to allow the Y2R model to consider more shift points than just the observed branching times. This function compares the AIC score of the best rate-constant model (AICrc) to the best rate-variable model (AICrv), with a positive ΔAICrc (AICrc – AICrv) implying that a rate-variable model is a better fit than a rate-constant model.

Extinction can dissipate signals of an early-burst and what looks like decreasing speciation rates over time could instead reflect an increase in extinction rate over time. To test whether speciation and extinction rates vary over time, we explored the following models: time-varying speciation with constant extinction (SPVAR), time-varying extinction with constant speciation (EXVAR) and both speciation and extinction varying over time (BOTHVAR) using the laser package.

Detecting among-lineage rate heterogeneity.—The recently developed Bayesian Analysis of Macroevolutionary Mixtures (BAMM; Rabosky 2014) software in combination with the R package BAMMtools (Rabosky et al. 2014) was used to estimate marginal distributions of speciation and extinction rates for each branch in the tree. Furthermore, we tested whether there are distinct rate regimes across the GMYC and DS reconstructed phylogenies. Unlike stepwise AIC models (e.g. MEDUSA; Alfaro et al. 2009) that simply compare models with different numbers of rate shifts, this method simulates posterior distributions of a large number of rate shift configurations and calculates posterior probabilities for these. BAMM was allowed to sample every 1000th generation of 5 million MCMC iterations, priors were configured based on the setBAMMprior function in BAMMtools and the initial values for λ and μ were set to the birth-death model estimates obtained from laser. The analysis using the GMYC tree assumed complete sampling, whereas the analysis using the DS tree was

supplemented with sampling fraction information for each genus. For each analysis, four independent runs were executed and convergence of the posterior probability densities were checked by visually inspecting the log-likelihood traces and computing the effecting sample sizes using the R package coda (Plummer et al. 2006). To compare the relative support of one rate regime model over another Bayes factors were calculated, including runs sampling only the priors as well.

Detecting trait-specific rate shifts.—The Multiple State Speciation and Extinction (MuSSE) model implemented in the R package diversitree v.0.9-6 (FitzJohn 2012) was used to examine whether shifts in discrete character states are associated with shifts in diversification rate. Speciation and extinction rates were estimated for lineages with different reproductive modes (free-swimming larva, free-swimming larva in micro water body, larva in terrestrial nest, lecithotrophic viviparity and matrotrophic viviparity; Online Appendix 6). Using a ML optimization approach, we compared speciation and extinction rates for a model where rates are constrained across all character states to a model where rates are free to vary. A likelihood ratio test based on a χ^2 distribution was then used to evaluate whether allowing different states to be associated with different rates significantly improved the fit of the model. The analysis was repeated using a Bayesian method of estimating posterior probability distributions of the rate parameters using and an exponential prior and Markov chain Monte Carlo (MCMC) simulations to account for uncertainty in parameter estimations. The tuning parameter *w*, which defines how much the MCMC process varies the parameter values in each step, functions well when using the width between the 5% and the 95% quantile marks of the marginal distributions for each parameter (FitzJohn 2012). This range was determined by running a preliminary MCMC search with *w* arbitrarily set to 0.1 across all parameters for 1000 iterations. The final run was then executed with the new tuning parameter estimates and iterated 10,000 times. Parameter traces were visually inspected and the first 1000 iterations were discarded as burn-in. This analysis run with the DS tree included sampling faction information to correct for biased undersampling. The ML search was carried out on the GMYC and the DS MCC trees, but then also looped over the 1000 randomly sampled posterior trees of each to accommodate phylogenetic uncertainty. Information on reproductive modes was obtained from the literature and species for which the reproductive mode is unknown, the most likely mode was assigned based on indirect inferences such as oviducal egg size and/or extrapolation of the assumption that species of the same genus or closely related

group have the same reproductive mode (see Online Appendix 6). This was favoured over the alternative of pruning the tree to only species with known breeding biology, to maintain as high a taxon sampling as possible.

Disparity in Life-History

To explore how life-history strategies diversified over time, the disparity of a clutch and egg size within and between clades was compared. Under an EO model, the divergence into different parts of the niche space should happen rapidly, early in the evolutionary history of a group after which point, disparity remains constant and low. Such patterns can be visualized by plotting disparity through time (DTT) using the dtt function in the geiger package. This is achieved by calculating disparity at each node by taking the average relative disparity (as Euclidian distances) of all subclades at that node and dividing it by the average of the whole clade, moving from the root of the tree to the tips (Harmon et al. 2003). These measures are standardized by dividing by the overall disparity of the entire tree so that values near zero imply that variation in the tested characters are partitioned more or less evenly across subclades whereas values near one suggest that individual subclades contain significant portions of the variation. The Morphological Disparity Index (MDI; Harmon et al. 2003) was also calculated by comparing the observed disparity values to a null model composed of 1000 simulations under a Brownian Motion model. This measure gives the area between the observed DTT and the median of the simulations, where a positive value indicates a greater overall disparity than expected and a negative value indicates less disparity than expected. The standard plot produced by the dtt function was modified to show DTT through absolute rather than relative time, the median instead of the mean line of the simulations and also to include the DTT lines for all 1000 posterior samples to incorporate phylogenetic uncertainty.

Clutch and egg size of toads are correlated with each other and with female body size (Fig. 1b; Liedtke et al. 2014) and therefore Principal Component scores of female body size (snout-vent length in mm), clutch size (number of eggs in a single clutch) and egg size (diameter of eggs in mm) were used. All measurements were natural log transformed and species for which traits were unknown were removed from the tree, resulting in a reduced dataset of 39 species (Tree F in Online Appendix 4). All genera except for *Churamiti* continued to be represented however (Online Appendix 7). All measurements were taken

from Liedtke et al. (2014) and references therein, and refer to maximum records per species as this is the most widely available measurement (see Liedtke et al. 2014).

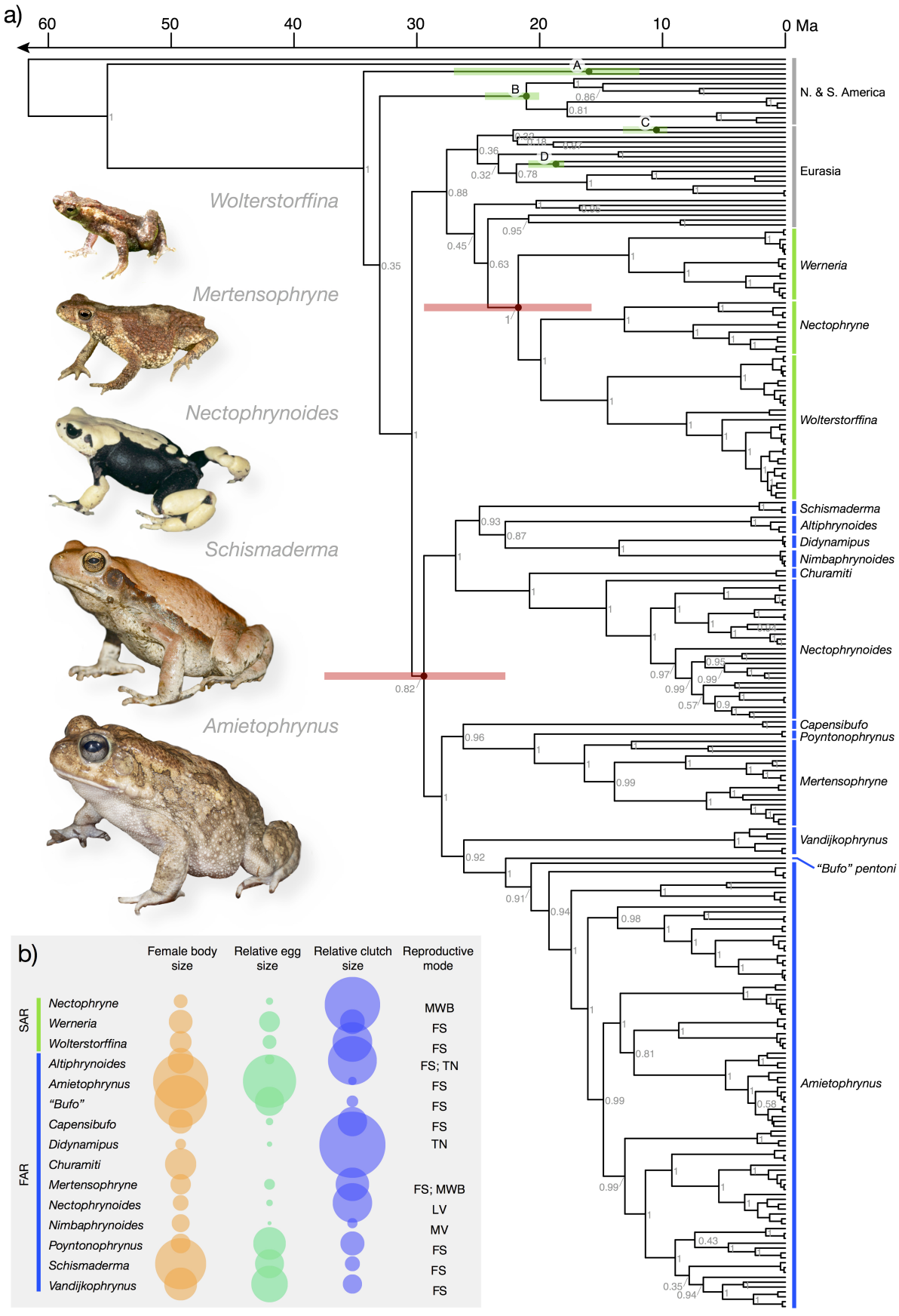
Results

Phylogenetic Inferences

For both the full tree (Fig. 1a; Online Appendix 8) and the FAR tree (Online Appendix 9), a birth-death speciation prior produced higher marginal log likelihood scores than a Yule prior, with log 10 Bayes Factors of 2.986 and 3.110 respectively. The full tree confirms that African bufonids are paraphyletic with two independent colonization events into Africa (Fig. 1a). Most relationships of Eurasian groups are poorly resolved, but for both African radiations, internal nodes are generally well supported. The full tree reconstruction dates the origin of the Old World radiation at 30.40 Ma (95% Highest Posterior Density interval; HPD=23.24,38.50), which is in concordance with previous estimates (Van Bocxlaer et al. 2010), with the two colonization events into Africa occurring shortly after, at 29.42 Ma (HPD=22.79, 37.53) and 21.74 Ma (95% HPD=15.77, 29.42) respectively.

All genera are recovered as monophyletic. An unexpectedly high number of candidate species were recovered for *Nectophryne*, *Wolterstorffina*, *Nectophrynoides*, *Mertensophryne* and in the *Amietophrynus gracilipes-kisoloensis-villiersi* complex, highlighting the need for taxonomic revisions of these groups. All major relationships were congruent in the full tree and the FAR tree, with the exception of the (((*Didynamipus, Nimbaphrynoides*), *Altiphrynoides*), *Schismaderma*) clade in the full tree which was recovered as ((*Didynamipus, Nimbaphrynoides*), (*Altiphrynoides*, *Schismaderma*)) in the FAR tree, but with lower node support. When pruning the FAR tree to only include a single representative of each described species (DS tree, Online Appendix 10), 60 out of the 89 known species are represented with the missing 29 belonging to the following genera: *Amietophrynus*—15, *Mertensophryne*—6, *Nectophrynoides*—2 and *Poyntonophrynus*—6.

FIGURE 1: a) MCC tree for Bufonidae recovered from time-calibrated Bayesian MCMC tree searches using BEAST under a birth-death uncorrelated lognormal relaxed clock model. Node support reflect posterior probabilities and node bars show the 95% highest posterior density of divergence times for key nodes; the origin of the two African clades and the fossil calibration points, A: The origin of the *Rhinella marina* clade, B: the most recent common ancestor for *Anaxyrus* and *Incilius* C: the origin of the *Bufo bufo* group and D: the origin of the *Bufotes viridis* group. The first African radiation (FAR) is colour-coded blue and the second African radiation (SAR) is colour-coded green. The inserted photographs show exemplary phenotypes of a selection of African bufonid genera. 1b) Depiction of the mean intergeneric relationships of maximum female body size, relative (to body size) maximum clutch size, relative maximum egg size and reproductive mode (where FS: free swimming larvae, MWB: free swimming larvae in micro water bodies, TN: larvae in terrestrial nests, MV: matrotrophic viviparity and LV: lecithotrophic viviparity). Measurements were taken from Liedtke et al. (2014).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Nectophrynoides
Capensibufo
Poyntonophrynus
Mertensophryne
Vandijkophrynus
"Bufo" pentoni
Amietophrynus
Wolterstorffina
Mertensophryne
Nectophrynoides
Schismaderma
Amietophrynus
b)
Female body size
Relative egg size
Relative clutch size
Reproductive mode
SAR
FAR
Nectophryne
Werneria
Wolterstorffina
Altiphrynoides
Amietophrynus
"Bufo"
Capensibufo
Didynamipus
Churamiti
Mertensophryne
Nectophrynoides
Nimbaphrynoides
Poyntonophrynus
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Species Delimitation

The BEAST chronogram of the FAR clade contained 500 ingroup terminals for which the GMYC model was a significantly better fit than the null model of constant diversification rates (likelihood ratio: 53.218, $p<0.001$). The GMYC-based delimitation set a threshold time at 1.081 Ma and recovered 118 most likely unique entities (Online Appendices 11-12). When comparing these entities to described species, additional units were recovered in the following genera: *Nimbaphrynoides*—1, *Schismaderma*—2, *Nectophrynoides*—17, *Capensibufo*—5, *Mertensophryne*—8, *Vandijkophrynus*—1 and *Amietophrynus*—26. Two pairs of species: *Mertensophryne howelli* and *M. usambarae* and *Amietophrynus pardalis* and *A. pantherinus*, were not recovered as distinct entities. Previous studies have shown that this method tends to overestimate species numbers (e.g. Miralles and Vences 2013) and indeed some of these seem unlikely to reflect biologically relevant divisions (e.g. *Nimbaphrynoides*; Sandberger et al. 2010). Regardless, qualitative assessments of the entities recovered suggest that overall, these numbers are not unreasonable, given the cryptic nature and large geographic ranges of many of these taxa.

Lineage Diversification

Rate shifts through time.—Lineage through time plots for the GMYC tree, the DS tree and a simulated set of pure-birth trees with 89 species are presented in Figure 2. For the GMYC tree, assuming complete taxon sampling, the net diversification rate was found to be 0.163 per Myr in the absence of extinction and decreased to 0.100 per Myr when assuming high rates of extinction ($\varepsilon=0.9$). Although γ was less than 0, the test statistic was not significantly different from the null hypothesis of constant rates through time (MCC tree: γ =-0.813, $p=0.416$, posterior trees: mean±SD γ= -0.553±0.576, $p=0.580$). For the DS tree, the net diversification rate when factoring in missing taxa was 0.151 per Myr in the absence of extinction and decreased to 0.089 per Myr when assuming high relative rates of extinction ($\varepsilon=0.9$). The observed γ statistic under the assumption of complete sampling was -2.230, which was significantly different from a constant rate model (one tail test $p=0.013$; posterior trees: mean±SD γ= -2.123±0.481, $p=0.034$). The MCCR γ test distribution that accounts for incomplete taxon sampling recovered a mean of -0.895 (SD=0.941) with a 5% critical value of -2.448.

For the GMYC tree, a two-rate model was a significantly better fit to the data than any constant rate model (ΔAICrc=35.836; Table 1), but the rate shift point proposed by this model was placed at 1.263 Ma, which may not be biologically meaningful as it roughly coincides with the cut-off for the species delimitation process (1.081 Ma). To account for this, the analyses were repeated on the tree after the terminal branches were truncated by the GMYC delimitation threshold time (tree D in Online Appendix 4). In doing so, a constant birth-death model performs best with a net diversification rate of 0.113 per Myr (Table 1). This supports the notion that the variable rate model preference is likely a reflection of the crude pruning of the tree via the GMYC delimitation method. The best constant rate model for the non-truncated tree was a pure-birth model, with a diversification rate of 0.164 per Myr (Table 1), comparable to the estimates calculated using the geiger package, and the best rate-constant model for the truncated tree was a birth-death model with r=0.113 (Table 1), closer to the geiger estimates for a model with relatively high extinction rates.

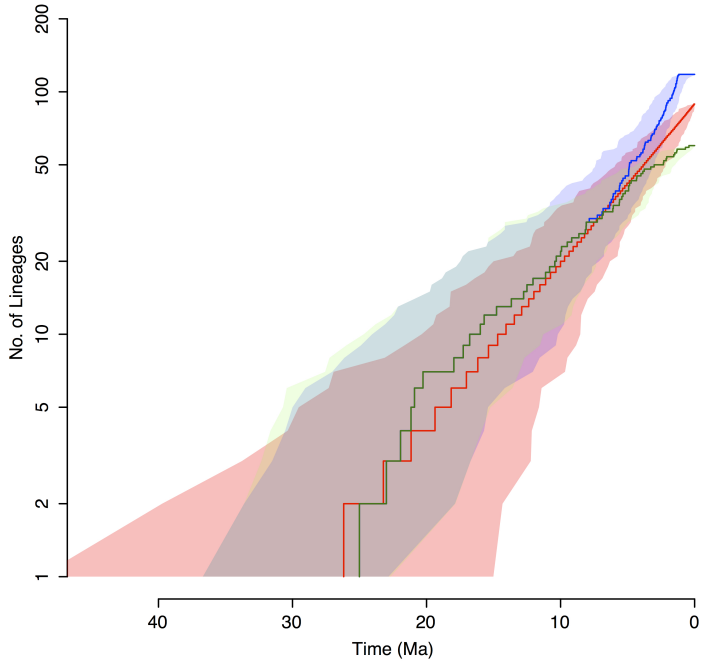

FIGURE 2: Lineage through time plots for the GMYC tree (blue) the DS tree (green) and the median of 1000 Yule simulations for a tree with 91 taxa and a speciation rate of 0.152 (red). Shaded areas mark the outlines of 1000 subsamples of posterior trees or simulated Yule trees.

The DS tree analyses show differing results, with all three rate-variable models (including the density dependent models indicative of an early burst) outperforming the two rate-constant models (Table 1). A two-rate Yule model performed best with an initial net diversification rate of 0.120, which drops off to 0.026 at 1.307 Ma. This suggests that even when correcting for incomplete sampling, a relatively greater proportion of diversification events occurred early in the history of the clade. This result should be treated with caution however, because our extensive sampling has revealed that there is a substantial

TABLE 1. Summary statistic of diversification models fitted to the branching times of the a) species delimited GMYC tree, b) truncated GMYC tree and c) DS tree. The models tested are Pure-Birth (PB), Birth-Death (BD), Density-Dependent, Exponential (DDX), Density-Dependent, Linear (DDL), Yule-2-Rate (Y2R), continuous-time varying speciation rates (SPVAR), continuous-time varying extinction rates (EXVAR) and continuous-time varying speciation and extinction rates (BOTHVAR). Parameters are a=extinction fraction, xp= magnitude of rate change, K=analogue to carrying capacity, lam0=initial speciation rate, mu0=final extinction rate, k=exponential change in speciation rate; z=exponential change in extinction rate.

Model	Rate	Parameters	Rate shift times	LH	AIC	ΔAIC
A) GMYC tree						
Rate-constant models						
PB	0.164			117.564	-233.129	35.837
BD	0.164	a=0		117.564	-231.129	37.837
Variable rate models						
DDX	0.177	xp=0.020		117.583	-231.166	37.800
DDL	0.191	K=432.256		118.039	-232.078	36.888
Y2R	0.204; 0.013		1.263	137.483	-268.966	0.000
Variable speciation/extinction models						
	Model Parameters					
SPVAR	lam0= 0.167; k=0.001; mu0=0.001			117.546	-229.091	39.875
EXVAR	lam0= 0.164; mu0=0.001; z=1.002			117.553	-229.106	39.860
BOTHVAR	lam0= 0.167; k=0.001; mu0=0.001; z=0.096			117.545	-227.090	41.876
B) Truncated GMYC tree						
Rate-constant models						
PB	0.200			140.581	-279.161	7.888
BD	0.113	a=0.647		145.525	-287.049	0.000
Variable rate models						
DDX	0.068	xp=-0.290		143.645	-283.290	3.760
DDL	0.200	K= 2077089.000		140.580	-277.160	9.889
Y2R	0.131; 0.246		5.404	145.454	-284.909	2.141
Variable speciation/extinction models						
	Model parameters					
SPVAR	lam0= 0.567; k=0.020; mu0=0.323			146.102	-286.205	0.844
EXVAR	lam0= 0.320; mu0=0.207; z=2466.427			145.525	-285.049	2.000
BOTHVAR	lam0= 0.373; k=0.003; mu0=0.298; z=0.125			146.198	-284.395	2.654
C) DS tree						
Rate-constant models						
PB	0.107			-3.182	8.365	3.532
BD	0.107	a=0		-3.182	10.365	5.532
Variable rate models						
DDX	0.380	xp=0.385		-0.603	5.205	0.373
DDL	0.152	K=119.999		-0.620	5.240	0.407
Y2R	0.120; 0.026		1.307	0.584	4.833	0.000
Variable speciation/extinction models						
	Model parameters					
SPVAR	lam0=0.231; k=0.044; mu0=0.001			-1.114	8.227	3.394
EXVAR	lam0= 0.107; mu0=0.001; z=1.003			-3.223	12.447	7.614
BOTHVAR	lam0= 0.229; k=0.044; mu0=0.001; z=0.001			-1.107	10.214	5.381

underestimation of true species numbers in the literature and the DS tree is underrepresenting recent diversification events. The GMYC tree, despite its possible overestimations, is therefore the better representation of the true diversity of the FAR species and this tree does not significantly depart from a pure-birth null model, with lineage accumulation being best characterized by rate-constant models, especially when truncating the tree to correct for the effects of the single-threshold GMYC pruning.

Among-lineage rate heterogeneity.—For the GMYC tree, BAMM found strong support for rate homogeneity; that is, a model with a single evolutionary rate regime had the highest posterior probability (PP=0.690; Fig. 3A) with a posterior odds ratio of 2.768 and a Bayes Factor score of 2.316 over the next best model, which was a two-process (i.e. one rate shift) model. Support diminishes with complexity of the models and models with more than six rate regimes were essentially never sampled (Fig. 3A). Scaling branch lengths to the posterior probability that the branch contains a rate shift shows that the probabilities across the entire tree are extremely low (note scale bar), with the basal branches of *Nectophrynoides* showing somewhat higher posterior probabilities for a rate shift (Fig. 3B).

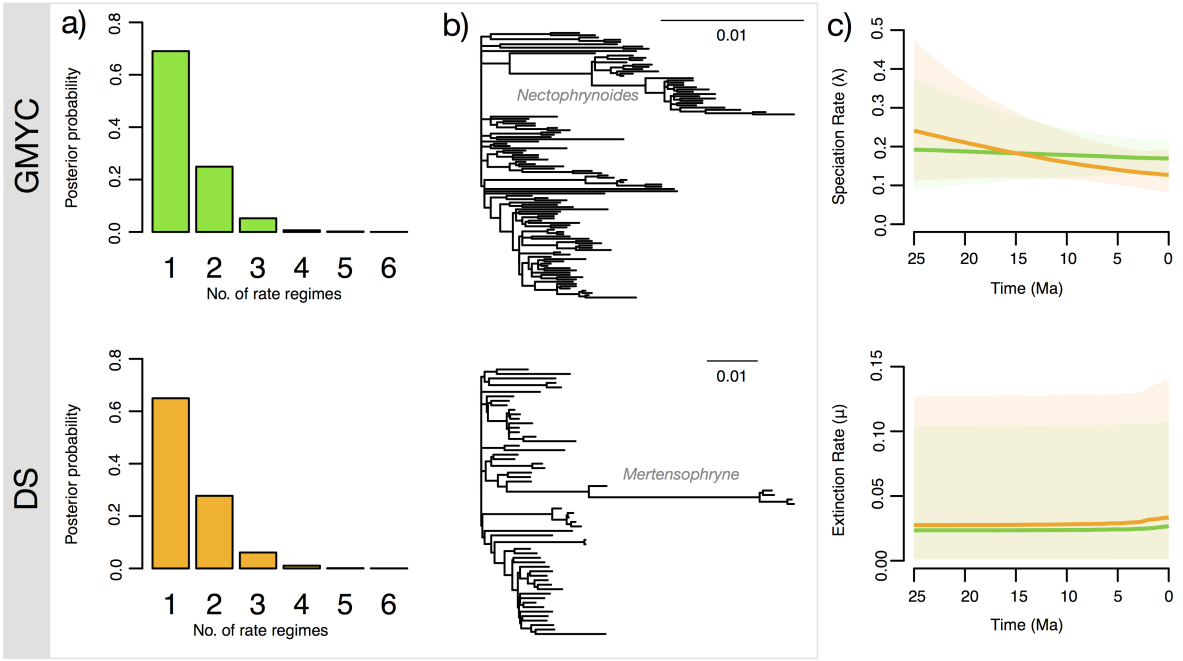

FIGURE 3: Diversification dynamics for the GMYC and the DS tree (correcting for known undersampling in the latter) using the BAMM software package. a) Posterior distribution of regimes with different numbers of rate processes (including the root process). b) Phylogenies with branch lengths transformed to correspond to the posterior probabilities of containing a rate shift. c) Speciation and extinction rates through time for the GMYC tree (green) and the DS tree (orange). Shaded areas denote the 95% quantiles on the posterior distribution of the rates at a given point in time.

The same rate homogeneity was recovered for the DS tree. A model with a single rate regime had the highest posterior probability (PP=0.650; Fig. 3A) with a posterior odds ratio of 3.343 and a Bayes Factor score of 1.898 over the next best model, which again was a two-process model. The transformed branch lengths to depict posterior probabilities for rate shifts shows that the probabilities are extremely low across the whole of the tree, with *Mertensophryne* showing the highest probabilities (Fig. 3B). The more likely shifts (longer branches) observed for *Mertensophryne* reflect the compensation for undersampling of this genus (only 35% of this genus is represented in the tree).

BAMM estimated speciation and extinction rates to be more or less constant over time for the GMYC tree and showing a consistent decrease in speciation rates for the DS tree (Fig. 3C). The steeper decline in speciation rate over time for the DS tree compared to the GMYC tree is likely driven by the current underestimation of species-level diversity in African bufonids, as demonstrated in this study.

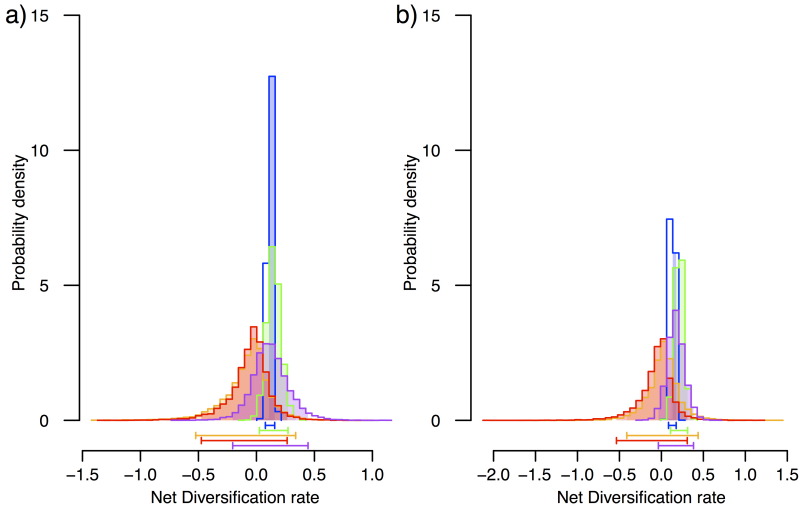

FIGURE 4: Probability density plots of posterior distribution of net diversification rates (r = speciation - extinction) associated with reproductive modes, estimated using MCMC-MuSSE for a) the GMYC tree and b) DS tree. Reproductive modes are blue: free swimming larvae, green: lecithotrophic viviparity, yellow: matrotrophic viviparity, orange: larvae in terrestrial nests and purple: free swimming larvae in micro water bodies. Shading and bars below the plot show the 95% quantile range.

TABLE 2. Parameter estimates under a MuSSE model using Maximum Likelihood on a) the GMYC tree assuming full sampling and b) the species tree pruned to all known species and assigning missing taxa to their most likely sister taxon. Values are those generated from the MCC tree with mean parameter estimates from 1000 random post burnin posterior trees given in parentheses.

Model	Speciation Rate					Extinction Rates					transition rate	LnLik	AIC
	λ1	*λ2*	*λ3*	*λ4*	*λ5*	*μ1*	*μ2*	*μ3*	*μ4*	*μ5*			
a) GMYC tree													
Constrained	0.163 (0.225)					<0.001 (0.050)					0.002 (0.002)	-357.088 (-358.005)	720.175
Unconstrained	0.151 (0.260)	0.233 (0.267)	0.166 (0.190)	0.025 (0.015)	0.226 (0.221)	0.001 (0.119)	<0.001 (<0.001)	0.115 (0.149)	<0.001 (0.015)	<0.001 (<0.001	0.002 (0.002)	-353.630 (-353.747)	729.260
likelihood ratio test (MCC tree): df=3,11 ; c^2=6.915; p=0.546; DAIC=9.085													
a) Described species tree													
Constrained	0.131 (0.133)					<0.000 (<0.001)					0.002 (0.002)	-214.294 (-214.794)	434.589
Unconstrained	0.127 (0.128)	0.178 (0.181)	0.026 (0.021)	0.023 (0.021)	0.233 (0.246)	<0.001 (<0.001)	<0.001 (<0.001)	0.021 (0.042)	<0.001 (0.015)	0.009 (0.036)	0.002 (0.003)	-212.162 (-212.542)	446.150
likelihood ratio test (MCC tree): df=3,11; c^2=4.266; p=0.832; DAIC=11.735													

Trait-specific rate shifts.—The ML approach in MuSSE suggested that there is no significant difference in the estimated parameters between the model where speciation and extinction rates are allowed to vary across character states and the model where speciation and extinction rates are constrained across character states, regardless of which tree is used (GMYC tree: χ^2=6.915, p=0.546; DS tree: c^2=4.266; p=0.832; Table 2). The MCMC approach produced concordant results with probability density for net diversification rates associated with all five character-states overlapping almost completely (Fig. 4). For all states, extinction rates are estimated to be almost negligible (except for the matrotrophic viviparous lineage) and the GMYC tree shows considerably higher speciation rates for lecithotrophic viviparous species than the DS tree, reflecting the large number of undescribed *Nectophrynoides* species not represented in the latter. Caution needs to be taken however when interpreting these results as tip ratio bias is high (less than 10% of tips share one state) and tip number is low (see Davis et al. 2013).

Disparity of Life-History

Life-history traits show a drastic drop in average subclade disparity early on in the history of bufonids, with little overlap in variation within species groups. The overall MDI score is below zero (-0.166) suggesting that the disparity of traits is less than expected under a Brownian Motion model with the observed disparity falling just below the 95% confidence intervals of the BM simulations throughout most of clade's history. The disparity plots indicate a peak in the last 5 million years, where disparity is greater than expected under a BM model, which is likely to be an artefact of under-sampling recent nodes (Harmon et al. 2003). This is therefore unlikely to be biological signal and is evident in other systems as well (Burbrink and Pyron 2009; Slater et al. 2010; Rowe et al. 2011; Derryberry et al. 2011).

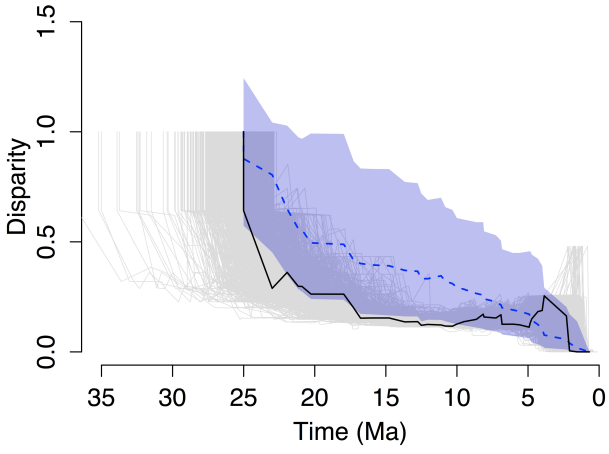

FIGURE 5: Disparity through time (DTT) plots for PCA scores of log transformed clutch size, egg size and body size. Black lines represent the observed DTT using the MCC tree and grey lines are the observed values for a subsample of 1000 post-burnin posterior trees. Dashed blue lines represent the median DTT under a Brownian Motion model simulation with 95% Confidence Intervals as the blue translucent polygon.

Discussion

African Bufonid Phylogeny

Evolutionary relationships among genera in the family Bufonidae remained relatively poorly known until multi-gene studies with relatively broad taxonomic coverage were undertaken (Frost et al. 2006; Pramuk et al. 2008; Van Bocxlaer et al. 2010; Pyron and Wiens 2011). Prior to these studies there was little consensus from morphology (e.g. Tihen 1960; Martins 1972; Grandison 1981), karyology (Bogart 1972), albumin cross reactions (Maxson 1984) and molecular sequence data (Graybeal 1997). The problems of these studies were multifaceted and what was particularly evident was lack of sufficient sampling of taxa, which more recent studies have begun to address. The more recent studies of bufonid phylogeny have in part resolved some of the outstanding phylogenetic uncertainties, revealing paraphyly of what was formerly considered the global genus '*Bufo*' (Graybeal 1997; Frost et al. 2006; Pramuk et al. 2008; Van Bocxlaer et al. 2010; Pyron and Wiens 2011), with many new generic names given to '*Bufo*' clades found in specific geographic areas such as *Poyntonophrynus*, *Vandijkophrynus* and *Amietophrynus* (Frost et al. 2006). However, as sampling was still lacking in many regions, especially in Africa, a full understanding of the bufonid radiation has so far proved to be elusive.

The published phylogeny that most resembles ours in terms of taxonomic focus and sampling of African species is that of Van Bocxlaer et al. (2010), yet we recovered differing intergeneric relationships. Van Bocxlaer et al. (2010) also recover a paraphyly of African genera, but instead of two clades as in our study, the *Schismaderma-Nimbaphrynoides-Didynamipus-Churamiti-Nectophrynoides* clade is recovered as a third, separate clade in their study. Crucial deeper nodes in their tree are not well supported however. In fact, the only clade that is consistently recovered across all major published molecular phylogenies (Frost et al. 2006; Van Bocxlaer et al. 2010; Pyron and Wiens 2011) including ours, is the *Nectophryne-Wolterstorffina-Werneria* clade. Our phylogeny differs from previous studies however, in that *Wolterstorffina*, not *Werneria* is sister to *Nectophryne*, a relationship that is well-supported and corresponds to the morphological relationships determined by Grandison (1981). Interestingly, this lineage appears to be most closely related to *Phrynoidis* and *Pedostibes* (although node support is low), two South East Asian genera that are loosely comparable in habitat preference and life history to at least one of the African genera; *Werneria*, inhabiting

montane or submontane forest and breeding in streams with stream adapted tadpoles (Amiet 1976; Rödel et al. 2004; Inger 2009).

We recover *Schismaderma* as a close relative of *Didynamipus* as did Van Bocxlaer et al. (2010) and we show that *Altiphrynoides* (cf. *osgoodi* and *malcolmi*) and *Nimbaphrynoides* also belong to this clade, two genera that have not been represented in previous molecular phylogenies. The inclusion of *Altiphrynoides* cf. *osgoodi* in our phylogeny must be highlighted as this species was formerly a monotypic genus (e.g. Largen 2001 see also Online Appendix 13 for further details). The recovered relationship of ((*Didynamipus*, *Nimbaphrynoides*), *Altiphrynoides*) again corresponds to what Grandison (1981) recovered in part of her tree based on morphological characters. Finally, in our phylogeny, *Vandijkophrynus* is not a member of the *Poyntonophrynus-Mertensophryne-Capensibufo* clade but is recovered as sister to *Amietophrynus* and "*Bufo*" *pentoni* instead, with better node support.

The phylogeny presented here is the most complete representation of African species of bufonids to date with greatly improved node support compared to previous phylogenies, yet a number of challenges remain. Firstly, the positioning of *Laurentophryne*, the only unsampled African genus not represented in our phylogeny. Secondly, *Poyntonophrynus* and *Mertensophryne* require more intensive surveying in specific geographic areas, despite the many additions made in this study already. Thirdly, the phylogenetic position of the secondary African radiation (SAR clade) remains unclear and more extensive sampling of Eurasian taxa is therefore needed. This includes species that are believed to belong to Eurasian clades, but occur in Africa such as *Duttaphrynus dodsoni* and *Barbarophryne brongersmai*.

No Ecological Opportunity on a Continental Scale?

The first radiation of bufonids to colonize Africa originated around 29.4 Ma, which was then followed by a second radiation around 21.7 Ma. The first radiation experienced a more or less constant rate of net diversification with estimated rates ranging from 0.113 to 0.164 lineages per Myr and no indication of a slowdown in rates. This estimate is considerably lower than the rates for classic examples of explosive radiations (>0.56 for Hawaiian silverswords; Baldwin and Sanderson 1998; ~0.36 for Lake Tanganyika cichlids; Day et al. 2008), but are comparable to rates estimated for continental radiations of a similar size and age (~0.16 for Neotropical ovenbirds and woodcreepers; Derryberry et al. 2011; 0.101-0.11 for African

catfish; Day et al. 2013). There is no significant lineage-specific variation in rates, neither is there a shift in diversification rate related to changes in reproductive modes. On the contrary, the disparity of the examined life history traits of clutch, egg and body size appears to be partitioned rapidly and early in the evolutionary history of this clade, deviating significantly from a Brownian Motion model of a constant accumulation of variance. In summary, the data suggest that despite their range-expansion abilities (Van Bocxlaer et al. 2010), African bufonids are unlikely to have experienced a period of rapid lineage expansion followed by a subsequent slowdown as expected under an EO model, although there is some indication that reproductive investment strategy partitioning occurred early on in their history.

Studies testing the EO hypothesis have predominantly focused on young lineages restricted to small, isolated areas. Comparatively fewer studies have focused on continent-wide radiations and recent studies on Neotropical ovenbirds and woodceepers (Derryberry et al. 2011), African catfish (Day et al. 2013), and African muroid rodents (Schenk et al. 2013), which parallel our study both in geographic and geological time scale, have recovered similar constant-rate patterns. Thus, there is a growing body of evidence to suggest that a generalized EO model may not be the norm for continental-scale colonization events or alternatively, that current methods do not adequately model the complex histories of such systems. The constant and homogenous lineage accumulation of bufonids, but the early partitioning of life history allows for interesting discussion of the processes that may have governed speciation in Africa and here we propose a number of explanations for these patterns.

Missed opportunity.— Simpson emphasized that opportunity alone may not be sufficient to promote invasion of adaptive zones if an evolutionary lineage is constrained or unable to 'take advantage' of evolutionary opportunities (Simpson 1953; Schluter 2000). Yoder et al. (2010) outline why some radiations fail to be explosive following ecological opportunity and highlight that the principle of evolution following 'genetic lines of least resistance' (Schluter 1996) may impede the exploitation of new habitats or niche space. Although this cannot be ruled out, there is little evidence to suggest this may be the case for toads. The ability of bufonids to colonize new habitats is well documented (Blair 1972; Van Bocxlaer et al. 2010) and the phenotypic and life history variation in this family is extensive. Bufonids are represented all across Africa and in all major biomes with specific lineages having deviated greatly from their likely ancestral *Bufo*-like form (e.g. *Nectophrynoides*, lecithotrophic viviparous dwarf toads restricted to moist montane forest habitats).

A further consideration to make is that bufonids were possibly one of the last major amphibian radiations to have become established in sub-Saharan Africa and so niches may not have been vacant – and therefore there was limited EO. All African amphibian families are relatively old, with most endemic to Africa (Andreone et al. 2008), and molecular (Cannatella and de Sá 1993; Duellman 1993; Vences et al. 2003; Van Bocxlaer et al. 2006; Roelants et al. 2007; Barej et al. 2014) and fossil (Duellman 1999) data support a long history of assemblages on the continent. Although the extent of niche overlap between bufonids and other anurans is debatable, some form of competition for resources is likely to have occurred. For arid-adapted bufonids, this includes competition with species such as *Tomopterna* and *Pyxicephalus* among others (e.g. tadpoles of *Schismaderma* co-occur in mixed swarms with *Pyxicephalus* tadpoles; Channing 2001). Equally, terrestrially breeding bufonids (e.g. *Nectophrynoides*) share humid forest habitats with other anurans with derived breeding strategies such as direct developing *Arthroleptis* (Müller et al. 2013). The co-occurrence of species that would have competed with bufonids therefore questions whether EO fully existed for colonizing bufonids. Interestingly, although EO might have been limited – the relative success of bufonids, as measured in species diversity, seems to be high. For example, for the (in some respects) ecologically similar Ranidae and Dicroglossidae that also colonized Africa more or less at the same time as bufonids (ca. 33 Ma for *Hylarana* and ca. 28 Ma for *Hoplobatrachus*; Alam et al. 2008; Wiens et al. 2009), current species estimates are substantially lower (*Hoplobatrachus* [N=1, but potentially slightly more (Bogart and Tandy 1976)], and *Hylarana* [N=11]). These differences highlight that although African bufonids have lower estimated diversification rates, comparably they were not unsuccessful.

