

# No ecological opportunity signal on a continental scale? Diversification and life-history evolution of African true toads (Anura: Bufonidae)

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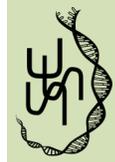
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The niche-filling process predicted by the “ecological opportunity” (EO) model is an often-invoked mechanism for generating exceptional diversity in island colonizers. Whether the same process governs lineage accumulation and trait disparity during continental colonization events is less clear. Here, we test this prediction by investigating the rate dynamics and trait evolution of one of Africa’s most widespread amphibian colonizers, the true toads (Bufonidae). By reconstructing the most complete molecular phylogeny of African Bufonidae to date, we find that the diversification of lineages in Africa best conforms to a constant rate model throughout time and across subclades, with little support for EO. Evolutionary rates of life-history traits have similarly been constant over time. However, an analysis of generalists and specialists showed a shift toward higher speciation rates associated with habitat specialization. The overall lack of EO signal can be interpreted in a number of ways and we propose several explanations. Firstly, methodological issues might preclude the detection of EO. Secondly, colonizers might not experience true EO conditions and due to the size, ecological heterogeneity and age of landmasses, the diversification processes might be more complex. Thirdly, lower speciation rates of habitat generalists may have affected overall proliferation of lineages.

**KEY WORDS:** Amphibia, BAMM, bGMYC, disparity, evolutionary rate dynamics, molecular phylogeny.



How species and species assemblages respond to a release from ecological competition is a key question in evolutionary biology (Simpson 1953; Schluter 2000; Losos 2010; Yoder et al. 2010). The colonization of islands (Robichaux et al. 1990; Grant 1999; Whittaker and Fernandez-Palacios 2007), mass extinction events (Sepkoski 1998), the availability of new resources (McKenna et al. 2009), or the evolution of key innovations (Hunter and Jernvall 1995; Jönsson et al. 2012) are classic examples of where a sudden intrinsic or extrinsic change has presented organisms with an “ecological opportunity” (EO; Simpson 1953). In response to this opportunity, lineages are predicted to rapidly diversify, unimpeded by competition until an ecological saturation point is reached inducing a slowdown in diversification (Nee et al. 1992; Rabosky 2009a). Phylogenies are a powerful tool for the inference of macroevolutionary processes (Mooers and Heard 1997) and the detection of diversity dependent lineage accumulation patterns in response to competitive release has been interpreted as a signal for EO, especially in relation to adaptive radiations (Losos and Mahler 2010). Diversifying into new niche space when presented with EO should also be reflected in the diversification and disparity of phenotypes (Simpson 1953; Schluter 2000; Harmon et al. 2003; Slater et al. 2010; Jönsson et al. 2012), especially in traits relevant to adaptation (Steelman and Danley 2003). Reconstructing the evolutionary history of phenotypes can therefore strongly complement our understanding of diversification from studying phylogenies (Mahler et al. 2010; Harmon et al. 2010; Slater et al. 2010).

Ecological opportunity is often cited as an important precondition for generating exceptional levels of biodiversity (Schluter 2000; Losos 2010), but most empirical studies on EO are focused on insular (Grant 1999; Harmon et al. 2008a; Jönsson et al. 2012), or localized mainland systems (Hughes and Eastwood 2006; Kozak and Wiens 2006; Pinto et al. 2008; Rabosky and Lovette 2008a; Price et al. 2014b; Slingsby et al. 2014). Yet, continental systems are often more diverse than their island counterparts (Whittaker and Fernandez-Palacios 2007; Pinto et al. 2008) and whether the same processes can generate bursts in biodiversity on a continent-wide scale has only recently begun to receive attention. Some of these studies have yielded support for EO as a key mechanism for producing exceptional biodiversity (Burbrink and Pyron 2009; Barker et al. 2013; Schenk et al. 2013, although not always; Price et al. 2014a), even showing multiple EO events nested across subclades (Drummond et al. 2012b; McGuire et al. 2014), while others have not detected EO signals (Derryberry et al. 2011; Claramunt et al. 2012b; Day et al. 2013; Schweizer et al. 2014; Alhajeri et al. 2015), and a general consensus on the role of ecological limits for diversification is lacking (Harmon and Harrison 2015; Rabosky and Hurlbert 2015).

In continent-wide studies of EO, detections of both rapid and early lineage and trait diversification has been attributed to the

biogeographic transition to new, underutilized areas as a result of a colonization event (Burbrink and Pyron 2009; Barker et al. 2013; Schenk et al. 2013; Price et al. 2014a). Conversely, a lack of signal has been attributed to the geographic and ecological complexity of continents, with ecological saturation unlikely to occur on such a scale (Derryberry et al. 2011; Day et al. 2013; Schweizer et al. 2014). Furthermore, the dispersal ability of ancestors that may have led to the continent-wide colonization of new habitats may in itself inhibit rapid speciation by preventing ecological isolation (Claramunt et al. 2012b). EO as a result of expansion into new geographic or ecological space may therefore be as much a driver for generating biodiversity in continent-wide clades as is the case for island or localized mainland radiations, but high dispersal ability or the magnitude of the ecological carrying capacity of continents could equally mean that the EO model is less applicable to such geographically and ecologically more complex systems. Alternatively, if indeed EO played a role in shaping diversification, these systems may be too old for early burst signals to be detectable if changes in rate over time were not drastic or if too much time has passed since rate equilibrium has been reached (Liow et al. 2010; Rabosky and Hurlbert 2015).

With 586 currently recognized species worldwide, Bufonidae is the third most species-rich family of amphibians (Frost 2016). Unlike most other amphibians, bufonids were able to colonize most parts of the world (Duellman 1999) and this species-rich and world-wide diversification across entire continents offers an excellent system for investigating how biodiversity accumulates on continents and whether early bursts in both lineage accumulation and trait disparity has occurred as a response to EO. African bufonids in particular are suitable for addressing EO, firstly, due to extreme trait disparity observed across species. Variable phenotypic traits include body size (19–163 mm; Liedtke et al. 2014), which is correlated with ecological factors and under strong selection in many systems (Davis 1938; Peters 1986). Furthermore, diverse modes of life history strategies (biphasic aquatic breeding to viviparity) are evident in African bufonids (Liedtke 2014; Liedtke et al. 2014) and components of these, such as fecundity (clutch size) and parental investment per offspring (egg size), are good indicators for adaptation to extrinsic factors (Dobzhansky 1950; Duellman and Trueb 1994; Roff 2002; Räsänen et al. 2008). A second qualification is the biogeographic transition African bufonids underwent. Both fossil and molecular evidence point to a Neotropical origin of Bufonidae (Tihen 1962; Blair 1972; Pramuk et al. 2008) at around 80–60 Ma (Pramuk et al. 2008; Van Bocxlaer et al. 2010) followed by a rapid global diversification earliest in the Late Eocene (40–30 Ma; Pramuk et al. 2008; Van Bocxlaer et al. 2010; Portik and Papenfuss 2015). By the Oligocene (~30 Ma) bufonids were established on all continents except Australia and Antarctica (Van Bocxlaer et al. 2010), neither of which host endemic bufonid lineages at present. Van Bocxlaer et al.

(2010) proposed that the evolution of an “optimal range-expansion phenotype” (robust, explosive breeders with high dispersal abilities) was crucial for their success, a phenotype they estimated as characteristic of the first lineage to colonize Africa as well. Their broad ecological tolerance may have been advantageous for allowing this group to disperse widely on the continent, but such habitat generalism may ultimately result in lower overall lineage proliferation (Claramunt et al. 2012a). Nonetheless, African bufonids display a rich array of phenotypes, reproductive modes, and habitat preferences (Tandy and Keith 1972; Clarke 2001; Liedtke et al. 2014), which raises the question whether this diversity was spurred by EO.

