The phylogenetic position and diversity of the enigmatic mongrel frog *Nothophryne* Poynton, 1963 (Amphibia, Anura)

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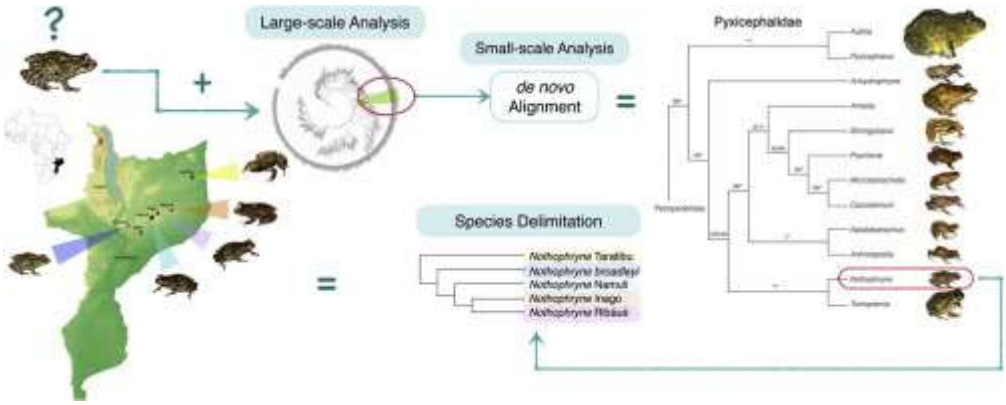
Highlights

• Using a two-tiered phylogenetic approach proves to be an efficient way to add new taxa into phylogenies.
• *Nothophryne* is a member of Pyxicephalidae and is the sister group of *Tomopterna.*
• First phylogeny to include representatives of all pyxicephalid genera.
• Cryptic diversity of *Nothophryne* species revealed.

Graphical abstract



Abstract

The phylogenetic relationships of the African mongrel frog genus *Nothophryne* are poorly understood. We provide the first molecular assessment of the phylogenetic position of, and diversity within, this monotypic genus from across its range — the Afromontane regions of Malawi and Mozambique. Our analysis using a two-tiered phylogenetic approach allowed us to place the genus in Pyxicephalidae. Within the family, *Nothophryne* grouped with *Tomopterna*, a hypothesis judged significantly better than alternative hypotheses proposed based on morphology. Our analyses of populations across the range of *Nothophryne* suggest the presence of several cryptic species, at least one species per mountain. Formal recognition of these species is pending but there is a major conservation concern for these narrowly distributed populations in an area impacted by major habitat change. The phylogenetic tree of pyxicephalids is used to examine evolution of life history, ancestral habitat, and biogeography of this group.

Keywords: Biogeography; Pyxicephalidae; Taxonomy; Ancestral reconstruction; Cryptic diversity; Mozambique; Malawi

1. Introduction

Knowledge of the African amphibian fauna is incomplete (e.g., Blackburn, 2008; Poynton, 1999), particularly in terms of taxonomic placement and delimitation across all taxonomic levels. Recent progress based on molecular data has revealed a wide-range of cryptic diversity of species (e.g., Blackburn, 2008; Channing et al., 2013; Loader et al., 2014; Tolley et al., 2010) and clarified phylogenetic relationships of many taxonomically uncertain groups (Barej et al., 2014; Frost et al., 2006; Pyron and Wiens, 2011; Scott, 2005; Siu-Ting et al., 2014; van der Meijden et al., 2011). Even though these studies have provided steps forward in our understanding of African amphibian diversity, many groups still require substantial investigation.