No saturation.—A key signature of the EO hypothesis is that as initially vacant niche space reaches saturation, diversification slows down in a density dependent fashion (Nee et al. 1992; Rabosky 2009a). The two tested density dependent models were always a worse fit than at least one of the constant rate models for both the full and truncated GMYC tree. The DS tree favoured both density dependent models over the constant rate models, however as discussed above, this is likely to be an artificial pattern resulting from the undersampling of recent (species-level) lineages. An explanation for a lack in density dependent declines could be that ecological limits for diversity may not easily be reached if an area is large (Kisel et al. 2011) or dispersal ability is high (Fritz et al. 2011). With an area of approximately 30 million km^2, the potential carrying capacity dictated by the species-area relationship alone

(MacArthur and Wilson 1967; Lomolino 2000) is exceedingly high and African toads might simply not be old enough to have surpassed the initial phase of lineage accumulation. Similarly, a continuous colonization of new areas across the continent, or a change in availability of suitable habitat due to climatic or geological fluctuations over the last 25 Myr may also have resulted in a succession of multiple ecological opportunities through time. As opposed to a single period of diversification, bufonids may thus have experienced a chain of such opportunities that have sustained the observed constant lineage accumulation. The dynamic formation of archipelagos in the Sunda shelf for example may have presented Asian shrews (*Crocidura*) with multiple, successive ecological opportunities which has maintained a similar pattern of consistent diversification rates over time (Esselstyn et al. 2009).

Africa as the odd man out.—The depauperate species richness, the unusually large distributions of species and the absence of certain radiations of flora all together when compared to South East Asia and South America has lead Richards (1973) to dub Africa as the 'odd man out'. Least in terms of continent-wide species richness, the same can be said for amphibians (Duellman 1993). Richards (1973) and his successors (Parmentier et al. 2007) have focused on climate as a key explanatory factor. Although tectonic movements continued to rearrange most major landmasses long into the Cenozoic, the African continent has drifted relatively little during this time and its current position is not far from the continent's location in the Cretaceous (Livingstone 1993). Regardless, Africa has experienced drastic climatic oscillations in the last 50-60 Myr as well as the reformation of major lakes and rivers, changing extent of the Sahara (e.g. Livingstone 1993) and shifts in vegetation patterns (e.g. Hamilton 1982). Perhaps most importantly for amphibians, Africa is, and most likely always has been, much drier than South America and South East Asia (Richards 1973; Livingstone 1993). Africa extends considerably farther north than South America and rainfall is governed by monsoonal winds from the Atlantic and Central Asia, both of which were weaker during ice ages, leading to severe droughts and the retraction of moist tropical forests (Flenley 1979; Livingstone 1993). For amphibians, and even dry adapted bufonids, Africa may therefore not have presented long-term ecological opportunities to begin with and the slow, constant increase in diversification is a result of varying, through time and space, niches.

Similarly, the geography of Africa may by less favourable for cladogenesis. Africa has fewer higher mountain ranges and peaks than other continents but has a proportionally higher overall altitude (McCarthy et al. 2005). There are thus few steep elevation gradients, which

have been show to stimulate speciation (e.g. Schneider et al. 1999; Schilthuizen 2000). Some indirect evidence for this comes from the fact that some of the most species rich areas of Africa are the ecological heterogeneous montane regions Cameroon, the Eastern Arc Mountains and the Ethiopian Highlands (Andreone et al. 2008) where such steep gradients do exist.

Loss of signal due to high rates of extinction.—The disparity of egg and clutch size through time shows an early partitioning of traits. Such a pattern is generally interpreted as a rapid segregation into different reproduction ecotypes in correspondence with the EO theory (Schluter 2000). This goes against the constant rates of diversification estimated for African bufonids, which could be an indication that signatures of the expected diversity-dependent lineage growth curve have been eroded by high rates of extinction (Rabosky and Lovette 2008b). Although we included models that try to fit varying extinction rates through time, estimating this parameter from phylogenies is problematic (Rabosky 2009b) and both γ and the MCCR test are known to be conservative with respect to extinction and have high type II errors (Pybus and Harvey 2000). A number of the models tested in this study return extinction rate estimates close to zero, a result that seems unlikely given the time span of ~30 Myr and the climatic oscillations during this time. A discordance between diversification rates and phenotypic disparity has also been observed in cetaceans (Slater et al. 2010) where the fossil record seemingly contradicts the estimated low extinction rates. Without a fossil record for African bufonids to speak of, direct evidence for an underestimation for extinction rates is lacking, but Raven and Axelrod (1974) suggest that the low species richness in angiosperms of Africa compared to South America are due to high extinction rates that occurred during the Tertiary and Quaternary, a history that if shaped by climate, might have been similar in amphibians.

Conclusion

Bufonids are renowned as one of the few amphibian radiations that has achieved near global diversification, with peaks in diversification rates during dispersal periods to new continents by the *Bufo*-like phenotype. Yet upon arriving in Africa, diversification rates are not exceptionally high and appear to have been constant over time, showing no early-burst as might be expected under an Ecological Opportunity model. This could be due to a number of factors pertaining to the immense geographic scale the radiation inhabits, the homogeneity of

environments with few areas of steep environmental gradients where speciation may occur, the accuracy of parameter estimates due to the long time scale over which diversification is estimated, the current, arid climatic conditions that are less suitable for amphibians and the past climatic oscillations that may have resulted in a succession of intermediate ecological opportunities. Although it remains elusive which of these processes has contributed most to shaping the diversity pattern of this continental radiation, this study adds to a growing list of cases of constant-rate, continent-wide diversification.

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Chapter IV

Evolution of Viviparity in African Anurans

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The Evolution of Viviparity in African Anurans

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Abstract

Viviparity is one of the most prolific examples of convergent evolution in vertebrate history. Although common in amniotes, the evolution of viviparity in amphibians is relatively rare, and in anurans, has evolved in only two families, in *Eleutherodactylidae* and *Bufonidae*. How viviparous lineages of bufonid occurring in Africa are related has remained largely unclear and therefore how this derived form of reproduction has evolved is consequently speculative. Here, we reconstruct the most complete species level molecular phylogeny for African bufonids to date, reconstruct ancestral states of reproductive modes, body size, clutch and egg size, and investigate potential environmental parameters that may have driven the evolution of viviparity. We find that viviparity has evolved twice, but from an ancestor that was preconditioned for viviparity by having a reduced body and clutch size. We also find that steep slope, a lack of standing water bodies and to some degree forest cover are important environmental variables for viviparous species and so viviparity may have evolved as a consequence of a lack of suitable aquatic breeding sites.

Introduction

Viviparity, the retention of eggs in the oviduct and the giving birth to live young, has evolved independently on multiple occasions in vertebrates and is considered one of the most impressive cases of convergent evolution in vertebrate history (Blackburn 2014). The reproductive strategy of viviparity is asymmetrically distributed across the tree of life however, with at least 115 occurrences in squamate reptiles and 22 in fish (9 times in chondrichthyes and 13 times osteichthyes), but only one in mammals (though this transition comprises the major therian radiation) and none in birds (Blackburn 1992; 2014). In amphibians, viviparity is rare, but has nonetheless evolved at least four times in caecilians (Gower et al. 2008; San Mauro et al. 2014), once in salamanders (Wells 2007; Buckley et al. 2007), although unconfirmed records indicate viviparity to be potentially more widespread (see Raffaëlli 2007), and at least twice in anurans: once in Eleutherodactylidae and at least once in Bufonidae (Wells 2007). Why and how viviparity evolved remains elusive and there is unlikely to be one single selective regime under which viviparity has evolved (Blackburn 2014).

It is commonly accepted that reproduction via aquatic oviparity and larval development is the plesiomorphic mode of reproduction of anurans, with viviparity being a highly derived

form of reproduction (Duellman and Trueb 1994; Wells 2007; Van Bocxlaer et al. 2010). A gradual model of evolution from oviparity to viviparity, where a series of semi-terrestrial and terrestrial breeding strategies represent intermediate steps has been proposed and largely accepted (Duellman and Trueb 1994), however there is evidence to suggest that at least the evolution of direct development (thought to be the most direct precursor to viviparity in anurans; Duellman and Trueb 1994) may not have required such transitional modes (Gomez-Mestre et al. 2012). Along with transitions to terrestrial breeding habits, a number of other adaptations are though to be necessary for viviparity to evolve. These include internal fertilization (Wake 1980), egg retention and elongated gestation periods (Wake 1993), small body size (Salthe and Duellman 1973; Wake 1978; Clarke 1996), reduced clutch size, increased egg size (Grandison 1978; Wake 1980) and increased parental care (Wake 1978). Physiological distinctions must also be made between the types of viviparity practiced by anurans. *Nectophrynoides* spp. and a single species of *Eleutherodactylus* (*E. jasperi*) undergo lecithotrophic viviparity, where nutrition to sustain the development of the young is derived solely from yolk provisions. *Nimbaphrynoides occidentalis* on the other hand practices matrotrophic viviparity, meaning the development of the young is sustained through supplements from the mother. It has been suggested that matrotrophic viviparity is derived from lecithotrophic viviparity in anurans (Xavier 1977; Blackburn 2006) as well as in salamanders (Wells 2007), but this evolutionary transition may be different in caecilians where unique reproductive strategies such as maternal dermatophagy have been suggested as potential precursors (Kupfer et al. 2006; Kouete et al. 2012; Wilkinson et al. 2013; San Mauro et al. 2014).

Evolutionary transitions from having free-living aquatic larvae to direct development and viviparity likely facilitated colonization of terrestrial environments. This change removed the previously stringent dependency on water bodies for reproduction and so hypotheses on the causal mechanism that drove the evolution of viviparity in amphibians have largely focused on abiotic factors. For example, in *Salamandra salamandra*, glaciation events during the Pleistocene are thought to have fragmented populations with some being restricted to areas of kastic limestone sediments where a lack of standing bodies of water may have selected for retention of eggs and developing embryos in the oviduct (García-París et al. 2003). In caecilians, it has been proposed that viviparity as a means for controlling ontochronological events is favoured in areas where climate fluctuates strongly so that giving birth can be timed

more effectively with the onset of rains (Giri et al. 2004; Gower et al. 2008). In anurans, two out of the three viviparous genera are high altitude inhabitants, potentially subjected to extreme climatic fluctuations and therefore similar hypotheses have been adopted (Wake 1980), but never empirically tested. Furthermore, if terrestrial egg deposition was an evolutionary precursor, tropical montane forests may have played a crucial role (Müller et al. 2013). Goin and Goin (1962) proposed that terrestrial forms of breeding may have been selected for in steep montane areas where standing bodies of water are scarce, and flow rates of streams are high. This hypothesis was refined by Poynton (1964) who suggested that high humidity and a dense undergrowth is key for permitting eggs to be laid on land without desiccating.

These sequences and scenarios for the evolution of viviparity in anurans remains largely speculative, primarily due to the uncertainty in phylogenetic relationships (Wake 1980). This is particularly the case for the two bufonid genera *Nectophrynoides* and *Nimbaphrynoides*, both occurring in Africa, but not comprising a monophyletic unit (Liedtke et al. submitted). Here, we reconstruct the phylogeny of African bufonids and explore character evolution and environmental parameters to further our understanding of how and under which conditions viviparity evolved in these lineages. Specifically we investigate whether environmental factors, such as forest habitat, surface gradient (slope), the availability of standing water bodies, humidity, and climatic fluctuations can explain the geographic distribution of these species. Furthermore, we reconstruct ancestral states for reproductive modes, to test whether other terrestrial or semi-terrestrial modes were likely precursors and we analyse the changes in body size, clutch size and egg size over time to establish whether shifts in these traits were indeed important prerequisites for viviparity to evolve.

Materials and Methods

Phylogenetic reconstruction

A time calibrated phylogeny of African bufonids with a selection of Eurasian and New World outgroups was generated for this study. The phylogenetic inference procedure is documented in detail in Appendix 2 and the sequence data comprised ~3439 base pairs across five nuclear and mitochondrial markers. Sequences were obtained from a previous study (Liedtke et al. submitted), with the exception of data for *Barbarophryne brongersmai* and *Poyntonophrynus lughensis*, which were generated *de novo* for this study. A single representative per described

species was included, totalling 116 species, of which 70 are African taxa. This covers ca. 70% of all described African species and all genera but *Laurentophryne*, a monotypic genus whose population status is unknown (IUCN SSC Amphibian Specialist Group 2013).

Joint posterior distribution of model parameters were estimated using Bayesian MCMC searches in BEAST v1.8.0 (Drummond et al. 2012). Molecular clock models were estimated separately for mitochondrial and nuclear markers using uncorrelated lognormal relaxed clock (ucld) priors (Drummond et al. 2006), a birth-death (Gernhard 2008) speciation tree priors was used and four fossil calibration constraints were implemented. A total of eight MCMC searches with 100 million generations, sampling every 5000th iterations were conducted to assess convergence and stability of parameters. Convergence, prior signal and effective sample sizes of parameters in the log files were visually inspected using Tracer (Rambaut and Drummond 2007), and AWTY (Wilgenbusch et al. 2004). Multiple tree files from the independent searches were combined using LogCombiner v1.8.0 (Rambaut and Drummond 2012a), and resampled at a lower frequency to obtain ca. 20,000 post-burning posterior trees. These trees were summarized as a maximum clade credibility tree (MCC tree) with median node heights and no limit on the posterior probability using TreeAnnotator v1.8.0 (Rambaut and Drummond 2012b).

Occurrence records and environmental parameters

Occurrence data for all African bufonid species included in the phylogeny were compiled from the open access databases of Global Biodiversity Information Facility (GBIF, www.gbif.org, accessed February 2013) and HerpNet (www.herpnet.org, access February 2013) and from non-open access sources including the Atlas and Red Book of South African Amphibians (Minter et al. 2004), records from The Natural History Museum, London (UK), South African National Biodiversity Institute (South Africa), Trento Museum of Natural History (Italy) and the Museum für Naturkunde, Berlin (Germany) and published, non-digitized sources (Joger 1981; Lanza 1981; Poynton and Broadley 1988; Largen 1997; Poynton and Clarke 1999; Largen 2001; Rödel et al. 2004; Din 2006; Weinberg 2008; Sandberger et al. 2010; Vasconcelos et al. 2010; Mercurio 2011; Barej et al. 2011; Hirschfeld et al. 2012). Duplicate records across data sources and multiple records per species from the same latitude and longitude were removed. Anecdotal records were geo-referenced where possible with the help of GeoNames (http://www.geonames.org/, Unxos GmbH,

Switzerland), and Google Earth (http://www.google.com/earth/, Google Inc., USA) was used to identify descriptive landscape features and to restrict locations to verbatim elevation references. Anecdotal records that could not accurately be assigned to a taxon or location were not included. Occurrence records per species were vetted by visual inspection aided by overlaying IUCN red list v2013.2 range maps (www.iucnredlist.org, IUCN, Switzerland) in ArcGIS v10.0 (ESRI, USA) and questionable records were removed.

Measures for forest cover, slope and topographic wetness, temperature and precipitation data per occurrence record were extracted from Global Information System layers at the maximum resolution available using ArcGIS. Forest cover, as a percentage of woody vegetation per grid cell, was measured using the Terra MODerate-resolution Imaging Spectroradiometer (MODIS) Vegetation Continuous Field layer for woody vegetation (2010 dataset, 250m resolution; www.landcover.org, University of Maryland, USA). Slope was calculated in degrees from a digital elevation model (250 m resolution; Jarvis et al. 2008) and topographic wetness information was obtained from the Topographic Wetness Index (TWI) layer of the African Soil Information Service (AfSIS; http://www.africasoils.net/; at 1 km resolution). TWI is calculated by combining effective drainage area information with slope (Beven and Kirkby 1979) and gives a measure of soil moisture based on where contributing runoff is high and slope is low. Climate information was extracted from the WorldClim database and derived BioClim layers (1 km resolution; www.worldclim.org, University of California, Berkeley, USA). As measures of climatic fluctuations, temperature and precipitation seasonality (BioClim layers BIO4 and BIO15) layers were used. As a measure of humidity, the aridity index 'Q' outlined in Tieleman (2003) was adopted, using mean annual precipitation (BIO12), and maximum and minimum temperature records (BIO5 and BIO6) so that $Q = \frac{BIO12}{(BIO5+BIO6)(BIO5-BIO6)} * 1000$. Median measurements per species are given in Table 1.

Life-history traits

We assigned six discrete reproductive modes to species of African bufonids: (1) aquatic oviparity with tadpoles developing in open bodies of water, including both permanent and temporary ponds, swamps, large puddles and ditches and large, slow flowing streams, (2) aquatic oviparity with tadpoles developing in micro water bodies such as water-filled tree holes, snail shells, or hollow coconuts, (3) aquatic oviparity with tadpoles developing in

torrential streams, (4) terrestrial oviparity with either complete or partial larval development undergone in the egg, (5) lecithotrophic viviparity defined as the retention of eggs in the oviduct of females where complete development is undertaken by the larva that are nourished only by the yolk of the ovum, and (6) matrotrophic viviparity where the embryonic development is supplemented by additional nutrients provided by the mother. Information on breeding biology was compiled from the IUCN red list database (www.iucnredlist.org, accessed in October 2013). Two important species for which breeding biology has not yet been confirmed are *Didynamipus sjostedti* and *Churamiti maridadi*. Grandison (1981) suggested that, based on its affinity to *Nimbaphrynoides* and extremely low complement of large eggs, *D. sjostedti* is most likely direct developing, a view also shared by Gartshore (1984). A recent report of a terrestrial clutch (Gonwouo et al. 2013) indeed suggests that this species deposits terrestrial eggs that possibly undergo direct development and therefore has been coded as such. *Churamiti maridadi*, despite its phylogenetic affinity with *Nectophrynoides* has been coded as breeding in open water bodies, based on the clutch characteristics described in Channing and Stanley (2002) and findings in this study.

Information for female body size (in snout-vent length), clutch size (as number of eggs per clutch) and egg size (diameter of egg without gelatinous layer in mm) was taken from Liedtke et al. (2014) with novel data for *Churamiti maridadi* collected for this study. As in Liedtke et al. (2014), maximum values per species were used as this produces the largest coherent dataset.

Environmental associations with reproductive modes

To visualize whether species practicing different reproductive modes occupy unique areas in environmental space and whether these are phylogenetically conserved, we projected the phylogeny onto the first two components of a phylogenetic principal component analysis (pPCA; Revell 2009) of median values for forest cover, slope, topographic wetness and humidity (Q) per species. Precipitation and temperature seasonality were not included as preliminary investigations rendered these to be the least informative. The components of the pPCA were subjected to a phylogenetic MANOVA (using the Pillai test statistic and 999 simulations in the R package geiger; Harmon et al. 2008) to test whether observed environmental preferences are significantly different for species of different reproductive modes. Each environmental parameter was also tested separately using phylogenetic

ANOVAs, including a posthoc test with Holm's adjustment method for multiple testing (Holm 1979) and 999 simulations using the phytools R package (Revell 2012). For all tests, *Nimbaphrynoides occidentalis* and therefore the category of matrotrophic viviparity was excluded due to a sample size of one.

TABLE 1. Median values for environmental variables per species used for comparative analysis

Species	BIO4	BIO15	Q	TWI	Slope (°)	Tree cover (%)
Altiphrynoides malcolmi	650	45	29.516	12	9.422	11
Altiphrynoides osgoodi	730	49	24.154	12	5.724	10
Amietophrynus brauni	1717	59	22.534	11.5	7.974	58.5
Amietophrynus camerunensis	727	56	28.571	13	1.382	39
Amietophrynus channingi	486.5	37	23.817	14	1.138	46.5
Amietophrynus garmani	2989.5	79	7.141	14	1.130	5
Amietophrynus gracilipes	792	59	29.196	13	1.740	37
Amietophrynus gutturalis	3108.5	69	10.679	13	1.830	8
Amietophrynus kisoloensis	286.5	38	29.944	12	4.811	32.5
Amietophrynus latifrons	914	68.5	46.959	13.5	3.673	47
Amietophrynus lemairii	1572	92	13.025	14	1.218	12
Amietophrynus maculatus	1183	70	17.421	13	1.633	13
Amietophrynus mauritanicus	5627	65	4.557	13	2.152	2
Amietophrynus pantherinus	2809	62	11.789	14	0.926	16
Amietophrynus pardalis	2633	19	10.374	13	2.326	13.5
Amietophrynus poweri	4918	76	4.133	14	0.581	2
Amietophrynus rangeri	3396	57	9.945	13	2.629	8
Amietophrynus regularis	1136	72	15.163	13	1.112	9
Amietophrynus steindachneri	1137	76	13.725	15	0.407	9
Amietophrynus superciliaris	861	61	32.848	12.5	2.056	30
Amietophrynus taiensis	899	55	28.246	14	1.499	39
Amietophrynus togoensis	933	63	24.492	13	1.663	39
Amietophrynus tuberosus	777	59	45.233	14	1.069	32
Amietophrynus villiersi	961	71	31.521	12	3.173	18
Amietophrynus xeros	2061.5	128	3.798	14	0.480	1
Bufo pentoni	2145.5	140	5.552	14	0.427	1.5
Capensibufo rosei	2962	56	15.587	12	5.459	23
Capensibufo tradouwi	3961	60	6.479	12	15.304	7
Churamiti maridadi	692	55.5	60.270	11	8.837	63
Didynamipus sjostedti	817.5	64.5	43.420	12.5	3.678	38
Mertensophryne anotis	1924	87	19.906	11.5	3.961	71.5
Mertensophryne howelli	2503	83	15.137	14	0.528	43
Mertensophryne lindneri	1472	91	15.376	13	1.051	6
Mertensophryne loveridgei	1645	91	22.286	13	1.723	69
Mertensophryne micranotis	1461.5	66	16.948	13	2.690	39.5
Mertensophryne taitana	1531.5	96	12.939	13	1.653	6.5
Mertensophryne usambarae	1666	92	20.108	12	3.548	75
Mertensophryne uzunguensis	1462	94.5	21.108	11	4.088	36
Nectophryne afra	810	62	44.904	13	1.850	37
Nectophryne batesii	819	58	45.376	12.5	3.168	23.5

Table 1 continued						
Nectophrynoides asperginis	1577.5	93	22.152	15	17.308	50.5
Nectophrynoides frontierei	1735	57	31.303	12	13.397	81
Nectophrynoides laticeps	1483	85	16.061	11	8.837	63
Nectophrynoides minutus	1574.5	85	18.167	11	12.435	60.5
Nectophrynoides paulae	1483	85	16.061	11	8.837	63
Nectophrynoides poyntoni	1527	94	21.794	10	28.854	58.5
Nectophrynoides pseudotornieri	1765	74	22.504	12	17.803	70
Nectophrynoides tornieri	1702.5	57	21.036	11	11.955	63
Nectophrynoides vestergaardi	1797	66.5	17.300	11	10.482	67
Nectophrynoides viviparus	1482	90	23.446	11	12.703	58
Nectophrynoides wendyae	1543	91	22.650	11.5	2.892	59.5
Nimbaphrynoides occidentalis	962	61	29.150	10	14.742	12
Poyntonophrynus damaranus	2241	114	2.500	14	1.786	1
Poyntonophrynus dombensis	2082	109	4.234	13	0.888	2
Poyntonophrynus fenoulheti	3427	78	7.258	13	1.802	6
Poyntonophrynus hoeschi	2521	122	1.539	13	3.415	0
Poyntonophrynus lughensis	882.5	90.5	4.075	14	0.394	1
Schismaderma carens	3241	74	8.574	13	1.758	8
Vandijkophrynus amatolicus	3011	41	18.928	13	3.596	11
Vandijkophrynus angusticeps	3439	61	7.296	14	1.341	7
Vandijkophrynus gariepensis	4218	47	3.965	13	1.914	3
Vandijkophrynus inyangae	2539	92	34.141	11	7.496	20
Vandijkophrynus robinsoni	3799	56	1.944	14	2.884	0
Werneria bambutensis	937.5	63.5	51.350	14	6.454	55.5
Werneria mertensiana	913.5	70.5	44.234	12	9.210	40
Werneria submontana	933	71	51.543	12.5	14.819	29
Werneria tandyi	913.5	68	47.044	12	5.960	42.5
Wolterstorffina chirioi	924	65	49.571	10	10.342	19
Wolterstorffina mirei	953	66.5	44.868	11	11.550	31.5
Wolterstorffina parvipalmata	897	67	47.124	12	9.597	42.5

Ancestral state reconstruction of reproductive modes

Ancestral states of discrete reproductive modes were reconstructed using three methods: A Maximum likelihood and a revers-jump MCMC method implemented in BayesTraits v2.0 (Pagel and Meade 2013) and a stochastic character mapping method (Huelsenbeck et al. 2003) with the R package phytools. Due to the uncertainty of deep nodes in the phylogeny, ancestral state reconstructions were restricted to the clade of interest (*Schismaderma, Nimbaphrynoides, Nectophrynoides, Altiphrynoides* and *Didynamipus*; see results) which is well supported, by pruning all other taxa from the MCC tree and a subset of 1000 post-burnin posterior trees of the BEAST inference.

Phylogenetic uncertainty was accounted for in BayesTraits by sampling trees from the posterior distribution and by using the AddMRCA method, which estimates state probabilities at the most recent common ancestor (MRCA) of a given set of taxa, instead of at a specific node in the tree. Only state probabilities for the MRCA of *Nectophrynoides-Churamiti*, *Altiphrynoides* spp, *Nimbaphrynoides-Didynamipus* and the MRCA of the entire clade were estimated, as these were the only well supported nodes of interest. For the likelihood approach, state probabilities were estimated for each posterior tree of the post-burnin subsample with 100 attempts per tree. For the MCMC analysis, a hyper exponential prior drawn from a uniform 0-1 distribution was set and the chain sampling the posterior distribution was run for 100 million iterations at a sampling rate of 10,000, discarding the first 10 million iterations as burnin. MCMC diagnostics in the form of parameter trace plots, effective sample size calculations and autocorrelation plots were carried out using the coda package v0.16-1 (Plummer et al. 2006) in R.

For the stochastic character mapping, a continuous-time reversible Markov model for the evolution of the reproductive modes was fitted to the data and then used to simulate stochastic character histories (Bollback 2006). We performed 999 simulations using the MCC tree with an equal rates empirical transition matrix used for fitting the Markov model and equal root node prior probabilities. Posterior probabilities at each node were then summarized as pie charts.

Ancestral state reconstruction of life history traits

The evolutionary trajectories of three continuous characters were investigated: body size, clutch size and egg size. These were visualized by plotting a 'traitgram' (Ackerly 2009) with the phytools package in R. Ancestral states are estimated for internal nodes using the Maximum Likelihood approach of Schluter et al. (1997), which minimizes the sum of squared changes along branches, assuming trait evolution under Brownian motion. All measurements were log10 transformed and for clutch and egg size, residuals of linear regressions on body size were used to obtain trait values relative to body size.

Results

Phylogenetic inference and relationship of viviparous lineages

The Bayesian inference supports the monophyly of all African genera (Figure 1a), with the exception of *Poyntonophrynus*. *Pontonophrynus lughensis* shows a phylogenetic affinity to *Mertensophryne* with high posterior probability support and we therefore propose that this species be transferred to *Mertensophryne* (*Mertensophryne lughensis* **comb. nov.)** for the monophyly of *Poyntonophrynus* to be upheld. Before the recognition of species status by Loveridge (1932), specimens were classified as *M. taitana* based on their morphological similarities (Loveridge 1932; Largen 2001). The genus '*Poyntonophrynus*' erected by Frost et al. (2006) to accommodate the species of Tandy and Keith's (1972) "*Bufo" vertebralis* group, but the Tandy and Keith expressed doubt about their inclusion of "*Bufo" lugehensis*, an uncertainty that our data confirms. Branch support for inter-generic relationships were relatively low, but the relationships of the African clades and divergence times roughly concur with the phylogeny of Liedtke et al. (submitted) which used a more extensive dataset.

Reproduction via aquatic oviparity with tadpole development in open water is the most common form of reproduction (albeit arguably the broadest category as well), with oviparity and tadpole development in micro water-bodies such as tree-holes or snail shells having evolved at least twice independently, possibly three times: once in *Nectophryne* and potentially twice in *Mertensophryne*. Adaptation of tadpoles to torrential streams appears to be confined to *Werneria*, but *Wolterstorffina parvipalmata* and *W. mirei* are known to breed near fast flowing streams as well. The tadpole habitat is uncertain, although for the former this is thought to be confined to small side-pools (Channing et al. 2012) with tadpoles having been found in a discarded tin can (Mertens 1939). Furthermore, *Wolterstorffina chirioi* has only been recorded from the summit of a single mountain in Cameroon, at altitudes higher than any water body and so it has been proposed that some form of terrestrial reproduction may occur (Boistel and Amiet 2001), yet this remains to be confirmed. It is coded as such for analyses here, but is not discussed in detail.

All confirmed terrestrial and viviparous forms of reproduction are practiced by closely related species, belonging to a well-supported group that comprises *Nectophrynoides*, *Churamiti*, *Altiphrynoides*, *Didynamipus*, *Nimbaphrynoides* and *Schismaderma*. Some internal

nodes are less well supported and especially the arrangement of *Altiphrynoides* (*Nimbaphrynoides*, *Didynamipus*) must remain speculative, but it can be said with certainty

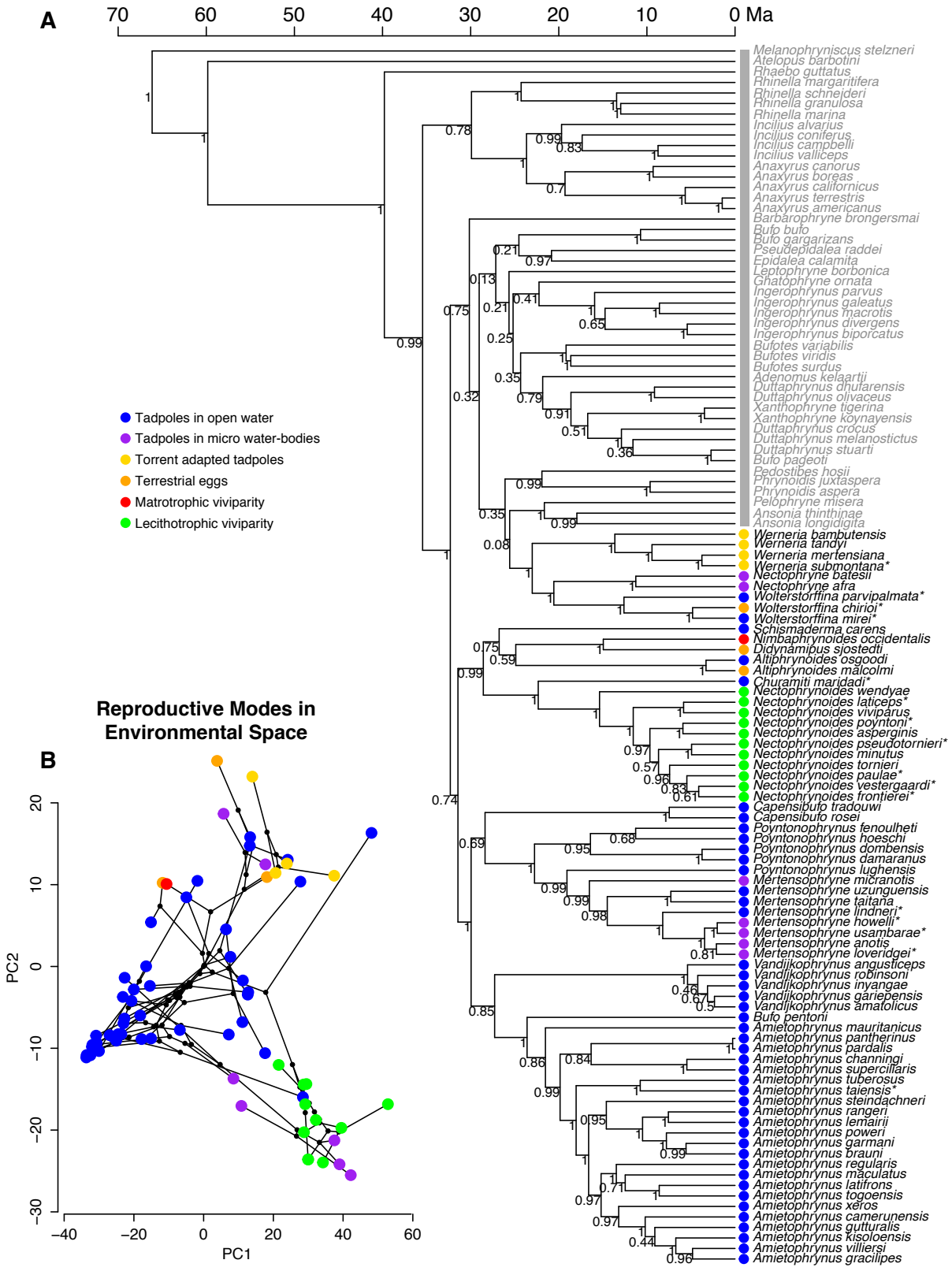

FIGURE 1:a) Maximum Clade Credibility tree from time calibrated Bayesian Inference with posterior

probabilities on branches and reproductive modes (for African taxa) as tip labels. For species, with asterisks, reproductive mode is assumed. B) Phylogenetic Principal component analysis on species medians of four environmental variables; forest cover, surface gradient (slope), topographic wetness and humidity.

that lecithotrophic and matrotrophic viviparous species do not form a clade and neither do terrestrial egg laying species.

One female *Churamiti maridadi* specimen (SVL 57.6) was dissected and the egg mass was removed, counted and egg diameters of three representative eggs were measured. The clutch contained approximately 240, pigmented eggs and the three egg diameters were 1.32, 1.34 and 1.36 mm. Given its body size, this species lays clutches that are too large with eggs that are too small to be either viviparous or direct developing and we hereby concur that this species most likely reproduces via aquatic oviparity with aquatic tadpoles (Channing and Stanley 2002).

Environmental associations with reproductive modes

The pPCA recovers clustering of reproductive modes along environmental axes (Figure 1b), the major contributing variables being tree cover and humidity respectively. There is also phylogenetic clustering (short branch lengths within reproductive mode clusters), which is not surprising given the conserved nature of reproductive modes (Figure 1a) and the strong phylogenetic signal in the data (Appendix 1). The two viviparous lineages do not occupy the same environmental space, neither do the two lineages breeding in micro-water bodies. The phylogenetic MANOVA confirmed a significant difference in environmental space between groups (approx. F=8.220; df=4,64; p=0.004) and plotting each variable separately (Figure 2) revealed that lecithotrophic viviparous species (*Nectophrynoides*) occur in highly forested areas with steep slopes and low topographic wetness. The matrotrophic viviparous *Nimbaphrynoides occidentalis* is also found on steep slopes and areas of low topographic wetness, but unlike *Nectophrynoides*, this species occurs in areas with little forest cover (Figure 2). Temperature and precipitation seasonality were largely uninformative and high humidity separates terrestrial egg laying and torrent adapted tadpole species from the other reproductive modes. The pANOVAs recovered significant differences for forest cover and slope (Table 2).

TABLE 2. Phylogenetic ANOVA results for environmental variables.

Variable	F	p-value
BIO4	2.055	0.806
BIO15	0.779	0.950
Q	6.862	0.282
TWI	5.519	0.394

slope	16.950	0.031
Tree cover	20.963	0.017

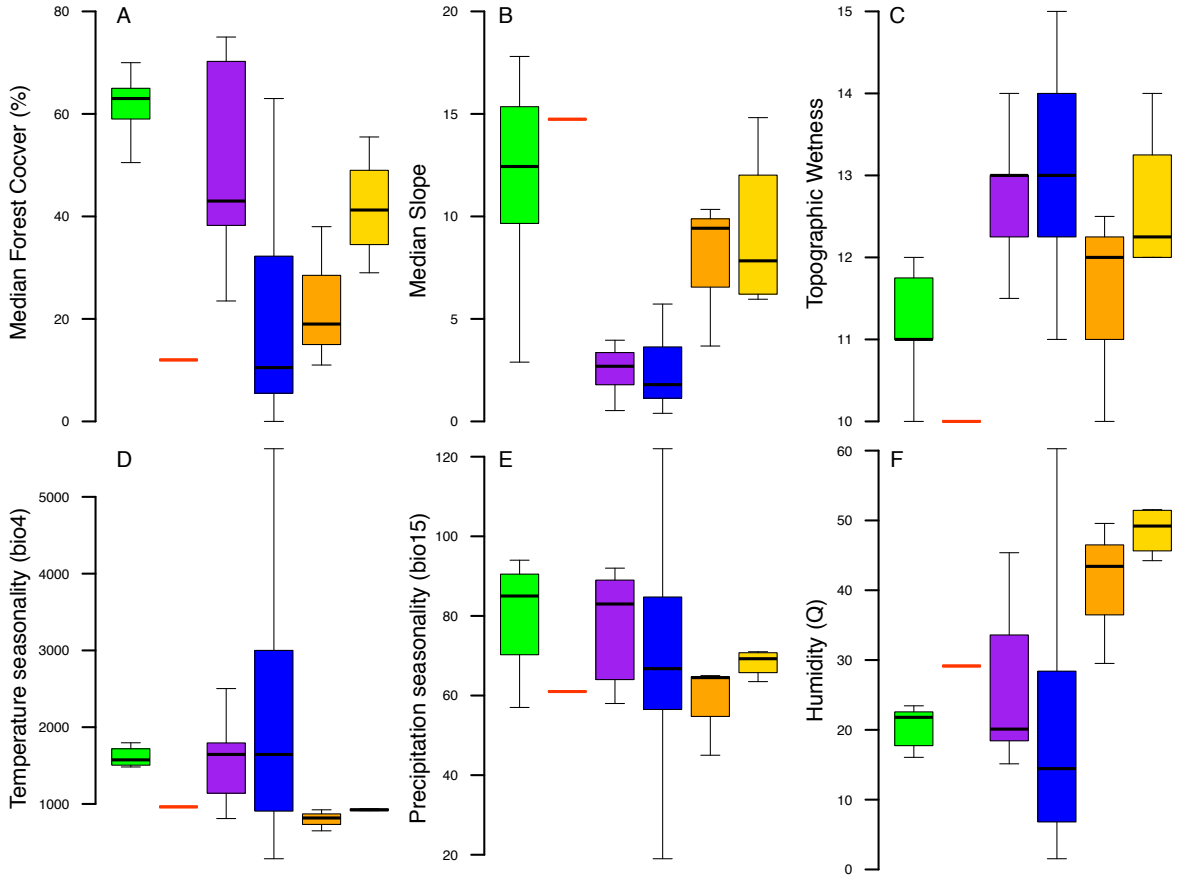

FIGURE 2: Median environmental variables per reproductive mode for a) forest cover, b) surface gradient (slope), c) topographic wetness, d) temperature seasonality, e) precipitation seasonality, f) humidity. Colours represent green: lecithotrophic viviparity, red: matrotrophic viviparity, purple: aquatic oviparity with tadpoles developing in micro water bodies, blue: aquatic oviparity with tadpoles developing in open water bodies, orange: terrestrial oviparity and yellow: aquatic oviparity in streams with torrent adapted tadpoles.

Ancestral state reconstruction of reproductive modes

All three methods show that the reproductive mode of the MRCA of the entire clade of interest is not lecithotrophic viviparity (Figure 3a). The stochastic character mapping (STM) on the consensus tree most frequently recovers aquatic oviparity with free swimming tadpoles as the ancestral state, but the BayesTraits ML (BT-ML) and MCMC (BT-MCMC) analyses, which sampled across the posterior distribution of trees showed equal maximum probability densities for aquatic oviparity, terrestrial egg laying and matrotrophic viviparity (Figure 3a). According to the STM, the *Churamiti-Nectophrynoides* ancestor was likely to practice either aquatic oviparity or lecithotrophic viviparity, but BT-ML and BT-MCMC rule out lecithotrophic viviparity, with the remaining three states showing equal probabilities with BT-ML favouring matrotrophic viviparity by a small margin (Figure 3b). All three

methods rule out lecithotrophic viviparity as the ancestral state for the MRCA of *Nimbaphrynoides-Didynamipus*, but BT-ML recovered matrotrophic viviparity with the highest probability whereas STM recovered terrestrial egg laying as the most probably state (Figure 3c). For the BT-MCMC, the remaining three states all converged on the same probability (Figure 3c). All three methods concur that the MRCA of the two *Altiphrynoides* species was not viviparous, and both BT-MCMC and BT-ML recover aquatic oviparity and terrestrial egg laying as equally probable ancestral states (Figure 3d). STM recovered aquatic oviparity as slightly more probable (Figure 3d).