By assembling the largest molecular and trait dataset for African bufonids to date, including numerous candidate taxa so far not formally described, we test whether the colonization of Africa by toads has left signals characteristic of saturation dynamics as part of the EO model. Under this model we expect to find an early burst of lineage accumulation and life-history trait disparity with subsequent saturation in the form of a slowdown in rates. We also investigate whether habitat generalists have experienced different speciation rates compared to habitat specialists.

## Methods

An extended version of the methods employed can be found as Supporting Information S1.

### TAXON SAMPLING AND DNA SEQUENCING

The number of currently recognized species of African Bufonidae (101, see extended methods S1) is unlikely to be the true number of species due to the questionable taxonomic validity of some (Tandy and Keith 1972; Poynton 1997; Rödel 2000; Rödel and Branch 2003) and the large number of candidate species awaiting formal taxonomic treatment (Tandy and Keith 1972; Poynton and Broadley 1988; Tolley et al. 2010). We have therefore sampled extensively to include at least one representative of every African genus and as many geographic localities as possible per species. 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 non-African 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 provide wider phylogenetic context and to allow for the inclusion of more fossil calibration points.

DNA was extracted from preserved tissue, 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 I (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). PCR products were sequenced by Microsynth AG (Balgris, CH), complementary strands were sequenced for proofreading and all sequences were deposited in GenBank (S2).

### PHYLOGENETIC INFERENCES

Sequences were processed using Codoncode Aligner v4.4.1 (Codoncode Cooperation, MA, USA) and Geneious Pro v5.6.7 (www.geneious.com; Kearse et al. 2012). Each gene region was aligned separately with MAFFT v7.017 (Katoh and Standley 2013), and GBLOCKS (Castresana 2000) was used to remove poorly aligned, ambiguous nucleotide, and gap positions in the 12S and 16S alignments. The coding genes were realigned and translated using TranslatorX (Abascal et al. 2010), and an optimal partitioning scheme and nucleotide substitution models for a concatenated alignment were determined using PartitionFinder v1.1.1 (Lanfear et al. 2012).

Previous molecular phylogenetic inferences have not recovered African bufonid species as monophyletic (Graybeal 1997; Frost et al. 2006; Pramuk et al. 2008; Van Bocxlaer et al. 2010; Pyron and Wiens 2011; Portik and Papenfuss 2015). To gain clarity on the phylogenetic relationship of African species and to allow for geological time calibration, a “global tree” inference was conducted first. Along with African species, representatives of Eurasian and New World genera were included in this inference, but only samples for which sequence data of all five gene regions was available (with the exception of *Incilius* spp. and *Bufo* *surdus*, included for calibration purposes despite missing COI sequences). Although only 60 of the 101 currently recognized species are covered, all African genera are represented in this tree, with the exception of *Laurentophryne*, a monotypic genus from eastern Democratic Republic of the Congo that has not been sighted since its original collection (Laurent 1950), despite recent efforts (Greenbaum and Kusamba 2012; IUCN SSC Amphibian Specialist Group 2013). For the purpose of getting a more complete understanding of the diversity of African lineages, a second alignment and phylogenetic reconstruction, restricted to include only African species, was conducted, using sequence data for as many individuals as possible, even if not all five genes were available. Due to the paraphyletic nature of African bufonids (see results), this reconstruction excluded genera that were not part of the first African radiation (FAR; applies to Central African genera: *Nectophryne*, *Werneria*, and *Wolterstorffina*), because their inclusion would (a) violate a number of assumptions related to

monophyly and complete taxon sampling for downstream analyses and (b) an EO signal in diversification is not expected for subsequent colonization events where the assumption of vacant niches no longer holds true (Schenk et al. 2013). The resulting nucleotide matrix for this second inference favors taxon sampling (covering 60 of the 89 currently recognized species of the FAR clade), but at the cost of missing sequence data, fossil calibration points, and species not belonging to the FAR clade.

Joint posterior distributions of all model parameters for both trees were estimated using Bayesian Markov Chain Monte Carlo (MCMC) searches in BEAST v1.7.5 (Drummond et al. 2012a). Molecular clock models were estimated for a linked set of the mitochondrial markers and for CXCR4 and RAG1 separately using uncorrelated lognormal relaxed clock (ucl) priors (Drummond et al. 2006) and birth-death tree prior (Gernhard 2008). The global tree was calibrated to geological units of time by including four fossil node constraints: the estimated origin of the *Rhinella marina* species-group (11.8 Ma; Estes and Wassersug 1963), the most recent common ancestor of *Anaxyrus* and *Incilius* (20 Ma; Tihen 1951), the oldest unambiguously identified member of the *Bufo bufo* group (9.6 Ma; Rage and Roček 2003) and the estimated age of the *Bufo viridis* lineage (18 Ma; Martín et al. 2012). As these fossils are not contained within the FAR clade, the crown age of the FAR tree ingroup was calibrated using the crown age distribution of the FAR clade in the global tree. A total of three MCMC searches with 100 million generations and three with 50 million generations, sampling every 2000<sup>th</sup> iteration, were conducted. For chain and parameter diagnostics, an additional MCMC search on priors only was conducted, convergence and effective sample sizes (ESS) of parameters in the log files as well as prior distributions were visually inspected using Tracer, and Are We There Yet (AWTY; Wilgenbusch et al. 2004) was used to assess convergence of tree topologies. Posterior trees were resampled and combined using LogCombiner v1.7.5 (Rambaut and Drummond 2012a) and summarized as a maximum clade credibility (MCC) tree using TreeAnnotator v1.7.5 (Rambaut and Drummond 2012b).

## SPECIES DISCOVERY

Two pruning methods were employed for deriving a tree with single representative tips per species. First, the FAR phylogeny was pruned to include only a single representative per currently recognized species (CRS; based on Frost 2016). However, 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 currently recognized species is therefore not a true representation of the phylogenetic diversity of these bufonids and to objectively obtain a tree that includes undescribed, but distinct, taxa, the Bayesian implementation of the General Mixed Yule-Coalescent model (bGMYC; Pons

et al. 2006; Reid and Carstens 2012) was used to identify suitable delimitation points on the chronogram to generate a second tree. Using the bGMYC package v1.0.2 (Reid 2014) in R (R core team 2013), the algorithm was run for 1 million MCMC iterations, sampling every 10,000<sup>th</sup> iteration after an initial 10,000 repetition burn-in. Point estimates for species limits were derived using a 0.01 posterior probability cutoff threshold and the FAR MCC tree as well as a random subset of 1000 posterior trees were pruned to include only a single representative terminal per delimited element. This pruning collapsed all divergences younger than 1.508 Ma resulting in an artificial flat-lining of diversification. As this may not be biologically meaningful, all analyses were repeated on the bGMYC tree with terminal branches truncated by 1.508 Myr (Fig. S1.1E and S7). The results did not differ substantially to those when using the bGMYC tree and are thus not discussed further.

## LINEAGE DIVERSIFICATION

Temporal and topological lineage diversification rate dynamics in the FAR clade (using both the bGMYC and the CRS tree) were investigated in order to detect an early burst followed by a slowdown in rate over time. The  $\gamma$  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 using the ape package v3.2 (Paradis et al. 2004). To account for missing taxa in the CRS tree, we employed a Monte Carlo Constant Rate (MCCR) test using the laser package v 2.4-1 (Rabosky 2006).

Using a likelihood approach, we then compared two constant rate models (a pure-birth [PB] and birth-death [BD] model with constant rates), to four variable rate models (PB with an exponential speciation rate [PB $\lambda$ exp], BD with a constant speciation rate and exponential extinction rates [BD $\lambda$ cst- $\mu$ exp], BD with an exponential speciation rate and constant extinction rate [BD $\lambda$ exp- $\mu$ cst] and BD with both exponential speciation and extinction rates [BD $\lambda$ exp- $\mu$ exp]), using the fit\_bd function of the RPANDA package v1.1 (Morlon et al. 2011, 2016). Model-fit was compared using Akaike Information Criterion adjusted for small sample sizes (AICc) and Akaike weights (Aw).