The mongrel frog *Nothophryne broadleyi* Poynton, 1963 is the only currently recognised member of its genus, and due to substantial threats within its restricted distribution (Mount Mulanje, Malawi and Mount Ribàué, Mozambique) is considered Endangered by the IUCN. *Nothophryne* is isolated on these inselbergs (isolated mountains), hiding under moss or other moist vegetation during the day. Eggs are laid in wet moss, and tadpoles develop in water seepages over exposed granitic outcrops. In the original diagnosis of the genus, Poynton (1963 p. 325) described the type series as presenting "a rather unexpected conglomeration of characters shown in a number of closely related genera, notably the external appearance of *Cacosternum capense*, a skeleton recalling *Anhydrophryne*, and a lingual papilla like that found in *Phrynobatrachus*. It is therefore placed in a new genus, and the odd assortment of characters gives the genus its name (Gk. *nothus* = mongrel)". Poynton (1963) considered the phylogenetic position of his new genus not altogether clear but evidently within the ranid subfamily Cacosterninae *sensu* Laurent (1961).

Scott (2005) made the first cladistic analysis of African ranids, including *Nothophryne*. In her thorough study that combined morphological and molecular data,

Scott retrieved *Nothophryne* as the sister group of *Cacosternum* Boulenger, 1887 and *Microbatrachella* Hewitt, 1926 (Cacosterninae, Ranidae). However, only morphological data were available for *Nothophryne* as well as some other taxa (i.e. *Ericabatrachus* Largen, 1991 and *Poyntonia* Channing and Boycott, 1989), and this produced some controversial relationships among groups. Since then, our understanding of the phylogenetic placement of *Nothophryne* has become further confused by the unstable phylogenetic relationships of higher taxa within ranids (e.g., Frost et al., 2006; Pyron and Wiens, 2011; van der Meijden et al., 2005). An example of this is its inclusion in Phrynobatrachidae *sensu* Dubois (1992), based exclusively on phenetic comparisons. In summary, the taxonomic placement of this enigmatic frog still remains unknown, due to the shifting taxonomy of ranids and lack of genetic data for *Nothophryne*. Hence, we expect that molecular data for *Nothophryne* will shed light on its phylogenetic placement, as has been the case with other recent examples of previously unsampled African ranids (e.g., Barej et al., 2014; Siu-Ting et al., 2014).

We obtained novel molecular data for *Nothophryne broadleyi* from recent fieldwork in the highlands of Mozambique and Malawi, including populations from several inselbergs where it had not been previously recorded. This allowed us to examine the phylogenetic placement of the genus, as well as the distribution and diversity of the populations on isolated inselbergs. We test whether these newly discovered populations represent the currently described species (i.e. *N. broadleyi*) or undescribed species, and make predictions regarding potential additional populations/species using ecological niche models. Using both the predictive modelling data and phylogenetic trees, we examine biogeographic hypotheses regarding ancient connections between the montane isolates. Lastly, our sampling of

Nothophryne has implications for life-history evolution in pyxicephalids, and we examine this by reconstructing ancestral life history traits with respect to habitat and breeding-mode shifts over time. We tentatively suggest that pyxicephalids originated in moist forest in Central or East Africa and had a semi-terrestrial breeding mode (i.e. eggs deposited out of water and aquatic larvae).

2. Material and methods

2.1. *Samples and sequencing*

Samples of *Nothophryne broadleyi* were collected from northern Mozambique and Malawi (Fig. 1) between 2009 and 2014 from the following field expeditions: Mount Namuli and Mount Inago (Bayliss et al., 2010; Timberlake et al., 2009), Mount Namuli (Farooq and Conradie, 2015), Mount Namuli and Mount Ribáuè (SANBI 2014 expedition) and Taratibu (H. Farooq 2014), with topotypic material collected from Mount Mulanje (M. Cunningham 2010). Individuals were found atop rock outcrops near water seepages and patches of moss. Tissue samples from thigh muscle and liver were obtained for eight specimens. Voucher specimens are deposited in the scientific collections of South African Institute for Aquatic Biodiversity (SAIAB), Port Elisabeth Museum (PEM) and Universidade Lúrio (UniLurio; Table S1 - Appendix A).