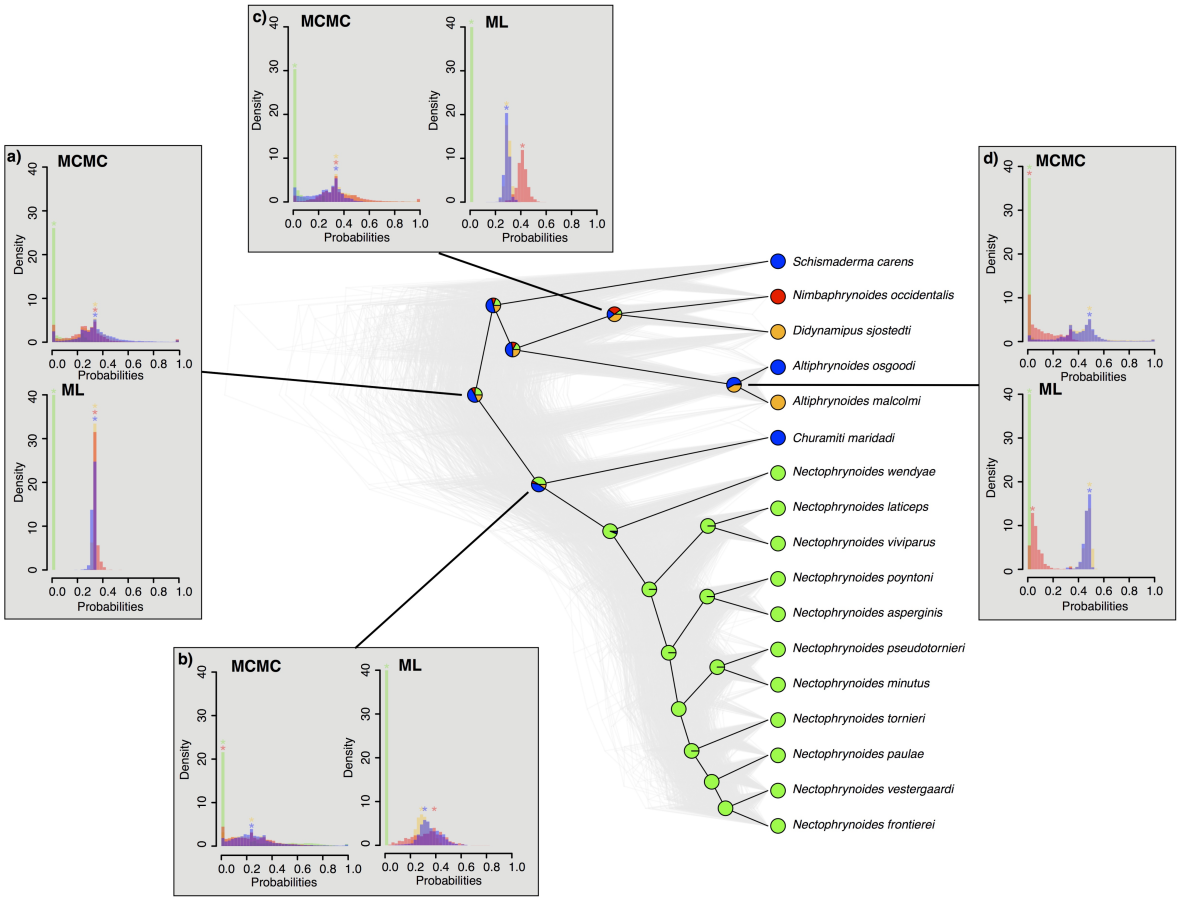

FIGURE 3: Ancestral state reconstructions for reproductive modes using three methods. The pie charts on nodes depict the results of Stochastic Character Mapping on a clade of the MCC tree and bar charts depict the results of Bayesian and Maximum Likelihood reconstructions for selected nodes, carried out in BayesTraits on a subsample of 1000 posterior trees (plotted as topologies in grey).

Ancestral state reconstruction of life history traits

The phenogram shows that viviparous and terrestrial breeding species are derived from small sized ancestors, with snout-vent lengths shorter than the ancestor of the entire group (Figure 4a). Clutch sizes relative to body sizes are more or less evenly spread with no clustering of reproductive modes (Figure 4b). Despite its unusually large body size, *N. viviparus* has a

smaller clutch size than expected given its body size (residual size below 0; Figure 4b). Egg sizes relative to body sizes are also not partitioned by reproductive mode, with *A. malcolmi* showing somewhat larger eggs than expected and *N. occidentalis* showing smaller eggs than expected given their body size.

The two bifurcations dividing viviparous lineages from lineages with different reproductive modes (i.e. *Nectophrynoides* from *Churamiti* and *Nimbaphrynoides* from *Didynamipus*) both show a reduction in body size and a reduction in clutch size, but only the lecithotrophic viviparous lineage shows an increase in egg size.

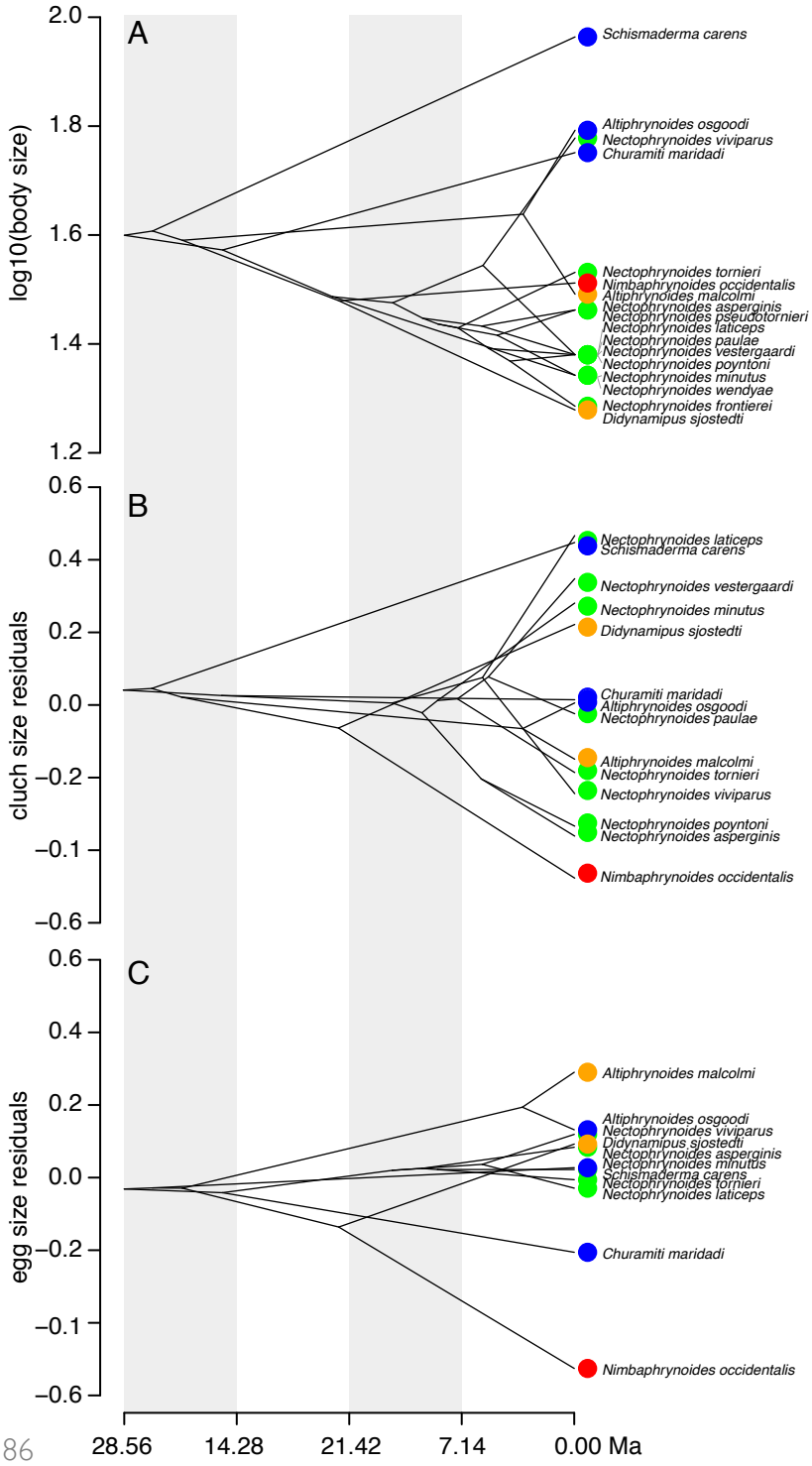

FIGURE 4: Traitgrams using a clade of the MCC tree for a) body size, b) clutch size and c) egg size. Body size is log 10

transformed and clutch and egg sizes are residuals from linear regressions on body size.

Discussion

Viviparity, the development of the embryo inside the mother and the giving birth to live young, is rare in amphibians compared to amniotes and fish (Blackburn 2014). In anurans, viviparity is practiced by only 15 species belonging to three genera, two of which are closely related Bufonidae (*Nectophrynoides* and *Nimbaphrynoides*) and occur in the African Tropics. How viviparous bufonids are related and whether there is a commonality in environmental conditions in which they occur has remained largely speculative (Wake 1978; 1980; Grandison 1981). In this study we reconstructed the most comprehensive species-level phylogeny of African bufonid species to date, measured habitat and climatic variables at collection sites of historical records for all species and reconstructed ancestral states for life history strategies as well as specific traits (body, clutch and egg size) for the clade containing the two viviparous genera.

Previous phylogenetic reconstructions for viviparous toads have been based on external morphology and life history characters (Wake 1980; Grandison 1981; Gauld and Underwood 1986; Graybeal and Cannatella 1995). More recent, large scale molecular phylogenetic reconstructions contained representatives of *Nectophrynoides*, but have not included *Nimbaphrynoides* or other key taxa such as *Altiphrynoides* (Frost et al. 2006; Van Bocxlaer et al. 2010; Pyron and Wiens 2011). The phylogeny in this study and in Liedtke et al. (submitted) are inferred from largely overlapping sequence data and are the first molecular studies to include both viviparous lineages in the same tree. The consensus topology of the viviparous lineages and close relatives largely concurs with that of the morphological tree reconstructed by Grandison (1981) and less so with recent molecular phylogenies (e.g. Pyron and Wiens 2011). Namely, we show that *Didynamipus* is indeed a close relative of *Nimbaphrynoides* and that *Altiphrynoides* is sister to this pair. We confirm that *Schismaderma* also belongs to this group (as already indicated in Van Bocxlaer et al. 2010) and show that other genera of Grandison's '*Nectophryne* line' (*Nectophryne*, *Wolterstorffina*, *Werneria* and *Capensibufo*) are only distantly related. The node support for the *Altiphrynoides* lineage was low however and a substantial proportion of the posterior distribution has this genus as sister to the *Churamiti-Nectophrynoides* group. Expanded genetic sampling will be needed to resolve the topology among these genera.

Our study shows that viviparity evolved twice in African bufonids. Although these two lineages are relatively closely related, they are separated by ca. 29 million years of evolution

and the type of viviparity is fundamentally different (lecithotrophic versus matrotrophic). We show that the clade containing these two lineages has diverged relatively early on in the history of bufonids on the continent, but the origins of viviparity most likely occurred much later, at least 15 million years ago, and approximately at the same time in both lineages. We recovered terrestrial egg laying has a potential precursor to matrotrophic viviparity, but this is less likely to be the case for lecithotrophic viviparity. A reduction in body size in ancestral lines leading to the viviparous clades is evident, especially compared to aquatic breeding conspecifics, but clutch sizes and egg sizes (relative to body sizes) are largely homogenous in this group, which is unexpected because aquatic breeding species tend to have considerably larger clutches and smaller eggs (Liedtke et al. 2014). Two exceptions to this pattern are the small egg sizes of *N. occidentalis* and *C. maridadi*. For *N. occidentalis*, this is due to the reduced yolk contents as a consequence of the matrotrophic nature of the embryo development (Angel and Lamotte 1944), but possible explanations for *C. maridadi*, whose egg size is comparable to large *Amietophrynus* species that lay very large clutches (Liedtke et al. 2014) remain elusive as very little is known about its breeding biology (Channing and Stanley 2002). Despite these anomalies, we can conclude that the ancestor of the entire group laid reduced number of eggs that's were larger and this is therefore the pleisiomorphic state, but body size reduction is a trait that is associated with terrestrial breeding (in *A. malcolmi* and *D. sjostedti*) and viviparity (*N. occidentalis* and *Nectophrynoides* spp.).

Interestingly, lecithotrophic and matrotrophic viviparous species do not show identical habitat preferences, but there were commonalities for some of the tested variables. As hypothesized, viviparous species occur in in areas with steep slopes. Terrestrial breeding species and species with torrent adapted tadpoles show similar habitat preferences, confirming the hypothesis that species must either adapt to torrential stream conditions or reproduce outside of water (Goin and Goin 1962; Campbell and Duellman 2000). Micro water body breeders, along with species breeding in open water bodies do not occur on steep slopes. For micro water body breeders this might be surprising given breeding in tree holes in montane forested habitats would be a potentially suitable alternative to inhospitable, fast flowing streams. Topographic wetness, an indictor for standing water bodies, was low for both viviparous and for some terrestrial breeding species, further strengthening Goin and Goin's (1962) 'broken topography hypothesis'. We found no evidence that viviparity is an adaptation to extreme climatic fluctuations as has been proposed for salamanders.

A number of other, potentially important traits associated with derived, terrestrial breeding modes in anurans were not treated in this study, largely due to the gaps in knowledge on breeding biology of African bufonids. Internal fertilization and parental care are thought to have played an important role in the evolution of viviparity for example (Wake 1978; 1980) and of the non-viviparous lineages, internal fertilization and parental care has been confirmed for only one other closely related taxon (in A. malcolmi Grandison 1978). Other more distantly related species, such as *Mertensophryne micranotis*, also practice internal fertilization (Grandison 1980) and *Nectophryne* spp. provide parental care (Scheel 1970). Neither trait is therefore unique to viviparous species and close relatives. Furthermore, internal fertilization is wide spread in salamanders and caecilians (Wake 2014), yet viviparity is not. To fully understand the importance of these traits in anurans, more basic breeding biology data is needed.

Besides the above tested or discussed correlated traits, viviparity also requires a number of endocrinological and physiological adaptations, such as the development of corpora lutea (Wake 1993). Although specific extrinsic conditions may favour a decreased dependency on laying eggs either directly in water or in moist undergrowth, this alone may not be sufficient to drive the evolution of live-bearing and it is apparent that a number of factors must coincide.

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SYNTHESIS

Discussion

"The role of environment in evolution may best be described by stating that the environment provides 'challenges' to which the organism 'responds' by adaptive changes" – Dobzhansky 1950; p 221.

The transition from aquatic to terrestrial reproduction in early tetrapods is viewed as a major adaptive change in the history of life (Romer, 1957; Tihen, 1960; Reisz, 1997). The transition to terrestrial habitats can be viewed, as stated by Dobzhansky (1950), with the environment providing the 'challenges' which species have to 'respond' to. Extant amphibians are an interesting group for testing this shift in habitat (Tihen, 1960; Carroll, 1969; Buchholz *et al.*, 2007), given the multiple, independent evolutionary changes towards terrestrial breeding in this group (Duellman & Trueb, 1994; Wells, 2007). Many amphibian species show partly or fully terrestrial modes of reproduction and their spatial distribution correlate with specific climatic and environmental factors (Goin & Goin, 1962; Poynton, 1964; Gomez-Mestre, Pyron & Wiens, 2012).

Despite the acknowledgement of the potential link between habitat and terrestrial breeding, few studies have empirically tested a causal link, and so, this thesis is dedicated to better understanding the interaction between terrestrial life history and geography. To achieve this, two strategies were employed. The first was to focus on a specific region, the Eastern Arc Mountains and adjacent lowlands of East Africa, and to investigate the distribution of terrestrial and aquatic breeding amphibians in relation to habitat types (chapter 1). The second strategy was to focus on a specific taxonomic group, the Bufonidae, and to investigate in more detail how specific life history traits are phylogenetically and spatially distributed. In particular how these traits have changed over time, whether lineages with different reproductive modes have diversified at different rates and to what extent the environment may have played a role in the evolution of viviparity (chapters 2-4). The work carried out for this thesis has also resulted in the assembly and publication of the most complete species list for the Eastern Arc Mountain area, the most complete list of reproductive modes, body, clutch and egg size for African species of Bufonidae and the most densely sampled phylogeny of Old World bufonids to date. In the sections below, the main findings of this thesis are outlined.

Forest is an important habitat for the evolution of terrestrial breeding

Poynton (1964) reasoned that forest permits the evolution of terrestrial breeding because 'unprotected amphibian egg[s] must be laid in a sheltered situation, and this sort of situation is provided by dense vegetation'. Our findings largely support this hypothesis, but suggest that the terrestrial breeding may have evolved outside forests too. Forest is indeed the best habitat predictor for the distribution of amphibians with terrestrial reproductive modes in East Africa and more specifically, the evolutionary transition to terrestrial egg laying is correlated with the transition to forest habitat (chapter 1). However, in bufonids, one of the two types of viviparity and one of the two species laying terrestrial eggs are associated with non-forest habitat (chapter 4).

The finding that viviparity is not always associated with forest does not however contradict Poynton's scenario of how terrestrial breeding evolved. As viviparity does not involve the deposition of eggs, Poynton's reasoning does not apply to *Nimbaphrynoides occidentalis*, a viviparous toad that occurs above the tree line on Mount Nimba (West Africa). Of the terrestrial egg-laying bufonids, *Didynamipus sjostedti* is most often found under closed canopy (Gonwouo *et al.*, 2013), but *Altiphrynoides malcolmi* occurs largely in open, Afro-alpine moor lands (Largen, 2001) and according to the optimal tree topology most likely did not have forest ancestors either (chapter 4). Although Poynton promotes forest as providing a wealth of suitable breeding sites for terrestrial reproduction, the key argument is that sheltered, humid oviposition sites are necessary and sites meeting these conditions can at times be found outside of forests too, or can be made to meet these conditions via nest building behaviour. For example, *A. malcolmi* larvae develop in nests at the base of dense grasses (Grandison, 1978) and *Breviceps* species inhabiting deserts lay terrestrial eggs in humid, subterranean burrows (Minter *et al.*, 2004).

The association of terrestrial breeding with forest or non-forest in combination with behavioural breeding site manipulation seen in Africa is likely to be the same elsewhere. In South America for example, major groups of terrestrial breeding anurans such as the genera *Eleutherodactylus* and *Pristimantis* are largely forest restricted, but a number of terrestrial egg laying *Leptodactylus* species inhabit non-forest habitats where they build foam nests, sometimes in combination with burrows (Prado *et al.*, 2002). Foam nests also protect eggs

from predator and microbial attacks (Fleming *et al.*, 2009) and are constructed by forest species too (Liao & Lu, 2010), but to my knowledge, no study has investigated whether the evolution of these nests in different lineages is the result or predatory or habitat induced selection.

The association of terrestrial breeding with forest recovered in this thesis may also be misleading. Small body sizes are suggested to be advantageous for 'reproductive experimentation' (Salthe & Duellman, 1973; Wake, 1978) and the reduction of body size is likely to be an evolutionary precursor for terrestrial breeding modes such as direct development in anurans (Duellman & Trueb, 1994). However, small body sizes also put amphibians at greater risk of desiccation and so the optimal body size for terrestrial breeding to evolve may constraint where adults can survive. It may therefore be the adults of terrestrial species that are restricted to humid forests, not the terrestrial eggs they lay that could be buried or kept moist in foam.

Steep topography may indeed play a role in the evolution of terrestrial breeding

The steep topography of mountains allows for few standing bodies of water to form and the strong current of low order streams may flush away aquatic amphibian eggs and larva (Goin & Goin, 1962). In line with this idea, Campbell and Duellman (2000) reported that montane slopes in the Neotropics are populated predominantly by species with direct development or torrent adapted tadpoles. In the Eastern Arc Mountains terrestrial larval development (including direct development) is correlated with montane forest (chapter 1). No specific test for steep surface gradients as a proxy for a lack of standing water bodies, was carried out, but the fact that montane grassland (predominantly flat plateaus in the Udzungwa Mountains) was not recovered as an important habitat for terrestrial breeders but steep, forested mountain flanks were suggests that steepness may indeed play a role in the evolution of terrestrial breeding. Poynton (1964) suggested that steep slopes tend to be forested and therefore this correlation could be misleading, but in bufonids, steepness is a better predictor for terrestrial breeding than forest cover and the species occurring in the steepest environments were either viviparous, terrestrial egg-layers or species with torrent adapted tadpoles (chapter 4). It is possible that steep topography selects *against* aquatic breeding and humid, dense vegetation in forest selects *for* terrestrial breeding.

More direct testing must be done to confirm Goin and Goin's (1962) theory. Topographic gradient is only a proxy for a lack of standing water bodies that can act as suitable breeding sites, and high resolution mapping of ponds and stream gradients are needed. Interestingly, the inclusion of the topographic wetness data (chapter 4) shows that viviparous species occur in areas of very low topographic wetness and humidity, whereas terrestrial egg-layers do not. Different environmental selective pressures are therefore likely to operate on different forms of terrestrial breeding. Saturated, moist soil is important for species where eggs are laid on the ground, but less relevant for species that carry the eggs in the oviduct. Based on this result, one could predict that the areas of high soil moisture and topographic wetness correlates with the distribution of South American direct developing species that lay eggs on the ground, but not with direct developing species that carry the eggs in specialized pouches (e.g. *Gastrotheca* spp.; Duellman & Trueb, 1994).

Terrestrial breeding does not promote higher diversification rates

Terrestrial breeding allows amphibians to become less dependent on open sources of water and thereby to expand into competitor-free habitats. Such an 'ecological opportunity' should lead to increased diversification (Simpson, 1953; Schluter, 2000), similar to the diversification burst in early terrestrial plants (Bateman *et al.*, 1998), and could explain the high number of species in the Neotropical 'Terrarana' amphibians (Hedges, Duellman & Heinicke, 2008). However, African bufonid lineages with terrestrial reproductive modes have not diversified at faster rates than aquatic breeders (chapter 3). Furthermore, habitat preferences and morphology are largely conserved in terrestrial breeding toads and there is little indication that terrestrial breeding is a 'key innovation' that has allowed for rapid phenotypic and ecological diversification. In fact, the entire bufonid radiation that colonized Africa, did so at a constant rate with no indication of early, high rates of cladogenesis as niche space is partitioned or a significant subsequent slow down as niche space becomes saturated. We propose several explanations for why this could be (chapter 3). Evidence for the ecological opportunity model most often comes from young, insular systems (e.g. Harmon *et al.*, 2008; Jønsson *et al.*, 2012) and recent continental-scale studies have also failed to detect a density dependent lineage accumulation pattern (Derryberry *et al.*, 2011; Day *et al.*, 2013). Such systems may therefore be too large or complex for ecological opportunity to occur or to be

accurately measured. Along this line of thinking, we propose that Africa is either too large or diverse of an area for lineages to quickly reach carrying capacity, was not a competitor-free landscape at the time of arrival of bufonids or that the historically dry climate has hindered the diversification amphibians in general. Alternatively, signals of an early burst may have been eroded over time, either by not effectively representing internal (extinct) lineages in the phylogeny or generalizing over multiple, repeated burst events (Esselstyn, Timm & Brown, 2009; Rabosky, 2009; Slater *et al.*, 2010). In support of this, we find that life history traits such as body size, clutch size and egg size did diverge early on and faster than expected by chance and so partitioning of niche space might have occurred (chapter 3).

Viviparity evolved twice

Viviparity evolved twice in bufonids, but in closely related lineages (chapter 4). In one lineage, embryonic development is sustained solely from yolk provisions in the egg (lecithotrophy in *Nectophrynoides*) and in the other, the embryos are nourished through specialized tissue in the uterus (matrotrophy in *N. occidentalis*) and yolk provisioning is therefore minimal. The only other confirmed terrestrial breeding bufonid species are close relatives, but ancestral state reconstructions suggest that at least lecithotrophic viviparity did not evolve from a terrestrial breeding ancestor and matrotrophic viviparity is not derived from lecithotrophic viviparity. Despite being of similar ages, the lecithotrophic lineage has diversified into ca. 30 species, whereas the matrotrophic lineage is monotypic. The difference in species diversity between these lineages might not be due to reproductive differences and instead have a biogeographic explanation. The *Nectophrynoides* group comprise a series of mountain endemics found along a fragmented mountain chain (Eastern Arc Mountains) whereas *N. occidentalis* occurs on a single, isolated mountain (Mount Nimba). Allopatry due to an expansion and contraction of suitable habitat may have been a more important driver of speciation in *Nectophrynoides* than *Nimbaphrynoides*.

Viviparous toads have smaller body sizes, smaller clutches and larger eggs (with the exception of the reduced egg size in *N. occidentalis*) compared to their aquatic breeding counterparts (chapter 2) and these traits segregated early on in the history of bufonids (chapter 3). The reduced body size, clutch size and the increased egg size of the most recent common ancestor of the two viviparous lineages might have been an important evolutionary

precursor for this kind of reproduction (chapter 4; Grandison, 1978; Wake, 1980; 1993), which could explain the repeated origin of viviparity in this clade. Interestingly, although the two viviparous lineages do not occur in identical habitats, steep slopes and low topographic wetness, suggestive of the absence of standing water bodies, are mutual environmental parameters for both and viviparity may therefore indeed represent an evolutionary alternative to torrent adapted tadpoles (as proposed by Goin & Goin, 1962; Campbell & Duellman, 2000).

Caveats

As with most scientific studies, there is a degree of uncertainty for some of the conclusions drawn in this thesis and these should be highlighted and discussed. The foremost limitation imposing uncertainty has been the poor state of knowledge of African amphibian taxonomy and ecology. In addition, the finite availability of tissue samples has meant taxonomic coverage remained incomplete. A long history of socio-political instability in many countries has hampered scientific progress and 21.2% of species are listed as data deficient on the IUCN red list (www.iucnredlist.org, accessed in May 2014). Often even the most basic aspects of biology are unknown. Although the number of data deficient species is lower than in other comparable regions (e.g. 31.5% in South America), the total number of species in Africa is likely to be severely underestimated and the taxonomy of many African groups await major revision (Andreone *et al.*, 2008). The comparative methods used in this body of work rely on near complete sampling of species, or at least an understanding of true species numbers and sampling biases. Fulfilling the assumptions associated to applying comparative methods could not be met with certainty in some cases. For example, in chapter 3 we uncover the wealth of undescribed species of bufonids in Africa, making sampling-fraction bias corrections, which rely on true species numbers impossible. This problem is confounded further by the low resolution of inter-generic relationships in the phylogeny. Although significant improvements in the phylogenetic understanding of bufonids have been made here, a number of key relationships await confirmation.

Breeding strategies in amphibians are generally coded as discrete traits (Duellman & Trueb, 1994; Wells, 2007; Vitt & Caldwell, 2009) and the coarseness of these coding bins can

strongly affect the results of statistical tests (see discussion in chapter 2). Due to the poor knowledge of life histories of many species, coding was limited to broad categories, which meant that potentially interesting details had to be omitted and biases may have been introduced. For example, whether aquatic breeding species deposit eggs in temporary or permanent, lentic or lotic water bodies could not be accurately coded, although these are known to be important differences that affect tadpole morphology, behaviour and developmental duration (Duellman & Trueb, 1994). In many cases, life history strategies had to be assumed based on phylogenetic positioning or indirect evidence, and intra-specific variances had to be largely ignored. More basic ecological field data are sorely needed to improve our knowledge of African amphibians.

Finally, rare occurrences in biology, such as the origin of life on earth itself, are intriguing, but their low sample sizes make them difficult to study. Derived, terrestrial breeding strategies in African amphibians are largely conserved and have evolved only a few times. Not surprisingly, the statistical power has therefore remained low for many of the tests, but the recovered trends have nonetheless been insightful.

Future Directions

A number of improvements can be made to address the problems and limitations discussed above. These are largely straightforward: more fieldwork and taxonomic revisions to improve our understanding of African amphibians and increased genetic sampling to improve the resolution of the phylogenetic reconstructions. The following section will therefore focus instead on interesting new directions to take that would build on the work presented in this thesis.

A broader approach – The current analyses have been restricted to African species, but bufonids are a global clade and the African lineages are not monophyletic. By restricting our analysis to Africa, a number of relevant variations in traits have been excluded. For example, there is a prominent radiation of toads with torrent adapted tadpoles on steep slopes in South East Asia (Inger, 1966) and South/Central America (Duellman & Lynch, 1969) and bromeliad breeding (e.g. *Dendrophryniscus*) and direct developing species (e.g. *Oreophrynella* and

Osornophryne) in South America (Duellman & Trueb, 1994). Similarly, African bufonids, although interesting due to the viviparous nature of some species, show limited diversity in reproductive modes with many semi-terrestrial and terrestrial alternatives to viviparity being largely under-represented. By repeating the analyses carried out in chapters 2, 3 and 4 with other major African groups such as Afrobatrachia, or by extending the analyses of chapter 1 to cover all of East Africa or even the whole of sub-Saharan Africa, a greater number of repeated occurrences of life history transitions and habitat correlations may be attainable.

Similarly, a number of traits associated with terrestrial breeding have not been addressed in much detail and should be explored further. These include mode of fertilization, gestation period, developmental mechanisms, extent and type of parental care and whether tadpoles are endotrophic or exotrophic (Wake, 1978; Hanken, 1992; Wake, 1993; Gomez-Mestre *et al.*, 2012; Wake, 2014). It should be noted however that this information is currently largely lacking for African taxa and so extensive collaborations and fieldwork would be required. Extrinsic, biotic factors such as predation or competitive exclusion, proposed as driving factors for the evolution of terrestrial breeding (Lutz, 1948; Tihen, 1960) have also not been addressed in this thesis and certainly deserve more attention as well.

A narrower approach – Alternatively, future studies could focus on the microevolution of terrestrial breeding by looking at plasticity in relevant life history traits (e.g. Vonesh, 2005; Gomez-Mestre, Wiens & Warkentin, 2008; Touchon & Warkentin, 2008; Eads, Mitchell & Evans, 2012). In *Salamandra salamandra* for example, the duration of egg retention is highly plastic (Wake, 1993; Buckley *et al.*, 2007), but whether such plasticity exists in *Nectophrynoides* remains to be investigated. Wake (1993) argues that the evolution of viviparity from direct developing ancestors need not require macro-steps in evolution and that an extension of the egg retention period reflecting environmental fluctuations may suffice. Whether the gestation period and the developmental stages of new-borns are plastic and whether this correlates with seasonal or site-specific fluctuations in climatic conditions remains to be investigated for a number of terrestrial breeding bufonids.

The improvement of genomic techniques means that elucidating the gene network and mutations that have lead to terrestrial eggs and development is becoming a possibility. Such an approach has recently been taken to better understand the multiple, independent evolution of skin secretions in amphibians for example (Roelants *et al.*, 2013), and so this may

prove to be a fruitful direction to take.

Conclusion

In an effort to understand whether specific variations in life history strategies have evolved as adaptations to the environment, this thesis has focused on the phylogenetic and geographic distribution of terrestrial breeding strategies in African amphibians. The chapters in this thesis reveal that terrestrial breeding and viviparity evolved frequently in forested and/or in topographically complex habitats, but also that these habitats are not exclusive to terrestrial breeders. Steep gradients appear to have a stronger effect than forest, but forest is nonetheless important. Furthermore, this thesis shows that diversification rates have remained constant across lineages of Bufonidae with different reproductive modes and therefore viviparity (a highly derived and rare life history strategy in amphibians) does not appear to have increased diversification rates compared to the plesiomorphic biphasic breeding strategy, though potentially it allowed the penetration into new habitats. The constant rate of diversification, without signs of temporal or clade specific bursts lends an explanations to why at least in bufonids, species richness is lower in Africa than in other tropical regions.

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Supplementary Materials

Chapter I

Forests as promoters of terrestrial life history strategies in East African amphibians

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1. Species lists, breeding biology and habitat categorizations

Alphabetical list of species included in this study and their corresponding breeding strategies and predominant habitat categories are given in Table 1. We used a simplified three state coding scheme to categorize breeding biology: 0 – aquatic eggs and larvae, 1 – terrestrial eggs, aquatic larvae and 2 – complete development on land. Habitat categories are condensations of IUCN habitat categories with modifications according to Poynton et al. [1]: CLO- "Coastal Lowland Others" (ICUN categories: savanna, shrubland, tropical dry lowland grassland), CLF- "Coastal Lowland Forest" (IUCN category: tropical moist lowland forest), MF- "Montane Forest" (IUCN category: tropical moist montane forest) and MG- "Montane Grassland" (IUCN category: tropical dry high altitude grassland). Species marked with an asterisk (*) are not listed on the IUCN Red List database and breeding biology and habitat categories were assigned based on personal experience and published data.

Supplementary Table 1. Species included in this study and their corresponding breeding biology (degree of terrestrialization) and habitat preferences.

Species	Terrestrialization	Habitat
Afrixalus cf. *uluguruensis**	1	MF
Afrixalus delicatus	1	CLO
Afrixalus dorsimaculatus	1	MF
Afrixalus fornasini	1	CLO
Afrixalus morerei	1	MG
*Afrixalus sp.*1*	1	MF
Afrixalus stuhlmanni	1	CLO
Afrixalus sylvaticus	1	CLF
Afrixalus uluguruensis	1	MF
Amietia angolensis	0	CLO
Amietia tenuoplicata	0	CLO
Amietia viridireticulata	0	CLO
Amietophrynus brauni	0	MF
Amietophrynus garmani	0	CLO
Amietophrynus gutturalis	0	CLO
Amietophrynus maculatus	0	CLO
Amietophrynus reesi	0	CLO
Amietophrynus xeros	0	CLO
Arthroleptis affinis	2	MF
*Arthroleptis anotis**	2	MF

Arthroleptis cf. *fichika**	2	MF
Arthroleptis cf. *xenodactyloides**	2	MF
Arthroleptis fichika	2	MF
*Arthroleptis kidogo**	2	MF
Arthroleptis lonnbergi	2	CLO
*Arthroleptis nguruensis**	2	MF
Arthroleptis nikeae	2	MF
Arthroleptis reichei	2	MF
Arthroleptis sp. 1*	2	MF
Arthroleptis sp. 2*	2	MF
Arthroleptis stenodactylus	2	CLO
Arthroleptis stridens	2	CLO
Arthroleptis tanneri	2	MF
Arthroleptis xenodactyloides	2	CLF
Arthroleptis xenodactylus	2	MF
Boulengerula boulengeri	2	MF
Boulengerula cf. *boulengeri**	2	MF
Boulengerula cf. *ulugurensis**	2	MF
Boulengerula changamwensis	2	CLF
Boulengerula niedeni	2	MF
Boulengerula taitanus	2	MF
Boulengerula uluguruensis	2	MF
Breviceps fichus	2	MG
Breviceps mossambicus	2	CLO
*Callulina dawida**	2	MF
*Callulina hanseni**	2	MF
*Callulina kanga**	2	MF
Callulina kisiwamsitu	2	MF
Callulina kreffti	2	MF
Callulina laphami	2	MF
Callulina shengena	2	MF
*Callulina meteora**	2	MF
Callulina sp. 2*	2	MF
*Callulina sp.*1*	2	CLF
*Callulina stanleyi**	2	MF
Chiromantis kelleri	1	CLO
Chiromantis petersii	1	CLO
Chiromantis xerampelina	1	CLO
Churamiti maridadi	NA	MF
Hemisus marmoratus	1	CLO
Hildebrandtia macrotympanum	0	CLO
Hildebrandtia ornata	0	CLO
Hoplophryne cf. *rogersi**	1	MF
Hoplophryne cf. *uluguruensis**	1	MF
Hoplophryne rogersi	1	MF
Hoplophryne sp. 1*	1	MF
Hoplophryne uluguruensis	1	MF
Hylarana galamensis	0	CLO
Hyperolius argus	0	CLO
Hyperolius cf. *puncticulatus**	1	MF
Hyperolius glandicolor	0	CLO
Hyperolius kihangensis	NA	MF
Hyperolius mariae	0	CLO
Hyperolius minutissimus	0	CLO
Hyperolius mitchelli	1	CLF
Hyperolius nasutus	0	CLO
Hyperolius parkeri	1	CLO
Hyperolius pictus	1	MG

Hyperolius pseudargus	0	MG
Hyperolius puncticulatus	1	CLO
Hyperolius pusillus	0	CLO
Hyperolius reesi	0	CLO
Hyperolius rubrovermiculatus	1	CLF
Hyperolius sp. 1*	NA	CLO
Hyperolius sp. 2*	NA	MF
Hyperolius spinigularis	1	MF
Hyperolius tanneri	NA	MF
Hyperolius tuberilinguis	1	CLO
Hyperolius viridiflavus	0	CLO
Kassina maculata	0	CLO
Kassina senegalensis	0	CLO
Kassina somalica	0	CLO
Leptopelis argenteus	1	CLO
Leptopelis barbouri	1	MF
Leptopelis bocagii	1	CLO
Leptopelis cf. *barbouri**	1	MF
Leptopelis cf. *uluguruensis**	1	MF
Leptopelis concolor	1	CLO
Leptopelis flavomaculatus	1	CLF
Leptopelis parbocagii	1	CLO
Leptopelis parkeri	1	MF
Leptopelis uluguruensis	1	MF
Leptopelis vermiculatus	1	MF
Mertensophryne (S.) loveridgei	0	CLF
Mertensophryne (S.) usambarae	0	CLF
Mertensophryne lindneri	0	CLO
Mertensophryne micranotis	0	CLF
Mertensophryne taitana	0	CLO
Mertensophryne uzunguensis	NA	MG
Nectophrynoides asperginis	2	MF
Nectophrynoides cryptus	2	MF
Nectophrynoides frontierei	2	MF
Nectophrynoides laevis	2	MF
Nectophrynoides laticeps	2	MF
Nectophrynoides minutus	2	MF
Nectophrynoides paulae	2	MF
Nectophrynoides poyntoni	2	MF
Nectophrynoides pseudotornieri	2	MF
Nectophrynoides sp. 1*	2	MF
Nectophrynoides sp. 2*	2	MF
Nectophrynoides sp. 3*	2	MF
Nectophrynoides sp. 4*	2	MF
Nectophrynoides sp. 5*	2	MF
Nectophrynoides sp. 6*	2	MF
Nectophrynoides sp. 7*	2	MF
Nectophrynoides tornieri	2	MF
Nectophrynoides vestergaardi	2	MF
Nectophrynoides viviparus	2	MF
Nectophrynoides wendyae	2	MF
Parhoplophryne usambarica	NA	MF
Petropedetes cf. *yakusini**	1	MF
Petropedetes martiensseni	1	MF
Petropedetes yakusini	1	MF
Phlyctimantis keithae	0	MF
Phrynobatrachus acridoides	0	CLO
Phrynobatrachus breviceps	0	MG

Phrynobatrachus krefftii	1	MF
Phrynobatrachus mababiensis	0	CLO
Phrynobatrachus natalensis	0	CLO
Phrynobatrachus pallidus	0	CLO
Phrynobatrachus parvulus	0	MG
Phrynobatrachus rungwensis	0	MG
Phrynobatrachus scheffleri	0	CLO
Phrynobatrachus sp. 1*	0	MG
Phrynobatrachus ukingensis	0	MG
Phrynobatrachus uzungwensis	0	MF
Phrynomantis bifasciatus	0	CLO
Probreviceps cf. *durirostris**	2	MF
Probreviceps durirostris	2	MF
Probreviceps loveridgei	2	MF
Probreviceps macrodactylus	2	MF
Probreviceps rungwensis	2	MF
Probreviceps uluguruensis	2	MF
Ptychadena anchietae	0	CLO
Ptychadena grandisonae	0	MG
Ptychadena mascareniensis	0	CLO
Ptychadena mossambica	0	CLO
Ptychadena oxyrhynchus	0	CLO
Ptychadena porosissima	0	MG
Ptychadena schillukorum	0	CLO
Ptychadena taenioscelis	0	CLO
Ptychadena uzungwensis	0	MG
Pyxicephalus adspersus	0	CLO
Pyxicephalus edulis	0	CLO
Schismaderma carens	0	CLO
Schistometopum gregorii	2	CLO
Scolecomorphus cf. *kirkii**	2	MF
Scolecomorphus cf. *vittatus**	2	MF
Scolecomorphus kirkii	2	MF
*Scolecomorphus sp.*1*	2	MF
Scolecomorphus uluguruensis	2	MF
Scolecomorphus vittatus	2	MF
Spelaeophryne methneri	2	CLF
Strongylopus fuelleborni	1	MG
Tomopterna cryptotis	0	CLO
Tomopterna luganga	0	CLO
Xenopus borealis	0	MG
Xenopus muelleri	0	CLO
Xenopus petersii	0	CLO
Xenopus victorianus	0	CLO

2. Phylogenetic Analysis

The comparative analysis outlined in this study required a species level phylogeny of East African amphibian species. However, for the majority of species included in this study (180 species; see Supplementary Table 1), molecular data remains unavailable. Using existing molecular data, we explored two different strategies for producing a comprehensive species level phylogeny of East African amphibians. Strategy 1 was to reconstruct a genus level phylogeny of East African amphibians using a mitochondrial and nuclear dataset. Species

were added manually as a polytomy during the final tree reconstruction step. The advantage of this approach is a complete phylogeny, although with unresolved nodes and equal branch lengths among species in each genus. While this strategy under-samples branch length differences among species, it provides a more accurate basis for analysing species across our study area. Strategy 2 was to utilize an existing phylogeny containing species that occur across the area and pruning out all species that do not inhabit the Eastern Arc Mountains and adjacent lowlands. This approach provides a better estimate of species level differences, but at the expense of completeness. Pyron and Wien [2] produced the most comprehensive analysis of amphibian relationships and we explored the suitability of this tree, pruned down to contain only East African taxa, to use in the comparative analyses here.

Strategy 1: Complete East African Tree

We compiled a data set for 33 amphibian ingroup species, including 30 frogs, and 3 caecilians using Genbank and previously published sequence data for the 16S rRNA and RAG1 genes (See Supplementary Table 2). The representative samples of each genus were not necessarily from specimens from the region. In two cases where there was an absence of one gene fragment for a species, we produced chimeric sequences for taxa using available sequences for presumably closely related taxa. Rag-1 sequences were not available for the following genera: *Churamiti, Hildebrandtia,* and *Phlyctimantis*. Based on previous studies, preliminary 16S trees, or BLAST searches, *Churamiti* shows closer relationships with *Nectophrynoides*, *Hildebrandtia* with *Ptychadena*, and *Phlyctimantis* with *Kassina* and Rag-1 data of these genera were used to form a chimeric sequence. In addition, analyses were conducted using alignments with missing data, rather than using chimeric sequences (e.g. for *Churamiti, Hildebrandtia,* and *Phlyctimantis*), to test how robust the phylogenies including and excluding such sequences were. No significant differences were noted. *Parhoplophryne usambarica* has not been collected since its original description [3] and data on its breeding biology and phylogenetic relationships are unknown. Therefore this taxon was excluded from all analyses.