Bayesian Analysis of Macroevolutionary Mixtures software (BAMM; Rabosky 2014) in combination with the R package BAMMtools v2.0 (Rabosky et al. 2014) was used to test whether subclades diversify under distinct rate regimes. BAMM was allowed to sample every 1000<sup>th</sup> generation of 5 million MCMC iterations, priors were configured based on the setBAMMprior function in BAMMtools. The analysis using the bGMYC tree assumed complete sampling (see extended methods; S1), whereas the analysis using the CRS tree was supplied with sampling proportion information for each genus. For each analysis, four independent runs were executed to check for convergence of the

posterior probability densities, and Bayes factors were calculated to compare the relative support of one rate regime model over another.

### LIFE HISTORY DIVERSIFICATION

To explore how life-history characters diversified over time, the rates of evolution and disparity of body, clutch, and egg size were investigated. Mature female body size (snout-vent length in mm), clutch size (number of eggs per clutch), and egg size (diameter of eggs in mm) were log<sub>10</sub> transformed to better conform to normality and size-free residuals were subsequently calculated for clutch and egg size data by regressing traits on body size using phylogenetic-generalized least squares (pGLS) regressions. Body, clutch, and egg size measurements were taken from Liedtke et al. (2014) and references therein, with new measurements for *Churamiti maridadi*. Traits were mapped on the CRS tree, pruning terminal branches (species) for which traits are unknown, resulting in a dataset of 60, 46, and 42 species for body, clutch, and egg size, respectively.

A likelihood approach was used to compare the fit of a series of six evolutionary models to the continuous trait data. Three constant rate models (Brownian motion [BM; Felsenstein 1973], Ornstein-Uhlenbeck [OU; Butler and King 2004], and Pagel's lambda [ $\lambda$ ; Pagel 1999]) were compared to three variable rate models (early-burst [EB; exponential rate change through time; Harmon et al. 2010], linear [LIN; linear rate change through time], and Pagel's time-dependent delta [ $\delta$ ; Pagel 1999] model). Models were fitted using the fitContinuous function of the geiger package v2.0 (Harmon et al. 2008b) and comparisons were based on AIC and  $A_w$ . We further investigated the temporal rate dynamics and rate heterogeneity of trait evolution using BAMM. For each trait, the rate shift configuration with the highest posterior probability was determined and the rate profile through time of the phenotypic rate parameter  $\beta$  was plotted. In addition, the disparity of traits within and between sub clades were investigated using the dtt function in the geiger package, and by calculating the morphological disparity index (MDI; Harmon et al. 2003) to test for deviation from a Brownian motion model.

### SPECIATION RATES OF HABITAT GENERALISTS VERSUS SPECIALISTS

Species were scored as being either habitat generalists or habitat specialists based on the habitat description provided by the IUCN red list database and the authors' first-hand experience (www.iucnredlist.org; Table S1.3; Fig. 7). Our evaluation of habitat comprised of a two-step process: (1) Constructing Table S1.3 based on IUCN data and checking for anomalies and/or mistakes. All authors participated in this process and have considerable experience working in the field observing/collecting African amphibians, and (2) Filtering data according to evaluation from

experts and grouping according to being generalist (more than two distinct habitat types) or specialist (two or less habitat types). Certain habitat types that could be considered synonymous were grouped (see S1). The Binary State Speciation and Extinction (BiSSE) model implemented in the diversitree v.0.9-6 package (FitzJohn 2012) was used to examine whether shifts in habitat specialization are associated with shifts in speciation rate using a maximum likelihood (ML) and Bayesian approach (10,000 iterations with 1000 iterations discarded as burn-in). The analysis run with the CRS tree included sampling fraction information to correct for biased undersampling (72% of habitat generalists, 66% of habitat specialist sampled) and the bGMYC tree, coding undescribed species based on sampling locality and habitat preferences of their closest relative. This method is known to be problematic when the number of terminals are low or when character ratios are biased (Davis et al. 2013; Rabosky and Goldberg 2015), and so simulation tests were performed (following Onstein et al. 2015) to ensure that there was sufficient power in the data (see S1).