Total genomic DNA was extracted using Qiagen DNeasy kit following the protocol for purification of total DNA from animal tissues. In order to make comparisons with other African ranid frogs (van der Meijden et al., 2011, 2005), we amplified and sequenced four widely used markers in amphibian phylogenetics, comprising segments of two partial mitochondrial genes 12SrRNA (*12S*) and 16SrRNA *(16S*) and two nuclear genes, rhodopsin exon 1 (*RHOD*), and

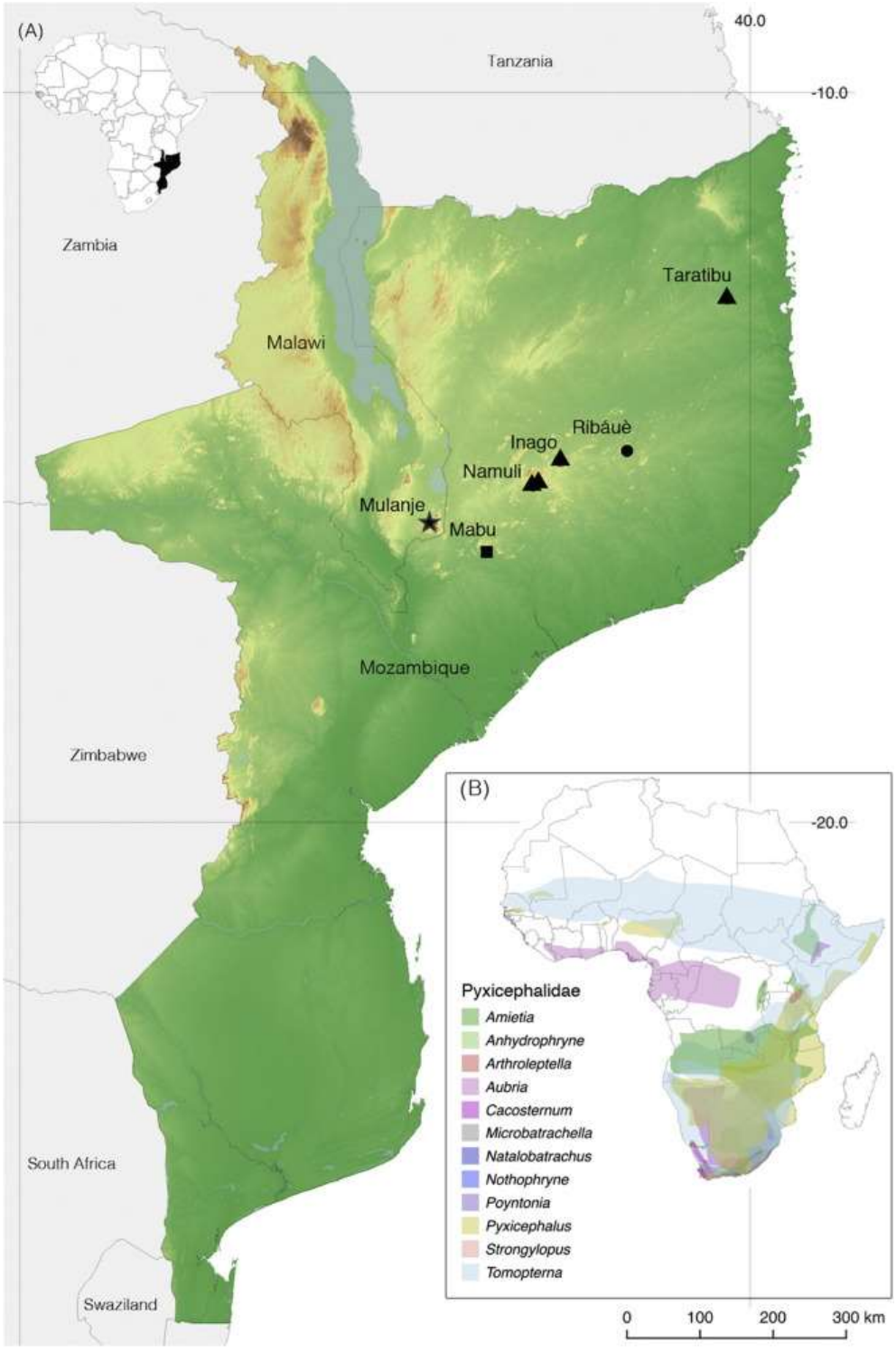


Figure 1. Distribution of *Nothophryne* and Pyxicephalidae. (A) Star indicates the type locality (Mount Mulanje). The other previously known locality (Mount Ribáuè) is marked with a black circle and new localities are shown as white circles. Black square represents Mount Mabu (where *Nothophryne* has been heard but not collected). (B) Distribution of pyxicephalids (downloaded from http://www.iucnredlist.org/ in November 2015).

recombination activating gene 1 (*RAG1*). PCR was performed using Illustra PuReTaq Ready-To-Go PCR Beads (Tables S2 and S3 - Appendix A), respectively. DNA sequences of both strands were sequenced by Microsynth AG (Balgrist, Switzerland).

2.2. *Data matrix and alignment*

Despite historical uncertainty in the taxonomic position of *Nothophryne*, there is little doubt that this taxon is a member of Ranoidea. Therefore we used a two-tiered approach similar to Siu-Ting et al. (2014) in order to determine its placement within the group. We first conducted a broad phylogenetic analysis using a large-scale published alignment, followed by a second targeted analysis with a subset of the taxa for a more precise and well-supported placement. Thus, for our large-scale analysis, we used part of the Siu-Ting et al. (2014) large-scale alignment, i.e. only including the four markers that we sequenced for our *Nothophryne* samples: *12S*, *16S*, *RHOD* and *RAG1* in order to minimise the amount