For phylogenetic inference we sampled one lepidosaur (*Lacerta lepida*) as an outgroup. The complete data set is a concatenation of one mitochondrial gene fragment (part of the 16S rRNA gene) and one nuclear protein-coding gene fragment (parts of Rag-1) totaling 1086 bp. Nucleotide sequences were aligned using MUSCLE [5] with default settings in the bioinformatics tool suite Geneious Pro 5.5.4 [6]. Alignment ambiguities for the

mitochondrial gene fragment were excluded using GBLOCKS version 0.19b [7] with default parameter settings for block selection (less stringent options were not selected). The resulting alignment is deposited in the Dryad repository: http://dx.doi.org/10.5061/dryad.8f74d [4]. For each gene partition, including codon position, the best-fit models of nucleotide substitution were identified using the Akaike information criterion (AIC;[8]) as implemented in Modeltest version 3.7 [9]. Best-fit models were estimated for each individual partition.

The datasets were analysed using maximum likelihood (ML; [10]), and Bayesian inference (BI; [11]). Both analyses were run using a constraint to find the optimal tree shown in Pyron and Wiens [2], given that this represents the most comprehensive analysis of species level relationships across all amphibians. ML analyses were conducted with RAxML version 7.0.4 [12] using the rapid hill climbing algorithm [13]. BI used MrBayes version 3.2.1 [14] running four simultaneous Markov chains for 10 million generations, sampling every 1000 generations, and discarding the first one million generations as burn-in to prevent sampling before reaching stationarity. Two independent BI runs were performed to identify convergence. For both ML and BI analyses, model parameters were independently optimized for each partition ("un-link" option in effect). Support for internal branches was evaluated by non-parametric bootstrapping [10] with 1000 replicates performed with RAxML (ML), and by posterior probabilities (BI). In order to produce a species level phylogeny for comparative analyses, all study species were inserted in appropriate genera with inter-relationships unresolved in a polytomy. This phylogeny is also deposited in the Dryad Digital Repository as a newick file: http://dx.doi.org/10.5061/dryad.8f74d [4]. For the BayesTraits analysis, 100 permuted trees were generated with polytomies resolved to a branch length of 0.0001 in Mesquite v2.74 [15].

Strategy 2: Pyron and Wiens' Tree

The phylogeny presented by Pyron and Wiens [2] is currently the most comprehensive analysis of amphibian relationships. It includes data from 2871 species, with an average of 2563 base pairs per species. This tree was used as a basis for conducting comparative analyses. A single Maximum likelihood tree was made available from the authors. This tree was pruned using the R package "APE" [16], removing all taxa not included in our analysis. The resulting tree was then used as a basis for conducting the comparative analyses. Supplementary Table 3 lists species coverage for both datasets (complete dataset and Pyron and Wiens data set)

Supplementary Table 2. African species used in the study with specimen-vouchers, localities, genbank accession numbers and origin.

Species	Voucher	Geographic origin	16S rRNA	RAG1	Origin
Afrixalus dorsalis	CAS 207523	Equatorial Guinea	DQ347296	DQ347236	[17]
Amietia angolensis	VUB0992	Subsaharan Africa	DQ347318	DQ347257	Genbank
Amietophrynus brauni	FMNH 251853	Tanzania	AF220886	DQ158361	[17]
Arthroleptis variabilis	CAS 207822	Equatorial Guinea	AY322263	AY364210	[17]
Boulengerula boulengeri	BMNH 2002.950	Tanzania	EF107199	EF107322	[17]
Breviceps mossambicus	VUB 1031	Subsaharan Africa	EF017947	EF018056	[17]
Callulina kreftti	TNHC 62491	Tanzania	DQ347339	DQ347281	[17]
Chiromantis rufescens	CAS "143502"	Subsaharan Africa	GQ204724	GQ204605	Genbank
Churamiti maridadi	MTSN 5584	Tanzania	FJ882769	EF107329	Genbank, RAG1 = EF107329 (*N. tornieri*)
Hemisus marmoratus	CAS 214843	Kenya	AY364372	AY364216	Roelants, et al 2007
Hildebrandtia ornata	"127641"	Subsaharan Africa	AF215402	DQ347245	Genbank, RAG1 = DQ347245 (*Ptychadena* spp)
Hoplophryne rogersi	MTSN 5158	Tanzania	EF017961	EF018050	[17]
Amnirana galamensis	VUB 0996	Subsaharan Africa	DQ347032	DQ347260	[17]
Hyperolius sp.	VUB 0924	Kenya	AF249033	AY364208	[17]
Kassina maculata	"8414"	Subsaharan Africa	AF215444	AY571651	[17]
Leptopelis kivuensis	CAS 201700	Uganda	AY322245	AY364211	[17]
Mertensophryne micranotis	BMNH 2002.343	Tanzania	EF107207	EF107330	[17]
Nectophrynoides tornieri	BMNH 2005.1375	Tanzania	EF107206	EF107329	[17]
Parhoplophryne usambarica	See MTSN 5158	Tanzania	EF017961	EF018050	[17] (assumed close *H. rogersi*)
Petropedetes cf. *parkeri*	VUB 0955	Subsaharan Africa	AY364369	AY364213	[17]
Phlyctimantis leonardi	DPL 4058 *		DQ283356 *	AY571651	Genbank, RAG1 = AY571651 (*K. senegalensis*)
Phrynobatrachus kreffti	VUB 1068	Tanzania	DQ347342	DQ347284	[17]
Phrynomantis bifasciatus	VUB 0541	Subsaharan Africa	AY948732	AY948918	[17]
Probreviceps macrodactylus	KMH 21399	Tanzania	AY531875	KC632525	[18]
Ptychadena anchietae	VUB 0958	Kenya	DQ347307	DQ347245	[17]
Pyxicephalus edulis	BMNH 2002.438	Tanzania	EF107211	EF107333	[17]
Schismaderma carens	MVZ 223386	Subsaharan Africa	DQ158424	DQ158350	Genbank
Schistometopum thomense	BMNH 2000.301	Sao Tomé	EF107204	EF107327	[17]
Scolecomorphus vittatus	CAS 168810	Tanzania	EF107171	EF107294	[17]
Spelaeophryne methneri	FMNH 255879	Tanzania	EF107167	EF107290	[17]
Strongylopus grayi	VUB 0991	Subsaharan Africa	DQ347317	DQ347256	Genbank
Tomopterna cf. *natalensis*	ZFMK 68815	Rep. South Africa	DQ347300	DQ347239	[17]
Xenopus cf. *muelleri*	VUB 0921	Kenya	AY523771	AY523743	[17]

3. Comparative analysis

3.1 Details on comparative trait analysis

Correlates of breeding strategy and habitat types were identified using a phylogenetic generalized least squares approach (pGLS; [19]), using the package APE [16] in R v.2.13.0 [20]. The regression model was constructed so as to test the effect of habitat as a categorical, explanatory variable on the breeding biology as the response variable, correcting for phylogenetic non-independence. Different models of evolution were implemented as error structures in three separate regressions, allowing traits to evolve via a Brownian Motion model, a Pagel's λ model or an Ornstein-Uhlenbeck model. AIC scores of each regression were compared and the best scoring model was considered the most appropriate (models with ΔAIC>2 were deemed as acceptable alternative models).

Our coding system for the breeding biology of amphibians is based on two traits: environment of egg deposition and environment of larval development. To investigate whether the evolution of these two traits are affected differently by the environment, any habitat that was recovered to have a significant effect on the breeding strategy was carried forward and correlated evolution of habitat and terrestrial ovipositioning, and of habitat and terrestrial larval development was tested using the DISCRETE module in BayesTraits ([21]; available at http://www.evolution.rdg.ac.uk/). This software models the evolution of two binary traits across a given phylogeny, allowing traits to evolve either independently or dependent of each other. Both a Likelihood and Bayesian approach was used (see below for details). The log-likelihood scores and harmonic means for each of the two models were then compared to test for evidence of correlated evolution of traits.

100 trees with randomly resolved polytomies were generated in Mesquite [15] to average the effects of varying topologies. 25 optimization attempts were used in the likelihood analyses and significant improvements of the dependent over the independent model (or vice versa) were measured using a log-likelihood ratio statistic (2[(log-likelihood (dependent model) – log-likelihood (independent model))]), which follows a χ^2 distribution with 4 degrees of freedom (calculated as the difference between the number of parameters between the two models, following Pagel [21]).

For the Markov chain Monte Carlo simulations, both models were run for 5 050 000 iterations, sampling every 100 chains, after a burn in period of 50 000 iterations. A reversible-jump hyperprior with a distribution of 0 to 30 was implemented, from which values to seed the exponential priors were drawn (rjhp exp 0 30; as recommended by the software authors) and the ratedev was adjusted to obtain acceptance rates between 20-40% [21]. A log-Bayes Factor (2log[harmonic mean (dependent model)] – log[harmonic mean (independent model)]) greater than 10 was considered as strong evidence in favour of one model over the other.

A number of different datasets were used to test the robustness of our results as described in detail below. All datasets have been deposited in the Dryad repository: http://dx.doi.org/10.5061/dryad.8f74d [4].

3.2 Comparison of data sets (strategies 1 and 2)

Compared to the complete dataset containing all 180 species, the phyogeny based on Pyron and Wiens [2] contained only 73 taxa. These 73 taxa are not an accurate representation of the four different habitat categories, with a bias in favour of Coastal Lowland non-forest species, when compared to the 180 taxa of our dataset (see Supplementary Table 3). For instance, whereas 50% of the species of the full dataset are montane forest associated species, the dataset from Pyron and Wiens [2] contains only 34.2% montane forest species. The results of the pGLS and BayesTraits analyses using the full dataset (strategy 1) and the Pyron and Wiens data (strategy 2) were nonetheless broadly comparable. However, only montane forest was recovered as being significant using the Pyron and Wiens dataset, as opposed to montane and lowland forest in our dataset.

Supplementary Table 3. Relative numbers and percentages of species included for main habitat categories.

	Pyron and Wiens [2]		*Full dataset using constrained tree*	
	No. of species	*Percentage of total number of species*	*No. of species*	*Percentage of total number of species*
CLO	42	57.5	64	35.6
CLF	3	4.1	11	6.1
MF	25	34.2	90	50.0
MG	3	4.1	15	8.3
Total	**73**	**100**	**180**	**100**

3.3 Results of the analyses of the full dataset (strategy 1)

Phylogenetic generalized least-squares regression implementing a Pagel's lambda model of evolution to test the effect of habitat on breeding biology

	coefficient ± SE	*t-value*	*p-value*
Pagel's lambda model; λ= 0.984			
Intercept	1.195 ± 0.700	1.557	*p=0.121*
Costal lowland forest	0.259 ± 0.080	3.582	*p<0.001*
Montane forest	0.159 ± 0.048	4.429	*p<0.001*
Montane grassland	0.025 ± 0.066	0.489	*p=0.625*

Correlated evolution of breeding strategy and habitat in BayesTraits-DISCRETE showing Log Likelihood scores and Harmonic Means for independent and dependent evolution of traits

	Log Likelihood		Likelihood Ratio	*p-value*	MCMC Harmonic mean		Bayes Factor
	Independent	Dependent			Independent	Dependent	
Terrestrial egg – Montane forest	-140.556	-122.445	36.221	*p<0.001*	-145.416	-134.189	22.454
Terrestrial egg – Coastal lowland forest	-92.491	-87.029	10.922	*p<0.05*	-104.587	-98.739	11.696
Terrestrial larva – Montane forest	-100.574	-94.318	12.512	*p<0.05*	-107.237	-108.125	-1.776
Terrestrial larva – Coastal lowland forest	-52.509	-52.432	0.154	*p=0.997*	-71.978	-69.916	4.125

3.4 Results of the analyses of the Pyron and Wiens [2] data set (strategy 2)

Phylogenetic generalized least-squares regression implementing a Pagel's lambda model of evolution to test the effect of habitat on breeding biology

	coefficient ± SE	*t-value*	*p-value*
Pagel's lambda model; λ= 1.000			
Intercept	0.862 ± 0.546	1.579	*p=0.119*
Costal lowland forest	0.194 ± 0.229	0.847	*p=0.400*
Montane forest	0.390 ± 0.116	3.353	*p<0.05*
Montane grassland	0.020 ± 0.201	0.099	*p=0.921*

Correlated evolution of breeding strategy and habitat in BayesTraits-DISCRETE showing Log Likelihood scores and Harmonic Means for independent and dependent evolution of traits

	Log Likelihood		Likelihood Ratio	*p-value*	MCMC Harmonic mean		Bayes Factor
	Independent	Dependent			Independent	Dependent	
Terrestrial egg – Montane forest	-60.979	-51.705	18.549	*p<0.001*	-66.557	-60.829	11.454
Terrestrial egg – Coastal lowland forest	-37.893	-37.619	0.548	*p=0.969*	-44.348	-44.074	0.549
Terrestrial larva – Montane forest	-50.026	-44.101	11.850	*p<0.05*	-56.690	-51.221	10.938
Terrestrial larva – Coastal lowland forest	-26.940	-25.876	2.128	*p=0.712*	-30.374	-31.541	-2.333

3.5 Comparison of the results of the analyses of the different datasets (strategies 1 and 2)

The overall similar results using the Pyron and Wiens tree as compared to our tree using a resolved, genus-level phylogenetic backbone with intrageneric polytomies shows that our phylogenetic approach is adequate for performing the comparative analyses. The one major difference is the lack of significance for lowland forest using the Pyron and Wiens dataset. A comparison of the datasets shows that the main difference between the two is essentially a greatly reduced number of species associated with lowland forests in the Pyron and Wiens dataset (3 vs. 11 in our original dataset; see Supplementary Table 3). The lack of significance for lowland forest for the Pyron and Wiens dataset is most likely a result of the diminished number of lowland forest species in the dataset. In general, there are fewer lowland forest associated species compared to the other habitat categories and this habitat is therefore particularly sensitive to a reduction in number of species in the analysis. Given that a comprehensive inclusion of terminals is more important for the comparative analyses than a fully resolved tree and seeing that the results recovered with the two datasets are comparable, we based our analyses on the full dataset instead of using the Pyron and Wiens tree.

3.6 Correcting for undescribed species and potential taxonomic inflation

The complete data set of 180 species contains a number of not yet formally named taxa. The overwhelming majority of these undescribed and provisionally assigned species (all "sp." and "cf." taxa in Supplementary Table 1) originate from the forests of the Eastern Arc Mountains and most are characterized by derived reproductive modes. These species await taxonomic verification but based on current expert opinion are putative new species (candidate species

sensu [22]). Because sampling in the region is probably biased towards montane habitats we investigated the robustness of our analyses to the high proportion of candidate species from montane forests compared to coastal lowlands and montane grasslands. This involved a re-analysis of all data using the above approaches but with potential new species removed. This conservative approach to species diversity estimation indicated no significant differences in the pGLS results recovered from an analysis including all putative new species (see results table below). In contrast to the analysis on the full dataset, the correlation between terrestrial larval development and montane forest habitat lost strength slightly in the BayesTraits analysis when applying a Likelihood method (*p=0.054*). Similarly, the Baysian method could no longer recover a significant improvement of the dependent over the independent model of evolution for terrestrial egg deposition in association with Coastal Lowland Forest (BF=7.180).

After removing candidate species the pGLS results continue to show a significant positive effect of both forest types on the occurrence of terrestrial breeding amphibians and the conclusions drawn from the BayesTraits analysis are comparable too: there is support for correlated evolution of terrestrial egg deposition predominantly with montane forest but also with coastal lowland forest. Although there is some indication of correlated evolution of terrestrial larval development and montane forest, this association is no longer statistically supported.

Phylogenetic generalized least-squares regression implementing a Pagel's lambda model of evolution to test the effect of habitat on breeding biology

	coefficient ± SE	*t-value*	*p-value*
Pagel's lambda model; λ= 0.981			
Intercept	1.204 ± 0.778	1.547	*p=0.124*
Costal lowland forest	0.257 ± 0.082	3.133	*p<0.05*
Montane forest	0.227 ± 0.063	3.611	*p<0.001*
Montane grassland	0.040 ± 0.069	0.575	*p=0.566*

Correlated evolution of breeding strategy and habitat in BayesTraits-DISCRETE showing Log Likelihood scores and Harmonic Means for independent and dependent evolution of traits

	Log Likelihood		Likelihood Ratio	*p-value*	MCMC Harmonic mean		Bayes Factor
	Independent	Dependent			Independent	Dependent	
Terrestrial egg – Montane forest	-121.202	-108.468	25.469	*p<0.001*	-126.885	-118.146	17.478
Terrestrial egg – Coastal lowland forest	-84.914	-80.013	9.802	*p<0.05*	-99.555	-95.965	7.180
Terrestrial larva – Montane forest	-84.156	-79.515	9.281	*p=0.054*	-90.569	-93.368	-5.598
Terrestrial larva – Coastal	-47.868	-47.870	-0.005	*p=1.000*	-68.501	-66.581	3.840

lowland forest

3.7 Influence of viviparous species

Both viviparity and ovoviviparity are highly derived reproductive modes that are generally rare among amphibians [23]. Within East Africa the caecilian genera *Scolecomorphus* and *Schistometopum* contain viviparous species [24] and the bufonid *Nectophrynoides* species are ovoviviparous [23]. When looking at the taxonomic composition of lowland forest and non-forest habitats, montane forest, and montane grassland, it is apparent that viviparous and ovoviviparous species are predominantly found in montane forest. Especially *Nectophrynoides* represents a species-rich radiation of small bufonids nearly exclusively confined to the montane forests of the EAM. To test the influence that viviparous and ovoviviparous species might have on the analyses, we performed a separate pGLS and BayesTraits analysis excluding viviparous and ovoviviparous species from the dataset. Both montane and lowland forest habitats were again recovered as containing significantly more species with terrestrialized breeding strategies than the other habitat categories for the pGLS analysis. In comparison to the results when using the full dataset, the BayesTraits analysis no longer recovers a significant association for terrestrial larval development and montane forest (LR=6.056; *p=0.195*). Furthermore the MCMC method could no longer recover a significant improvement of the dependent over the independent model of evolution for terrestrial egg deposition in association with coastal lowland forest although the log Bayes Factor is only marginally below the significance threshold (BF=9.218).

As is the case for the analyses using the conservative dataset (above), the pGLS and BayesTraits analysis suggest that even when removing the viviparous lineages, the general association of terrestrial breeding with forest habitat (especially montane forest) remains significant. However, no statistically significant support could be found for correlated evolution of terrestrial larval development and either forest habitat types. Given the relatively high number of viviparous and ovoviviparous species in the original dataset, the latter result is not surprising.

Phylogenetic generalized least-squares regression implementing a Pagel's lambda model of evolution to test the effect of habitat on breeding biology

	coefficient ± SE	*t-value*	*p-value*
Pagel's lambda model; λ= 0.969			
Intercept	0.994 ± 0.676	1.470	*p=0.144*

Costal lowland forest	0.256 ± 0.078	3.259	*p<0.05*
Montane forest	0.224 ± 0.057	3.957	*p<0.001*
Montane grassland	0.040 ± 0.067	0.601	*p=0.549*

Correlated evolution of breeding strategy and habitat in BayesTraits-DISCRETE showing Log Likelihood scores and Harmonic Means for independent and dependent evolution of traits

	Log Likelihood		Likelihood Ratio	*p-value*	MCMC Harmonic mean		Bayes Factor
	Independent	Dependent			Independent	Dependent	
Terrestrial egg – Montane forest	-130.676	-113.450	34.452	p<0.001	-139.213	-126.319	25.788
Terrestrial egg – Coastal lowland forest	-88.566	-83.007	11.117	p<0.05	-102.530	-97.921	9.218
Terrestrial larva – Montane forest	-89.472	-86.444	6.056	p=0.1950	-97.720	-98.569	-1.699
Terrestrial larva – Coastal lowland forest	-47.361	-46.838	1.046	p=0.9027	-66.991	-63.776	6.429

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Supplementary Materials

Chapter II

Appendix

Table 1S. Egg diameter (in mm), clutch size and body size (snout vent length in mm) of dissected female toads housed in museum collections. In cases where egg size data is missing, ova were unusually small and likely to not be matured and therefore not measured, and where clutch size is missing, the females have been previously dissected and not the full clutch was preserved. BMNH numbers refer to vouchers housed in the Natural History Museum, London and ZMB numbers are housed at the Museum für Naturkunde, Berlin.

Species	Voucher Number	Egg Diameter (in mm)	Clutch Size	Snout Vent Length	Eggs pigmented?
Amietophrynus brauni	BMNH1974.428	1.0	~9000	91.5	pigmented
Amietophrynus brauni	BMNH1974.430		~4000	75.3	pigmented
Amietophrynus camerunensis	BMNH1975.181	1.7	~2000	78.1	pigmented
Amietophrynus camerunensis	BMNH1982.130	1.7	~2100	66	pigmented
Amietophrynus camerunensis	BMNH1984.239	1.6		62	pigmented
Amietophrynus kisoloensis	BMNH1934.12.15.272	1.9	~1800	74.2	pigmented
Amietophrynus kisoloensis	BMNH1934.12.15.274	1.6	~2100	78.4	pigmented
Amietophrynus kisoloensis	BMNH1957.1.13.51	1.7	~2400	75.1	pigmented
Amietophrynus lemairii	BMNH1932.9.9.2	1.5	~2500	58.5	pigmented
Amietophrynus lemairii	BMNH1932.9.9.2	1.4	~2400	65.6	pigmented
Amietophrynus lemairii	BMNH1932.9.9.6	1.2	~1600	55.8	pigmented
Amietophrynus pardalis	BMNH11.4.21.10.11	1.5	~14000	132.8	pigmented
Amietophrynus tuberosus	BMNH1969.508	1.1	~4200	59	pigmented
Amietophrynus tuberosus	BMNH58.11.2.154	1.5	~2700	68.5	pigmented
Amietophrynus urunguensis	BMNH1985.1006	0.6	~60	25.2	unpigmented
Amietophrynus xeros	BMNH1952.1.7.39	1.0	~5000	73.4	pigmented
Amietophrynus xeros	BMNH1984.172		~2400	68.1	pigmented
Didynamipus sjostedti	BMNH1969.1637	2.4	18	20.2	unpigmented
Duttaphrynus dodsoni	BMNH1931.7.20.55	1.5	410	63.3	pigmented
Duttaphrynus dodsoni	BMNH1931.7.20.60	1.3	470	57.2	pigmented
Duttaphrynus stuarti	BMNH1940.6.2.26	2.8	~2200	92.5	pigmented
Mertensophryne lindneri	BMNH1978.611	2.1	81	25	unpigmented
Mertensophryne lindneri	BMNH2000.729	2.1	57	24.6	unpigmented
Mertensophryne loveridgei	BMNH1988.246	1.9	82	32.8	unpigmented
Mertensophryne loveridgei	BMNH1988.7	2.1	131	32.4	unpigmented
Mertensophryne micranotis	BMNH1980.198	1.7	70		unpigmented
Mertensophryne micranotis	BMNH1982.85	1.8			unpigmented
Mertensophryne uzunguensis	BMNH2002.157	0.8	188	33.2	unpigmented
Nectophryne batesii	BMNH1978.805	2.1	23	23.6	unpigmented
Nectophrynoides vestergaardi	BMNH1982.499		46		unpigmented
Nectophrynoides viviparus	BMNH2005.822	2.9	160	49.4	unpigmented
Nectophrynoides viviparus	BMNH2005.827	2.6	96	37.6	unpigmented
Werneria bambutensis	ZMB76850	1.6	380	385	unpigmented
Werneria bambutensis	ZMB76698	1.9	344	420	unpigmented

Table 2S. References from which female body sizes, clutch size and egg size information listed in Table 1 was obtained.

Species	Max. Female Body Size (Snout Vent Length in mm)	Maximum Recorded Clutch Size	Maximum Recorded Egg Size (Diameter in mm)
Altiphrynoides malcolmi	Largen and Sprawls, 2010	Grandison, 1978	Wake, 1980
Altiphrynoides osgoodi	Largen and Sprawls, 2010	Wake, 1980	Grandison, 1978
Amietophrynus brauni	Channing and Howell, 2006	this study	this study
Amietophrynus camerunensis	Frétey et al., 2011	this study	this study
Amietophrynus channingi	Barej et al., 2011	Barej et al., 2011	Barej et al., 2011
Amietophrynus funereus	Channing and Howell, 2006		Perret, 1966
Amietophrynus garmani	Channing and Howell, 2006	Channing and Howell, 2006	Channing and Howell, 2006
Amietophrynus gracilipes	Perret, 1966		Perret, 1966
Amietophrynus gutturalis	Channing and Howell, 2006	Channing and Howell, 2006	Channing and Howell, 2006
Amietophrynus kisoloensis	Channing and Howell, 2006	this study	this study
Amietophrynus lemairii	Channing, 2001	this study	this study
Amietophrynus maculatus	Channing and Howell, 2006	Rödel, 1996	Rödel, 1996
Amietophrynus mauritanicus	Schleich et al., 1996	Schleich et al., 1996	Schleich et al., 1996
Amietophrynus pantherinus	Preez et al., 2009	Channing, 2001	
Amietophrynus pardalis	Channing, 2001	this study	this study
Amietophrynus poweri	Channing, 2001	Channing, 2001	
Amietophrynus rangeri	Channing, 2001	Minter et al., 2004	Channing, 2001
Amietophrynus regularis	Largen and Sprawls, 2010	Schleich et al., 1996	Barbault, 1984
Amietophrynus superciliaris	Barej et al., 2011	Barej et al., 2011	Barej et al., 2011
Amietophrynus tuberosus	Frétey et al., 2011	this study	this study
Amietophrynus xeros	Channing and Howell, 2006	this study	this study
Barbarophryne brongersmai	Hoogmoed, 1972	this study	this study
Bufo pentoni	Rödel, 1996	Rödel, 1996	Rödel, 1996
Capensibufo rosei	Channing, 2001	Grandison, 1980	Grandison, 1980
Capensibufo tradouwi	Preez et al., 2009	Channing, 2001	Channing, 2001
Didynamipus sjostedti	Grandison, 1981	Grandison, 1981	Grandison, 1981
Duttaphrynus dodsoni	Largen and Sprawls, 2010	this study	this study
Laurentophryne parkeri	Laurent, 1950	Tihen, 1960	Grandison, 1981
Mertensophryne anotis	Channing, 2001	Channing, 2001	Channing, 2001
Mertensophryne howelli	Channing and Howell, 2006	Poynton and Clarke, 1999	Poynton and Clarke, 1999
Mertensophryne lindneri	Channing and Howell, 2006	this study	this study
Mertensophryne lonnbergi	Poynton and Broadley, 1988	Channing and Howell, 2006	
Mertensophryne loveridgei	Channing and Howell, 2006	this study	this study
Mertensophryne melanopleura	Poynton and Broadley, 1988	Tihen, 1960	Tihen, 1960
Mertensophryne micranotis	Channing and Howell, 2006	this study	this study
Mertensophryne taitana	Channing and Howell, 2006	Ngwava et al., 2009	Ngwava et al., 2009
Mertensophryne usambarae	Channing and Howell, 2006	Poynton and Clarke, 1999	Poynton and Clarke, 1999
Mertensophryne uzunguensis	Channing and Howell, 2006	this study	Poynton et al., 2005
Nectophryne afra	Perret, 1966	Perret, 1966	Perret, 1966
Nectophryne batesii	Perret, 1966	Perret, 1966	Perret, 1966
Nectophrynoides asperginis	Channing and Howell, 2006	Channing and Howell, 2006	Poynton et al., 1998
Nectophrynoides cryptus	Channing and Howell, 2006	Channing and Howell, 2006	Perret, 1972
Nectophrynoides laticeps	Harper et al., 2010	Channing et al., 2005	Channing et al., 2005

Nectophrynoides minutus	Channing and Howell, 2006	Channing and Howell, 2006	Perret, 1972
Nectophrynoides paulae	Harper et al., 2010	Menegon et al., 2007	
Nectophrynoides poyntoni	Channing and Howell, 2006	Menegon et al., 2004	
Nectophrynoides tornieri	Channing and Howell, 2006	Gallien, 1959	Gallien, 1959
Nectophrynoides vestergaardi	Channing and Howell, 2006	this study	
Nectophrynoides viviparus	Channing and Howell, 2006	this study	this study
Nimbaphrynoides occidentalis	Sandberger et al., 2010 and Sandberger pers. comm.	Angel and Lamotte, 1944	Gallien, 1959
Poyntonophrynus dombensis	Channing, 2001	Channing, 2001	Channing, 2001
Poyntonophrynus fenoulheti	Channing, 2001	Lambiris, 1989	Lambiris, 1989
Schismaderma carens	Channing, 2001	Channing, 2001	Channing, 2001
Vandijkophrynus amatolicus	Channing, 2001		Channing, 2001
Vandijkophrynus angusticeps	Channing, 2001	Channing, 2001	Channing, 2001
Vandijkophrynus gariepensis	Channing, 2001		Channing, 2001
Vandijkophrynus robinsoni	Channing, 2001	Minter et al., 2004	
Werneria bambutensis	Rödel et al., 2004	Amiet, 1976	Amiet, 1976
Werneria tandyi	Rödel et al., 2004	Amiet, 1976	Amiet, 1976
Wolterstroffina parvipalmata	Perret, 1966	Mertens, 1939	Mertens, 1939

References for Table 2S

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SUPPLEMENTARY MATERIALS

Chapter III

Online Appendix 1. Primers and PCR conditions used for generating the sequence data for this study.

Gene	Primer	Length	Source	Cycling profile
12S	L1091: 5'-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3' R1478: 5'-TGACTGCAGAGGGTGACGGGCGGTGTGT-3'	~380bp	Kocher et al. 1989	95°C – 5.00 min [95°C – 1.00 min 51°C – 1.00 min 72°C – 1.30 min] x35 72°C – 7.00 min
16S	FWD: 5'-CGCCTGTTACCAAAAACAT-3' REV: 5'-CCGGTTTGAACTCAGATCA-3'	~570bp	Palumbi 1996	
COI	P3F*: 5'-CAATACCAAACCCCCTTRTTYGTWTGATC-3' P3R: 5'-GCTTCTCARATAATAAATATYAT-3'	841bp	San Mauro et al. 2004	95°C – 5.00 mins [95°C – 45.0 sec 50°C – 45.0 sec 72°C – 1.30 min] x35 72°C – 7.00 min
	co1f: 5'-CCTGCAGGAGGAGGAGAYCC-3' COIa: 5'-AGTATAAGCGTCTGGGTAGTC-3'	639bp	Kessing et al. 1989; Palumbi et al. 2002	
CXCR4	CXCR4-C: 5'-GTCATGGGCTAYCARAAGAA-3' CXCR4-F: 5'-TTGAATTTGGCCCRAGGAARGC-3'	711bp	Biju and Bossuyt 2003	95°C – 5.00 mins [95°C – 1.00 min 52°C – 1.00 min 72°C – 1.30 min] x45 72°C – 7min
	CXCR4-E: 5'-AGGACAATGACWGAYAAGTA-3' CXCR4-G: 5'-AGGCAACAGTGGAARAANGC-3'	687bp	Biju and Bossuyt 2003	
RAG1	RAG1.Mart.FL1: 5'-AGCTGCAGYCARTAYCAYAARATGTA-3' RAG1.AMP.R1: 5'-AACTCAGCTGCATTKCCAATRTCA-3'	933bp	Páez-Moscoso and Guayasamin 2012	95°C – 5.00 mins [95°C – 20.0 sec 50°C – 25.0 sec 72°C – 2.00 min] x40 72°C – 7.00 min
	RAG1 C: 5'-GGAGATGTTAGTGAGAARCAYGG-3' RAG1 E: 5'-TCCGCTGCATTTCCRATGTCRCA-3'	558bp	Biju and Bossuyt 2003	

* Identical primers were used for sequencing reactions, with the exception of P3F for which a modified, shorter version of the primer was used (P3F seq: 5'-TACCAAACCCCCTTRTTYG-3').

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ONLINE APPENDIX 2. GenBank numbers and voucher information for individuals included in the phylogenetic reconstructions.

Species	Voucher ID	Tree	Country	12S	16S	COI	CXCR4	RAG1
Altiphrynoides cf. *osgoodi*	MW6306	FAR tree & Full tree	Ethiopia	KF664637	KF665309	KF665726	KF665885	KF666313
Altiphrynoides malcolmi	MW6331	FAR tree & Full tree	Ethiopia	KF665005	KF665145	KF665785	KF665916	KF666436
Altiphrynoides malcolmi	MW6333	FAR tree	Ethiopia		KF665264	KF665741		
Altiphrynoides malcolmi	SL004	FAR tree & Full tree	Ethiopia	KF664830	KF665218	KF665715	KF665971	KF666287
Altiphrynoides malcolmi	SL079	FAR tree & Full tree	Ethiopia	KF664681	KF665285	KF665720	KF665965	KF666181
Amietophrynus brauni	BM2002.350	FAR tree & Full tree	Tanzania	KF664985	EF107208	KF665644	EF107492	EF107331
Amietophrynus brauni	brauA	FAR tree	Tanzania	AF220840	AF220886			
Amietophrynus brauni	FMNH 251853	FAR tree	Tanzania	DQ158437	DQ158437		DQ306514	DQ158361
Amietophrynus brauni	KMH21154	FAR tree	Tanzania	KF664774	KF665207			
Amietophrynus brauni	KMH21184	FAR tree	Tanzania	KF664782	KF665163			
Amietophrynus brauni	KMH21527	FAR tree & Full tree	Tanzania	KF664650	KF665239	KF665608	KF665991	KF666342
Amietophrynus brauni	KMH22583	FAR tree	Tanzania	KF664999	KF665161		KF665847	
Amietophrynus brauni	KMH23757	FAR tree	Tanzania	KF664796	KF665244			
Amietophrynus brauni	KMH23781	FAR tree & Full tree	Tanzania	KF664688	KF665339	KF665582	KF665999	KF666407
Amietophrynus brauni	KMH25754	FAR tree	Tanzania	KF664880	KF665186		KF665912	
Amietophrynus brauni	MCZ-23158	FAR tree & Full tree	Tanzania	KF664775	KF665128	KF665621	KF666085	KF666397
Amietophrynus brauni	MCZ:A-138507	FAR tree	Tanzania		HM754618			
Amietophrynus brauni	MCZ:A-138552	FAR tree	Tanzania		HM754617			
Amietophrynus brauni	MTSN 5237	FAR tree	Tanzania	KF664956	KF665273			
Amietophrynus brauni	MTSN 5258	FAR tree	Tanzania	KF664691	KF665424	KF665635		
Amietophrynus brauni	MVZ:Herp:233789	FAR tree	Tanzania	KF664915	KF665211	KF665794		KF666201
Amietophrynus brauni	MVZ:Herp:233790	FAR tree	Tanzania	KF664987	KF665416	KF665827		
Amietophrynus camerunensis	cameA	FAR tree	Equatorial Guinea	AF220846	AF220893			
Amietophrynus camerunensis	CAS 199137	FAR tree	Cameroon	GU226836	GU226836	KF665533		
Amietophrynus camerunensis	CAS 207288	FAR tree	Equatorial Guinea	DQ158439	DQ158439		DQ306555	DQ158363
Amietophrynus camerunensis	DS 81	FAR tree & Full tree	Central African Republic	KF664869	KF665123	KF665563	KF665865	KF666461

Amietophrynus camerunensis	NCSM 76800	FAR tree & Full tree	Gabon	KF665022	KF665404	KF665730	KF665920	KF666271
Amietophrynus camerunensis	NCSM 77612	FAR tree & Full tree	Gabon	KF664686	KF665368	KF665705	KF666067	KF666404
Amietophrynus channingi	ZFMK 63894	FAR tree	DRC		Q882842			
Amietophrynus channingi	ZFMK 62573	FAR tree	DRC	KF664735	HQ882843		KF666006	
Amietophrynus garmani	16BTspA	FAR tree	South Africa		AF220885			
Amietophrynus garmani	AACRG 0069?	& Full tree	South Africa	KF664721	KF665115	KF665525	KF665980	KF666415
Amietophrynus garmani	AACRG 1592	FAR tree & Full tree	South Africa	KF664668	KF665078	KF665541	KF665836	KF666466
Amietophrynus garmani	MCZ38808	FAR tree & Full tree	South Africa	KF664684	KF665281	KF665707	KF666109	KF666160
Amietophrynus cf. *garmani*	MCZFS-A-15501	FAR tree & Full tree	Ethiopia	KF664872	KF665314	KF665675	KF666077	KF666256
Amietophrynus cf. *garmani*	MCZFS-A-15545	FAR tree	Ethiopia	KF664921	KF665233		KF665958	KF666284
Amietophrynus cf. *garmani*	MCZFS-Z-37784	FAR tree & Full tree	Ethiopia	KF664767	KF665446	KF665548	KF666093	KF666157
Amietophrynus cf. *garmani*	MVZ:Herp:234095	FAR tree	Kenya	KF664633	KF665151	KF665739		
Amietophrynus cf. *garmani*	SL164	FAR tree & Full tree	Ethiopia	KF664903	KF665096	KF665687	KF665914	KF666352
Amietophrynus gracilipes	vgCG12-009	FAR tree	Congo Rep.	KF664627	KF665063		KF665837	KF666242
Amietophrynus gracilipes	vgCG12-103	FAR tree	Congo Rep.	KF664631	KF665384		KF665903	
Amietophrynus cf. *gracilipes*	831LG	FAR tree & Full tree	Cameroon	KF664690	KF665147	KF665520	KF665886	KF666402
Amietophrynus cf. *gracilipes*	CAS 207620	FAR tree & Full tree	Equatorial Guinea	FJ882824	FJ882824	KF665561	FJ882724	DQ158378
Amietophrynus cf. *gracilipes*	DS 07	FAR tree	Central African Republic	KF664682	KF665388			
Amietophrynus cf. *gracilipes*	DS 08	FAR tree	Central African Republic	KF664972	KF665478			KF666318
Amietophrynus cf. *gracilipes*	DS 66	FAR tree & Full tree	Central African Republic	KF664793	KF665459	KF665530	KF666081	KF666451
Amietophrynus cf. *gracilipes*	DS 74	FAR tree	Central African Republic	KF664748	KF665190			
Amietophrynus cf. *gracilipes*	DS 80	FAR tree	Central African Republic	KF665001	KF665406			
Amietophrynus cf. *gracilipes*	DS 98	FAR tree & Full tree	Central African Republic	KF664953	KF665414	KF665683	KF665968	KF666239
Amietophrynus cf. *gracilipes*	NCSM 76801	FAR tree & Full tree	Gabon	KF664874	KF665287	KF665534	KF666103	KF666364
Amietophrynus cf. *gracilipes*	vg09-046	FAR tree & Full tree	Cameroon	KF664893	KF665206	KF665779	KF666016	KF666280
Amietophrynus cf. *gracilipes*	vgCAR089	FAR tree	Central African Republic		KF665410			
Amietophrynus gutturalis	AACRG 1015	FAR tree	Botswana	KF664926	KF665453		KF665917	KF666316
Amietophrynus gutturalis	AC2362	FAR tree	Tanzania	KF664644	KF665126			
Amietophrynus gutturalis	AC2809	FAR tree & Full tree	South Africa	KF664945	KF665219	KF665606	KF665973	KF666453

Amietophrynus gutturalis	AC2914	FAR tree	Tanzania	KF664860	KF665463			
Amietophrynus gutturalis	AC2933	FAR tree & Full tree	Tanzania	KF665004	KF665280	KF665551	KF665986	KF666301
Amietophrynus gutturalis	BM2000.980	FAR tree	Tanzania	KF664928	KF665295			
Amietophrynus gutturalis	BM2005.1542	FAR tree	Tanzania	KF664723	KF665159	KF665804		KF666336
Amietophrynus gutturalis	FMNH 251386	FAR tree	Tanzania	KF664706	KF665467			
Amietophrynus gutturalis	FMNH 274838	FAR tree	Malawi	KF664602	KF665044			
Amietophrynus gutturalis	FMNH 274839	FAR tree	Malawi	KF664835	KF665183			
Amietophrynus gutturalis	FMNH 274864	FAR tree	Malawi	KF664837	KF665330			
Amietophrynus gutturalis	FMNH 274865	FAR tree	Malawi	KF664792	KF665369			
Amietophrynus gutturalis	FMNH 274866	FAR tree	Malawi	KF664727	KF665297			
Amietophrynus gutturalis	FMNH 274910	FAR tree	Malawi	KF664785	KF665204			
Amietophrynus gutturalis	FMNH 274911	FAR tree & Full tree	Malawi	KF664843	KF665402	KF665729	KF665879	KF666217
Amietophrynus gutturalis	guttA	FAR tree	South Africa	AF220831	AF220875			
Amietophrynus gutturalis	guttB	FAR tree	Mozambique	AF220832	AF220876			
Amietophrynus gutturalis	guttC	FAR tree	South Africa		AF220877			
Amietophrynus gutturalis	guttD	FAR tree	South Africa		AF220878			
Amietophrynus gutturalis	HM 1589	FAR tree	Malawi		KF665275			
Amietophrynus gutturalis	M 250	FAR tree	Malawi	KF664980	KF665317		KF666032	
Amietophrynus gutturalis	MTSN 7315	FAR tree & Full tree	Tanzania	KF664675	KF665437	KF665545	KF666021	KF666266
Amietophrynus gutturalis	MTSN 7401	FAR tree & Full tree	Tanzania	KF664799	KF665389	KF665772	KF666090	KF666260
Amietophrynus gutturalis	MTSN 9749	FAR tree & Full tree	DRC	KF664940	KF665124	KF665694	KF666013	KF666209
Amietophrynus gutturalis	MTSN 9763	FAR tree & Full tree	DRC	KF664907	KF665101	KF665652	KF665866	KF666143
Amietophrynus gutturalis	MTSN 9969	FAR tree & Full tree	DRC	KF664738	KF665160	KF665775	KF666033	KF666203
Amietophrynus gutturalis	MVZ:Herp:223357	FAR tree	Zimbabwe	U52746				
Amietophrynus gutturalis	MVZ:Herp:233792	FAR tree & Full tree	Kenya	KF664742	KF665221	KF665543	KF666120	KF666460
Amietophrynus gutturalis	MVZ:Herp:234057	FAR tree & Full tree	Uganda	KF664910	KF665364	KF665828	KF665926	KF666438
Amietophrynus gutturalis	MVZ:Herp:265837	FAR tree	Mozambique		KF665360			
Amietophrynus gutturalis	MVZ:Herp:265838	FAR tree	Mozambique		KF665487			
Amietophrynus gutturalis	MVZ:Herp:265840	FAR tree	Mozambique		KF665290			