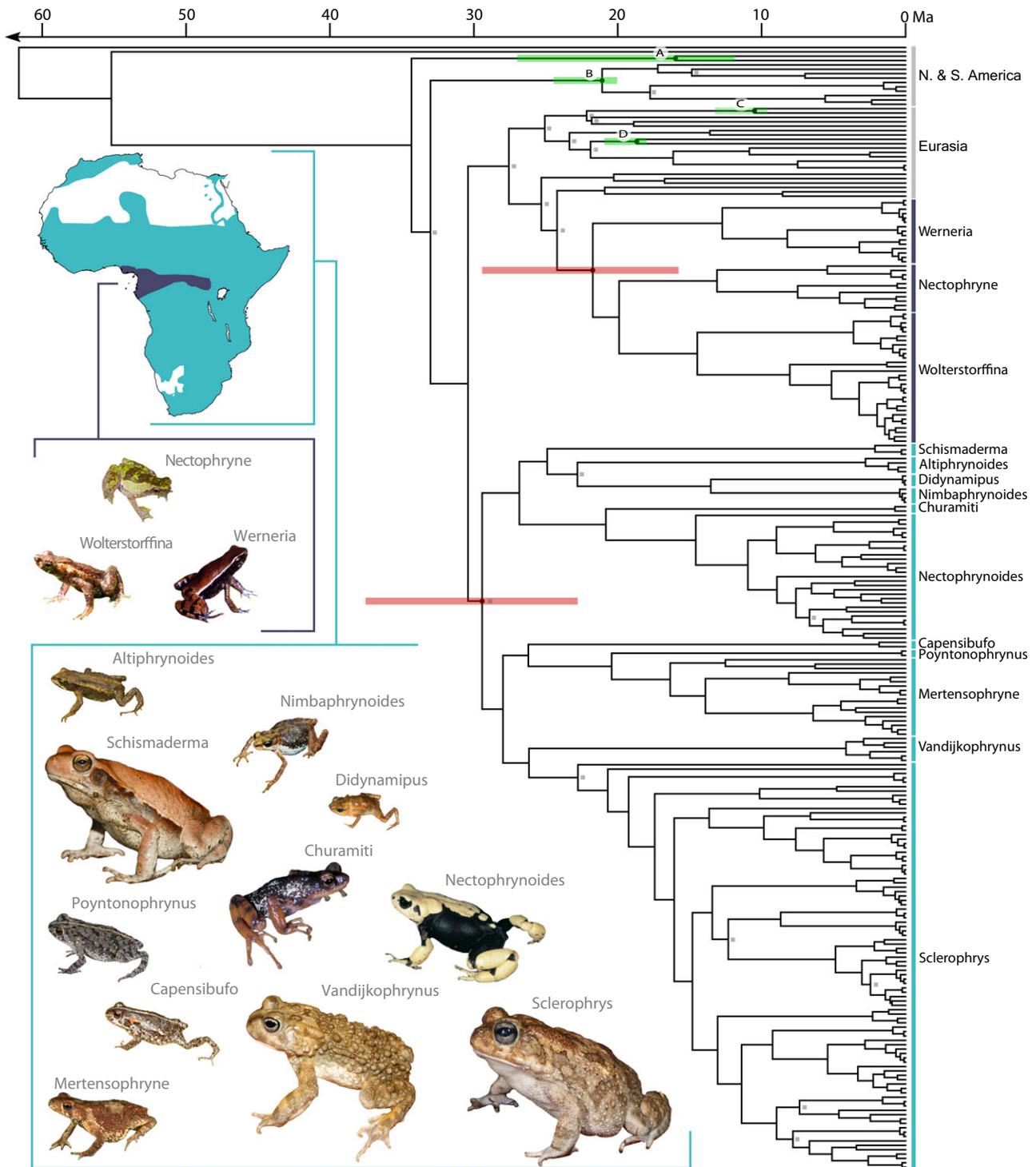
## Results

### PHYLOGENETIC INFERENCE

The global tree (Fig. 1; S3) supports previous claims that African bufonids are paraphyletic (Pramuk et al. 2008; Van Bocxlear et al. 2010; Portik and Papenfuss 2015), here recovering two independent colonization events into Africa. Most relationships of Eurasian groups are poorly resolved, but for both African radiations, internal nodes are generally well supported (posterior probabilities >0.9). The global tree reconstruction dates the origin of the Old World radiation at 30.4 Ma (95% Highest Posterior Density interval; HPD = 23.2–38.5), with the two colonization events into Africa occurring shortly after, at 29.4 Ma (95% HPD = 22.8–37.5) and 21.7 Ma (95% HPD = 15.8–29.4), respectively. An unexpectedly high degree of genetic divergences, especially within *Nectophryne*, *Wolterstorffina*, *Nectophrynoidea*, *Mertensophryne*, and in the *Sclerophrys gracilipes-kisoloensis-villiersi* complex were recovered, highlighting the need for taxonomic revisions of these groups. All major relationships were congruent in the global tree and the FAR tree (S3 and S4). When pruning the FAR tree to only include a single representative of each currently recognized species (CRS tree; S5), 60 out of the 89 known species are represented with the missing 29 belonging to the following genera: *Sclerophrys*–15 (out of 38), *Mertensophryne*–6 (out of 11), *Nectophrynoidea*–2 (out of 15), and *Poyntonophrynus*–6 (out of 10).

### SPECIES DISCOVERY

The bGMYC species discovery with a posterior probability threshold of 0.01 recovered 102 delimited entities (S4 and S6).



**Figure 1.** 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 below posterior probabilities of 0.9 are indicated by gray squares and node bars show the 95% highest posterior density of divergence times for key nodes; the origin of the two African clades (red) and the fossil calibration points (green), (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 color-coded blue and the second African radiation (SAR) is color-coded purple, with the insert depicting the geographic distribution of these two clades and representative photo vouchers per genus to highlight their morphological diversity (sizes approximately to relative scale).

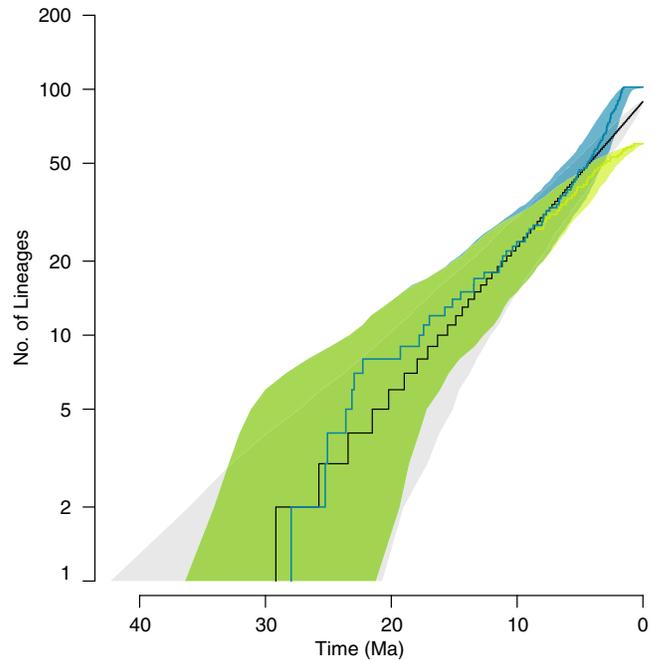
Almost all currently recognized species of this clade were delimited consistently, with the exception of three species pairs: *Mertensophryne howelli* and *M. usambarae*, *Poyntonophrynus damaranus*, and *P. dombensis* and *Sclerophrys pardalis* and *S. pantherinus*, which were not identified as distinct entities. This echoes previous difficulties in discerning the species status of at least the latter species pair (Poynton and Lambiris 1998; Measey and Tolley 2009). In addition, units phylogenetically distinct from currently recognized species were recovered in the following genera: *Schismaderma*–2, *Nectophrynooides*–13, *Capensibufo*–4, *Mertensophryne*–8, and *Sclerophrys*–18. Cryptic diversity has previously been recognized (Poynton and Broadley 1988; Tolley et al. 2010), and qualitative and quantitative assessments (acoustic calls, distribution, genetics, and morphology) of the entities recovered suggest that overall, delimited elements are likely to represent valid species (e.g., Poynton et al. 2016). We investigated the degree to which potential oversplitting would affect our results by tracing the erosion of Pybus and Harvey’s  $\gamma$  as delimited units are sequentially dropped from the bGMYC tree to approach the CRS tree (S8). From this, we can conclude that only if ~58% or more of the delimited units are not true species, does  $\gamma$  deviate significantly from the below reported results. It is therefore unlikely that overestimation of cryptic species numbers is impacting the diversification results.

**LINEAGE DIVERSIFICATION**

Lineage through time plots for the bGMYC tree (102 terminals), the CRS tree (60 terminals) were compared to a simulated set of 1000 pure-birth trees based on the total number of currently recognized species (89 terminals; Fig. 2). For the bGMYC tree, a negative  $\gamma$  was recovered, but the relative distribution of splitting events was not significantly different from the null hypothesis of constant rates through time ( $\gamma_{MCC} = -1.061$ ,  $p_{MCC} = 0.144$ ; mean  $\pm$  SD  $\gamma_{posterior} = -0.951 \pm 0.537$ ,  $P = 0.171$ ). Similarly, the observed  $\gamma$  recovered for the CRS tree was negative ( $\gamma_{MCC} = -1.834$ ; mean  $\pm$  SD  $\gamma_{posterior} = -1.826 \pm 0.447$ ), but again was not significantly different from the null distribution (MCCR test 5% critical value =  $-2.449$ ;  $P_{MCC} = 0.158$ ;  $P_{posterior} = 0.153$ ).

For both trees (bGMYC and CRS tree), the best-fitting models to describe lineage diversification were constant rate, pure-birth models (Table 1). For the bGMYC tree, this model was a considerably better fit than any variable rate model ( $Aw = 0.501$ ;  $\Delta AICc > 2.069$ ; Table 1), but for the CRS tree, support for constant diversification over variable rate models was less substantial ( $Aw = 0.452$ ;  $\Delta AICc > 1.460$ ; Table 1).

For both the bGMYC and CRS tree, BAMM found strong support for rate homogeneity. A model with a single evolutionary rate regime had the highest posterior probability (PP = 0.650 and 0.630 for the two trees, respectively; Fig. 3A) with posterior odds ratios of 2.390 (bGMYC) and 2.234 (CRS) and Bayes factor



**Figure 2.** Lineage through time plots for the bGMYC tree (blue) the CRS tree (green) and the median of 1000 Yule simulations for a tree with 89 taxa (gray/black). Shadings mark the 95% quantiles of posterior or simulated trees.

scores of 1.624 (bGMYC) and 1.519 (CRS) over the next best models, which in both cases were two-rate regime models (i.e., one rate shift). Support diminished with complexity of the models (Fig. 3A). BAMM estimated extinction rates to be more or less constant (and low) over time for both the bGMYC and CRS tree, with a slight increase in extinction rates in recent history in the CRS tree (Fig. 3B). The CRS tree showed a marginal decline in speciation rate over time, whereas the bGMYC tree showed an initial increase followed by a flattening out of the curve over time (Fig. 3B).