of missing data. The alignment extracted from Siu-Ting et al. (2014) constitutes our "start alignment" containing 860 taxa, which we updated for recent taxonomic revisions (see Appendix B). For instance *Rana megatympanum* was removed because it is a synonym of *Odorrana tiannanensis* (Yang and Li, 1980), and according to Channing et al. (2013) the sequences related to *Cacosternum platys* Rose, 1950 actually are of *Microbatrachella capensis* (Boulenger, 1910). *Rana pretiosa* Baird and Girard, 1853 was excluded because there was no sequence available for the selected markers (see below). We updated the matrix with 306 novel sequences available on GenBank (until 01 August 2015, see Appendix B). In addition to *Nothophryne*, two other taxa were added to the alignment: *Odontobatrachus natator* (Boulenger, 1905), a member of the newly described family Odontobatrachidae, and *Tomopterna* cf. *tandyi*. The latter was

included despite its unconfirmed specific identity because it is the only member of this genus with the complete selected set of genes sequenced and available on GenBank. Finally, we included our newly sequenced sample of *Nothophryne broadleyi* collected near the type locality on Mount Mulanje, Malawi.

All new sequences were added to our start alignment using the profile alignment method in Muscle v.3.8 (Edgar, 2004) and the resulting alignments were then inspected and adjusted manually using Geneious v.7.1 (Kearse et al., 2012). TranslatorX (Abascal et al., 2010) was also used to improve and maintain the correct reading frames for the alignments of protein-encoding nuclear genes (*RAG1* and *RHOD*). The total number of species included per marker was 786 (*12S*), 840 (*16S*), 366 (*RAG1*) and 419 (*RHOD*), with the most of our species overlap being in our *12S* and *16S* markers. Our final large-scale concatenated alignment included a total of 858 taxa and was 4157 bp long.