Amietophrynus gutturalis	MVZ:Herp:265843	FAR tree	Mozambique		KF665315			
Amietophrynus gutturalis	MVZ:Herp:265844	FAR tree	Mozambique		KF665051			
Amietophrynus gutturalis	MVZ:Herp:265846	FAR tree	Mozambique		KF665042			
Amietophrynus gutturalis	MVZ:Herp:265847	FAR tree	Mozambique		KF665366			
Amietophrynus gutturalis	MVZ:Herp:265856	FAR tree	Mozambique		KF665493			
Amietophrynus gutturalis	MVZ:Herp:265857	FAR tree	Mozambique		KF665198			
Amietophrynus gutturalis	MVZ:Herp:265867	FAR tree	Mozambique		KF665054			
Amietophrynus gutturalis	MW4174	FAR tree	Tanzania	FJ882851	FJ882851		FJ882725	
Amietophrynus gutturalis	MW6389	FAR tree	Ethiopia	KF664620	KF665283	KF665637		
Amietophrynus gutturalis	PK045	FAR tree & Full tree	Kenya	KF664593	KF665474	KF665760	KF666074	KF666424
Amietophrynus gutturalis	SL 1104	FAR tree	DRC	KF664803	KF665199			
Amietophrynus gutturalis	SL481	FAR tree			GQ183567			
Amietophrynus gutturalis	STG001	FAR tree & Full tree	Tanzania	KF664857	KF665494	KF665497	KF666128	KF666265
Amietophrynus gutturalis	STG002	FAR tree & Full tree	Tanzania	KF664978	KF665112	KF665666	KF666096	KF666303
Amietophrynus kisoloensis	CAS 201948	FAR tree & Full tree	Uganda	GU226837	GU226837	KF665519	GU226834	KF666361
Amietophrynus kisoloensis	CAS 202005	FAR tree	Uganda	DQ158464	DQ158464		DQ306560	
Amietophrynus kisoloensis	kisoA	FAR tree	Uganda	AF220844	AF220891			
Amietophrynus kisoloensis	MTSN 6879	FAR tree & Full tree	DRC	KF664753	KF665266	KF665696	KF666003	KF666183
Amietophrynus kisoloensis	MTSN 7219	FAR tree & Full tree	Rwanda	KF664725	KF665248	KF665752	KF665982	KF666233
Amietophrynus kisoloensis	MVZ:Herp:223361	FAR tree	Uganda	AY325995	AY325995			
Amietophrynus kisoloensis	SL482	FAR tree			GQ183568			
Amietophrynus kisoloensis	TNHC 61999	FAR tree	North Africa	AY680264	AY680264			
Amietophrynus cf. *kisoloensis*	MTSN 6882	FAR tree & Full tree	DRC	KF664807	KF665434	KF665627	KF666079	KF666255
Amietophrynus cf. *kisoloensis*	MTSN 7348	FAR tree & Full tree	Rwanda	KF664836	KF665291	KF665591	KF665998	KF666261
Amietophrynus cf. *kisoloensis*	MTSN 7355	FAR tree & Full tree	Rwanda	KF664673	KF665192	KF665734	KF666011	KF666425
Amietophrynus latifrons	AMC319	FAR tree & Full tree	Cameroon	KF664884	KF665484	KF665546	KF666022	KF666222
Amietophrynus latifrons	MC11_035	FAR tree & Full tree	Cameroon	KF664929	KF665409	KF665647	KF666004	KF666272
Amietophrynus latifrons	MH0206	FAR tree & Full tree	Cameroon	KF664962	KF665217	KF665737	KF666112	KF666154
Amietophrynus latifrons	MH0233	FAR tree & Full tree	Cameroon	KF664617	KF665091	KF665630	KF666133	KF666146

Amietophrynus latifrons	MH0423	FAR tree	Cameroon	KF664737	KF665249			
Amietophrynus lemairii	AACRG 1052	FAR tree & Full tree	Botswana	KF664873	KF665036	KF665803	KF666038	KF666396
Amietophrynus lemairii	lemaA	FAR tree	Botswana	AF220847	AF220895			
Amietophrynus maculatus	AACRG 0684	FAR tree & Full tree	South Africa	KF664989	KF665241	KF665506	KF666064	KF666205
Amietophrynus maculatus	AMC002	FAR tree	Cameroon	KF664704	KF665257	KF665678	KF665831	
Amietophrynus maculatus	AMC012	FAR tree	Cameroon	KF664605	KF665213		KF666078	
Amietophrynus maculatus	AMC041	FAR tree	Cameroon	KF664817	KF665048		KF666136	
Amietophrynus maculatus	AMC084	FAR tree & Full tree	Cameroon	KF664896	KF665243	KF665709	KF666134	KF666389
Amietophrynus maculatus	AMC147	FAR tree & Full tree	Cameroon	KF664902	KF665456	KF665526	KF665938	KF666432
Amietophrynus maculatus	AMI 1	FAR tree & Full tree	Cameroon	KF664786	KF665308	KF665566	KF665961	KF666248
Amietophrynus maculatus	BE 39	FAR tree	Benin	KF664954	KF665492			
Amietophrynus maculatus	CAS 229969	FAR tree	Sierra Leone	KF664702	KF665166			
Amietophrynus maculatus	CAS 229986	FAR tree & Full tree	Sierra Leone	KF665024	KF665184	KF665770	KF665921	KF666277
Amietophrynus maculatus	CAS 229987	FAR tree	Sierra Leone	KF664658	KF665113			
Amietophrynus maculatus	CAS 229988	FAR tree & Full tree	Sierra Leone	KF664671	KF665251	KF665769	KF665989	KF666234
Amietophrynus maculatus	CAS 230064	FAR tree	Sierra Leone	KF664679	KF665411	KF665562	KF665851	
Amietophrynus maculatus	DS 83	FAR tree & Full tree	Central African Republic	KF664744	KF665090	KF665761	KF665929	KF666368
Amietophrynus maculatus	GS 196	FAR tree & Full tree	Sierra Leone	KF665023	KF665334	KF665661	KF665948	KF666428
Amietophrynus maculatus	HM 1626	FAR tree	Malawi		KF665361			
Amietophrynus maculatus	HM 1648	FAR tree	Malawi		KF665443			
Amietophrynus maculatus	HM 1652	FAR tree	Malawi		KF665230			
Amietophrynus maculatus	HM 1746	FAR tree	Malawi		KF665059			
Amietophrynus maculatus	LE 36	FAR tree & Full tree	Ghana	KF664838	KF665175	KF665748	KF666139	KF666349
Amietophrynus maculatus	M 263	FAR tree & Full tree	Malawi	KF664841	KF665454	KF665733	KF665835	KF666420
Amietophrynus maculatus	macuA	FAR tree	Swaziland	AF220837	AF220883			
Amietophrynus maculatus	macuB	FAR tree	Uganda	AF220838	AF220884			
Amietophrynus maculatus	MVZ:Herp:233791	FAR tree	Uganda	KF664787	KF665136			
Amietophrynus maculatus	MVZ:Herp:234551	FAR tree	Uganda	KF664607	KF665439	KF665578	KF665868	
Amietophrynus maculatus	MVZ:Herp:253187	FAR tree & Full tree	Nigeria	KF664888	KF665307	KF665639	KF665848	KF666291

Amietophrynus maculatus	MVZ:Herp:265841	FAR tree	Mozambique		KF665329			
Amietophrynus maculatus	MVZ:Herp:265845	FAR tree	Mozambique		KF665031			
Amietophrynus maculatus	MVZ:Herp:265863	FAR tree	Mozambique		KF665393			
Amietophrynus maculatus	MVZ:Herp:265864	FAR tree	Mozambique		KF665189			
Amietophrynus maculatus	MW6140	FAR tree	Sierra Leone	GU183858	GU183858		GU183851	
Amietophrynus maculatus	Ni 105	FAR tree	Mozambique	KF664645	KF665173			
Amietophrynus maculatus	NI 42	FAR tree	Mozambique	KF664957	KF665343	KF665749		KF666216
Amietophrynus maculatus	PK126	FAR tree & Full tree	Kenya	KF664726	KF665111	KF665789	KF666017	KF666417
Amietophrynus maculatus	SA 128	FAR tree	Senegal	KF664698	KF665073			
Amietophrynus maculatus	ZFMK 75443	FAR tree & Full tree	Cameroon	KF664959	KF665107	KF665556	KF665883	KF666305
Amietophrynus maculatus	ZFMK 92986	FAR tree & Full tree	Ivory Coast	KF664832	KF665196	KF665553	KF666073	KF666462
Amietophrynus maculatus	ZFMK 92987	FAR tree & Full tree	Ivory Coast	KF664797	KF665371	KF665594	KF665897	KF666144
Amietophrynus maculatus	ZFMK 92988	FAR tree & Full tree	Ivory Coast	KF664802	KF665102	KF665809	KF665966	KF666204
Amietophrynus mauritanicus	isolate Algeria	FAR tree	Morocco	FJ609232				
Amietophrynus mauritanicus	isolate Argana	FAR tree	Morocco	FJ609236				
Amietophrynus mauritanicus	isolate Tunisia	FAR tree	Tunisia	FJ609238	FJ609239			
Amietophrynus mauritanicus	MNCN/ADN15.707	FAR tree	Morroco	KF664616	KF665089		KF665934	KF666315
Amietophrynus mauritanicus	MVZ:Herp:164714	FAR tree	Morocco	AY680265	AY680265			
Amietophrynus mauritanicus	NP B-22-1	FAR tree	Morocco	FJ882826	FJ882826		FJ882727	
Amietophrynus mauritanicus	vg07-025	FAR tree & Full tree	Morocco	KF664780	KF665428	KF665723	KF666116	KF666227
Amietophrynus pantherinus	MH_0276	FAR tree & Full tree	South Africa	KF664917	KF665321	KF665614	KF666024	KF666226
Amietophrynus pantherinus	MH0309	FAR tree & Full tree	South Africa	KF664685	KF665451	KF665822	KF666060	KF666180
Amietophrynus pantherinus	pantA	FAR tree	South Africa	AF220848	AF220896			
Amietophrynus pantherinus	pathC	FAR tree	South Africa	AF220849				
Amietophrynus pardalis	HB035	FAR tree & Full tree	South Africa	KF664840	KF665337	KF665527	KF665852	KF666241
Amietophrynus pardalis	HB036	FAR tree	South Africa		KF665227			
Amietophrynus pardalis	pardA	FAR tree	South Africa	AF220850	AF220897			
Amietophrynus poweri	AACRG 0795	FAR tree & Full tree	South Africa	KF664609	KF665365	KF665776	KF665949	KF666328
Amietophrynus poweri	AACRG 0803	FAR tree	Namibia	KF664652	KF665349	KF665766		KF666152

Amietophrynus poweri	CAS 193854	FAR tree	Namibia	U52745				
Amietophrynus poweri	CAS 193857	FAR tree	Namibia	FJ882771	KF665399		FJ882722	
Amietophrynus poweri	CAS 193885	FAR tree	Namibia	DQ158482	DQ158482		DQ306559	DQ158401
Amietophrynus poweri	garmA	FAR tree	South Africa	AF220833	AF220879			
Amietophrynus poweri	poweA	FAR tree	Namibia	AF220834	AF220880			
Amietophrynus poweri	poweB	FAR tree	Botswana	AF220835	AF220881			
Amietophrynus poweri	poweC	FAR tree	Botswana	AF220836	AF220882			
Amietophrynus poweri	VC080	FAR tree & Full tree	South Africa	KF664862	KF665138	KF665565	KF665839	KF666324
Amietophrynus rangeri	AC2471	FAR tree	South Africa	KF664707	KF665327	KF665783	KF665946	
Amietophrynus rangeri	AC2473	FAR tree & Full tree	South Africa	KF664760	KF665268	KF665806	KF665871	KF666416
Amietophrynus rangeri	AC2727	FAR tree & Full tree	South Africa	KF664731	KF665289	KF665763	KF666138	KF666238
Amietophrynus rangeri	rangA	FAR tree	South Africa	AF220828	AF220868			
Amietophrynus rangeri	rangB	FAR tree	South Africa	AF220829	AF220869			
Amietophrynus rangeri	rangC	FAR tree	South Africa	AF220830	AF220870			
Amietophrynus rangeri	rangD	FAR tree	South Africa		AF220871			
Amietophrynus rangeri	rangE	FAR tree	South Africa		AF220872			
Amietophrynus rangeri	rangF	FAR tree	South Africa		AF220873			
Amietophrynus rangeri	rangG	FAR tree	South Africa		AF220874			
Amietophrynus regularis	DS 82	FAR tree & Full tree	Central African Republic	KF664618	KF665408	KF665651	KF666072	KF666405
Amietophrynus regularis	E102	& Full tree	Egypt	KF664821	KF665120	KF665552	KF665904	KF666166
Amietophrynus regularis	E21	FAR tree	Egypt	KF664995	KF665201			
Amietophrynus regularis	E36	FAR tree	Egypt	KF664789	KF665490			
Amietophrynus regularis	E56	FAR tree	Egypt	KF664773	KF665139			
Amietophrynus regularis	FMNH 262252	FAR tree	Niger	KF664632	KF665473			
Amietophrynus regularis	FMNH 262253	FAR tree & Full tree	Niger	KF664728	KF665104	KF665599	KF666092	KF666292
Amietophrynus regularis	GS 193	FAR tree & Full tree	Sierra Leone	KF664635	KF665421	KF665756	KF665876	KF666427
Amietophrynus regularis	isolate 001	FAR tree	Capa Verde	HM769992	HM770010			
Amietophrynus regularis	isolate 002	FAR tree	Capa Verde	HM769993	HM770011			
Amietophrynus regularis	isolate 003	FAR tree	Capa Verde	HM769994	HM770012			

Amietophrynus regularis	isolate 004	FAR tree	Capa Verde	HM769995	HM770013			
Amietophrynus regularis	isolate 005	FAR tree	Capa Verde	HM769996	HM770014			
Amietophrynus regularis	isolate 006	FAR tree	Capa Verde	HM769997	HM770015			
Amietophrynus regularis	isolate 007	FAR tree	Capa Verde	HM769998	HM770016			
Amietophrynus regularis	isolate 008	FAR tree	Capa Verde	HM769999	HM770017			
Amietophrynus regularis	isolate 009	FAR tree	Capa Verde	HM770000	HM770018			
Amietophrynus regularis	isolate 010	FAR tree	Capa Verde	HM770001	HM770019			
Amietophrynus regularis	isolate 410	FAR tree	Niger	HM769984	HM770002			
Amietophrynus regularis	isolate 411	FAR tree	Niger	HM769985	HM770003			
Amietophrynus regularis	isolate 417	FAR tree	Burkina Faso	HM769986	HM770004			
Amietophrynus regularis	isolate 423	FAR tree	Burkina Faso	HM769987	HM770005			
Amietophrynus regularis	isolate 424	FAR tree	Burkina Faso	HM769988	HM770006			
Amietophrynus regularis	isolate 460	FAR tree	Mali	HM769989	HM770007			
Amietophrynus regularis	isolate B1	FAR tree	Guinea-Bissau	HM769990	HM770008			
Amietophrynus regularis	isolate B2	FAR tree	Guinea-Bissau	HM769991	HM770009			
Amietophrynus regularis	KU 290435	FAR tree	Ghana	DQ158485	DQ158485		DQ306523	DQ158404
Amietophrynus regularis	LM 137	FAR tree	Ghana	AY028486				
Amietophrynus regularis	MVZ:Herp:223372	FAR tree	Uganda	U52728	U52762			
Amietophrynus regularis	MVZ:Herp:245396	FAR tree & Full tree	Ghana	KF664708	KF665303	KF665569	KF665932	KF666362
Amietophrynus regularis	reguA	FAR tree	Kenya	AF220843	AF220889			
Amietophrynus regularis	reguB	FAR tree	Uganda		AF220890			
Amietophrynus regularis	SA 016	FAR tree	Senegal	KF664747	KF665098		KF665905	
Amietophrynus regularis	SA 118	FAR tree & Full tree	Senegal	KF664812	KF665356	KF665716	KF665956	KF666163
Amietophrynus regularis	SIH-04	FAR tree		AY330899	AY330891			AY323763
Amietophrynus regularis	SL501	FAR tree			GQ183570			
Amietophrynus regularis	ZFMK 75630	FAR tree & Full tree	Cameroon	KF664966	KF665304	KF665824	KF665954	KF666384
Amietophrynus regularis	ZFMK 75631	FAR tree	Cameroon	KF664740	KF665069		KF665878	KF666299
Amietophrynus sp.	AC2905	FAR tree & Full tree	Tanzania	KF664615	KF665405	KF665815	KF666076	KF666167
Amietophrynus sp.	MTSN 9840	FAR tree & Full tree	DRC	KF664960	KF665182	KF665584	KF666028	KF666412

Amietophrynus sp.	ZFMK 75769	FAR tree & Full tree	Cameroon	KF664636	KF665305	KF665746	KF665959	KF666240
Amietophrynus steindachneri	CAS 214839	FAR tree & Full tree	Kenya	FJ882825	FJ882825	KF665771	FJ882726	DQ158406
Amietophrynus steindachneri	MVZ:Herp:223373	FAR tree	Kenya	AY325981	AY325981			
Amietophrynus steindachneri	MVZ:Herp:223374	FAR tree	Kenya		U52763			
Amietophrynus steindachneri	VW596	FAR tree	Kenya	KF664750	KF665420		KF665937	KF666409
Amietophrynus steindachneri	VW614	FAR tree	Kenya	KF664765	KF665379		KF666083	KF666376
Amietophrynus superciliaris	E182.11	FAR tree	Cameroon	KF664963	HQ882848			KF666343
Amietophrynus superciliaris	E184.1	FAR tree	Guinea		HQ882846			
Amietophrynus superciliaris	E184.2	FAR tree	Guinea	KF664666	KF665234		KF666054	KF666442
Amietophrynus superciliaris	E184.3	FAR tree	Guinea	KF664629	HQ882845		KF666110	KF666281
Amietophrynus superciliaris	E184.4	FAR tree	Liberia	KF664754	HQ882847		KF665887	
Amietophrynus superciliaris	E187.2	FAR tree	Nigeria	KF664988	HQ882849			
Amietophrynus taiensis	GS 146	FAR tree	Sierra Leone	KF664883	KF665083		KF665963	KF666190
Amietophrynus taiensis	GS 147	FAR tree & Full tree	Sierra Leone	KF664745	KF665288	KF665686	KF666087	KF666321
Amietophrynus taiensis	GS 148	FAR tree & Full tree	Sierra Leone	KF664621	KF665302	KF665583	KF666027	KF666381
Amietophrynus taiensis	GS 149	FAR tree & Full tree	Sierra Leone	KF664851	KF665205	KF665643	KF666005	KF666346
Amietophrynus togoensis	ANK 53	FAR tree & Full tree	Ghana	KF664712	KF665445	KF665701	KF666035	KF666359
Amietophrynus togoensis	GS 109	FAR tree	Sierra Leone	KF664853	KF665433			
Amietophrynus togoensis	GU 146	FAR tree	Guinea	KF664771	KF665197			
Amietophrynus togoensis	GU 151	FAR tree & Full tree	Guinea	KF664974	KF665100	KF665662	KF666041	KF666408
Amietophrynus togoensis	GU 192	FAR tree & Full tree	Guinea	KF664899	KF665466	KF665542	KF666020	KF666221
Amietophrynus tuberosus	UTA A52375	FAR tree	Cameroon	DQ283362	DQ283362			
Amietophrynus tuberosus	vg10-221	FAR tree & Full tree	Cameroon	KF664779	KF665246	KF665810	KF665977	KF666290
Amietophrynus tuberosus	ZFMK 75441	FAR tree	Cameroon	KF664604	KF665419		KF665845	KF666150
Amietophrynus cf. *tuberosus*	vg10-222	FAR tree & Full tree	Cameroon	KF664761	KF665359	KF665502	KF666063	KF666286
Amietophrynus villiersi	LG0572	FAR tree & Full tree	Cameroon	KF664762	KF665353	KF665536	KF666001	KF666212
Amietophrynus villiersi	MH0340	FAR tree & Full tree	Cameroon	KF664845	KF665202	KF665792	KF666056	KF666353
Amietophrynus xeros	AC1989	FAR tree & Full tree	Tanzania	KF664612	KF665174	KF665754	KF665841	KF666275
Amietophrynus xeros	AMNH 109826	FAR tree	Mali	DQ158499	DQ158499		DQ306561	DQ158414

Amietophrynus xeros	BX1827	FAR tree	Mauritania		GQ868494			
Amietophrynus xeros	BX2211	FAR tree	Mauritania		GQ868491			
Amietophrynus xeros	BX2676	FAR tree	Mauritania		GQ868493			
Amietophrynus xeros	BX368	FAR tree	Nigeria		GQ868485			
Amietophrynus xeros	BX369	FAR tree	Nigeria		GQ868486			
Amietophrynus xeros	BX456	FAR tree	Mali		GQ868489			
Amietophrynus xeros	BX462	FAR tree	Mali		GQ868487			
Amietophrynus xeros	BX473	FAR tree	Senegal		GQ868490			
Amietophrynus xeros	BX994	FAR tree	Mali		GQ868488			
Amietophrynus xeros	CAS 214829	FAR tree & Full tree	Kenya	FJ882823	FJ882823	KF665511	FJ882723	DQ158375
Amietophrynus xeros	FMNH 262256	FAR tree & Full tree	Niger	KF665000	KF665140	KF665564	KF666137	KF666456
Amietophrynus xeros	FMNH 262289	FAR tree & Full tree	Niger	KF664724	KF665131	KF665670	KF666131	KF666430
Amietophrynus xeros	MHNG 2650.038	FAR tree	Mali	KF664878	KF665177	KF665523	KF666102	
Amietophrynus xeros	xeroA	FAR tree	Tanzania	AF220841	AF220887			
Amietophrynus xeros	xeroB	FAR tree	Tanzania	AF220842	AF220888			
Anaxyrus americanus	CAS 207258	Full tree	USA	FJ882827	FJ882827	KF665817	FJ882730	KF666350
Anaxyrus americanus	CAS 223832	Full tree	USA	KF664881	KF665122	KF665823	KF665863	KF666426
Anaxyrus boreas	CAS 176529	Full tree	USA	FJ882830	FJ882830	KF665820	FJ882732	KF666377
Anaxyrus boreas	CAS 201586	Full tree	USA	KF664930	KF665480	KF665518	KF665864	KF666312
Anaxyrus canorus	CAS 209233	Full tree	USA	KF664990	KF665178	KF665524	KF665840	KF666431
Anaxyrus terrestris	CAS 207171	Full tree	USA	FJ882829	FJ882829	KF665667	FJ882731	KF666176
Ansonia longidigita	VUB 0666	Full tree	Malaysia	FJ882796	FJ882796	KF665812	FJ882698	KF666400
Ansonia thinthinae	CAS 243945	Full tree	Myanmar	KF664734	KF665162	KF665611	KF665854	KF666367
Atelopus barbotini	BPN 1697	Full tree	French Guiana	GU183859	GU183859	KF665712	GU183852	KF666236
Bufo bufo	vg06-282	FAR tree & Full tree	Czech Republic	KF664601	KF665394	KF665517	KF666057	KF666388
Bufo gargarizans	CAS 228184	Full tree	China	FJ882808	FJ882808	KF665641	FJ882708	KF666177
Bufo pageoti	CAS 233251	Full tree	Myanmar	KF664905	KF665335	KF665626	KF665978	KF666231
"Bufo" pentoni	BE 20	FAR tree & Full tree	Benin	KF664969	KF665129	KF665512	KF666058	KF666258
Bufotes surdus	ZMMSU A-4027	Full tree	China	FJ882810	FJ882810		FJ882711	

Bufotes viridis	vg07-187	Full tree	Czech Republic	KF664594	KF665464	KF665616	KF665913	KF666439
Capensibufo rosei	AC2963	FAR tree	South Africa	KF664900	KF665475		KF666111	KF666251
Capensibufo rosei	AdV1	FAR tree	South Africa		FN652326			
Capensibufo rosei	AdV16	FAR tree	South Africa		FN652330			
Capensibufo rosei	AdV17	FAR tree	South Africa		FN652331			
Capensibufo rosei	AdV18	FAR tree	South Africa		FN652332			
Capensibufo rosei	AdV19	FAR tree	South Africa		FN652333			
Capensibufo rosei	AdV2	FAR tree	South Africa		FN652327			
Capensibufo rosei	AdV21	FAR tree	South Africa		FN652334			
Capensibufo rosei	AdV22	FAR tree	South Africa		FN652335			
Capensibufo rosei	AdV23	FAR tree	South Africa		FN652336			
Capensibufo rosei	AdV24	FAR tree	South Africa		FN652337			
Capensibufo rosei	AdV25	FAR tree	South Africa		FN652338			
Capensibufo rosei	AdV29	FAR tree	South Africa		FN652339			
Capensibufo rosei	ADV32	FAR tree	South Africa		FN652340			
Capensibufo rosei	ADV34	FAR tree	South Africa		FN652341			
Capensibufo rosei	AdV6	FAR tree	South Africa		FN652328			
Capensibufo rosei	AdV9	FAR tree	South Africa		FN652329			
Capensibufo rosei	crosA	FAR tree	South Africa	AF220864	AF220911			
Capensibufo rosei	KTH09-330	FAR tree & Full tree	South Africa	KF664778	KF665447	KF665585	KF666069	KF666395
Capensibufo rosei	KTH09-335	FAR tree & Full tree	South Africa	KF664868	KF665294	KF665706	KF665976	KF666159
Capensibufo rosei	MH_0233	FAR tree	South Africa		FN652325			
Capensibufo rosei	MH0197	FAR tree	South Africa		FN652324			
Capensibufo rosei	MH0201	FAR tree	South Africa		FN652323			
Capensibufo tradouwi	CF018	FAR tree	South Africa		FN652317			
Capensibufo tradouwi	CTGV1	FAR tree	South Africa		FN652321			
Capensibufo tradouwi	CTGV2	FAR tree	South Africa	KF664849	KF665072			
Capensibufo tradouwi	ctraA	FAR tree	South Africa	AF220865	AF220912			
Capensibufo tradouwi	KTH296	FAR tree	South Africa		FN652315			

Capensibufo tradouwi	KTH302	FAR tree	South Africa		FN652316			
Capensibufo tradouwi	MH0225	FAR tree	South Africa		FN652318			
Capensibufo tradouwi	MH0861	FAR tree	South Africa		FN652319			
Capensibufo tradouwi	MH0898	FAR tree	South Africa		FN652322			
Churamiti maridadi	MTSN 5584	FAR tree & Full tree	Tanzania	FJ882769	FJ882770	KF665516	KF666088	KF666213
Churamiti maridadi	MTSN 5585	FAR tree & Full tree	Tanzania	KF664661	KF665195	KF665768	KF665935	KF666268
Didynamipus sjostedti	0822LG	FAR tree & Full tree	Cameroon	KF664935	KF665469	KF665793	KF666047	KF666289
Didynamipus sjostedti	0824LG	FAR tree	Cameroon	KF664649	KF665099			
Didynamipus sjostedti	0825LG	FAR tree & Full tree	Cameroon	KF664600	KF665372	KF665550	KF665941	KF666294
Didynamipus sjostedti	0827LG	FAR tree & Full tree	Cameroon	KF664606	KF665485	KF665618	KF666012	KF666314
Didynamipus sjostedti	AG 259	FAR tree	Cameroon	AY325991	AY325991			
Didynamipus sjostedti	didyA	FAR tree	Cameroon	AF220867	AF220914			
Didynamipus sjostedti	MOR 0163	FAR tree	Nigeria	KF664805	KF665105			
Duttaphrynus crocus	CAS 220193	Full tree	Myanmar	FJ882789	FJ882789	KF665657	FJ882690	KF666270
Duttaphrynus dhufarensis	CAS 227584	Full tree	Oman	FJ882837	KF665085	KF665821	FJ882679	KF666330
Duttaphrynus olivaceus	CAS 232073	Full tree	Pakistan	KF664676	KF665215	KF665805	KF666043	KF666298
Duttaphrynus olivaceus	CAS 232138	Full tree	Pakistan	KF664603	KF665193	KF665735	KF666052	KF666419
Duttaphrynus stuarti	CAS 221485	Full tree	Myanmar	FJ882788	FJ882788	KF665503	FJ882689	KF666269
Epidalea calamita	vg07-119	Full tree	Czech Republic	KF664850	KF665137	KF665813	KF665981	KF666155
Incilius alvarius	UTA:A-53924	Full tree	USA	HM563818	HM563860		HM563891	HM563977
Incilius campbelli	UTA:A-50902	Full tree	Guatemala	HM563825	HM563866		HM563898	HM563984
Incilius coniferus	MVZ:Herp:203775	Full tree	Costa Rica	HM563829	HM563870		HM563902	HM563988
Incilius valliceps	MZFC:JRM-3868	Full tree	Mexico	HM563854	AY008211		HM563927	HM564013
Ingerophrynus divergens	VUB 0602	Full tree	Malaysia	FJ882802	FJ882802	KF665713	FJ882701	KF666187
Ingerophrynus macrotis	CAS 230357	Full tree	Myanmar	FJ882803	FJ882803	KF665540	KF666117	KF666244
Leptophryne borbonica	VUB 0673	Full tree	Malaysia	FJ882799	FJ882799	KF665688	EF107450	KF666468
Melanophryniscus stelzneri	VUB 0985	Full tree		FJ882853	FJ882853	KF665744	AY948784	KF666223
Mertensophryne anotis	anotA	FAR tree	Zimbabwe	AF220862	AF220910			
Mertensophryne anotis	anotB	FAR tree	Zimbabwe	AF220863				

Mertensophryne howelli	MTSN-T2202	FAR tree & Full tree	Tanzania	KF664964	KF665247	KF665531	KF666045	KF666383
Mertensophryne lindneri	BM2002.394	FAR tree & Full tree	Tanzania	KF664736	KF665426	KF665790	KF665953	KF666333
Mertensophryne lindneri	BM2005.930	FAR tree	Tanzania	KF665021	KF665153			
Mertensophryne lindneri	lindA	FAR tree	Mozambique	AF220861	AF220909			
Mertensophryne loveridgei	KMH26653	FAR tree & Full tree	Tanzania	FJ882820	FJ882820	KF665555	KF665834	KF666356
Mertensophryne loveridgei	MCZ-32084	FAR tree & Full tree	Tanzania	KF664924	KF665338	KF665572	KF665947	KF666463
Mertensophryne micranotis	BM2002.343	FAR tree & Full tree	Tanzania	KF664823	KF665194	KF665498	EF107491	EF107330
Mertensophryne micranotis	BM2002.364	FAR tree & Full tree	Tanzania	KF664784	KF665132	KF665825	KF666007	KF666414
Mertensophryne micranotis	BM2002.428	FAR tree	Tanzania	KF664822	KF665074	KF665814		
Mertensophryne micranotis	BM2005.135	FAR tree & Full tree	Tanzania	KF664976	KF665300	KF665697	KF665890	KF666354
Mertensophryne micranotis	MCZ-32087	FAR tree & Full tree	Tanzania	KF665020	KF665240	KF665579	KF666123	KF666378
Mertensophryne micranotis	MCZ-32088	FAR tree & Full tree	Tanzania	KF664672	KF665255	KF665632	KF665849	KF666457
Mertensophryne micranotis	MTSN 5443	FAR tree	Tanzania	KF664815	KF665209	KF665659	KF665939	
Mertensophryne micranotis	MTSN 5444	FAR tree	Tanzania	KF664783	KF665080	KF665731		
Mertensophryne micranotis	MTSN 5445	FAR tree	Tanzania	KF664833	KF665400	KF665623	KF665858	
Mertensophryne micranotis	MTSN 9558	FAR tree	Tanzania	KF664696	KF665200	KF665598	KF665927	
Mertensophryne micranotis	PK064	FAR tree & Full tree	Kenya	KF664729	KF665094	KF665654	KF666114	KF666387
Mertensophryne micranotis	PK118	FAR tree	Kenya	KF664947	KF665225			
Mertensophryne micranotis	VW00462	FAR tree	Kenya	KF664720	KF665041	KF665786		KF666435
Mertensophryne micranotis	VW00465	FAR tree & Full tree	Kenya	KF664824	KF665222	KF665796	KF666122	KF666338
Mertensophryne micranotis	VW679	FAR tree & Full tree	Kenya	KF664710	KF665250	KF665510	KF665856	KF666390
Mertensophryne micranotis	VW680	FAR tree & Full tree	Kenya	KF664891	KF665381	KF665610	KF666015	KF666232
Mertensophryne sp.	BM2002.158	FAR tree	Tanzania	KF664622	KF665060	KF665601	KF666055	
Mertensophryne sp.	BM2005.1541	FAR tree & Full tree	Tanzania	KF664938	KF665093	KF665645	KF665842	KF666145
Mertensophryne taitana	BM2005.1540	FAR tree	Tanzania	FJ882845	FJ882845			
Mertensophryne taitana	JM 773	FAR tree & Full tree	Kenya	KF664809	KF665047	KF665612	KF665995	KF666310
Mertensophryne taitana	JM0174	FAR tree	Kenya	KF664955	KF665088	KF665676	KF666050	
Mertensophryne taitana	MW4094	FAR tree	Kenya	KF664642	KF665491		KF665843	
Mertensophryne taitana	TNHC 53893	FAR tree		U52729				

Mertensophryne usambarae	MTSN 9541	FAR tree & Full tree	Tanzania	KF665026	KF665336	KF665800	KF666115	KF666360
Mertensophryne usambarae	MTSN 9570	FAR tree & Full tree	Tanzania	KF664699	KF665229	KF665575	KF665984	KF666174
Mertensophryne uzunguensis	BM2002.151	FAR tree	Tanzania	KF664697	KF665279	KF665692		
Mertensophryne uzunguensis	BM2002.157	FAR tree & Full tree	Tanzania	KF664717	KF665170	KF665699	FJ882720	KF666366
Mertensophryne uzunguensis	MTSN 5439	FAR tree	Tanzania		KF665231			
Mertensophryne uzunguensis	MTSN 5440	FAR tree	Tanzania	KF665028	KF665277			
Mertensophryne uzunguensis	MTSN 8712	FAR tree	Tanzania		KF665236			
Mertensophryne uzunguensis	MTSN 8783	FAR tree	Tanzania		KF665367			
Nectophryne afra	N41ROHO	Full tree	Nigeria	KF664625	KF665311	KF665682	KF666002	KF666418
Nectophryne afra	NCSM 77617	Full tree	Gabon	KF664971	KF665127	KF665698	KF665994	KF666182
Nectophryne cf. *afra*	MVZ:Herp:234689	Full tree	Cameroon	KF664806	KF665325	KF665791	KF665838	KF666171
Nectophryne cf. *afra*	MVZ:Herp:234857	Full tree	Cameroon	KF664711	KF665181	KF665829	KF665867	KF666446
Nectophryne batesii	887	Full tree		KF664719	KF665134	KF665649	KF665972	KF666296
Nectophryne batesii	0369LG	Full tree	Cameroon	KF664733	KF665242	KF665622	KF666124	KF666398
Nectophryne batesii	MTSN 5891	Full tree	Cameroon	KF664743	KF665452	KF665708	KF665850	KF666449
Nectophryne cf. *batesii*	0767LG	Full tree	Cameroon	KF664936	KF665413	KF665702	KF666108	KF666375
Nectophryne cf. *batesii*	MVZ:Herp:234688	Full tree	Cameroon	KF665012	KF665479	KF665571	KF666037	KF666225
Nectophryne cf. *batesii*	N43ROHO	Full tree	Nigeria	KF664912	KF665403	KF665725	KF665909	KF666247
Nectophryne cf. *batesii*	NCSM 76799	Full tree	Gabon	KF664979	KF665313	KF665581	KF666142	KF666311
Nectophrynoides asperginis	KMH 15150	Full tree	Tanzania	KF664776	KF665171	KF665547	KF665900	KF666319
Nectophrynoides frontierei	KMH16100	FAR tree	Tanzania	KF665015	KF665158	KF665751		
Nectophrynoides frontierei	KMH16367	FAR tree	Tanzania	KF664628	KF665223	KF665602		
Nectophrynoides laticeps	MTSN 5635	FAR tree & Full tree	Tanzania	KF664871	KF665263	KF665802	KF666119	KF666370
Nectophrynoides laticeps	MTSN 5637	FAR tree & Full tree	Tanzania	KF664863	KF665351	KF665710	KF665880	KF666172
Nectophrynoides laticeps	MTSN 5641	FAR tree & Full tree	Tanzania	KF664858	KF665261	KF665758	KF665957	KF666423
Nectophrynoides minutus	MW3309	FAR tree & Full tree	Tanzania	FJ882814	FJ882814	KF665588	KF665907	KF666454
Nectophrynoides minutus	MW7339	FAR tree	Tanzania	KF664755	KF665156	KF665634		
Nectophrynoides minutus	RO2007	FAR tree	Tanzania	KF664909	KF665391	KF665587		
Nectophrynoides minutus	RO2019	FAR tree & Full tree	Tanzania	KF664870	KF665429	KF665656	KF666019	KF666215

Nectophrynoides paulae	MTSN 5621	FAR tree	Tanzania	KF664855	KF665358	KF665620	KF666070	
Nectophrynoides paulae	MTSN 5622	FAR tree	Tanzania	KF664715	KF665188	KF665742		
Nectophrynoides paulae	MTSN 5623	FAR tree	Tanzania	KF664752	KF665235	KF665722		
Nectophrynoides paulae	MTSN 5624	FAR tree	Tanzania	KF664763	KF665238	KF665521		
Nectophrynoides paulae	MTSN 5626	FAR tree & Full tree	Tanzania	KF664950	KF665118	KF665801	KF666034	KF666169
Nectophrynoides paulae	MTSN 5630	FAR tree	Tanzania	KF664709	KF665328	KF665773		
Nectophrynoides poyntoni	MTSN 5075	FAR tree	Tanzania	KF664804	KF665468	KF665747	KF665870	
Nectophrynoides poyntoni	MTSN 5076	FAR tree & Full tree	Tanzania	KF664920	KF665092	KF665755	KF665910	KF666413
Nectophrynoides poyntoni	MTSN 5080	FAR tree	Tanzania	KF664701	KF665293	KF665724	KF665844	
Nectophrynoides pseudotornieri	MTSN 7782	FAR tree	Tanzania	KF664730	KF665296		KF665985	KF666317
Nectophrynoides pseudotornieri	RO2020	FAR tree & Full tree	Tanzania	KF664844	KF665392	KF665653	KF665906	KF666410
Nectophrynoides pseudotornieri	RO2143	FAR tree	Tanzania	KF664859	KF665417	KF665672	KF666105	
Nectophrynoides pseudotornieri	RO2157	FAR tree	Tanzania	KF664722	KF665082	KF665727	KF665943	
Nectophrynoides sp.	MTSN 8149	FAR tree	Tanzania	KF664770	KF665259	KF665609		
Nectophrynoides sp.	MTSN 8155	FAR tree	Tanzania	KF664646	KF665141	KF665797	KF665908	
Nectophrynoides sp.	MTSN 8175	FAR tree & Full tree	Tanzania	KF664864	KF665495	KF665819	KF666071	KF666164
Nectophrynoides sp.	MW6798	FAR tree	Tanzania	KF664643	KF665422	KF665642	KF666061	
Nectophrynoides sp.	KMH26262	FAR tree	Tanzania	KF664811	KF665108	KF665677	KF665832	
Nectophrynoides sp.	KMH26650	FAR tree & Full tree	Tanzania	KF664941	KF665191	KF665638	KF666099	KF666188
Nectophrynoides sp.	KMH35967	FAR tree & Full tree	Tanzania	KF664932	KF665441	KF665782	KF665936	KF666179
Nectophrynoides sp.	KMH35969	FAR tree	Tanzania	KF665007	KF665110	KF665671	KF666042	
Nectophrynoides sp.	MW1822	FAR tree	Tanzania	KF664759	KF665146		KF666082	EF107329
Nectophrynoides sp.	MW6695	FAR tree	Tanzania	KF664946	KF665050	KF665808	KF666059	
Nectophrynoides sp.	MW7011	FAR tree	Tanzania	KF664694	KF665401	KF665691	KF666098	
Nectophrynoides tornieri	KMH16085	FAR tree	Tanzania	KF665003	KF665438	KMH16085	KF666023	
Nectophrynoides tornieri	RDS951	FAR tree	Tanzania	KF664714	KF665256			
Nectophrynoides tornieri	TZ213	FAR tree	Tanzania	KF664861	KF665169	KF665513		
Nectophrynoides tornieri	TZ214	FAR tree & Full tree	Tanzania	KF664834	KF665046	KF665669	KF666125	KF666192
Nectophrynoides cf. *tornieri*	MTSN 5334	FAR tree	Tanzania	KF665006	KF665053		KF665992	