**LIFE-HISTORY DIVERSIFICATION**

Likelihood model fitting for rates of body, clutch, and egg size evolution, consistently recovered constant rate models outperforming variable rate models (Table 2). For body and egg size, Pagel’s  $\lambda$  model performed best, but only for body size was this a noticeably better fit than the next best model (body size  $Aw_{\lambda} = 0.950$  over  $Aw_{OU} = 0.019$ ; egg size  $Aw_{\lambda} = 0.280$  over  $Aw_{OU} = 0.226$ ; Table 2). For clutch size, a Brownian motion model was the best fit ( $Aw_{BM} = 0.328$  over the next best  $Aw_{\lambda} = 0.171$ ). Despite marginal differences in top model performances, the early burst model was consistently ranked lowest for all three traits (body size  $Aw_{EB} = 0.003$ ; clutch size  $Aw_{EB} = 0.121$ ; egg size  $Aw_{EB} = 0.042$ ). BAMM also recovered single-rate regimes as the best shift configurations for body, clutch, and egg size although differences

**Table 1.** Model fit comparison for diversification models fitted to the branching times of the (A) bGMYC tree and (B) CRS tree.

Model	$\lambda$ Parameters	$\mu$ Parameters	LH	AICc	$\Delta$ AICc	Aw
<b>A) bGMYC tree</b>						
<i>Constant rate models</i>						
PB	0.137		−298.691	599.421	–	0.501
BD	0.137	<0.001	−298.691	601.503	2.081	0.177
<i>Variable rate models</i>						
PB $\lambda$ .exp	0.136; 0.002		−298.685	601.491	2.069	0.178
BD $\lambda$ .cst- $\mu$ .exp	0.137	<0.001; −0.218	−298.691	603.626	4.205	0.061
BD $\lambda$ .exp- $\mu$ .cst	0.136; 0.002	<0.001	−298.685	603.614	4.193	0.062
BD $\lambda$ .exp- $\mu$ .exp	0.135; 0.002	<0.001; −0.056	−298.685	605.782	6.360	0.021
<b>B) CRS tree</b>						
<i>Constant rate models</i>						
PB	0.121		−191.348	384.765	–	0.452
BD	0.121	<0.001	−191.348	386.907	2.142	0.155
<i>Variable rate models</i>						
PB $\lambda$ .exp	0.106; 0.015		−191.008	386.226	1.460	0.218
BD $\lambda$ .cst- $\mu$ .exp	0.146	378.188; −2225.174	−190.891	388.210	3.445	0.081
BD $\lambda$ .exp- $\mu$ .cst	0.106; 0.015	<0.001	−191.008	388.444	3.679	0.072
BD $\lambda$ .exp- $\mu$ .exp	0.106; 0.015	<0.001; 0.005	−191.008	390.743	5.977	0.023

The models tested are pure-birth (PB) and birth-death (BD) with constant rates, PB with an exponential speciation rate (PB $\lambda$ .exp), BD with a constant speciation rate and exponential extinction rates (BD $\lambda$ .cst- $\mu$ .exp), BD with an exponential speciation rate and constant extinction rate (BD $\lambda$ .exp- $\mu$ .cst) and BD with both exponential speciation and extinction rates (BD $\lambda$ .exp- $\mu$ .exp). Parameters refer to the estimated rates at the tips and the corresponding time-variation parameter.

from more complex regime models were marginal (posterior probabilities: 0.38; 0.53; 0.50, Bayes factors: 1.29; 0.84; 0.84, respectively). In line with the likelihood model fitting, all three characters show relatively constant rates over time, with the arguable exception of an initial increase in clutch size (Fig. 4).

The trait disparity analysis corroborates homogeneity in trait evolution. The average subclade disparities of all three investigated life-history traits did not significantly deviate from expectation under Brownian motion (MDI scores of −0.022, −0.095, and 0.077 for body, clutch, and egg size respectively), but clutch size, and to some degree body size, show a more defined drop in subclade disparity early on in the history of bufonids than expected (Fig. 5). The disparity plots for body size and egg size indicate peaks during the last 5 million years, where disparity is greater than expected under a BM model, which may be an artifact of undersampling recent nodes (Harmon et al. 2003) and unlikely to be a biological signal (see similar pattern in: Burbink and Pyron 2009; Slater et al. 2010; Derryberry et al. 2011; Rowe et al. 2011).

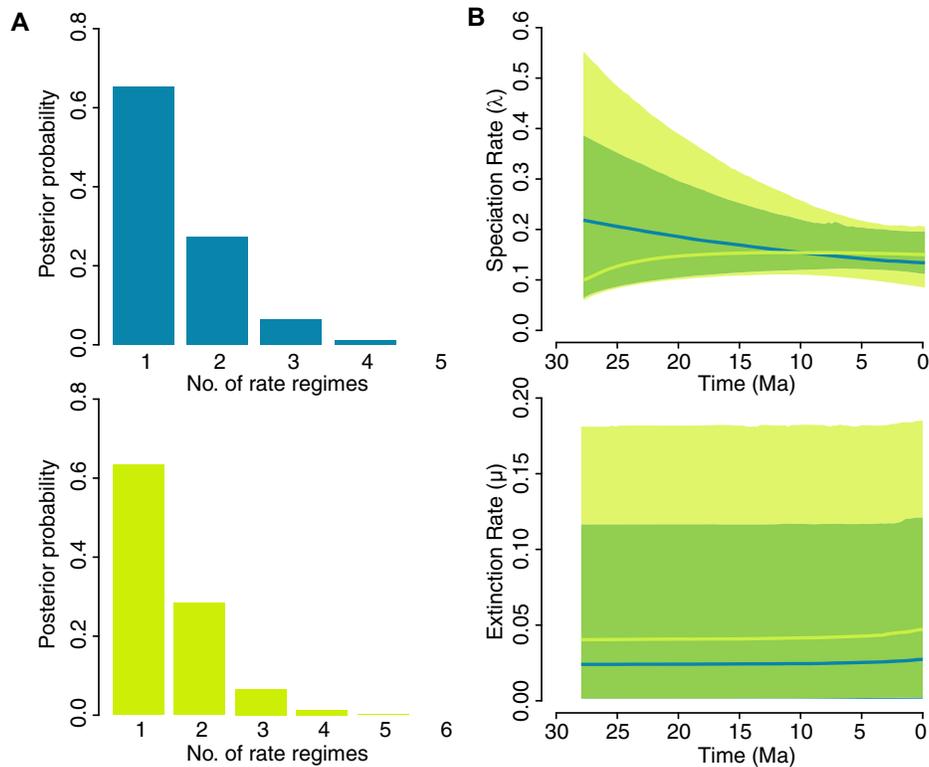
#### SPECIATION RATES OF HABITAT GENERALISTS VERSUS SPECIALISTS

Based on our coding, habitat specialists are more common than habitat generalists (bGMYC tree: 73%, CRS tree: 83%), but habitat generalists widely dispersed across the tree except in the *Nectophrynoides* subclade. Both the ML and Bayesian approaches in BiSSE suggested a shift toward higher speciation rates

associated with shifts from habitat generalists to habitat specialists (Fig. 6). The ML approach recovers the shift in speciation rates as significant when using the CRS tree (and incorporating sampling fraction information), but not when using the bGMYC tree (CRS tree:  $\chi^2 = 4.779$ ,  $P = 0.029$ ; bGMYC tree:  $\chi^2 = 0.508$ ,  $P = 0.576$ ). Transitions from generalist to specialists (q01) were higher than vice versa (q10) for both the bGMYC (medians: q01 = 0.060; q10 = 0.019) and the CRS tree (medians: q01 = 0.113; q10 = 0.074).