2.3. *Phylogenetic analyses*

For our large-scale phylogenetic analysis, we performed a maximum likelihood (ML) analysis with non-parametric bootstrapping in RAxML v.8.2 (Stamatakis, 2014). Phylogenetic trees were rooted at Hemisotidae + Brevicipitidae, because this node is considered basal within Ranoidea (following Frost et al., 2006; Pyron and Wiens, 2011; Siu-Ting et al., 2014; Zhang et al., 2013). We used the unlinked GTR + GAMMA (GTR + G) model implemented in RAxML across the different genes and codon partitions. The partition scheme used for the dataset was per gene (in the non protein-coding markers) and per codon position (for the protein-coding markers). For this analysis we included *Nothophryne* sequences only from the type locality (Mount Mulanje, Malawi).

Small-scale analyses were performed using a subset of taxa chosen based on the large-scale ML analysis and their relative completeness and stability. The small-scale dataset (42 taxa and 3984 bp) included representatives of *Nothophryne* from near the type locality of *N. broadleyi* and five additional localities (in total eight samples), and all species of Pyxicephalidae from our large-scale alignment, with the exception of *Tomopterna natalensis* (Smith, 1849), which was found from the large-scale analysis to be an unstable taxon (see results). Three representatives from Petropedetidae (chosen to minimise missing data) were included as outgroup taxa. Analysing the small-scale dataset allowed us to investigate the relationships among different populations of *Nothophryne broadleyi* and to test monophyly of this taxon. A *de novo* alignment was performed for each gene using MAFFT v.7.0 (Katoh and Standley, 2013) applying the algorithm E-INS-i (recommended for less then 200 sequences with multiple conserved domains and long gaps), and nucleotide substitution models were selected using PartitionFinder (Lanfear et al., 2012). The following models were applied to each partition in the concatenated data: GTR + I + G (12S + 16S, RAG1_p1), GTR + I (RAG1_p2), SYM + G (RAG1_p3), GTR + G (RHOD_p1 + RHOD_p2) and HKY + G (RHOD_p3). MrBayes v.3.2 (Ronquist and Huelsenbeck, 2003) was used for the Bayesian inference (BI). Two runs were executed using four chains, and 20 million generations were sampled every 1000 generations with the initial 10% discarded as burn-in, and examination of the effective sample sizes (ESS) for convergence of parameters was done using Tracer (Rambaut et al., 2014). For comparison, a ML analysis was performed in RAxML using the GTR + G model, and support values were estimated using non-parametric bootstrap (100 replicates). Phylogenetic trees were visualized in iTOL v.2.1 (Letunic and Bork, 2011).

Approximately Unbiased (AU) tests (Shimodaira, 2002) were used to evaluate the fit of our new and Scott's (2005) previously proposed hypothesis of the relationships of *Nothophryne* to the small-scale dataset. The tree with the best overall fit was our ML GTR + G tree. This tree was then used as a backbone to generate (by manually editing the position of *Nothophryne* and other taxa) trees representing Scott's (2005) hypothesis. As explained in Siu-Ting et al. (2014), by using the tree that provided the best fit to the data, we avoided a potential bias against the contrasting hypotheses. Four trees were tested in total: our Bayesian and ML (GTR + G) trees, a tree representing Scott's (2005) proposed relationships, and a tree where we only moved *Nothophryne* to portray Scott's (2005) placement of this taxon (i.e. as sister taxon of the *Microbatrachella* + *Cacosternum* clade); see summary of tested topologies in Figure 2. Additionally, in order to facilitate comparison, we pruned out the taxa that were not included in Scott's work, namely *Strongylopus fasciatus*