Nectophrynoides cf. *tornieri*	MTSN 5429	FAR tree	Tanzania	KF664718	KF665168	KF665567	MTSN5429	
Nectophrynoides cf. *tornieri*	MTSN 5432	FAR tree	Tanzania	KF664598	KF665179			
Nectophrynoides cf. *tornieri*	MTSN 5434	FAR tree	Tanzania	KF664716	KF665271	KF665674		
Nectophrynoides cf. *tornieri*	MTSN 5435	FAR tree	Tanzania	KF664984	KF665226			
Nectophrynoides cf. *tornieri*	MTSN 7725	FAR tree	Tanzania	KF664996	KF665431		KF666018	KF666335
Nectophrynoides cf. *tornieri*	MTSN 7751	FAR tree	Tanzania	KF664848	KF665116			KF666288
Nectophrynoides cf. *tornieri*	MTSN 7780	FAR tree	Tanzania	KF664973	KF665348			KF666228
Nectophrynoides cf. *tornieri*	MTSN 7781	FAR tree	Tanzania	KF664958	KF665109			KF666322
Nectophrynoides cf. *tornieri*	MTSN 8544	FAR tree	Tanzania	KF664751	KF665346	KF665787	KF665924	
Nectophrynoides cf. *tornieri*	MTSN 8545	FAR tree & Full tree	Tanzania	KF664801	KF665269	KF665660	KF665969	KF666326
Nectophrynoides cf. *tornieri*	MTSN 8546	FAR tree	Tanzania	KF664683	KF665385	KF665711		
Nectophrynoides cf. *tornieri*	MTSN 9080	& Full tree	Tanzania	KF664882	KF665322	KF665684	KF665875	KF666348
Nectophrynoides cf. *tornieri*	RO2078	FAR tree & Full tree	Tanzania	KF664975	KF665457	KF665528	KF665979	KF666422
Nectophrynoides cf. *tornieri*	RO2083	FAR tree	Tanzania	KF664689	KF665319	KF665624	KF666097	
Nectophrynoides cf. *tornieri*	RO2088	FAR tree	Tanzania	KF664813	KF665058	KF665633	KF666062	
Nectophrynoides cf. *tornieri*	RO2134	FAR tree	Tanzania	KF664788	KF665152	KF665774	KF666075	
Nectophrynoides cf. *tornieri*	TZ263	FAR tree & Full tree	Tanzania	KF664795	KF665396	KF665522	KF665990	KF666173
Nectophrynoides vestergaardi	MW3211	FAR tree & Full tree	Tanzania	KF665017	KF665310	KF665767	KF665853	KF666151
Nectophrynoides viviparus	H 20	FAR tree	Tanzania	KF664656	KF665316	KF665664		KF666161
Nectophrynoides viviparus	MTSN 9365	FAR tree & Full tree	Tanzania	KF664965	KF665079	KF665595	KF665951	KF666259
Nectophrynoides viviparus	MTSN 9383	FAR tree & Full tree	Tanzania	KF664886	KF665442	KF665799	KF665931	KF666158
Nectophrynoides cf. *viviparus*	KMH26637	FAR tree	Tanzania	KF665009	KF665097	KF665777	KF665974	
Nectophrynoides cf. *viviparus*	KMH26638	FAR tree	Tanzania	KF664951	KF665436	KF665718		
Nectophrynoides cf. *viviparus*	KMH26641	FAR tree & Full tree	Tanzania	KF664942	KF665387	KF665619	KF665933	KF666332
Nectophrynoides cf. *viviparus*	KMH26644	FAR tree	Tanzania	KF664839	KF665373	KF665740	KF665896	
Nectophrynoides cf. *viviparus*	KMH26998	FAR tree	Tanzania	KF664916	KF665180	KF665736	KF665855	
Nectophrynoides cf. *viviparus*	KMH27949	FAR tree	Tanzania	KF664939	KF665114	KF665826		
Nectophrynoides cf. *viviparus*	KMH27952	FAR tree	Tanzania	KF664669	KF665087	KF665807		
Nectophrynoides cf. *viviparus*	KMH27999	FAR tree	Tanzania	KF664687				

Nectophrynoides cf. *viviparus*	KMH28000	FAR tree	Tanzania	KF664901	KF665344			
Nectophrynoides cf. *viviparus*	KMH36201	FAR tree & Full tree	Tanzania	KF664624	KF665260	KF665628	KF665884	KF666191
Nectophrynoides cf. *viviparus*	MTSN 5248	FAR tree	Tanzania	KF664827	KF665274	KF665501	KF666030	
Nectophrynoides cf. *viviparus*	MTSN 5249	FAR tree	Tanzania	KF664639	KF665030	KF665596	KF666048	
Nectophrynoides cf. *viviparus*	MTSN 5253	FAR tree	Tanzania	KF664614	KF665150	KF665685		
Nectophrynoides cf. *viviparus*	MTSN 5339	FAR tree	Tanzania	KF664653	KF665067	KF665695		
Nectophrynoides cf. *viviparus*	MTSN 5340	FAR tree	Tanzania	KF664914	KF665444	KF665568	KF665902	
Nectophrynoides cf. *viviparus*	MTSN 5341	FAR tree	Tanzania	KF664626	KF665312	KF665505	KF666091	
Nectophrynoides cf. *viviparus*	MTSN 5342	FAR tree	Tanzania	KF664692	KF665064	KF665573	KF666010	
Nectophrynoides cf. *viviparus*	MTSN 7573	FAR tree	Tanzania	KF664982	KF665470			
Nectophrynoides cf. *viviparus*	MTSN 7798	FAR tree	Tanzania	KF664892	KF665320		KF666104	KF666184
Nectophrynoides cf. *viviparus*	MTSN 7811	FAR tree & Full tree	Tanzania	KF664977	KF665299	KF665590	KF665964	KF666246
Nectophrynoides cf. *viviparus*	MTSN 7812	FAR tree & Full tree	Tanzania	KF664876	KF665301	KF665574	KF666121	KF666206
Nectophrynoides cf. *viviparus*	MTSN 7815	FAR tree	Tanzania	KF664818	KF665380		KF665830	KF666282
Nectophrynoides cf. *viviparus*	MTSN 8404	FAR tree	Tanzania	KF664944	KF665117		KF665919	
Nectophrynoides cf. *viviparus*	MTSN 8405	FAR tree & Full tree	Tanzania	KF664856	KF665318	KF665679	KF665962	KF666465
Nectophrynoides cf. *viviparus*	MW1894	FAR tree & Full tree	Tanzania	FJ882816	FJ882816	KF665717	FJ882716	KF666202
Nectophrynoides cf. *viviparus*	MW1896	FAR tree & Full tree	Tanzania	KF664846	KF665142	KF665681	KF665895	KF666307
Nectophrynoides cf. *viviparus*	TZ391	FAR tree	Tanzania	KF664983	KF665455	KF665559	KF666140	
Nectophrynoides cf. *viviparus*	TZ88	FAR tree & Full tree	Tanzania	KF664677	KF665061	KF665529	KF666094	KF666308
Nectophrynoides cf. *viviparus*	TZ89	FAR tree	Tanzania	KF664611	KF665398	KF665757	KF666044	
Nectophrynoides wendyae	MTSN 5642	FAR tree & Full tree	Tanzania	KF664769	KF665374	KF665795	KF665882	KF666285
Nectophrynoides wendyae	MTSN 5644	FAR tree	Tanzania	KF664808	KF665370	KF665570	KF665925	
Nectophrynoides wendyae	MTSN 5647	FAR tree	Tanzania	KF664800	KF665324	KF665544	KF665918	
Nimbaphrynoides occidentalis	GU89	FAR tree	Guinea	KF664908	KF665149		KF665892	
Nimbaphrynoides occidentalis	MOR MTN15	FAR tree	Guinea	GU322833	GU322845			
Nimbaphrynoides occidentalis	MOR MTN16	FAR tree	Guinea	GU322834	GU322846			
Nimbaphrynoides occidentalis	MOR MTN22	FAR tree	Guinea	GU322835	GU322847			
Nimbaphrynoides occidentalis	MOR MTN245	FAR tree	Liberia	GU322823	GU322840			

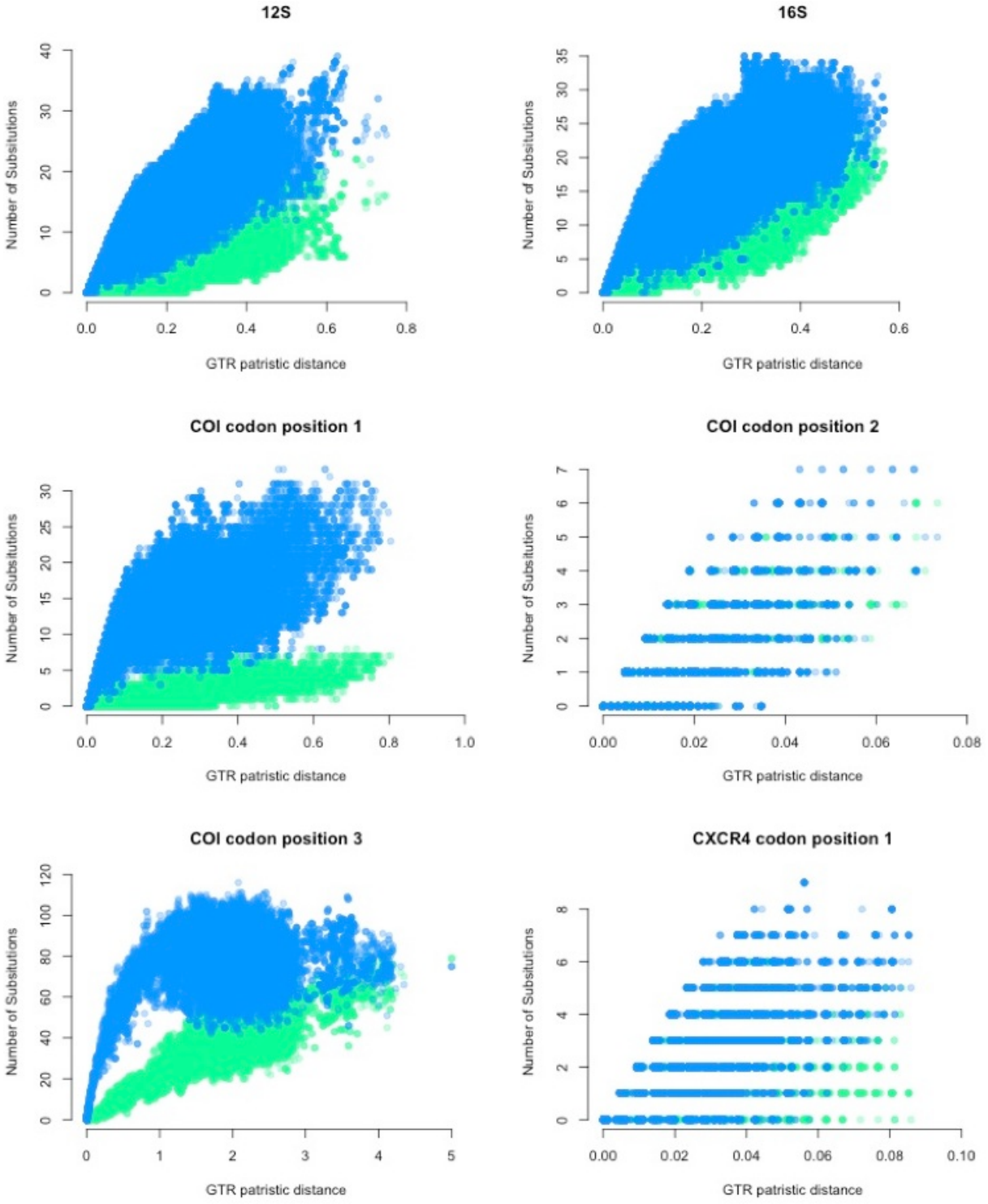
Nimbaphrynoides occidentalis	MOR MTN246	FAR tree	Liberia	GU322824	GU322841			
Nimbaphrynoides occidentalis	MOR MTN247	FAR tree	Liberia	GU322825	GU322842			
Nimbaphrynoides occidentalis	MOR MTN248	FAR tree	Liberia	GU322826				
Nimbaphrynoides occidentalis	MOR MTN78	FAR tree	Guinea	GU322836	GU322848			
Nimbaphrynoides occidentalis	MOR NI211	FAR tree	Guinea	GU322827				
Nimbaphrynoides occidentalis	MOR NL204	FAR tree	Liberia	GU322828				
Nimbaphrynoides occidentalis	MOR NL205	FAR tree	Liberia	GU322829				
Nimbaphrynoides occidentalis	MOR NL215	FAR tree	Liberia	GU322830				
Nimbaphrynoides occidentalis	MTN 23	FAR tree & Full tree	Guinea	KF665010	KF665040	KF665538	KF665967	KF666193
Nimbaphrynoides occidentalis	MTN 230	FAR tree & Full tree	Guinea	KF665011	KF665482	KF665719	KF665923	KF666148
Nimbaphrynoides occidentalis	MTN 52	FAR tree & Full tree	Guinea	KF665027	KF665377	KF665665	KF666040	KF666279
Nimbaphrynoides occidentalis	MTN 81	FAR tree & Full tree	Guinea	KF664680	KF665143	KF665554	KF665901	KF666165
Nimbaphrynoides occidentalis	ZMB73875	FAR tree	Liberia	GU322821	GU322838			
Nimbaphrynoides occidentalis	ZMB73876	FAR tree	Liberia	GU322822	GU322839			
Nimbaphrynoides occidentalis	ZMB73881	FAR tree	Guinea	GU322831	GU322843			
Nimbaphrynoides occidentalis	ZMB73882	FAR tree	Guinea	GU322832	GU322844			
Nimbaphrynoides occidentalis	ZMB73886	FAR tree	Guinea	GU322837	GU322849			
Pedostibes hosii	VUB 0661	Full tree	Malaysia	FJ882804	FJ882804	KF665818	EF107449	KF666369
Pelophryne misera	VUB 0641	Full tree	Malaysia	FJ882800	FJ882800	KF665680	FJ882700	KF666300
Phrynoidis aspera	CAS 248116	Full tree	Myanmar	KF664660	KF665483	KF665743	KF665952	KF666437
Phrynoidis juxtaspera	VUB 0649	Full tree	Malaysia	FJ882805	FJ882805	KF665605	FJ882710	KF666210
Poyntonophrynus damaranus	damaB	FAR tree	Namibia		AF220906			
Poyntonophrynus dombensis	dombA	FAR tree	Namibia	AF220857	AF220907			
Poyntonophrynus fenoulheti	AACRG 1598	FAR tree & Full tree	South Africa	KF664732	KF665265	KF665592	KF666066	KF666249
Poyntonophrynus fenoulheti	AACRG 1599	FAR tree & Full tree	South Africa	KF664816	KF665081	KF665728	KF665911	KF666357
Poyntonophrynus fenoulheti	fenoA	FAR tree	South Africa	AF220859	AF220908			
Poyntonophrynus hoeschi	jordA	FAR tree		AF220858				
Strauchbufo raddei	CAS 238862	Full tree	Mongolia	KF664854	KF665477	KF665558	KF666101	KF666186
Rhinella granulosa	VUB 1960	Full tree	Uruguay	FJ882774	FJ882775	KF665648	FJ882728	KF666195

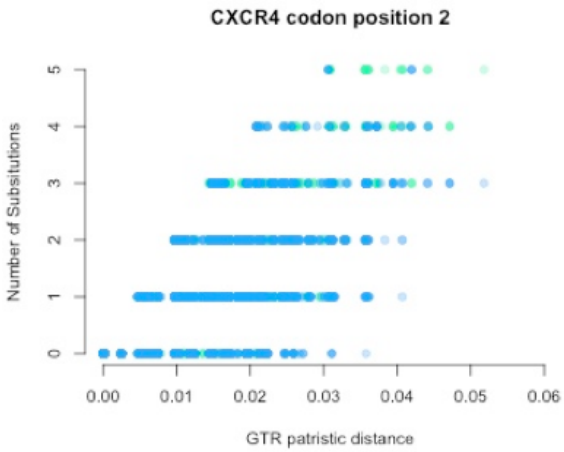
Rhinella cf. *marina*	VUB 1965	Full tree	Suriname	FJ882831	FJ882831	KF665615	KF665869	KF666345
Schismaderma carens	16scarA	FAR tree	South Africa	AF220866	AF220913			
Schismaderma carens	AACRG 1607	FAR tree & Full tree	South Africa	KF664825	KF665363	KF665798	KF665894	KF666196
Schismaderma carens	AACRG 1608	FAR tree & Full tree	South Africa	KF664829	KF665176	KF665515	KF666049	KF666220
Schismaderma carens	MOR Pe1	FAR tree & Full tree	Mozambique	KF664897	KF665121	KF665600	KF665988	KF666363
Schismaderma carens	MVZ:Herp:223386	FAR tree	Zimbabwe	DQ158424	DQ158424		DQ306519	DQ158350
Schismaderma carens	MW4279	FAR tree	Tanzania	FJ882849	FJ882849		FJ882717	
Schismaderma carens	RdS796	FAR tree	Tanzania	DQ283425	DQ283425			
Vandijkophrynus amatolicus	amatA	FAR tree	South Africa	AF220851	AF220898			
Vandijkophrynus angusticeps	AC2692	FAR tree & Full tree	South Africa	KF664791	KF665432	KF665693	KF666025	KF666237
Vandijkophrynus angusticeps	anguA	FAR tree	South Africa	AF220852	AF220899			
Vandijkophrynus angusticeps	KTH286	FAR tree	South Africa		FN652342			
Vandijkophrynus angusticeps	KTH404	FAR tree	South Africa	KF664819	KF665254	KF665721	KF666106	
Vandijkophrynus angusticeps	VC005	FAR tree & Full tree	South Africa	KF665016	KF665390	KF665539	KF666141	KF666393
Vandijkophrynus angusticeps	VC123	FAR tree	South Africa	KF664695	KF665412	KF665631		KF666382
Vandijkophrynus gariepensis	AC2831	FAR tree	South Africa	KF664705	KF665119	KF665607		
Vandijkophrynus gariepensis	AC2960	FAR tree	South Africa	KF664879	KF665037			
Vandijkophrynus gariepensis	CAS 193962	FAR tree	South Africa	U52731	U52768			
Vandijkophrynus gariepensis	gariA	FAR tree	South Africa	AF220853	AF220900			
Vandijkophrynus gariepensis	VC178	FAR tree & Full tree	South Africa	KF664828	KF665376	KF665613	KF665889	KF666339
Vandijkophrynus gariepensis	XRP3	FAR tree & Full tree	South Africa	KF664641	KF665465	KF665689	KF665960	KF666189
Vandijkophrynus inyangae	inyaA	FAR tree	Zimbabwe	AF220856	AF220904			
Vandijkophrynus robinsoni	AACRG 0068?	& Full tree		KF664648	KF665375	KF665788	KF665893	KF666198
Vandijkophrynus robinsoni	CAS 193549	FAR tree	South Africa	KF664911	KF665331	KF665617		
Vandijkophrynus robinsoni	gariC	FAR tree	South Africa	AF220855	AF220902			
Vandijkophrynus robinsoni	robiA	FAR tree	South Africa		AF220903			
Vandijkophrynus sp.	AC2690	FAR tree & Full tree	South Africa	KF664630	KF665062	KF665778	KF665975	KF666467
Werneria bambutensis	0328LG	Full tree	Cameroon	KF664703	KF665267	KF665508	KF665891	KF666421
Werneria bambutensis	652LG	Full tree	Cameroon	KF664667	KF665476	KF665784	KF665898	KF666214

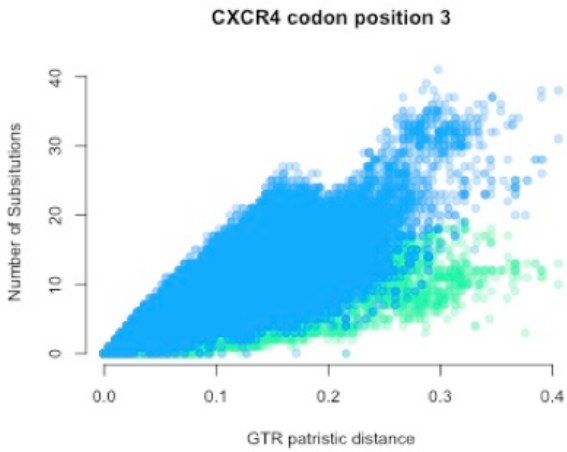
Werneria bambutensis	no3	Full tree	Cameroon	KF665002	KF665148	KF665597	KF665997	KF666394
Werneria bambutensis	no7	Full tree	Cameroon	KF665008	KF665298	KF665738	KF665860	KF666325
Werneria bambutensis	no80	Full tree	Cameroon	KF665013	KF665155	KF665700	KF665928	KF666197
Werneria bambutensis	vg05-PIV	Full tree	Cameroon	KF664647	KF665252	KF665577	KF665881	KF666257
Werneria mertensiana	0132LG	Full tree	Cameroon	KF664904	KF665033	KF665535	KF665945	KF666411
Werneria mertensiana	MTSN 5893	Full tree	Cameroon	KF664937	KF665065	KF665560	KF666129	KF666452
Werneria submontana	MHNG 2716.051	Full tree	Cameroon	KF664678	KF665284	KF665658	KF666080	KF666429
Werneria submontana	MHNG 2716.052	Full tree	Cameroon	KF664906	KF665172	KF665762	KF666009	KF666374
Werneria submontana	MHNG 2716.053	Full tree	Cameroon	KF664993	KF665187	KF665636	KF665915	KF666434
Werneria submontana	vg09-304	Full tree	Cameroon	KF664890	KF665130	KF665780	KF666084	KF666293
Werneria tandyi	0054LG	Full tree	Cameroon	KF664713	KF665220	KF665765	KF665873	KF666207
Werneria tandyi	0244LG	Full tree	Cameroon	KF664967	KF665095	KF665589	KF665888	KF666218
Werneria tandyi	MH0276	Full tree	Cameroon	KF664619	KF665489	KF665663	KF666100	KF666365
Wolterstorffina cf. *chirioi*	MCZ:A-138012	Full tree	Cameroon	KF664998	KF665448	KF665499	KF666014	KF666263
Wolterstorffina cf. *chirioi*	MCZ:A-138013	Full tree	Cameroon	KF664992	KF665397	KF665604	KF665940	KF666297
Wolterstorffina cf. *chirioi*	MCZ:A-138014	Full tree	Cameroon	KF664655	KF665068	KF665537	KF665874	KF666262
Wolterstorffina cf. *chirioi*	WC7	Full tree	Cameroon	KF664599	KF665056	KF665745	KF665833	KF666200
Wolterstorffina cf. *chirioi*	WOL1	Full tree	Cameroon	KF664610	KF665357	KF665580	KF665987	KF666219
Wolterstorffina cf. *chirioi*	WOLT-T2290	Full tree	Cameroon	KF664757	KF665203	KF665732	KF665899	KF666337
Wolterstorffina mirei	LG0003	Full tree	Cameroon	KF664820	KF665341	KF665500	KF666036	KF666230
Wolterstorffina mirei	LG0004	Full tree	Cameroon	KF664923	KF665228	KF665668	KF665944	KF666399
Wolterstorffina mirei	LG0006	Full tree	Cameroon	KF664986	KF665462	KF665557	KF666031	KF666208
Wolterstorffina mirei	LG0007	Full tree	Cameroon	KF664741	KF665323	KF665507	KF666132	KF666340
Wolterstorffina mirei	MCZ:A-138001	Full tree	Cameroon	KF664634	KF665352	KF665593	KF666051	KF666283
Wolterstorffina cf. *parvipalmata*	0099LG	Full tree	Cameroon	KF664961	KF665332	KF665646	KF665970	KF666440
Wolterstorffina cf. *parvipalmata*	0113LG	Full tree	Cameroon	KF664889	KF665210	KF665714	KF665942	KF666433
Wolterstorffina cf. *parvipalmata*	0116LG	Full tree	Cameroon	KF664952	KF665306	KF665759	KF665862	KF666464
Wolterstorffina cf. *parvipalmata*	0137LG	Full tree	Cameroon	KF664991	KF665427	KF665753	KF666113	KF666276
Wolterstorffina cf. *parvipalmata*	0236LG	Full tree	Cameroon	KF664831	KF665039	KF665750	KF665861	KF666380

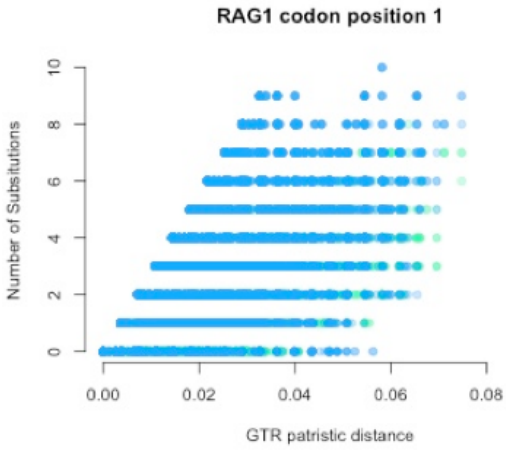
Wolterstorffina cf. *parvipalmata*	0679LG	Full tree	Cameroon	KF664898	KF665164	KF665673	KF666089	KF666273
Wolterstorffina cf. *parvipalmata*	0806LG	Full tree	Cameroon	KF664608	KF665144	KF665650	KF665950	KF666235
Wolterstorffina cf. *parvipalmata*	0828 N	Full tree	Cameroon	KF664918	KF665472	KF665532	KF665857	KF666156
Wolterstorffina cf. *parvipalmata*	0829 N	Full tree	Cameroon	KF664595	KF665326	KF665629	KF666065	KF666162
Wolterstorffina cf. *parvipalmata*	0830 N	Full tree	Cameroon	KF664826	KF665165	KF665514	KF666046	KF666385
Wolterstorffina cf. *parvipalmata*	618LG	Full tree	Cameroon	KF664798	KF665458	KF665703	KF666029	KF666373
Wolterstorffina cf. *parvipalmata*	AMC334	Full tree	Cameroon	KF664670	KF665237	KF665549	KF665930	KF666401
Wolterstorffina cf. *parvipalmata*	AMC335	Full tree	Cameroon	KF664913	KF665086	KF665640	KF666026	KF666379
Wolterstorffina cf. *parvipalmata*	CUMV 15186	Full tree	Equatorial Guinea	KF664794	KF665125	KF665509	KF666126	KF666254
Wolterstorffina cf. *parvipalmata*	MC11_185	Full tree	Cameroon	KF664925	KF665185	KF665764	KF666008	KF666327
Wolterstorffina cf. *parvipalmata*	MCZ:A-136748	Full tree	Cameroon	KF665029	KF665224	KF665504	KF666039	KF666295
Wolterstorffina cf. *parvipalmata*	MOR 41214	Full tree	Nigeria	KF664927	KF665106	KF665781	KF666095	KF666320
Wolterstorffina cf. *parvipalmata*	MTSN 5895	Full tree	Cameroon	KF664758	KF665440	KF665603	KF665846	KF666224
Wolterstorffina cf. *parvipalmata*	MTSN 5896	Full tree	Cameroon	KF664749	KF665461	KF665576	KF666086	KF666267

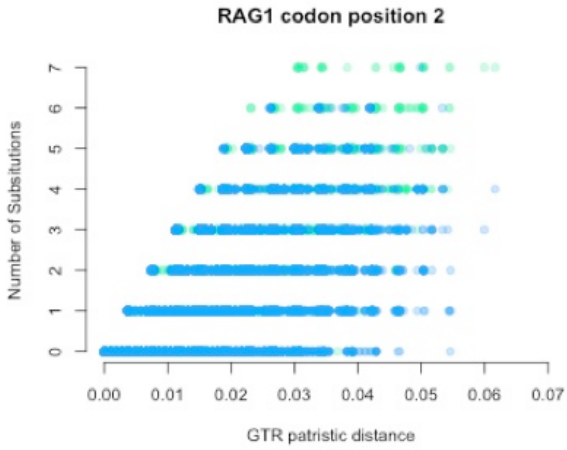
Online Appendix 3. Saturation plots of patristic distances recovered from a Maximum Likelihood GTR+G model implemented in RAxML v7.2.8, against the number of substitutions for each gene partition. Blue dots show transitions, green dots show transversions.

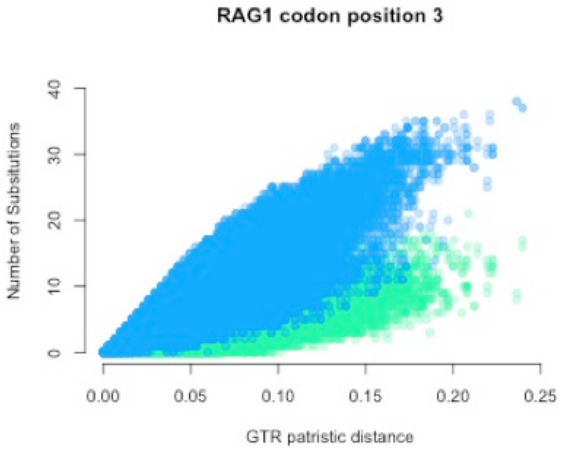



CXCR4 codon position 2
Number of Substitutions
0
1
2
3
4
5
0.00
0.01
0.02
0.03
0.04
0.05
0.06
GTR patristic distance

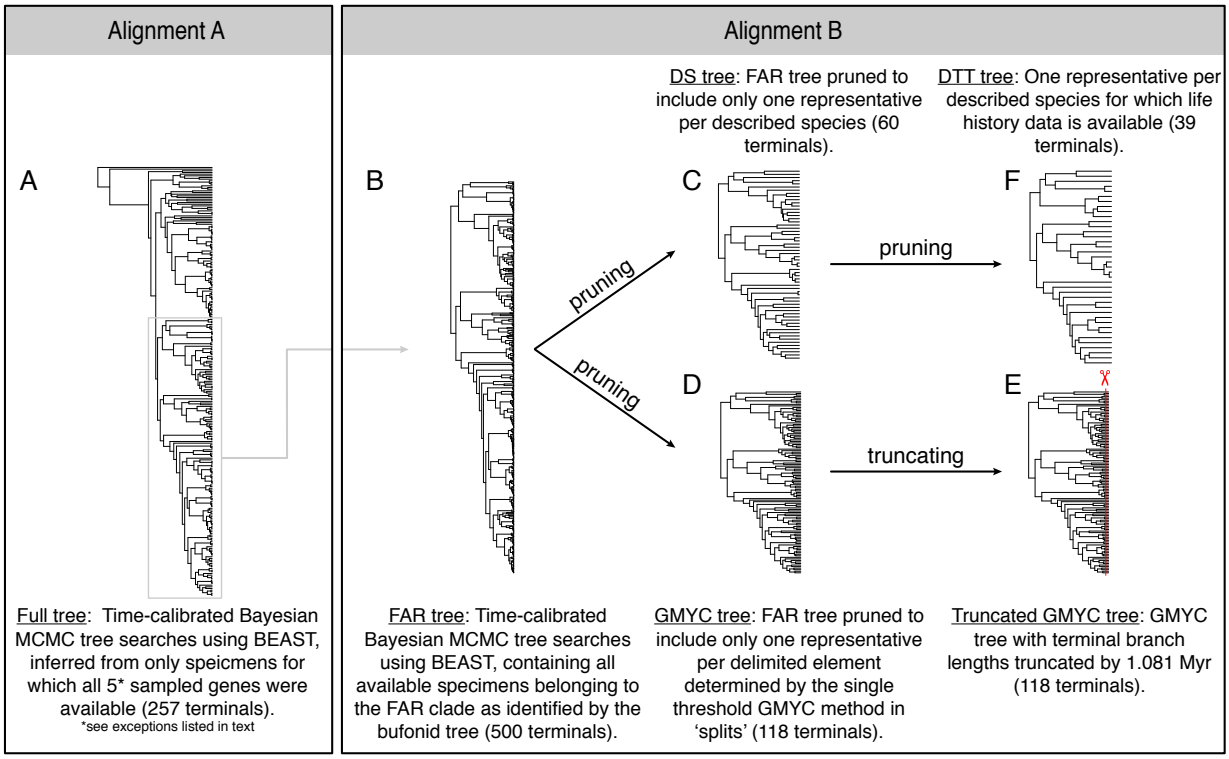

CXCR4 codon position 3
Number of Substitutions
0
10
20
30
40
0.0
0.1
0.2
0.3
0.4
GTR patristic distance


RAG1 codon position 1
Number of Substitutions
0
2
4
6
8
10
0.00
0.02
0.04
0.06
0.08
GTR patristic distance


RAG1 codon position 2
Number of Substitutions
0
1
2
3
4
5
6
7
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
GTR patristic distance


RAG1 codon position 3
Number of Substitutions
0
10
20
30
40
0.00
0.05
0.10
0.15
0.20
0.25
GTR patristic distance

Online Appendix 4. Graphic depiction of the phylogenetic inferences workflow, outlining how each tree used in this study was derived.



Online Appendix 5. Fossil calibration points

Four fossil calibration points were used to set a minimum age on the time to most recent common ancestor (tmrca) of extant clades. Before setting any constraints on calibrated nodes, an unconstraint analysis was carried out with MrBayes v3.2.2 to confirm that the nodes are well supported.

Rhinella marina.—The origin of the *Rhinella marina* species-group was dated to at least 11.8 Ma based on a fossil from the La Venta fauna of Colombia from the mid Miocene (Laventan age: 13.8 to 11.8 Ma; Estes and Wassersug 1963; www.fossilworks.org). The immediate sister species to the *R. marina* group is *R. crucifer* (sensu Maciel et al. 2010), however this species is not represented in the phylogeny and therefore a lognormal prior distribution was chosen over an exponential prior distribution for the tmrca of *R. marina* and *R. granulosa* (mean=2; SD=1; offset=11.8).

Anaxyrus-Incilius.—The tmrca for *Anaxyrus* and *Incilius* was set based on a fossil of *Bufo praevius* (Tihen 1951; now Incilius praevius sensu Martín et al. 2012) from Thomas Farm a site belonging to the Alchua Formation of the Hemingfordian stage (20.4-16.0 Ma ; www.fossilworks.org). The fossil shares skeletal features with *A. terrestris* and *I. valliceps* (Tihen 1951) and probably belonged to a group from which extant *Anaxyrus* and *Incilius* species are derived (Tihen 1972). Tihen (1951) writes that mammal and bird fossils from the same locality suggest that the deposits are from the older rather than the newer age of the Alchua Formation and adds that it seems likely that the genus "*Bufo*" was well established in the area as far back as the end of the Oligocene. A lognormal prior distribution was therefore chosen with an offset of 20 Ma and a mean of 2 (SD=1) to accommodate a wider age range for the most recent common ancestor of the genera *Anaxyrus* and *Incilius*.

Bufo bufo.—The oldest unambiguously identified *B. bufo* fossil was found in the Czech Republic and dates to MN 9 zone in the mid Miocene (Rage and Roček 2003) and so a hard minimum was set at 9.6 Ma for an exponential prior distribution (mean=2) for the most recent common ancestor of *B. bufo* complex and the *B. gargarizans* complex, based on the phylogenetic relationship sensu Van Bocxlaer et al. (2009) and preliminary unconstrained phylogenetic reconstructions.

Bufotes viridis.—The age of *B. viridis* was calibrated based on fossils of members of the *B. viridis* group discovered in Spain, France and Germany from the Burdigalian stage (Martín et al. 2012; MN 4b to MN 4a at 20.43 to 15.98 Ma; www.fossilworks.org). A fossil of *B. priscus*

(Špinar et al. 1993) from the mid Miocene, Devínska Nová Ves (Bonanza site; Astracian age; MN 6; 15.97 to 11.608 Ma) in Slovakia has since been determined to also belong to the *B. viridis* group (Martín et al. 2012), confirming that the origin of this lineage to have occurred before this time. Previous chronograms have constrained a node for *Strauchbufo raddei* to all other *Bufotes* lineages as the most recent common ancestor of that clade (e.g. Van Bocxlaer et al. 2010). However, recent findings (Dubois and Bour 2010) and our own uncalibrated trees do not support a close relationship between these two genera and therefore the split of *B. surdus* from the *B. viridis* group was sampled from an exponential prior distribution with an offset of 18 and a mean of 2 instead.

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Online Appendix 6. Reproductive mode coding for MuSSE analysis. All coding was based on information from the IUCN red list online database unless otherwise stated. Cases where breeding is unknown, but inferred, are indicated.

Species	MuSSE state	Comments
Altiphrynoides malcolmi	larva in terrestrial nest	
Altiphrynoides osgoodi	free-swimming larva	
Amietophrynus brauni	free-swimming larva	
Amietophrynus camerunensis	free-swimming larva	
Amietophrynus channingi	free-swimming larva	(Orts 1970)
Amietophrynus garmani	free-swimming larva	
Amietophrynus gracilipes	free-swimming larva	
Amietophrynus gutturalis	free-swimming larva	
Amietophrynus kisoloensis	free-swimming larva	
Amietophrynus latifrons	free-swimming larva	
Amietophrynus lemairii	free-swimming larva	inferred (IUCN SSC Amphibian Specialist Group 2013)
Amietophrynus maculatus	free-swimming larva	
Amietophrynus mauritanicus	free-swimming larva	
Amietophrynus pantherinus	free-swimming larva	
Amietophrynus pardalis	free-swimming larva	
Amietophrynus poweri	free-swimming larva	
Amietophrynus rangeri	free-swimming larva	
Amietophrynus regularis	free-swimming larva	
Amietophrynus steindachneri	free-swimming larva	
Amietophrynus superciliaris	free-swimming larva	
Amietophrynus taiensis	free-swimming larva	inferred from close relationship with *A. togoensis* (Rödel and Ernst 2000)
Amietophrynus togoensis	free-swimming larva	
Amietophrynus tuberosus	free-swimming larva	
Amietophrynus villiersi	free-swimming larva	
Amietophrynus xeros	free-swimming larva	
"Bufo" pentoni	free-swimming larva	
Capensibufo rosei	free-swimming larva	
Capensibufo tradouwi	free-swimming larva	
Churamiti maridadi	free-swimming larva	inferred from pigmented eggs (Channing and Stanley 2002)
Didynamipus sjostedti	larva in terrestrial nest	inferred from terrestrial clutch (Gonwouo et al. 2013)
Mertensophryne anotis	free-swimming larva	
Mertensophryne howelli	free-swimming larva in micro water body	inferred (IUCN red list)
Mertensophryne lindneri	free-swimming larva	inferred (IUCN red list)
Mertensophryne loveridgei	free-swimming larva in micro water body	inferred (IUCN red list)
Mertensophryne micranotis	free-swimming larva in micro water body	
Mertensophryne taitana	free-swimming larva	
Mertensophryne usambarae	free-swimming larva in micro water body	inferred (IUCN red list)
Mertensophryne uzunguensis	free-swimming larva	

Nectophrynoides asperginis	lecithotrophic viviparity	
Nectophrynoides frontierei	lecithotrophic viviparity	inferred (IUCN red list)
Nectophrynoides laticeps	lecithotrophic viviparity	inferred (IUCN red list)
Nectophrynoides minutus	lecithotrophic viviparity	
Nectophrynoides paulae	lecithotrophic viviparity	inferred (IUCN red list)
Nectophrynoides poyntoni	lecithotrophic viviparity	inferred (IUCN red list)
Nectophrynoides pseudotornieri	lecithotrophic viviparity	inferred (IUCN red list)
Nectophrynoides tornieri	lecithotrophic viviparity	
Nectophrynoides vestergaardi	lecithotrophic viviparity	inferred (IUCN red list)
Nectophrynoides viviparus	lecithotrophic viviparity	
Nectophrynoides wendyae	lecithotrophic viviparity	
Nimbaphrynoides occidentalis	matrotrophic viviparity	
Poyntonophrynus damaranus	free-swimming larva	inferred (IUCN red list)
Poyntonophrynus dombensis	free-swimming larva	
Poyntonophrynus fenoulheti	free-swimming larva	
Poyntonophrynus hoeschi	free-swimming larva	
Schismaderma carens	free-swimming larva	
Vandijkophrynus amatolicus	free-swimming larva	
Vandijkophrynus angusticeps	free-swimming larva	
Vandijkophrynus gariepensis	free-swimming larva	
Vandijkophrynus inyangae	free-swimming larva	
Vandijkophrynus robinsoni	free-swimming larva	

*IUCN red list: www.iucnredlist.org, last accessed on 6th February 2014

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Online Appendix 7. Life-history traits used in DTT analysis. Maximum female body size was measured as Snout-Vent-Length in mm, clutch size refers to the maximum number of eggs/offspring laid in a single clutch/born and egg size refers to the diameter of the egg in mm. All measurements were taken from Liedtke et al. (2014)* or references therein.