#### Discussion

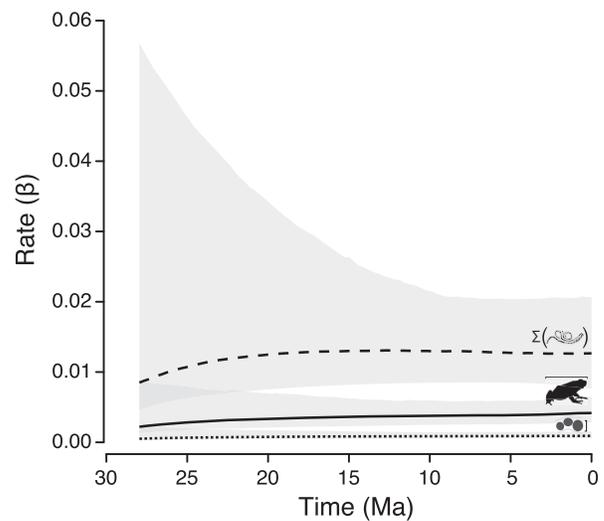
According to the EO model, expansion into new geographic areas should lead to a rapid diversification both in lineages and in phenotypic traits. Once niches become saturated in the newly colonized areas, rates should decrease in a diversity-dependent manner. Studies testing the EO model have predominantly focused on young lineages restricted to small, isolated areas such as islands, archipelagos, or insular mainland systems. Whether the same niche filling principles can lead to bursts in biodiversity in continent-wide systems, or if such burst and subsequent saturation dynamics can even be detected is less clear. By investigating the diversification history of one of Africa's most species-rich amphibian colonizers, we tested whether signals characteristic of the EO model can be recovered for this geographically and ecologically more complex, continental system.



**Figure 3.** Diversification dynamics for the bGMYC (blue) and the CRS tree (lime green) using the BAMM software package. (A) Posterior distribution of regimes with different numbers of rate processes (including the root process). (B) Speciation and extinction rates through time, where shaded areas denote the 95% quantiles on the posterior distribution of the rates.

**LINEAGE AND TRAIT DIVERSIFICATION OF AFRICAN BUFONIDS**

Key for accurately estimating diversification rates is the thorough sampling of species (Nee et al. 1994; Cusimano and Renner 2010; Brock et al. 2011), which is difficult when dealing with a geographically expansive radiation. Our extensive sampling and analyses of bufonids have revealed a sizable number of undescribed species, up to 45 phylogenetically delimited units. These undescribed, mostly cryptic lineages represent recent, species-level divergences and their exclusion from diversification analyses creates an erroneous overestimation of early divergences relative to recent ones (Figs. 2, 3B; Cusimano and Renner 2010). Critically, the inclusion of this cryptic diversity shows more apparently that the lineage accumulation curve of African toads does not significantly differ from a simulated constant rate curve (Pybus and Harvey’s  $\gamma$  close to zero; Fig. 2) and that the diversification rate is best described by a constant, pure-birth process (slightly outperforming a variable pure-birth process with a marginally exponentially decreasing speciation rate over time; Table 1). Furthermore, there are no significant rate regime shifts between subclades and thus, our data suggest that the first wave of African bufonids as a whole, or any subclades therein, have not experienced a period of rapid lineage expansion followed by a subsequent slowdown as expected under an EO mode (Fig. 3).



**Figure 4.** Rate dynamics (beta) through time for body size (full line) and size-free clutch (long dashes) and egg (short dashes) size. Shaded areas denote the 95% quantiles on the posterior distribution of rates.

An early burst in diversification under the EO scenario would indicate the rapid filling of available niches through adaptation and speciation. Analyses of trait evolution should reflect this in the form of early partitioning of traits (Simpson 1953; Mahler

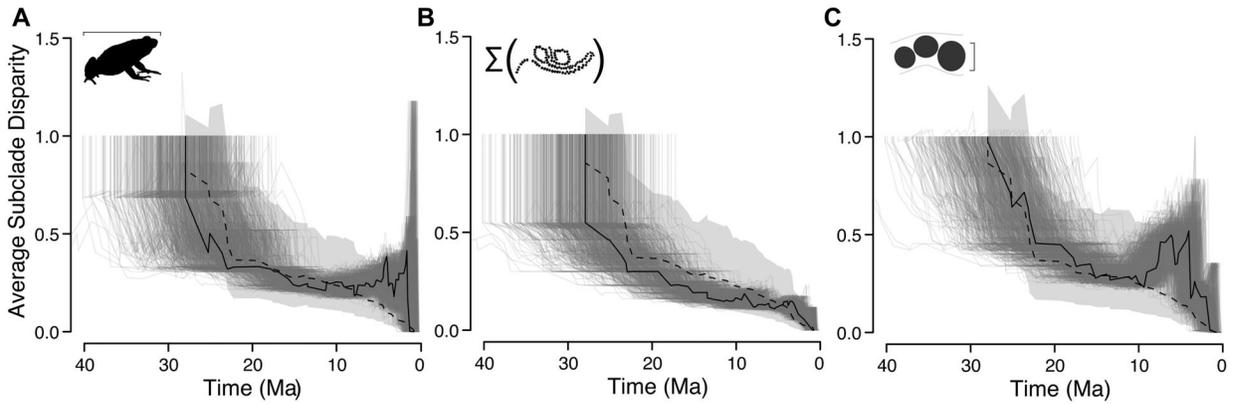
**Table 2.** Model fit comparison for evolutionary dynamics of life-history traits (A) body size, (B) size-corrected clutch size, and (C) size-corrected egg size.

Model	Rate	Parameters	lnL	AIC	$\Delta$ AIC	AW
<i>a) Body size</i>						
BM	Constant	$z0 = 1.692$	13.392	-22.785	9.666	0.008
OU	Constant	$z0 = 1.697; \alpha = 0.034$	15.339	-24.677	7.774	0.019
$\lambda$	Constant	$z0 = 1.689; \lambda = 0.808$	19.226	-32.451	-	0.950
EB	Variable	$z0 = 1.692; a = -1e-6$	13.392	-20.785	11.666	0.003
LIN	Variable	$z0 = 1.698; b = 0.217$	14.745	-23.490	8.961	0.011
$\delta$	Variable	$z0 = 1.702; \delta = 1.849$	14.635	-23.271	9.180	0.010
<i>b) Clutch size</i>						
BM	Constant	$z0 = 5.160e-11$	-18.984	41.969	-	0.328
OU	Constant	$z0 = 0.005; \alpha = 0.007$	-18.908	43.817	1.848	0.130
$\lambda$	Constant	$z0 = -0.001; \lambda = 0.965$	-18.635	43.270	1.301	0.171
EB	Variable	$z0 = -3.169e-7; a = -1e-6$	-18.984	43.969	2.000	0.121
LIN	Variable	$z0 = 0.004; b = 0.015$	-18.92	43.840	1.871	0.129
$\delta$	Variable	$z0 = 0.003; \delta = 1.079$	-18.97	43.940	1.971	0.122
<i>c) Egg size</i>						
BM	Constant	$z0 = -1.707e-11$	37.909	-71.818	1.785	0.115
OU	Constant	$z0 = -0.013; \alpha = 0.041$	39.588	-73.175	0.428	0.226
$\lambda$	Constant	$z0 = -0.001; \lambda = 0.8$	39.801	-73.603	-	0.280
EB	Variable	$z0 = -1.180e-7; a = -1e-6$	37.909	-69.818	3.785	0.042
LIN	Variable	$z0 = -0.013; b = 98.913$	39.271	-72.542	1.061	0.165
$\delta$	Variable	$z0 = -0.016; \delta = 2.254$	39.31	-72.620	0.983	0.171