Species	Body Size	Clutch size	Egg size
Altiphrynoides malcolmi	31.0	31	3.9
Altiphrynoides osgoodi	62.0	307	3.0
Amietophrynus brauni	110.0	9000	1.0
Amietophrynus camerunensis	91.0	2100	1.7
Amietophrynus channingi	143.0	4500	2.0
Amietophrynus garmani	115.0	20000	1.2
Amietophrynus gutturalis	120.0	25000	1.5
Amietophrynus kisoloensis	87.0	2400	1.9
Amietophrynus lemairii	70.0	2500	1.5
Amietophrynus maculatus	80.0	8000	1.5
Amietophrynus mauritanicus	150.0	10000	1.5
Amietophrynus pardalis	147.0	14000	1.5
Amietophrynus rangeri	115.0	10760	1.3
Amietophrynus regularis	130.0	11000	1.3
Amietophrynus superciliaris	163.0	4000	2.0
Amietophrynus tuberosus	74.0	4200	1.5
Amietophrynus xeros	97.0	5000	1.0
"Bufo" pentoni	95.0	2600	2.0
Capensibufo rosei	39.0	90	2.5
Capensibufo tradouwi	48.0	60	2.0
Didynamipus sjostedti	19.3	18	2.3
Mertensophryne anotis	46.0	105	2.5
Mertensophryne howelli	45.0	60	2.5
Mertensophryne lindneri	34.0	81	2.1
Mertensophryne loveridgei	38.0	131	2.1
Mertensophryne micranotis	24.0	70	1.8
Mertensophryne taitana	33.0	350	2.0
Mertensophryne usambarae	45.0	60	2.4
Mertensophryne uzunguensis	30.0	188	2.0
Nectophrynoides asperginis	29.0	16	2.4
Nectophrynoides laticeps	24.0	60	1.8
Nectophrynoides minutus	22.0	31	2.0
Nectophrynoides tornieri	34.0	37	2.0
Nectophrynoides viviparus	60.0	160	2.9
Nimbaphrynoides occidentalis	32.5	17	0.6
Poyntonophrynus dombensis	40.0	900	1.8
Poyntonophrynus fenoulheti	43.0	2000	1.8
Schismaderma carens	92.0	2500	2.5
Vandijkophrynus angusticeps	58.0	3000	2.0

*Liedtke H.C., Müller H., Hafner J., Nagel P., Loader S.P. 2014. Interspecific patterns for egg and clutch sizes of African Bufonidae (Amphibia: Anura). Zool. Anz. 253(4): 308-315.

Online Appendix 8. MCC tree for Bufonidae recovered from time-calibrated Bayesian MCMC tree searches using BEAST under a birth-death uncorrelated lognormal relaxed clock model. Nodes are annotated with posterior probabilities.


60
50
40
30
20
10
0 Ma
Melanophryniscus stelzneri (VUB 0985)
Atelopus barbotini (BPN 1697)
Rhinella granulosa (VUB 1960)
Rhinella marina (VUB 1965)
Incilius alvarius (UTA:A–53924)
Incilius coniferus (MVZ:Herp:203775)
Incilius valliceps (MZFC:JRM–3868)
Incilius campbelli (UTA:A–50902)
Anaxyrus terrestris (CAS 207171)
Anaxyrus americanus (CAS 223832)
Anaxyrus americanus (CAS 207258)
Anaxyrus boreas (CAS 176529)
Anaxyrus canorus (CAS 209233)
Anaxyrus boreas (CAS 201586)
Bufo bufo (vg06–282)
Bufo gargarizans (CAS 228184)
Leptophryne borbonica (VUB 0673)
Strauchbufo raddei (CAS 238862)
Epidalea calamita (vg07–119)
Ingerophrynus macrotis (CAS 230357)
Ingerophrynus divergens (VUB 0602)
Bufotes surdus (ZMMSU A–4027)
Bufotes viridis (vg07–187)
Duttaphrynus crocus (CAS 220193)
"Bufo" pageoti (CAS 233251)
Duttaphrynus stuarti (CAS 221485)
Duttaphrynus dhufarensis (CAS 227584)
Duttaphrynus olivaceus (CAS 232138)
Duttaphrynus olivaceus (CAS 232073)
Pelophryne misera (VUB 0641)
Ansonia thinthinae (CAS 243945)
Ansonia longidigita (VUB 0666)
Pedostibes hosii (VUB 0661)
Phrynoidis juxtaspera (VUB 0649)
Phrynoidis aspera (CAS 248116)
Werneria bambutensis (0328LG)
Werneria bambutensis (652LG)
Werneria bambutensis (vg05–PIV)
Werneria bambutensis (no3)
Werneria bambutensis (no80)
Werneria bambutensis (no7)
Werneria tandyi (0244LG)
Werneria tandyi (MH0276)
Werneria tandyi (0054LG)
Werneria mertensiana (MTSN 5893)
Werneria mertensiana (0132LG)
Werneria submontana (vg09–304)
Werneria submontana (MHNG 2716.053)
Werneria submontana (MHNG 2716.051)
Werneria submontana (MHNG 2716.052)
Nectophryne afra (NCSM 77617)
Nectophryne afra (N41ROHO)
Nectophryne afra (MVZ:Herp:234689)
Nectophryne afra (MVZ:Herp:234857)
Nectophryne batesii (887)
Nectophryne batesii (NCSM 76799)
Nectophryne batesii (0767LG)
Nectophryne batesii (MTSN 5891)
Nectophryne batesii (0369LG)
Nectophryne batesii (MVZ:Herp:234688)
Nectophryne batesii (N43ROHO)
Wolterstorffina mirei (LG0007)
Wolterstorffina mirei (LG0006)
Wolterstorffina mirei (MCZ:A–138001)
Wolterstorffina mirei (LG0004)
Wolterstorffina mirei (LG0003)
Wolterstorffina cf. chirioi (MCZ:A–138012)
Wolterstorffina cf. chirioi (WOLT–T2290)
Wolterstorffina cf. chirioi (WOL1)
Wolterstorffina cf. chirioi (MCZ:A–138014)
Wolterstorffina cf. chirioi (WC7)
Wolterstorffina cf. chirioi (MCZ:A–138013)
Wolterstorffina cf. parvipalmata (0806LG)
Wolterstorffina cf. parvipalmata (618LG)
Wolterstorffina cf. parvipalmata (MC11_185)
Wolterstorffina cf. parvipalmata (0116LG)
Wolterstorffina cf. parvipalmata (0137LG)
Wolterstorffina cf. parvipalmata (0099LG)
Wolterstorffina cf. parvipalmata (0113LG)
Wolterstorffina cf. parvipalmata (0679LG)
Wolterstorffina cf. parvipalmata (AMC335)
Wolterstorffina cf. parvipalmata (AMC334)
Wolterstorffina cf. parvipalmata (MCZ:A–136748)
Wolterstorffina cf. parvipalmata (CUMV 15186)
Wolterstorffina cf. parvipalmata (0236LG)
Wolterstorffina cf. parvipalmata (MTSN 5895)
Wolterstorffina cf. parvipalmata (0828 N)
Wolterstorffina cf. parvipalmata (0829 N)
Wolterstorffina cf. parvipalmata (MTSN 5896)
Wolterstorffina cf. parvipalmata (0830 N)
Wolterstorffina cf. parvipalmata (MOR 41214)
Schismaderma carens (MOR Pe1)
Schismaderma carens (AACRG 1608)
Schismaderma carens (AACRG 1607)
Altiphrynoides osgoodi (MW6306)
Altiphrynoides malcolmi (MW6331)
Altiphrynoides malcolmi (SL004)
Altiphrynoides malcolmi (SL079)
Didynamipus sjostedti (0827LG)
Didynamipus sjostedti (0822LG)
Didynamipus sjostedti (0825LG)
Nimbaphrynoides occidentalis (MTN 23)
Nimbaphrynoides occidentalis (MTN 52)
Nimbaphrynoides occidentalis (MTN 230)
Nimbaphrynoides occidentalis (MTN 81)
Churamiti maridadi (MTSN 5585)
Churamiti maridadi (MTSN 5584)
Nectophrynoides wendyae (MTSN 5642)
Nectophrynoides sp. (MTSN 7812)
Nectophrynoides sp. (MTSN 7811)
Nectophrynoides sp. (KMH36201)
Nectophrynoides sp. (MW1896)
Nectophrynoides sp. (MW1894)
Nectophrynoides sp. (KMH26641)
Nectophrynoides viviparus (MTSN 9383)
Nectophrynoides viviparus (MTSN 9365)
Nectophrynoides sp. (TZ88)
Nectophrynoides sp. (MTSN 8405)
Nectophrynoides laticeps (MTSN 5637)
Nectophrynoides laticeps (MTSN 5641)
Nectophrynoides laticeps (MTSN 5635)
Nectophrynoides sp. (RO2078)
Nectophrynoides sp. (MTSN 9080)
Nectophrynoides sp. (MTSN 8545)
Nectophrynoides vestergaardi (MW3211)
Nectophrynoides paulae (MTSN 5626)
Nectophrynoides sp. (KMH35967)
Nectophrynoides sp. (MTSN 8175)
Nectophrynoides poyntoni (MTSN 5076)
Nectophrynoides asperginis (KMH 15150)
Nectophrynoides pseudotornieri (RO2020)
Nectophrynoides minutus (MW3309)
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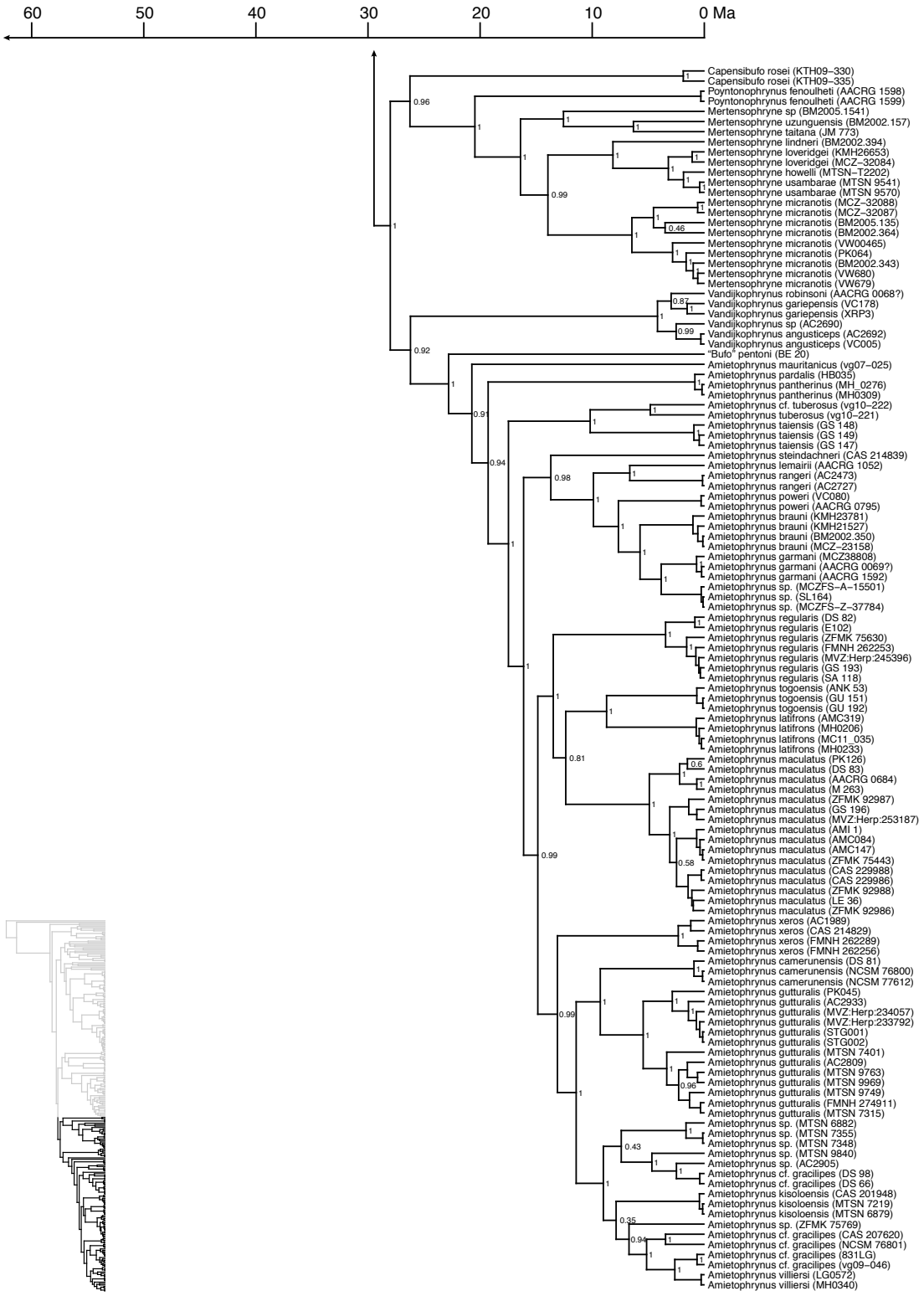
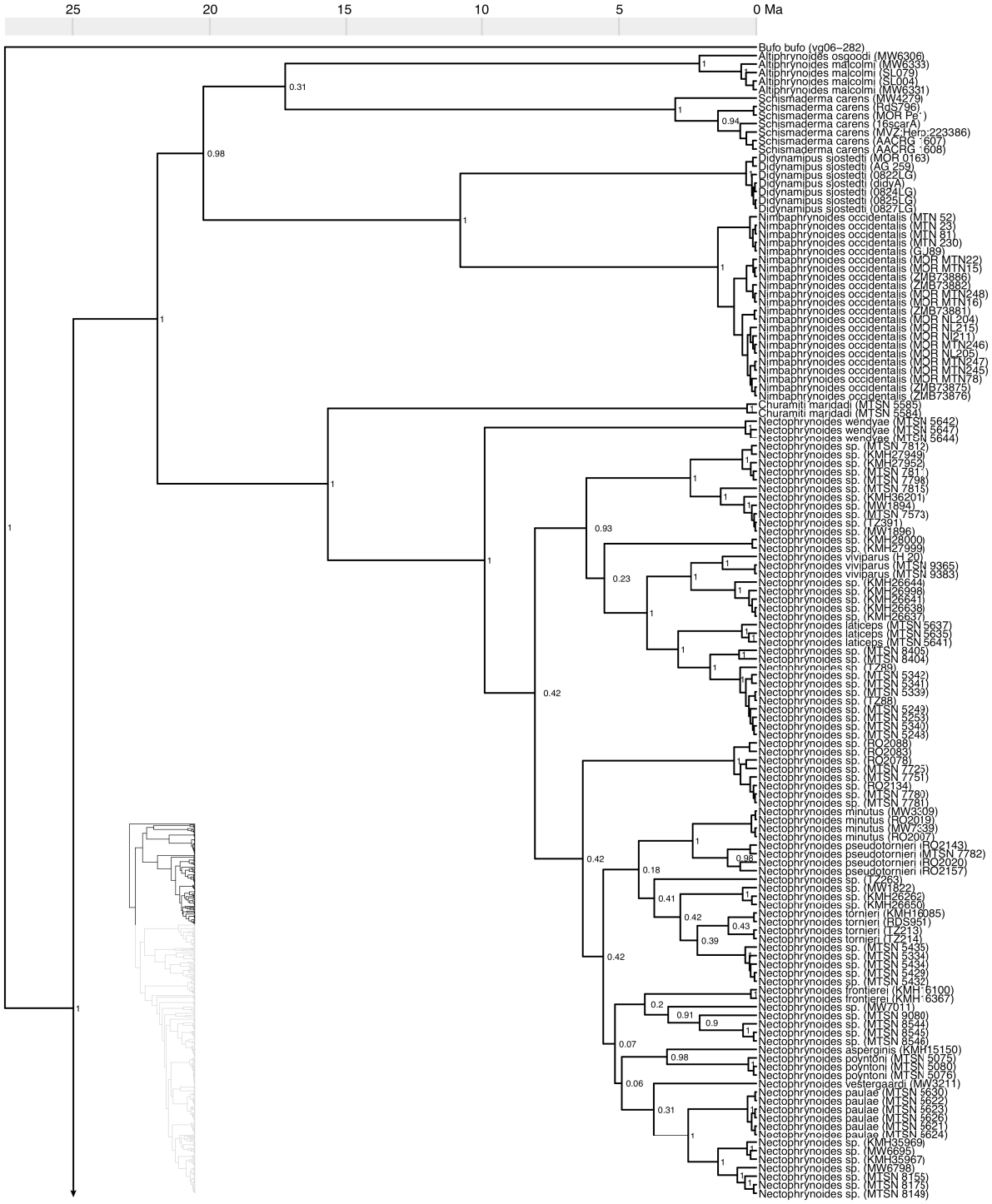


Next page- **Online Appendix 9.** MCC tree for the first African radiation (FAR tree) of bufonids, recovered from time-calibrated Bayesian MCMC tree searches using BEAST under a birth-death uncorrelated lognormal relaxed clock model. Nodes are annotated with posterior probabilities.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Altiphrynoides malcolmi (SL079)
Altiphrynoides malcolmi (SL004)
Altiphrynoides malcolmi (MW6331)
Schismaderma carens (MW4279)
Schismaderma carens (RdS796)
Schismaderma carens (MOR Pe')
Schismaderma carens (16scarA)
Schismaderma carens (MVZ Herp:223386)
Schismaderma carens (AACRG :607)
Schismaderma carens (AACRG :608)
Didynamipus sjostedti (MOR 0163)
Didynamipus sjostedti (AG 259)
Didynamipus sjostedti (0822LG)
Didynamipus sjostedti (didyA)
Didynamipus sjostedti (0824LG)
Didynamipus sjostedti (0825LG)
Didynamipus sjostedti (0827LG)
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Nimbaphrynoides occidentalis (MTN 23)
Nimbaphrynoides occidentalis (MTN 81)
Nimbaphrynoides occidentalis (MTN 230)
Nimbaphrynoides occidentalis (GJ89)
Nimbaphrynoides occidentalis (MOR MTN22)
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Nimbaphrynoides occidentalis (ZMB73886)
Nimbaphrynoides occidentalis (ZMB73882)
Nimbaphrynoides occidentalis (MOR MTN248)
Nimbaphrynoides occidentalis (MOR MTN16)
Nimbaphrynoides occidentalis (ZMB73881)
Nimbaphrynoides occidentalis (MOR NL204)
Nimbaphrynoides occidentalis (MOR NL215)
Nimbaphrynoides occidentalis (MOR NI211)
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Nimbaphrynoides occidentalis (MOR MTN247)
Nimbaphrynoides occidentalis (MOR MTN245)
Nimbaphrynoides occidentalis (MOR MTN78)
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Churamiti maridadi (MTSN 5584)
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Nectophrynoides wendyae (MTSN 5644)
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Nectophrynoides viviparus (MTSN 9383)
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Nectophrynoides sp. (KMH26641)
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Nectophrynoides sp. (KMH26637)
Nectophrynoides laticeps (MTSN 5637)
Nectophrynoides laticeps (MTSN 5635)
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Nectophrynoides sp. (MTSN 5341)
Nectophrynoides sp. (MTSN 5339)
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Nectophrynoides sp. (MTSN 5249)
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Nectophrynoides minutus (RO2007)
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Nectophrynoides pseudotornieri (MTSN 7782)
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Nectophrynoides pseudotornieri (RO2157)
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Nectophrynoides sp. (KMH26262)
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Nectophrynoides tornieri (RDS951)
Nectophrynoides tornieri (TZ213)
Nectophrynoides tornieri (TZ214)
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Nectophrynoides sp. (MTSN 5434)
Nectophrynoides sp. (MTSN 5429)
Nectophrynoides sp. (MTSN 5432)
Nectophrynoides frontierei (KMH 6100)
Nectophrynoides frontierei (KMH 6367)
Nectophrynoides sp. (MW7011)
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Nectophrynoides sp. (MTSN 8544)
Nectophrynoides sp. (MTSN 8545)
Nectophrynoides sp. (MTSN 8548)
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Nectophrynoides poyntoni (MTSN 5075)
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Nectophrynoides paulae (MTSN 5623)
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Nectophrynoides paulae (MTSN 5621)
Nectophrynoides paulae (MTSN 5624)
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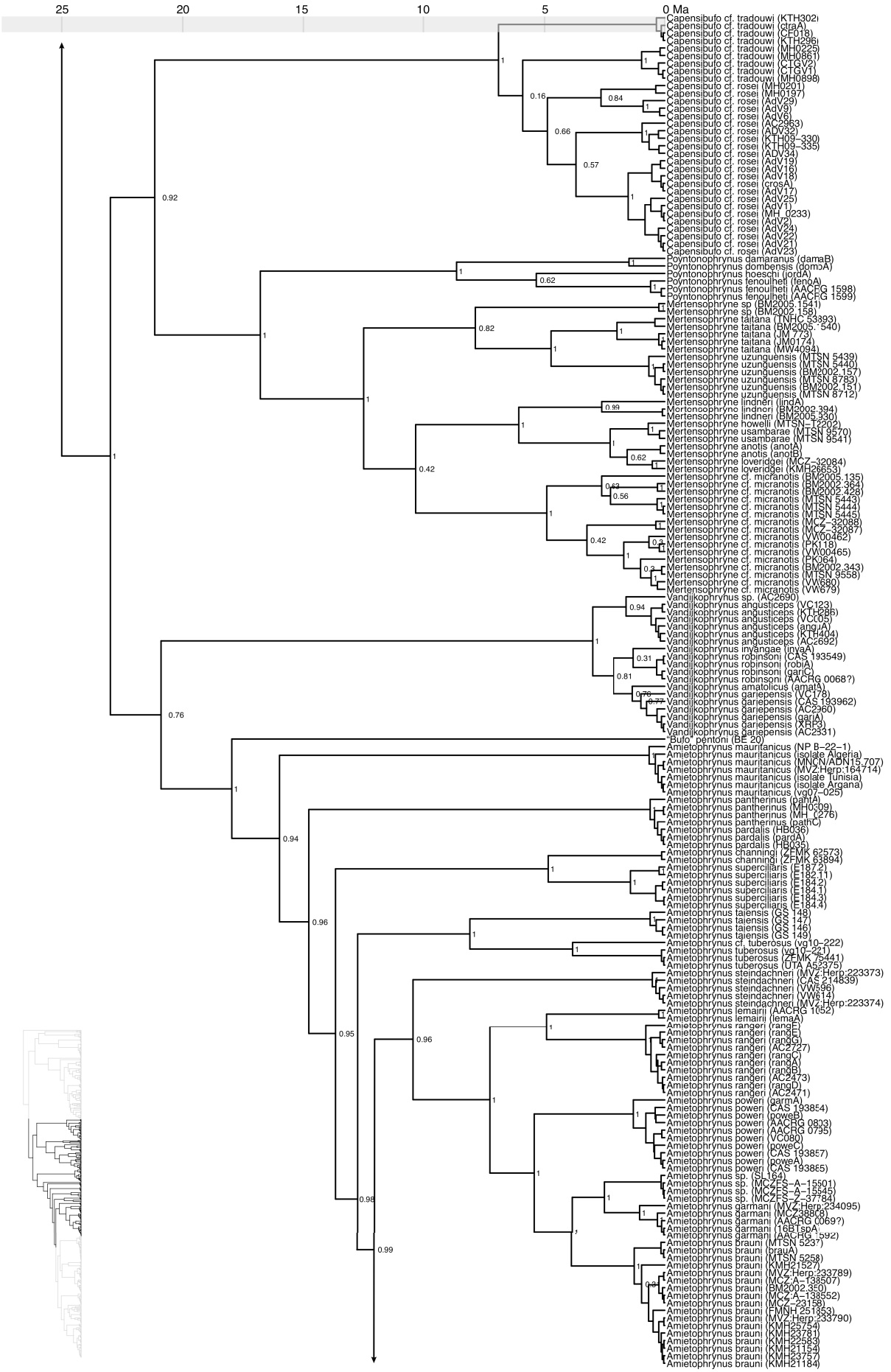
25
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Capensibufo cf. tradouwi (ctraA)
Capensibufo cf. tradouwi (CF018)
Capensibufo cf. tradouwi (KTH296
Capensibufo cf. tradouwi (MH0225)
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Capensibufo cf. tradouwi (CTGV2)
Capensibufo cf. tradouwi (CTGV1)
Capensibufo cf. tradouwi (MH0898)
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Capensibufo cf. rosei (MH0197)
Capensibufo cf. rosei (AdV29)
Capensibufo cf. rosei (AdV9)
Capensibufo cf. rosei (AdV6)
Capensibufo cf. rosei (AC2963)
Capensibufo cf. rosei (ADV32)
Capensibufo cf. rosei (KTH09–330)
Capensibufo cf. rosei (KTH09–335)
Capensibufo cf. rosei (ADV34)
Capensibufo cf. rosei (AdV19)
Capensibufo cf. rosei (AdV16)
Capensibufo cf. rosei (AdV18)
Capensibufo cf. rosei (crosA)
Capensibufo cf. rosei (AdV17)
Capensibufo cf. rosei (AdV25)
Capensibufo cf. rosei (AdV1)
Capensibufo cf. rosei (MH_0233)
Capensibufo cf. rosei (AdV7)
Capensibufo cf. rosei (AdV24)
Capensibufo cf. rosei (AdV22)
Capensibufo cf. rosei (AdV21)
Capensibufo cf. rosei (AdV23)
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Poyntonophrynus dombensis (domA)
Poyntonophrynus hoeschi (jordA)
Poyntonophrynus fenoulheti (fenoA)
Poyntonophrynus fenoulheti (AACRG 1598)
Poyntonophrynus fenoulheti (AACRG 1599)
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Mertensophryne sp (BM2002.158)
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Mertensophryne taitana (BM2005.540)
Mertensophryne taitana (JM 772)
Mertensophryne taitana (JM0174)
Mertensophryne taitana (MW4094)
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Mertensophryne uzunguensis (BM2002.151)
Mertensophryne uzunguensis (MTSN 8712)
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Mertensophryne lindneri (BM2002.394)
Mertensophryne lindneri (BM2005.330)
Mertensophryne howelli (MTSN–T2202)
Mertensophryne usambarae (MTSN 9570)
Mertensophryne usambarae (MTSN 9541)
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Mertensophryne anotis (anotB)
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Mertensophryne loveridgei (KMH28653)
Mertensophryne cf. micranotis (BM2005.135)
Mertensophryne cf. micranotis (BM2002.364)
Mertensophryne cf. micranotis (BM2002.428)
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Mertensophryne cf. micranotis (VW679)
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Vandijkophrynus angusticeps (KTH286)
Vandijkophrynus angusticeps (VC005)
Vandijkophrynus angusticeps (angA)
Vandijkophrynus angusticeps (KTH404)
Vandijkophrynus angusticeps (AC2692)
Vandijkophrynus inyangae (invaA)
Vandijkophrynus robinsoni (CAS 193549)
Vandijkophrynus robinsoni (robiA)
Vandijkophrynus robinsoni (garlC)
Vandijkophrynus robinsoni (AACRG 0068?)
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Vandijkophrynus gariepensis (VC178)
Vandijkophrynus gariepensis (CAS 193962)
Vandijkophrynus gariepensis (AC2360)
Vandijkophrynus gariepensis (garlA)
Vandijkophrynus gariepensis (XR53)
Vandijkophrynus gariepensis (AC2331)
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Amietophrynus mauritanicus (isolate Algeria)
Amietophrynus mauritanicus (MNCN/ADN15.707)
Amietophrynus mauritanicus (MVZ:Herp:164714)
Amietophrynus mauritanicus (isolate Tunisia)
Amietophrynus mauritanicus (isolate Argana)
Amietophrynus mauritanicus (vg07–025)
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Amietophrynus pantherinus (pathC)
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Amietophrynus pardalis (HB035)
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Amietophrynus superciliaris (E184.1)
Amietophrynus superciliaris (E184.3)
Amietophrynus superciliaris (E184.4)
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Amietophrynus taiensis (GS 146)
Amietophrynus taiensis (GS 149)
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Amietophrynus tuberosus (vg10–221)
Amietophrynus tuberosus (ZFMK 75441)
Amietophrynus tuberosus (UTA A52375)
Amietophrynus steindachneri (MVZ:Herp:223373)
Amietophrynus steindachneri (CAS 214839)
Amietophrynus steindachneri (VW596)
Amietophrynus steindachneri (VW614)
Amietophrynus steindachneri (MVZ:Herp:223374)
Amietophrynus lemairii (AACRG 1052)
Amietophrynus lemairii (lemaA)
Amietophrynus rangeri (rangE)
Amietophrynus rangeri (rangF)
Amietophrynus rangeri (rangG)
Amietophrynus rangeri (AC2727)
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Amietophrynus rangeri (rangB)
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Amietophrynus poweri (CAS 193854)
Amietophrynus poweri (poweB)
Amietophrynus poweri (AACRG 0803)
Amietophrynus poweri (AACRG 0795)
Amietophrynus poweri (VC080)
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Amietophrynus sp. (MCZFS–A–15546)
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Amietophrynus garmani (MCZ38808)
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Amietophrynus brauni (KMH22583)
Amietophrynus brauni (KMH21154)
Amietophrynus brauni (KMH23757)
Amietophrynus brauni (KMH21184)


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Amietophrynus regularis (requA)
Amietophrynus regularis (E36)
Amietophrynus regularis (SIH-04)
Amietophrynus regularis (E56)
Amietophrynus regularis (DS 82)
Amietophrynus regularis (E102)
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Amietophrynus regularis (GS 193)
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Amietophrynus regularis (isolate 002)
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Amietophrynus maculatus (macuB)
Amietophrynus maculatus (MVZ:Herp:234551)
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Amietophrynus maculatus (ACRG 0684)
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Amietophrynus kisoloensis (CAS 201948)
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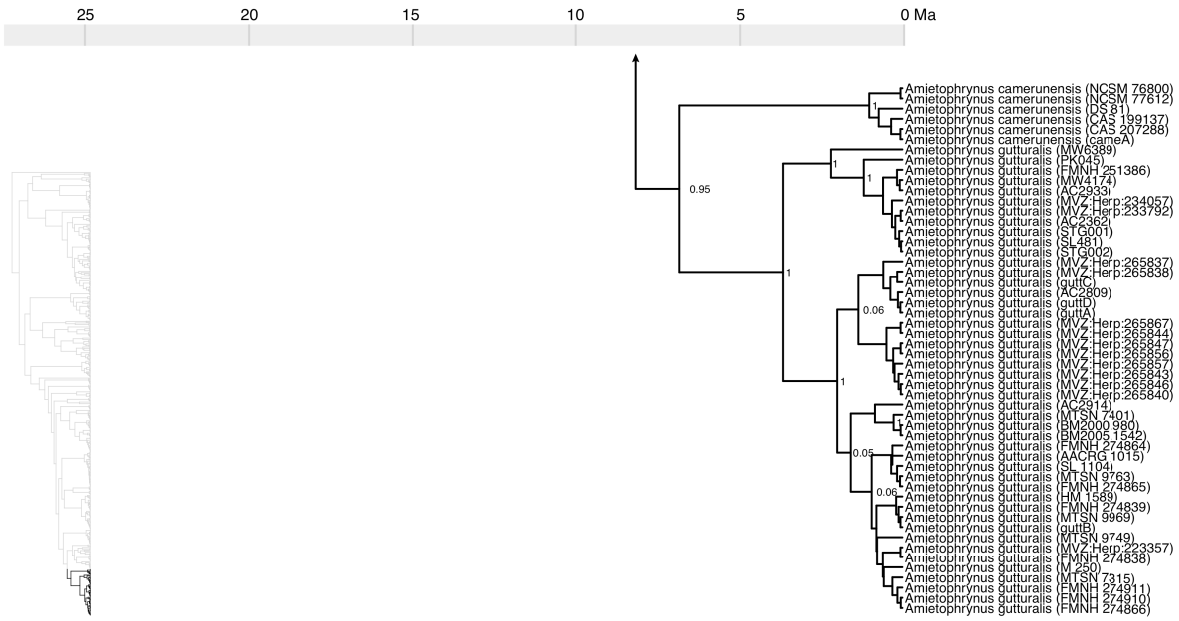
0.32
0.97
0.99
0.74
0.72
0.95


25
20
15
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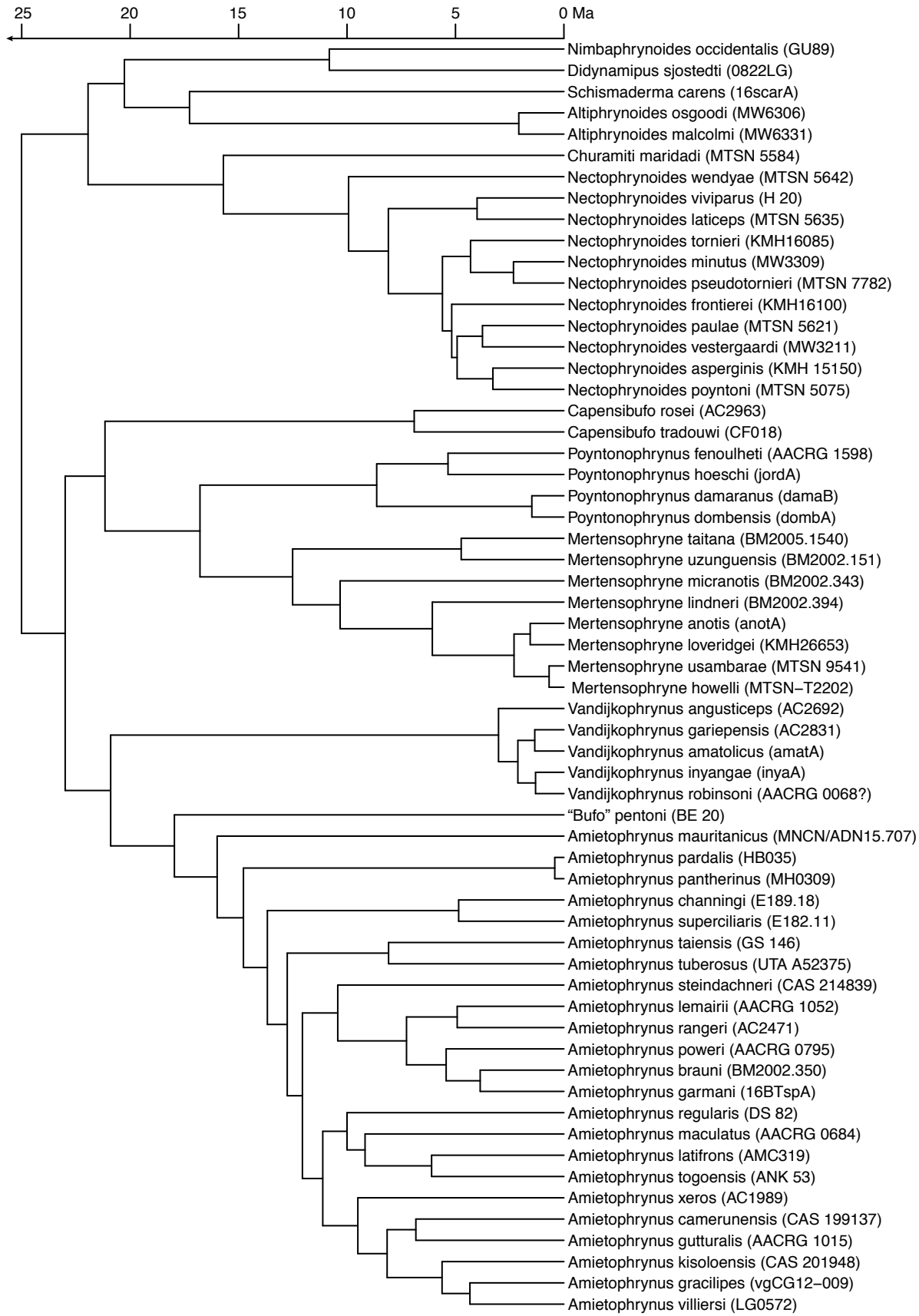
1
1
0.06
1
0.05
0.06
Amietophrynus camerunensis (NCSM 76800)
Amietophrynus camerunensis (NCSM 77612)
Amietophrynus camerunensis (DS 81)
Amietophrynus camerunensis (CAS 199137)
Amietophrynus camerunensis (CAS 207288)
Amietophrynus camerunensis (cameA)
Amietophrynus gutturalis (MW6389)
Amietophrynus gutturalis (PK045)
Amietophrynus gutturalis (FMNH 251386)
Amietophrynus gutturalis (MW4174)
Amietophrynus gutturalis (AC2933)
Amietophrynus gutturalis (MVZ:Herp:234057)
Amietophrynus gutturalis (MVZ:Herp:233792)
Amietophrynus gutturalis (AC2362)
Amietophrynus gutturalis (STG001)
Amietophrynus gutturalis (SL481)
Amietophrynus gutturalis (STG002)
Amietophrynus gutturalis (MVZ:Herp:265837)
Amietophrynus gutturalis (MVZ:Herp:265838)
Amietophrynus gutturalis (guttC)
Amietophrynus gutturalis (AC2809)
Amietophrynus gutturalis (guttD)
Amietophrynus gutturalis (guttA)
Amietophrynus gutturalis (MVZ:Herp:265867)
Amietophrynus gutturalis (MVZ:Herp:265844)
Amietophrynus gutturalis (MVZ:Herp:265847)
Amietophrynus gutturalis (MVZ:Herp:265856)
Amietophrynus gutturalis (MVZ:Herp:265857)
Amietophrynus gutturalis (MVZ:Herp:265843)
Amietophrynus gutturalis (MVZ:Herp:265846)
Amietophrynus gutturalis (MVZ:Herp:265840)
Amietophrynus gutturalis (AC2914)
Amietophrynus gutturalis (MTSN 7401)
Amietophrynus gutturalis (BM2000:980)
Amietophrynus gutturalis (BM2005:1542)
Amietophrynus gutturalis (FMNH 274864)
Amietophrynus gutturalis (AACRG 1015)
Amietophrynus gutturalis (SL_1104)
Amietophrynus gutturalis (MTSN 9763)
Amietophrynus gutturalis (FMNH 274865)
Amietophrynus gutturalis (HM 1589)
Amietophrynus gutturalis (FMNH 274839)
Amietophrynus gutturalis (MTSN 9969)
Amietophrynus gutturalis (guttB)
Amietophrynus gutturalis (MTSN 9749)
Amietophrynus gutturalis (MVZ:Herp:223357)
Amietophrynus gutturalis (FMNH 274838)
Amietophrynus gutturalis (M 250)
Amietophrynus gutturalis (MTSN 7315)
Amietophrynus gutturalis (FMNH 274911)
Amietophrynus gutturalis (FMNH 274910)
Amietophrynus gutturalis (FMNH 274866)

Online Appendix 10. Phylogenetic tree recovered from pruning the FAR tree to include only a single representative of each described species (DS tree).



Online Appendix 11. Table of unique entities recovered using the GYMC method implemented in the r package *splits* using the single threshold model.

Voucher ID	Species based on field identifications	GMYC delimited entity
MW6331 MW6333 SL004 SL079	*Altiphrynoides malcolmi*	*Altiphrynoides malcolmi*
MW6306	*Altiphrynoides* cf. *osgoodi*	*Altiphrynoides* cf. *osgoodi*
BM2002.350 KMH21154 KMH21184 KMH21527 KMH22583 KMH23757 KMH23781 KMH25754 MCZ:A-138507 MCZ:A-138552 MCZ:A-23158 MTSN 5237 MVZ:Herp:233790 brauA	*Amietophrynus brauni*	*Amietophrynus brauni* (1)
FMNH 251853 MTSN 5258 MVZ:Herp:233789		*Amietophrynus brauni* (2)
CAS 199137 CAS 207288 DS 81 NCSM 76800 NCSM 77612 cameA	*Amietophrynus camerunensis*	*Amietophrynus camerunensis*
E189.18 E189.19	*Amietophrynus channingi*	*Amietophrynus channingi*
16BTspA AACRG 0069? AACRG 1592 MCZ38808 MVZ:Herp:234095	*Amietophrynus garmani*	*Amietophrynus garmani*
vgCG12-009 vgCG12-103	*Amietophrynus gracilipes*	*Amietophrynus gracilipes*
DS 07 DS 08 DS 66 DS 74 DS 80 DS 98 vgCAR089	*Amietophrynus* cf. *gracilipes*	*Amietophrynus* cf. *gracilipes* (1)
831LG vg09-046	*Amietophrynus* cf. *gracilipes*	*Amietophrynus* cf. *gracilipes* (2)
CAS 207620	*Amietophrynus* cf. *gracilipes*	*Amietophrynus* cf. *gracilipes* (3)
NCSM 76801		*Amietophrynus* cf. *gracilipes* (4)
AC2362 AC2933 FMNH 251386 MVZ:Herp:265843 MVZ:Herp:265844 SL 1104	*Amietophrynus gutturalis*	*Amietophrynus gutturalis* (1)

guttB guttC guttD		
AC2914 BM2000.980 BM2005.1542 MVZ:Herp:233792		*Amietophrynus gutturalis* (2)
AACRG 1015 FMNH 274838 FMNH 274839 FMNH 274864 FMNH 274865 FMNH 274866 FMNH 274910 FMNH 274911 MTSN 7315 MTSN 9763 MTSN 9969 MVZ:Herp:223357 MVZ:Herp:234057 MVZ:Herp:265837 MVZ:Herp:265838 MVZ:Herp:265840 guttA		*Amietophrynus gutturalis* (3)
AC2809 HM 1589 MTSN 7401 MTSN 9749 MVZ:Herp:265846 MVZ:Herp:265847		*Amietophrynus gutturalis* (4)
MVZ:Herp:265856 MVZ:Herp:265857 MVZ:Herp:265867 MW4174 MW6389 M 250 PK045 SL481		*Amietophrynus gutturalis* (5)
STG001		*Amietophrynus gutturalis* (6)
STG002		*Amietophrynus gutturalis* (7)
CAS 201948 CAS 202005 MTSN 6879 MTSN 7219 MVZ:Herp:223361 SL482 TNHC 61999 kisoA	*Amietophrynus kisoloensis*	*Amietophrynus kisoloensis*
AMC319 MC11_035 MH0206 MH0233 MH0423	*Amietophrynus latifrons*	*Amietophrynus latifrons*
AACRG 1052 lemaA	*Amietophrynus lemairii*	*Amietophrynus lemairii*
DS 83 MVZ:Herp:253187 MVZ:Herp:265841	*Amietophrynus maculatus*	*Amietophrynus maculatus* (1)

MVZ:Herp:265845 ZFMK 75443		
AACRG 0684 HM 1626 HM 1648 HM 1652 HM 1746 MVZ:Herp:233791 MVZ:Herp:234551 MVZ:Herp:265864 MW6140 M 263 NI 42 PK126 SA 128		*Amietophrynus maculatus* (2)
AMC002 AMC041 AMC084 AMC147 AMI 1 GS 196 ZFMK 92987		*Amietophrynus maculatus* (3)
AMC012 BE 39 CAS 229969 CAS 229986 CAS 229987 CAS 229988 CAS 230064 LE 36 MVZ:Herp:265863 Ni 105 ZFMK 92986 ZFMK 92988 macuB		*Amietophrynus maculatus* (4)
macuA		*Amietophrynus maculatus* (5)
MNCN/ADN15.707 MVZ:Herp:164714 NP B-22-1 isolate Algeria isolate Argana isolate Tunisia vg07-025	*Amietophrynus mauritanicus*	*Amietophrynus mauritanicus*
MH0309 MH_0276 pantA pathC	*Amietophrynus pantherinus*	*Amietophrynus pantherinus/pardalis*
HB035 HB036 pardA	*Amietophrynus pardalis*	
AACRG 0795 AACRG 0803 CAS 193854 CAS 193857 CAS 193885 poweC garmA poweA	*Amietophrynus poweri*	*Amietophrynus poweri* (1)

poweB		
VC080		*Amietophrynus poweri* (2)
AC2471 AC2473 AC2727 rangA rangB rangC rangD rangE rangF rangG	*Amietophrynus rangeri*	*Amietophrynus rangeri*
E21 FMNH 262252 FMNH 262253 GS 193 KU 290435 LM 137 MVZ:Herp:223372 MVZ:Herp:245396 SA 016 SA 118 SIH-04 SL501 ZFMK 75630 ZFMK 75631 isolate 001 isolate 002 isolate 003 isolate 004 isolate 005 isolate 006 isolate 007 isolate 008 isolate 009 isolate 010 isolate 411 isolate 424 isolate 460 isolate B2 reguB	*Amietophrynus regularis*	*Amietophrynus regularis* (1)
isolate 410 reguA		*Amietophrynus regularis* (2)
DS 82 E102 E36 E56 isolate 417 isolate 423 isolate B1		*Amietophrynus regularis* (3)
vg10-222	*Amietophrynus* cf. *tuberosus*	*Amietophrynus* cf. *tuberosus*
ZFMK 75769	*Amietophrynus* sp.	*Amietophrynus* sp.
MTSN 9840	*Amietophrynus* sp.	*Amietophrynus* sp.
MTSN 6882 MTSN 7348 MTSN 7355	*Amietophrynus* sp.	*Amietophrynus* sp.
AC2905	*Amietophrynus* sp.	*Amietophrynus* sp.
CAS 214839	*Amietophrynus steindachneri*	*Amietophrynus steindachneri*

MVZ:Herp:223373 MVZ:Herp:223374 VW596 VW614		
E182.11 E187.2		*Amietophrynus superciliaris* (1)
E184.1 E184.2 E184.3 E184.4	*Amietophrynus superciliaris*	*Amietophrynus superciliaris* (2)
GS 146 GS 147 GS 148 GS 149	*Amietophrynus taiensis*	*Amietophrynus taiensis*
ANK 53 GS 109 GU 146 GU 151 GU 192	*Amietophrynus togoensis*	*Amietophrynus togoensis*
UTA A52375 ZFMK 75441 vg10-221	*Amietophrynus tuberosus*	*Amietophrynus tuberosus*
LG0572 MH0340	*Amietophrynus villiersi*	*Amietophrynus villiersi*
AMNH 109826 BX1827 BX2211 BX2676 BX368 BX369 BX456 BX462 BX473 BX994 CAS 214829 FMNH 262256 FMNH 262289 MHNG 2650.038 xeroB	*Amietophrynus xeros*	*Amietophrynus xeros* (1)
AC1989 xeroA		*Amietophrynus xeros* (1)
BE 20	*"Bufo" pentoni*	*"Bufo" pentoni*
AC2963 AdV25 AdV29 KTH09-335 MH0197		*Capensibufo rosei* (1)
ADV34 AdV1 AdV16 AdV17 KTH09-330	*Capensibufo rosei*	*Capensibufo rosei* (2)
ADV32 AdV18 AdV19 AdV2 AdV21 AdV22		*Capensibufo rosei* (3)

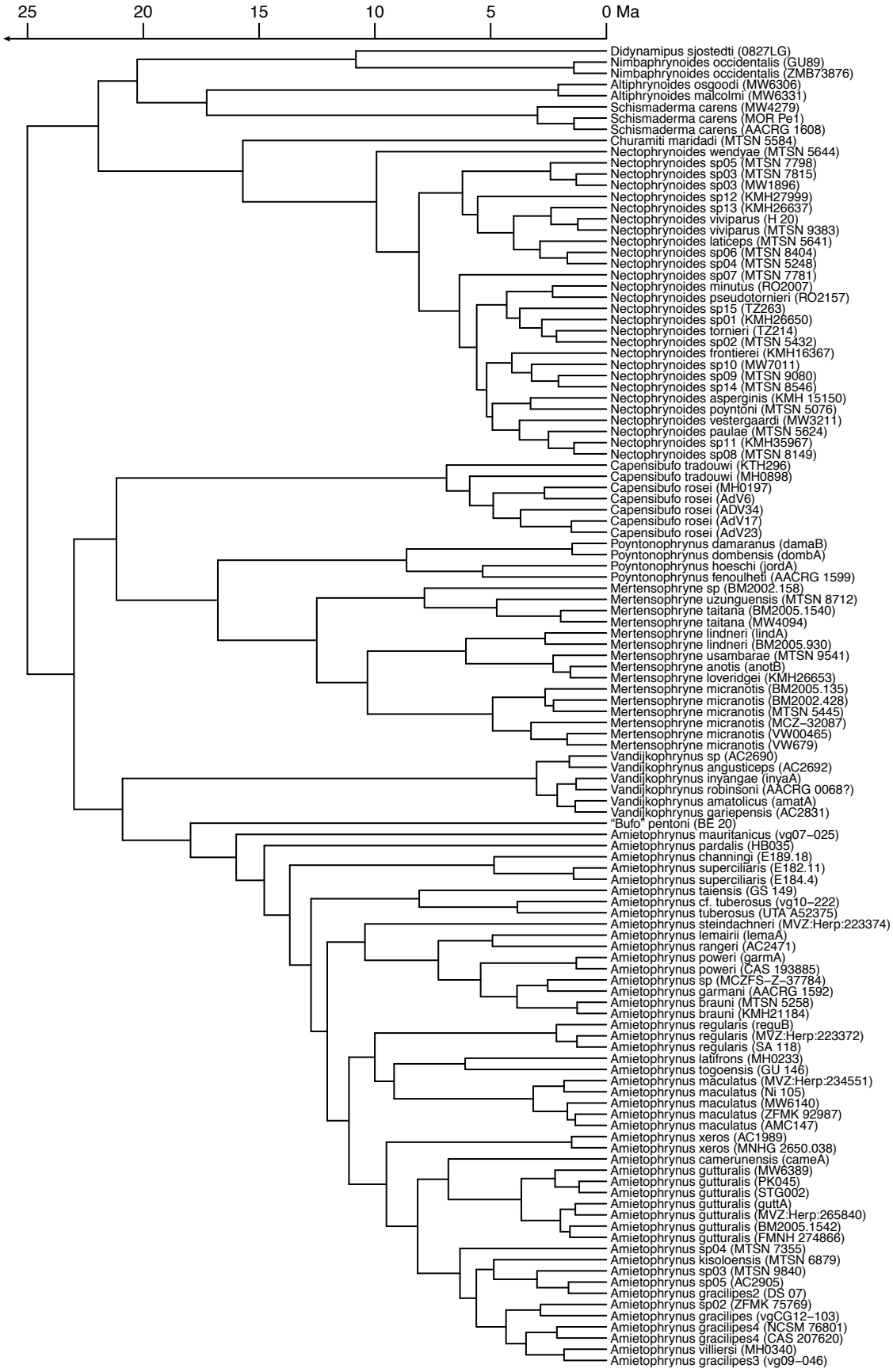
AdV23 MH0201		
MH_0233 crosA		*Capensibufo rosei* (4)
AdV24 AdV6 AdV9		*Capensibufo rosei* (5)
CF018 KTH296 KTH302 MH0225		*Capensibufo tradouwi* (1)
CTGV1 CTGV2 MH0861 MH0898 ctraA	*Capensibufo tradouwi*	*Capensibufo tradouwi* (2)
MTSN 5584 MTSN 5585	*Churamiti maridadi*	*Churamiti maridadi*
0822LG 0824LG 0825LG 0827LG AG 259 MOR 0163 didyA	*Didynamipus sjostedti*	*Didynamipus sjostedti*
MCZFS-A-15501 MCZFS-A-15545 MCZFS-Z-37784 SL164	*Amietophrynus* sp.	*Amietophrynus* sp.
anotA anotB	*Mertensophryne anotis*	*Mertensophryne anotis*
MTSN-T2202	*Mertensophryne howelli*	*Mertensophryne howelli/usambarae*
BM2002.394 BM2005.930	*Mertensophryne lindneri*	*Mertensophryne lindneri* (1)
lindA		*Mertensophryne lindneri* (2)
KMH26653 MCZ:A-32084	*Mertensophryne loveridgei*	*Mertensophryne loveridgei*
MTSN 5443 MTSN 5444 MTSN 5445	*Mertensophryne micranotis*	*Mertensophryne micranotis* (1)
BM2002.364 BM2002.428		*Mertensophryne micranotis* (2)
MCZ:A-32087 MCZ:A-32088		*Mertensophryne micranotis* (3)
BM2002.343 MTSN 9558 PK064 VW679 VW680		*Mertensophryne micranotis* (4)
PK118 VW00462 VW00465		*Mertensophryne micranotis* (5)
BM2005.135		*Mertensophryne micranotis* (6)
BM2002.158 BM2005.1541	*Mertensophryne* sp	*Mertensophryne* sp
BM2005.1540 TNHC 53893	*Mertensophryne taitana*	*Mertensophryne taitana* (1)
JM 773		*Mertensophryne taitana* (2)

JN0174 MW4094		
MTSN 9541 MTSN 9570	*Mertensophryne usambarae*	*Mertensophryne howelli/usambarae*
BM2002.151 BM2002.157 MTSN 5439 MTSN 5440 MTSN 8712 MTSN 8783	*Mertensophryne uzunguensis*	*Mertensophryne uzunguensis*
KMH 15150	*Nectophrynoides asperginis*	*Nectophrynoides asperginis*
KMH16100 KMH16367	*Nectophrynoides frontierei*	*Nectophrynoides frontierei*
MTSN 5635 MTSN 5637 MTSN 5641	*Nectophrynoides laticeps*	*Nectophrynoides laticeps*
MW3309 MW7339 RO2007 RO2019	*Nectophrynoides minutus*	*Nectophrynoides minutus*
MTSN 5621 MTSN 5622 MTSN 5623 MTSN 5624 MTSN 5626 MTSN 5630	*Nectophrynoides paulae*	*Nectophrynoides paulae*
MTSN 5075 MTSN 5076 MTSN 5080	*Nectophrynoides poyntoni*	*Nectophrynoides poyntoni*
MTSN 7782 RO2020 RO2143 RO2157	*Nectophrynoides pseudotornieri*	*Nectophrynoides pseudotornieri*
KMH26262 KMH26650 MW1822	*Nectophrynoides* sp.	*Nectophrynoides* sp.
MTSN 5334 MTSN 5429 MTSN 5432 MTSN 5434 MTSN 5435	*Nectophrynoides* sp.	*Nectophrynoides* sp.
KMH36201 MTSN 7573 MW1894 MW1896 TZ391	*Nectophrynoides* sp.	*Nectophrynoides* sp. (1)
MTSN 7815		*Nectophrynoides* sp (2)
MTSN 5248 MTSN 5249 MTSN 5253 MTSN 5339 MTSN 5340 MTSN 5341 MTSN 5342 TZ88 TZ89	*Nectophrynoides* sp.	*Nectophrynoides* sp.
KMH27949 KMH27952	*Nectophrynoides* sp.	*Nectophrynoides* sp.

MTSN 7798 MTSN 7811 MTSN 7812 MTSN 8404 MTSN 8405	*Nectophrynoides* sp.	*Nectophrynoides* sp.
MTSN 7725 MTSN 7751 MTSN 7780 MTSN 7781 RO2078 RO2083 RO2088 RO2134	*Nectophrynoides* sp.	*Nectophrynoides* sp.
MTSN 8149 MTSN 8155 MTSN 8175 MW6798	*Nectophrynoides* sp.	*Nectophrynoides* sp.
MTSN 9080	*Nectophrynoides* sp.	*Nectophrynoides* sp.
MW7011	*Nectophrynoides* sp.	*Nectophrynoides* sp.
KMH35967 KMH35969 MW6695	*Nectophrynoides* sp.	*Nectophrynoides* sp.
KMH27999 KMH28000	*Nectophrynoides* sp.	*Nectophrynoides* sp.
KMH26637 KMH26638 KMH26641 KMH26644 KMH26998	*Nectophrynoides* sp.	*Nectophrynoides* sp.
MTSN 8544 MTSN 8545 MTSN 8546	*Nectophrynoides* sp.	*Nectophrynoides* sp.
TZ263	*Nectophrynoides* sp.	*Nectophrynoides* sp.
KMH16085 RDS951 TZ213 TZ214	*Nectophrynoides tornieri*	*Nectophrynoides tornieri*
MW3211	*Nectophrynoides vestergaardi*	*Nectophrynoides vestergaardi*
H 20	*Nectophrynoides viviparus*	*Nectophrynoides viviparous* (1)
MTSN 9365 MTSN 9383		*Nectophrynoides viviparous* (2)
MTSN 5642 MTSN 5644 MTSN 5647	*Nectophrynoides wendyae*	*Nectophrynoides wendyae*
GU89 MTN 23 MTN 230 MTN 52 MTN 81	*Nimbaphrynoides occidentalis*	*Nimbaphrynoides occidentalis* (1)
MOR MTN15 MOR MTN16 MOR MTN22 MOR MTN245 MOR MTN246 MOR MTN247 MOR MTN248 MOR MTN78 MOR NI211		*Nimbaphrynoides occidentalis* (2)

MOR NL204 MOR NL205 MOR NL215 ZMB73875 ZMB73876 ZMB73881 ZMB73882 ZMB73886		
damaB	*Poyntonophrynus damaranus*	*Poyntonophrynus damaranus*
dombA	*Poyntonophrynus dombensis*	*Poyntonophrynus dombensis*
AACRG 1598 AACRG 1599 fenoA	*Poyntonophrynus fenoulheti*	*Poyntonophrynus fenoulheti*
jordA	*Poyntonophrynus hoeschi*	*Poyntonophrynus hoeschi*
16scarA AACRG 1607 AACRG 1608 MVZ:Herp:223386	*Schismaderma carens*	*Schismaderma carens* (1)
MOR Pe1 RdS796		*Schismaderma carens* (2)
MW4279		*Schismaderma carens* (3)
amatA	*Vandijkophrynus amatolicus*	*Vandijkophrynus amatolicus*
AC2692 KTH286 KTH404 VC005 VC123 anguA	*Vandijkophrynus angusticeps*	*Vandijkophrynus angusticeps*
AC2831 AC2960 CAS 193962 VC178 XRP3 gariA	*Vandijkophrynus gariepensis*	*Vandijkophrynus gariepensis*
inyaA	*Vandijkophrynus inyangae*	*Vandijkophrynus inyangae*
AACRG 0068? CAS 193549 gariC robiA	*Vandijkophrynus robinsoni*	*Vandijkophrynus robinsoni*
AC2690	*Vandijkophrynus* sp.	*Vandijkophrynus* sp.

Online Appendix 12. Tree recovered from pruning the FAR tree to include only a single representative of each GMYC delimited element (GMYC tree).



Online Appendix 13: *Altiphrynoides* cf. *osgoodi*

During sampling in the Bale Mountains, Ethiopia (as outlined in Gower et al. 2013) we found a single juvenile of uncertain identity in a locality (near to Goba) where no other bufonids were collected. We assume this juvenile to be *Altiphrynoides osgoodi* given new molecular data collected on this specimen, which indicated substantial molecular differences from adult *A. malcomi* collected from a different location (Harenna). Morphological characters separating these two species (formerly separate genera) are not easy (see Largen 2001) and are mainly based on differences in breeding biology. Until adult specimens of *A. osgoodi* are secured and tested against these samples this finding is tentative. An alternative explanation would be that it is another species, a congener of *A. malcomi*, however given the substantial molecular difference and the close geographical distance of samples confidently identified as *A. malcomi* (from Harenna Forest) we suspect this alternative explanation to be unlikely.

References

Gower D.J., Aberra R.K., Schwaller S., Largen M.J., Collen B., Spawls S., Menegon M., Zimkus B.M., de Sá R., Mengistu A.A., Gebresenbet F., Moore R.D., Saber S.A., Loader S.P. 2013. Long-term data for endemic frog genera reveal potential conservation crisis in the Bale Mountains, Ethiopia. Oryx. 47:59–69.

Largen M.J. 2001. Catalogue of the amphibians of Ethiopia, including a key for their identification. Trop. Zool. 14:307–402.

Supplementary Materials

Chapter IV

Appendix 1: Supplementary Tables

Table S1. Genbank accession numbers of sequences used

Species	Voucher ID	12S	16S	COI	CXCR4	RAG1
Adenomus kelaartii	VUB 0171	FJ882780	FJ882780		EF107447	
Altiphrynoides malcolmi	MW6331	KF665005	KF665145	KF665785	KF665916	KF666436
Altiphrynoides osgoodi	MW6306	KF664637	KF665309	KF665726	KF665885	KF666313
Amietophrynus brauni	KMH21527	KF664650	KF665239	KF665608	KF665991	KF666342
Amietophrynus camerunensis	NCSM 76800	KF665022	KF665404	KF665730	KF665920	KF666271
Amietophrynus channingi	E189.19	KF664735	HQ882843		KF666006	
Amietophrynus garmani	MCZ38808	KF664684	KF665281	KF665707	KF666109	KF666160
Amietophrynus gracilipes4	NCSM 76801	KF664874	KF665287	KF665534	KF666103	KF666364
Amietophrynus gutturalis	MTSN 9969	KF664738	KF665160	KF665775	KF666033	KF666203
Amietophrynus kisoloensis	CAS 201948	GU226837	GU226837	KF665519	GU226834	KF666361
Amietophrynus latifrons	MC11_035	KF664929	KF665409	KF665647	KF666004	KF666272
Amietophrynus lemairii	AACRG 1052	KF664873	KF665036	KF665803	KF666038	KF666396
Amietophrynus maculatus	AMC147	KF664902	KF665456	KF665526	KF665938	KF666432
Amietophrynus mauritanicus	vg07-025	KF664780	KF665428	KF665723	KF666116	KF666227
Amietophrynus pantherinus	MH_0276	KF664917	KF665321	KF665614	KF666024	KF666226
Amietophrynus pardalis	HB035	KF664840	KF665337	KF665527	KF665852	KF666241
Amietophrynus poweri	AACRG 0795	KF664609	KF665365	KF665776	KF665949	KF666328
Amietophrynus rangeri	AC2473	KF664760	KF665268	KF665806	KF665871	KF666416
Amietophrynus regularis	DS 82	KF664618	KF665408	KF665651	KF666072	KF666405
Amietophrynus steindachneri	CAS 214839	FJ882825	FJ882825	KF665771	FJ882726	DQ158406
Amietophrynus superciliaris	E184.3	KF664629	HQ882845		KF666110	KF666281
Amietophrynus taiensis	GS 148	KF664621	KF665302	KF665583	KF666027	KF666381
Amietophrynus togoensis	GU 151	KF664974	KF665100	KF665662	KF666041	KF666408
Amietophrynus tuberosus	vg10-221	KF664779	KF665246	KF665810	KF665977	KF666290
Amietophrynus villiersi	MH0340	KF664845	KF665202	KF665792	KF666056	KF666353
Amietophrynus xeros	FMNH 262289	KF664724	KF665131	KF665670	KF666131	KF666430
Anaxyrus americanus	CAS 223832	KF664881	KF665122	KF665823	KF665863	KF666426
Anaxyrus boreas	CAS 176529	FJ882830	FJ882830	KF665820	FJ882732	KF666377
Anaxyrus californicus	CAS 175636	FJ882828	KF665292	KF665811		KF666250
Anaxyrus canorus	CAS 209233	KF664990	KF665178	KF665524	KF665840	KF666431
Anaxyrus terrestris	CAS 207171	FJ882829	FJ882829	KF665667	FJ882731	KF666176
Ansonia longidigita	VUB 0666	FJ882796	FJ882796	KF665812	FJ882698	KF666400
Ansonia thinthinae	CAS 243945	KF664734	KF665162	KF665611	KF665854	KF666367
Atelopus barbotini	BPN 1697	GU183859	GU183859	KF665712	GU183852	KF666236
Barbarophryne brongersmai	IBES3045	pending	pending	pending	pending	pending
Bufo bufo	vg06-282	KF664601	KF665394	KF665517	KF666057	KF666388
Bufo gargarizans	CAS 228184	FJ882808	FJ882808	KF665641	FJ882708	KF666177
Bufo pageoti	CAS 233251	KF664905	KF665335	KF665626	KF665978	KF666231
Bufo pentoni	BE 20	KF664969	KF665129	KF665512	KF666058	KF666258
Bufotes surdus	ZMMSU A-4027	FJ882810	FJ882810		FJ882711	
Bufotes variabilis	VUB 1813	FJ882812	FJ882812		FJ882713	
Bufotes viridis	vg07-187	KF664594	KF665464	KF665616	KF665913	KF666439
Capensibufo rosei	KTH09-335	KF664868	KF665294	KF665706	KF665976	KF666159
Capensibufo tradouwi	CTGV2	KF664849	KF665072			

Churamiti maridadi	MTSN 5585	KF664661	KF665195	KF665768	KF665935	KF666268
Didynamipus sjostedti	0827LG	KF664606	KF665485	KF665618	KF666012	KF666314
Duttaphrynus crocus	CAS 220193	FJ882789	FJ882789	KF665657	FJ882690	KF666270
Duttaphrynus dhufarensis	CAS 227584	FJ882837	KF665085	KF665821	FJ882679	KF666330
Duttaphrynus melanostictus	CAS 247174	KF664640	KF665340		KF665993	KF666243
Duttaphrynus olivaceus	CAS 232073	KF664676	KF665215	KF665805	KF666043	KF666298
Duttaphrynus stuarti	CAS 221485	FJ882788	FJ882788	KF665503	FJ882689	KF666269
Epidalea calamita	vg07-119	KF664850	KF665137	KF665813	KF665981	KF666155
Ghatophryne ornata	SDB 435	FJ882797	FJ882797		FJ882694	
Incilius alvarius	UTA:A-53924	HM563818	HM563860		HM563891	HM563977
Incilius campbelli	UTA:A-50902	HM563825	HM563866		HM563898	HM563984
Incilius coniferus	MVZ:Herp:203775	HM563829	HM563870		HM563902	HM563988
Incilius valliceps	MZFC:JRM-3868	HM563854	AY008211		HM563927	HM564013
Ingerophrynus biporcatus	TNHC 53890	U52732	U52770			
Ingerophrynus divergens	VUB 0602	FJ882802	FJ882802	KF665713	FJ882701	KF666187
Ingerophrynus galeatus	FMNH 256443	DQ158452	DQ158452		DQ306506	DQ158374
Ingerophrynus macrotis	CAS 230357	FJ882803	FJ882803	KF665540	KF666117	KF666244
Ingerophrynus parvus	CAS 236086	KF664931	KF665415		KF665955	KF666331
Leptophryne borbonica	VUB 0673	FJ882799	FJ882799	KF665688	EF107450	KF666468
Melanophryniscus stelzneri	VUB 0985	FJ882853	FJ882853	KF665744	AY948784	KF666223
Mertensophryne anotis	anotA	AF220862	AF220910			
Mertensophryne howelli	MTSN-T2202	KF664964	KF665247	KF665531	KF666045	KF666383
Mertensophryne lindneri	BM2002.394	KF664736	KF665426	KF665790	KF665953	KF666333
Mertensophryne loveridgei	MCZ-32084	KF664924	KF665338	KF665572	KF665947	KF666463
Mertensophryne micranotis	MCZ-32087	KF665020	KF665240	KF665579	KF666123	KF666378
Mertensophryne taitana	JM 773	KF664809	KF665047	KF665612	KF665995	KF666310
Mertensophryne usambarae	MTSN 9541	KF665026	KF665336	KF665800	KF666115	KF666360
Mertensophryne uzunguensis	BM2002.157	KF664717	KF665170	KF665699	FJ882720	KF666366
Nectophryne afra	MVZ:Herp:234857	KF664711	KF665181	KF665829	KF665867	KF666446
Nectophryne batesii	MVZ:Herp:234688	KF665012	KF665479	KF665571	KF666037	KF666225
Nectophrynoides asperginis	KMH 15150	KF664776	KF665171	KF665547	KF665900	KF666319
Nectophrynoides frontierei	KMH16367	KF664628	KF665223	KF665602		
Nectophrynoides laticeps	MTSN 5641	KF664858	KF665261	KF665758	KF665957	KF666423
Nectophrynoides minutus	MW3309	FJ882814	FJ882814	KF665588	KF665907	KF666454
Nectophrynoides paulae	MTSN 5626	KF664950	KF665118	KF665801	KF666034	KF666169
Nectophrynoides poyntoni	MTSN 5076	KF664920	KF665092	KF665755	KF665910	KF666413
Nectophrynoides pseudotornieri	RO2020	KF664844	KF665392	KF665653	KF665906	KF666410
Nectophrynoides tornieri	TZ214	KF664834	KF665046	KF665669	KF666125	KF666192
Nectophrynoides vestergaardi	MW3211	KF665017	KF665310	KF665767	KF665853	KF666151
Nectophrynoides viviparus	MTSN 9383	KF664886	KF665442	KF665799	KF665931	KF666158
Nectophrynoides wendyae	MTSN 5642	KF664769	KF665374	KF665795	KF665882	KF666285
Nimbaphrynoides occidentalis	MTN 23	KF665010	KF665040	KF665538	KF665967	KF666193
Pedostibes hosii	VUB 0661	FJ882804	FJ882804	KF665818	EF107449	KF666369
Pelophryne misera	VUB 0641	FJ882800	FJ882800	KF665680	FJ882700	KF666300
Phrynoidis aspera	CAS 248116	KF664660	KF665483	KF665743	KF665952	KF666437
Phrynoidis juxtaspera	VUB 0649	FJ882805	FJ882805	KF665605	FJ882710	KF666210
Poyntonophrynus damaranus	damaB		AF220906			
Poyntonophrynus dombensis	dombA	AF220857	AF220907			
Poyntonophrynus fenoulheti	AACRG 1598	KF664732	KF665265	KF665592	KF666066	KF666249
Poyntonophrynus hoeschi	jordA	AF220858				

Poyntonophrynus lughensis	VG001	pending	pending	pending	pending	pending
Pseudepidalea raddei	CAS 238862	KF664854	KF665477	KF665558	KF666101	KF666186
Rhaebo guttatus	MW10096	KF664651	KF665347		KF666068	KF666304
Rhinella granulosa	VUB 1960	FJ882774	FJ882775	KF665648	FJ882728	KF666195
Rhinella margaritifera	MW10041	KF665019	KF665423	KF665704		KF666178
Rhinella marina	VUB 1965	FJ882831	FJ882831	KF665615	KF665869	KF666345
Rhinella schneideri	KU 289057	DQ158480	DQ415572		DQ306528	DQ158399
Schismaderma carens	MOR Pe1	KF664897	KF665121	KF665600	KF665988	KF666363
Vandijkophrynus amatolicus	amatA	AF220851	AF220898			
Vandijkophrynus angusticeps	AC2692	KF664791	KF665432	KF665693	KF666025	KF666237
Vandijkophrynus gariepensis	VC178	KF664828	KF665376	KF665613	KF665889	KF666339
Vandijkophrynus inyangae	inyaA	AF220856	AF220904			
Vandijkophrynus robinsoni	AACRG 0068?	KF664648	KF665375	KF665788	KF665893	KF666198
Werneria bambutensis	0328LG	KF664703	KF665267	KF665508	KF665891	KF666421
Werneria mertensiana	0132LG	KF664904	KF665033	KF665535	KF665945	KF666411
Werneria submontana	vg09-304	KF664890	KF665130	KF665780	KF666084	KF666293
Werneria tandyi	MH0276	KF664619	KF665489	KF665663	KF666100	KF666365
Wolterstorffina chirioi	WOL1	KF664610	KF665357	KF665580	KF665987	KF666219
Wolterstorffina mirei	LG0003	KF664820	KF665341	KF665500	KF666036	KF666230
Wolterstorffina parvipalmata	618LG	KF664798	KF665458	KF665703	KF666029	KF666373
Xanthophryne koynayensis	SDB 2004-012	FJ882782	FJ882782		FJ882691	
Xanthophryne tigerina	SDB 4758	FJ882783	FJ882783		FJ882692	

Table S2. Phylogenetic signal of environmental variables.

	Blomberg's K	*p(K)*	*Pagel's Lambda*	*p(lam)*
BIO4	0.704	0.001	0.972	<0.001
BIO15	0.208	0.677	0.657	<0.001
Q_	0.835	0.001	0.883	<0.001
TWI	0.324	0.122	0.332	<0.001
Slope	0.808	0.001	0.786	<0.001
Tree cover	0.797	0.001	0.838	<0.001

Appendix 2: Phylogenetic reconstruction

A time calibrated phylogeny of African bufonids with a selection of Eurasian and New World outgroups was generated for this study. A total of ~3439 base pairs comprising five markers including partial sequences of two ribosomal RNA genes; 12S and 16S rRNA (~380 and ~575 bp), and three coding regions: cytochrome-oxidase subunit 1 (COI; mitochondrial, ~840 bp), C-X-C chemokine receptor type 4 (CXCR4; nuclear, 711 bp), and recombination activating gene-1 (RAG1; nuclear, ~933 bp) were aligned to form a concatenated data matrix (see Liedtke et al. for details). Sequences were obtained from a previous study (Liedtke et al.), with the addition of data for *Barbarophryne brongersmai* and *Poyntonophrynus lughensis* which were generated *de novo* for this study (list of specimens and GenBank accession numbers are provided in Table S1). A single representative per described species was included, totalling 116 species, of which 70 are African taxa. This covers ca. 70% of all described African species and all genera but *Laurentophryne*, a monotypic genus whose population status is unknown (IUCN SSC Amphibian Specialist Group 2013).

The alignments per locus were processed using the bioinformatics platform Geneious Pro v5.6.7 (created by Biomatters, available from http://www.geneious.com) and the MAFFT v7.017 (Katoh and Standley 2013) plugin using the auto setting for all coding genes and the E-INS-i algorithm for 12S and 16S. The alignments were manually checked and poorly aligned positions and divergent regions of DNA in the 12S and 16S alignments were removed using Gblocks (Castresana 2000) with the options set to allow for smaller final blocks and less strict flanking positions, but no gap positions. The coding genes were realigned and translated using TranslatorX (Abascal et al. 2010) to find the open reading frame. All five genes were concatenated and an optimal partitioning scheme and nucleotide substitution models were determined using partitionfinder v1.1.1 (Lanfear et al. 2012) based on Akaike Information Criterion scores (AIC) implementing the greedy search algorithm. Non-coding genes and each codon position for coding genes were treated as individual partitions (totalling to 11 potential partitions). The 3rd codon position of COI was omitted due to a high degree of nucleotide saturation (see Liedtke et al.).

Joint posterior distribution of all model parameters were estimated using Bayesian MCMC searches in BEAST v1.8.0 (Drummond et al. 2012). Partitionfinder recovered a ten-partition scheme as optimal (nine after excluding CO1-cp3) with the following substitution

models: GTR+Γ+I (12S and 16S), SYM+Γ+I (COI-cp1), GTR+Γ+I (COI-cp2), SYM+Γ+I (CXCR4-cp1), GTR+Γ+I (CXCR4-cp2), TrN+Γ (CXCR4-cp3), GTR+Γ+I (RAG1-cp1), GTR+Γ+I (RAG1-cp2) and HKY+Γ (RAG1-cp3). +Γ+I schemes were reduced to +Γ to avoid over-parameterization due to non-independence of estimates for the proportion of invariable sites and among-site rate variations (Yang 2006). Molecular clock models were estimated for a linked set of mitochondrial markers (12S, 16S and COI) and for CXCR4 and RAG1 separately using uncorrelated lognormal relaxed clock (ucld) priors (Drummond et al. 2006). A birth-death (Gernhard 2008) speciation tree priors as used and four fossil calibration constraints were implemented (Liedtke et al.)

A total of eight MCMC searches with 100 million generations, sampling every 5000th iterations were conducted to assess convergence and stability of parameters. An additional MCMC search on priors only (i.e. with an empty alignment) was also executed to assess whether the signal in the data for estimating parameters is overwhelmed by the prior settings. Convergence and effective sample sizes of parameters in the log files were visually inspected using Tracer, and AWTY (Wilgenbusch et al. 2004) was used to assess whether the MCMC analyses were run long enough to allow the tree topologies to be adequately sampled in proportion to their true posterior probability distribution. All tree searches were conducted on the Linux-HPC cluster of the Computing Centre of the University of Basel (Universitätsrechenzentrum Basel).

Multiple tree files from the independent searches were combined using LogCombiner v1.8.0 (Rambaut and Drummond 2012a). Appropriate burn-in thresholds were set for each run based on the inspection of the chain in Tracer and states were resampled at a lower frequency to obtain ca. 20,000 posterior trees. These trees were then summarized on a maximum clade credibility tree (MCC tree) using TreeAnnotator v1.8.0 (Rambaut and Drummond 2012b) using median node heights and no limit on the posterior probability.

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Curriculum Vitae

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	University of Basel		
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EDUCATION AND ACADEMIC QUALIFICATIONS

2011–2014 **Doctor of Philosophy (Ph.D.)**
University of Basel, Switzerland
Zoology: summa cum laude
Thesis: "Evolution of Terrestrial Breeding in African Amphibians"
Supervisors: Dr. Simon Loader
Dr. Hendrik Müller
Prof. Dr. Peter Nagel

2009–2010 **Master of Science (M.Sc.)**
Lund University, Sweden
Biology with Specialization in Ecology: Pass with Distinction
Thesis: "Descending the Andes: the Biogeography and Diversification of the *Pristimantis conspicillatus* Species Group (Anura: Strabomantidae)"
Supervisors: Dr. José M. Padial
Prof. Dr. Staffan Bensch

2005–2008 **Bachelor of Science (B.Sc.)**
University College London, United Kingdom
Zoology: Upper Second-class Honours (2,i)
Thesis: "Investigating Paternity in a Wild Population of Stalk-eyed Fly, *Teleopsis dalmanni*"
Supervisors: Prof. Dr. Andrew Pomiankowski
Dr. Jennifer Small

2004–2005 **International Baccalaureate (IB)**
International School of Penang. Malaysia

RELEVANT SKILLS

Computing

Competent user of phylogenetic software including RAxML, MrBayes, BEAST, Geneious, BAMM, BayesTraits and Mesquite; ArcGIS; R, Unix shell scripting and cluster operations; SPSS; FileMaker Pro. Some familiarity with: python and java.

Communication

Fluent in English and German, intermediate in Spanish some knowledge of French

Additional Skills and Certifications

German (EU) Driver's license (for class B vehicles)

Certified PADI Advanced Open Water diver

PRESENTATIONS, POSTERS AND PRIZES

2014	Presentation: 33rd Willi Hennig Society meeting, Trento, Italy
2013	Presentation: 13th Joint meeting of the Swiss Zoological and Swiss Systematics Society. Basel, Switzerland
2012	Prize: Best presentation at "Multivariate data analysis in ecology and evolution in R" course. CIBIO, Portugal
2012	Presentation: 7th World Congress of Herpetology, Vancouver, Canada.
2012	Poster: 15th African Amphibian Working Group meeting, Trento, Italy.
2012	Presentation: Swiss Systematics Society annual meeting, Bern, Switzerland.

GRANTS

2014	Freiwillige Akademische Gesellschaft Basel, PhD extension Grant (CHF 12,000)
2012	Swiss Zoological Society Travel Grant (CHF 1,300)
2012	University of Basel Travel Grant (CHF 420)
2010	British Ecological Society Research Grant (£200)

MENTORING AND TEACHING EXPERIENCE

I have co-supervised the thesis of one M.Sc. student and I have taught one-day workshops for 'molecular sequencing lab techniques', 'introduction to R' and 'introduction to comparative methods in R'.

PROFESSIONAL SERVICES

I am an associate Editor for the journal Herpetology Notes and I have acted as a reviewer for the following Journals: Frontiers in Biogeography, Biotropica, Journal of Herpetology, Herpetology Notes. In 2012 I have assisted in an IUCN Red List conservation assessment for East African amphibians.

FIELDWORK

Peninsular Malaysia (2007), Spain (2007), United Kingdom (2007), Paraguay (2008), Kenya (2009), Sweden (2009, 2010), Uganda (2010), Rwanda (2011), Cameroon (2011), Malawi (2012)

MEMBERSHIPS

2012–	Swiss Zoological Society
2012–	Swiss Systematics Society
2011–	Society for the Study of Amphibians and Reptiles (SSAR)
2007–2008	Zoological Society of London

WORK EXPERIENCE AND FURTHER EDUCATION

2014	Workshop: Computational Methods in Macroevolutionary Analysis, Zurich. Focus: diversification rate analyses. Organizer: Dr. D. Rabosky.
2013	Workshop: Applied Phylogenetics, Bodega CA. Focus: phylogenetic methods; Bayesian statistics; comparative analyses. Organizer: Dr. B. Moore
2012	Workshop: Mulitvariate data analysis for Ecology and Evolution in R. Focus: multivariate statistics; model selection; R. Organizer: Dr. D. Adams
2012	Workshop: Applying Phylogenetic Generalized Least Squares. Focus: pGLS; R. Organizer: Dr. A. Gonzalez-Voyer
2011	Summer school: Evolutionary Ecology and Systematics. Focus: 'Phylogenetics –new applications, pitfalls and challenges'. Organizer: Ludwig-Maximilian University of Munich.
2010	Field course: Tropical Biology Association field course; Uganda. Focus: Tropical biology field training.
2009	Laboratory Research Assistant. Employer: Lund University and Sverige Lantbruksuniversitet, Alnarp, Sweden. Tasks: Gas Chromatograpy Electro-antennographic detection (GC-EAD), Single Sensillum Recording (GC-SSR). Referee: Dr. G. Svensson
2009	Field Assistant. Employer: Dr. J. T. Knudsen, Lund University. Tasks: pollinator (*Xylocopa* spp.) experiment coordinator in Mombasa, Kenya.
2008	Intern for Amphibian Research. Host: Instituto de Investigación Biológica del *Paraguay (IIBP)*. Tasks: Amphibian survey. Referee: F. Brusquetti
2007	Field and Laboratory Research Assistant. Employer: University College London. Tasks: Fieldwork in Malaysia with *Teleopsis* spp., microsatellite lab work in London. Referee: Prof. Dr. A. Pomiankowski

INTERESTS

In my free time I enjoy participating in team sports such as football and basketball, outdoor sports such as mountain biking and hiking, and wildlife photography.

PUBLICATIONS

Onadeko AB, Rödel M-O, Liedtke HC, Barej M (2014). The rediscovery of Perret's toad, *Amietophrynus perreti* (Schiøtz, 1963) after more than 40 years, with comments on the species' phylogenetic placement and conservation status. Zoosystematics and Evolution 90(2): 113-119

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Liedtke HC, Åbjörnsson K, Harraca V, Knudsen JT, Wallin EA, Hedenström E, Ryne C (2011). Alarm pheromones and chemical communication in nymphs of the tropical bed bug *Cimex hemipterus* (Hemiptera: Cimicidae). PLoS ONE 6(3): e18156

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Svensson GP, Liedtke C, Hedenström E, Breistein P, Bång J, Larsson MC (2011). Chemical ecology and insect conservation: optimizing pheromone-based monitoring of the threatened saphroxylic click beetle *Elater ferrugineus*. Journal of Insect Conservation 16(4): 549-555