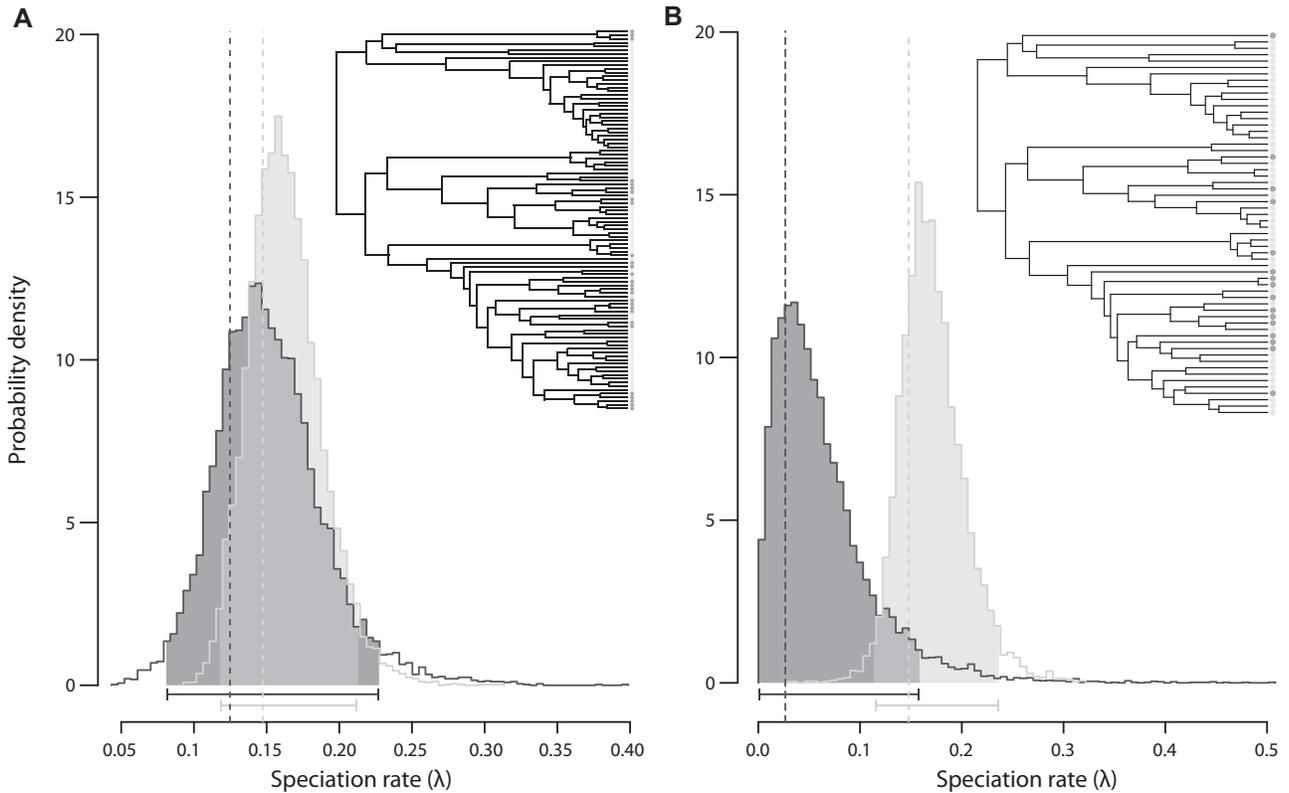
The models tested are Brownian motion (BM), Ornstein-Uhlenbeck (OU), Pagel's lambda ( $\lambda$ ), early burst (EB), linear variable rate (LIN), and Pagel's delta ( $\delta$ ). Parameters refer to trait evolution rate estimates at the root ( $z0$ ), attraction strength of the OU model ( $\alpha$ ), Pagel's branch length transformation ( $\lambda$ ), rate change parameter for the EB model (a), slope parameter for the LIN model (b), and Pagel's delta ( $\delta$ ).

et al. 2010), which has even been suggested to be a more reliable signal for EO than lineage diversification (e.g., Slater et al. 2010; Schweizer et al. 2014; but see Harmon et al. 2010). In African bufonids we find little deviation from constant rates and subclade disparity through time in the evolution of life history traits (Table 2; Figs. 4 and 5). At most, clutch size is partitioned more rapidly than expected, indicating that a division between explosive and low-fecundity breeders likely occurred early on in the history of African toads. However it should be noted that here too, deviation in trait evolution from expectations under Brownian motion is not substantial. This is mirrored in the rate estimates over time that show a steeper increase in rates closer to the root of the tree, but the overall rate change is minimal. Despite Simpson's predictions (Simpson 1953; shown also in more recent comparative studies, e.g., Rabosky et al. 2013), trait and lineage diversification need not always be coupled (Kozak et al. 2006; Ruta et al. 2013) and whether the constant lineage and trait diversification rates are correlated in bufonids requires further testing. Nonetheless, the combined molecular and trait evidence from both analyses provides stronger support for gradual evolutionary patterns on the continent of Africa—not an intuitively clear outcome from previous studies and predictions (e.g., Van Bocxlaer et al. 2010).

High dispersal ability can facilitate geographic expansions while maintaining gene flow among populations and thereby inhibit speciation (Claramunt et al. 2012a). Given the high-dispersal nature of many bufonids (Van Bocxlaer et al. 2010), we predicted that the colonization of Africa by toads need not result in the proliferation of ecological specialists, but instead can result in a lower number of generalists. Based on our coding, the majority of species (at least 73%) show narrow habitat preferences and such specialists have experienced (albeit marginally) higher speciation rates than their generalist counterparts (Fig. 6). This pattern is frequently observed in other groups too (Hernández and Vrba 2005 and references therein). Moreover, the BiSSE transition rate estimates suggest that specialist bufonids are most frequently derived from generalist ancestors. We note that such analyses are strongly subjected to the coding of traits, and determining whether species are indeed habitat specialists is not trivial and our analysis provides only the first assessment of this. Nonetheless, the BAMM analysis places the highest probability for rate shifts to have occurred along the basal branches of *Nectophrynoides* (data not shown), a highly specialized genus restricted to montane environments (Müller and Liedtke et al. 2013) and the largest subclade with no habitat generalists.



**Figure 5.** Disparity through time (DTT) plots for (A) body size and (B) size-corrected clutch size and (C) size-corrected egg size. Solid lines represent the observed DTT using the MCC tree and gray lines are the observed values for a subsample of 1000 postburnin posterior trees. Dashed lines represent the median DTT under a Brownian motion model simulation with 95% confidence intervals as the light gray polygon.



**Figure 6.** Probability density plots of posterior distribution of speciation rates associated with shifts from habitat generalists (dark) to specialists (light), estimated using MCMC-BiSSE using the (A) bGMYC tree and (B) CRS tree. Dashed lines are maximum likelihood rate estimates and inserted phylogenies depict the coding of tip states.

Because generalist lineages have persisted in most other subclades of African bufonids they may have contributed to a buffering of the explosive early niche-filling that is central to the EO model. However, we caution against an over interpretation of

this finding, as differences in speciation rates between generalists and specialists were marginal, and the overall number of generalist species was comparatively low. Nonetheless, a similar scenario has been proposed for furnariid birds of South America

(Claramunt et al. 2012a,b) and the exceptional ability of some organisms to colonize large areas may ultimately inhibit their lineage proliferation.

### **IS THE EO MODEL A GOOD FRAMEWORK FOR EXPLAINING DIVERSIFICATION IN COLONIZERS OF CONTINENTAL SYSTEMS?**

A growing number of studies on a diverse range of vertebrates have failed to find evidence for diversity-dependent rate curves (Neotropical birds: Derryberry et al. 2011; Claramunt et al. 2012b; Schweizer et al. 2014; African catfish: Day et al. 2013; murid rodents: Schenk et al. 2013). A trend is seemingly emerging that EO, frequently considered a precursor for generating diversity in insular systems (Arbogast et al. 2006; Hughes and Eastwood 2006; Harmon et al. 2008a; Jønsson et al. 2012; but see Esselstyn et al. 2009; Losos and Mahler 2010) may not always be an appropriate model for explaining diversification patterns in continent-wide radiations. Here, we discuss, using our data on toads, possible reasons why constant diversification rates may indeed be more common for continental systems and why some studies continue to find EO signals. Reasons why EO might simply not be detectable are discussed in the ensuing sections.

#### *Tabula rasa?*

Most African amphibian families are endemic and relatively old (Andreone et al. 2008), with 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; Blackburn et al. 2015; Gardner and Rage 2016) data suggesting that there is a long history of amphibian assemblages on the continent. Bufonids were late arrivals on the continent of Africa and may not have been presented with the opportunity of “vacant” niches. Furthermore, competition for resources need not be restricted to interactions only with other amphibians; macroinvertebrates for example, directly compete for resources with tadpoles (Morin et al. 1988). Schenk et al. (2013), comparing successive colonization events in rodents, have shown that indeed, secondary lineages, on average, diversify to a lesser extent regardless of whether or not primary colonizers exhibit early burst diversification patterns. In our system too, the second African toad radiation (although not treated in detail here) appears to be much less diverse than the first, despite comparable clade ages (12, relative to 89 recognized species resulting in 0.553 compared to 3.027 species per million years respectively, when dividing number of extant species by clade age). The concept of “vacant” niches may therefore be unrealistic for continental systems that tend to have much older histories of supporting life than unbalanced assemblages in insular systems. Only in cases where a specific “ecomorph” is underrepresented may colonizers

continue to be truly presented with EO on continental systems (e.g., Burbrink and Pyron 2009).

#### *Scale and complexity*

The ecological limits for diversity may not easily be reached if an area is large (Kisel et al. 2011) and dispersal ability of organisms is high (Fritz et al. 2011). Continents tend to be larger than insular systems, and high dispersal ability would be important for successful, continent-wide colonization. With an area of approximately 30 million km<sup>2</sup>, the potential carrying capacity of Africa dictated by the species-area relationship alone (MacArthur and Wilson 1967; Lomolino 2000) is exceedingly high and it seems plausible that a saturation point of resources, causing a diversity-dependent decline in diversification, may never be reached by toads, or at least has not been reached yet, as has been speculated for African catfish (Day et al. 2013).

The dynamic geographic changes that impact the history of life are likely to produce highly complex conditions for diversification of lineages under the assumptions of an EO model. For example, the successive rising of new islands in Southeast Asia is thought to have produced repeated EO for shrews to diversify, sustaining a constant diversification rate, instead of reaching the expected diversity-dependent slowdown (Esselstyn et al. 2009). Since the Oligocene, Africa has experienced a fluctuating climate (Richards 1973; Flenley 1979; Livingstone 1993; Parmentier et al. 2007) and one can imagine that the resulting expansion and contraction of habitats and species ranges (Nakazawa and Peterson 2015) could equally have resulted in a pattern of repeated regional opportunity, concealing any singular continent-wide EO signal. Geographic range expansions into the Andes for example has been attributed to promoting repeated bursts of diversification within continent-wide radiations of legumes (Drummond et al. 2012b) and hummingbirds (McGuire et al. 2014). In line with the notion of continuous opportunity, the slow decline of competing lineages (as seen in Quental and Marshall 2013) may have similarly presented bufonids with gradual niche-filling opportunities over its entire history, not just immediately after its colonization of Africa.

#### *Broad habitat tolerance limits EO diversification*

Habitat generalists are likely to be less hindered by ecological barriers to dispersal, which is an important trait for successful long distance dispersal and colonization (Baur and Bengtsson 1987; Van Bocxlaer et al. 2010; Dennis et al. 2012). This dispersal ability of habitat generalists would however, potentially limit genetic isolation caused by geographic fragmentation through fluctuating ecological conditions or geographic barriers, resulting in lower diversification than in specialists (Price and Wagner 2004; Phillimore et al. 2006; Von Rintelen et al. 2010; Claramunt et al. 2012a; Salisbury et al. 2012; but see Moyle et al. 2009). Therefore,

an alternative to an explosive, niche-filling diversification history resulting in large numbers of habitat specialists as predicted by the EO model may be a diversification history dominated by less explosive, habitat generalist lineages.

### Caveats

Based on rates of evolution in traits and lineages our data suggest bufonids did not experience an early burst followed by a diversity dependent slowdown. Simpson (1953) pointed out that opportunity alone may not be sufficient to promote invasion of new ecological space. As Simpson outlined, if an evolutionary lineage is constrained or unable to “take advantage” of evolutionary opportunities (Simpson 1953; Schluter 2000) some radiations may simply fail to be explosive if the necessary traits do not exist/evolve (Steelman and Danley 2003; Losos 2010; Yoder et al. 2010). Studies on the selective pressures acting on toads and competition with other species are required to better evaluate potential constraints on EO, and whether such factors form the basis for explaining non-EO evolutionary patterns.

Alternatively, EO may indeed have been presented to bufonids upon colonizing Africa, but we have failed to detect it. Diversification rate estimates require a number of assumptions concerning estimates of speciation and extinction, which question the veracity of interpreting diversification patterns outlined in this study. For example, high rates of extinction can erode signals of early bursts in phylogenies (Rabosky and Lovette 2008b). Although we included models that fit varying extinction rates through time, estimating this parameter from molecular phylogenies is problematic (Rabosky 2009b) and both  $\gamma$  and the MCCR test are known to be conservative with respect to extinction, and produce high type II errors (Pybus and Harvey 2000; Brock et al. 2011). If extinction rates were indeed low (as estimated by our analyses), but sufficient time has elapsed since the equilibrium diversity has been reached, traces of initial diversity-dependent lineage accumulation may again be lost (Liow et al. 2010; Rabosky and Hurlbert 2015). With a limited fossil record for African bufonids, direct evidence for estimating extinction rates is lacking, but Raven and Axelrod (1974) suggested that angiosperms in Africa have experienced high extinction rates during the Tertiary and Quaternary, a history that if shaped by climate, might have been similar for amphibians.

### Conclusion

Bufonids are renowned as one of the few amphibian radiations that have achieved a near global distribution, with peaks in diversification rates during dispersal periods to new continents facilitated by the evolution of the “toad-like” phenotype. Yet despite impressive present-day diversity, upon arriving in Africa, lineage and trait diversification rates appear to have been constant

over time, showing no early burst signal as might be expected under an EO model. Based on the findings presented here and recent studies in other groups, we conclude that constant-rate lineage and trait diversification might be the more pervasive model for continent-scale radiations in general. The constant overall rate might be due to more complex geographic and climatic histories of continents coupled with lineage-specific traits, such as those promoting habitat generalism, which might buffer against rapid EO-driven diversification. Limitations of current methods to detect early burst signals for old radiations and a depauperate fossil record makes an adequate evaluation of these factors difficult at this point, but our initial investigations into the role habitat generalism may have in buffering clade-wide diversification is encouraging for further investigations into understanding how habitat tolerance affects large-scale colonization success and diversification rates.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Supporting Information S1.** Extended version of Methods section.

**Supporting Information S2.** GenBank numbers and voucher information for individuals included in the phylogenetic reconstructions.

**Supporting Information S3.** MCC tree for Bufonidae (Global Tree) recovered from time-calibrated Bayesian MCMC tree searches using BEAST under a birth-death uncorrelated lognormal relaxed clock model.

**Supporting Information S4.** 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.

**Supporting Information S5.** Phylogenetic tree recovered from pruning the FAR tree to include only a single representative of each currently recognized species (CRS tree).

**Supporting Information S6.** Tree recovered from pruning the FAR tree to include only a single representative of each bGMYC delimited element (bGMYC tree).

**Supporting Information S7.** Repeat of rate dynamics analyses (BAMM and BiSSE) using truncated bGMYC tree.

**Supporting Information S8.** Investigating the effects of bGMYC oversplitting.