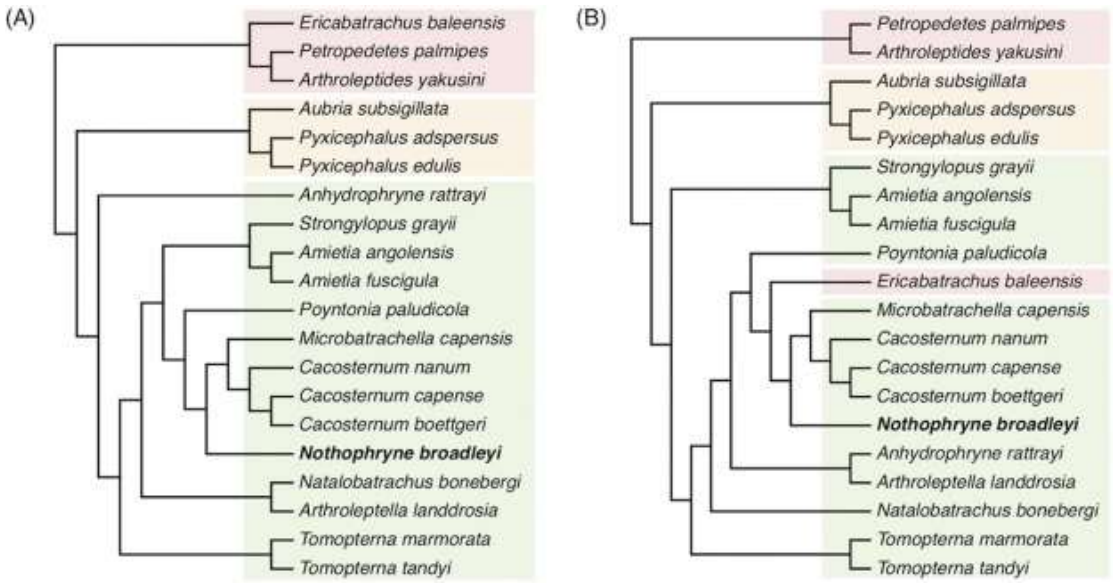


Figure 2. Alternative hypothesis of the relationship of *Nothophryne* and its putative sister groups. (A) Scott (2005) hypothesis, which represents the full set of relationships for that area of the tree; (B) Scott (2005) *Nothophryne* hypothesis represents only the phylogenetic relationship for *Nothophryne* in Scott's work. For more details about how this test was performed, refer to the Methods section.

(Smith, 1849), *S. bonaespei* (Dubois, 1981), *Artholeptella villiersi* Hewitt, 1935, *A. lightfooti* (Boulenger, 1910), *A. drewesii* Channing, Hendricks, and Dawood, 1994, *A. subvoce* Turner, de Villiers, Dawood, and Channing, 2004, and *A. bicolor* Hewitt, 1926. Per-site log-likelihoods were calculated for each of the tested topologies under GTR + G model in RAxML; then, these likelihood values were used to estimate statistical significance in CONSEL v.0.2 (Shimodaira and Hasegawa, 2001).

2.4. *Divergence-time estimation*

In order to estimate the approximate times of divergences within Pyxicephalidae, we generated an ultra-metric tree using BEAST v.2.1.3 (Bouckaert et al., 2014) with the Yule tree prior (as recommended for species-level analyses) and a lognormal relaxed molecular clock. As secondary calibration points we used two splits estimated by van der Meijden et al. (2005): the "African endemic clade", which was estimated to be ca. 69.9 mya (million years ago); and the "African endemic clade excluding *Pyxicephalus*" (and *Aubria*) estimated to be ca. 61.7 mya. We performed two runs of 100 million generations, sampling every 1000 generations, and ESS was examined using Tracer. The resulting trees were combined with the first 10% discarded as burn-in using LogCombiner v1.7.5, and the maximum clade credibility tree using the posterior mean node heights for the clades was obtained using TreeAnnotator v1.7.5 (both programs are part of the BEAST package).

2.5. *Ancestral-state reconstruction*

Pyxicephalids may have originated in Southern Africa in either savannah or forest (van der Meijden et al., 2011), but an analysis of ancestral habitat states has not been previously conducted. In addition, the ancestral breeding mode has not been

previously investigated for this family. We therefore selected two ecological traits, habitat and breeding mode, and mapped them onto our Pyxicephalidae phylogeny. Because many species inhabit other biomes (e.g., fynbos, lowland and montane grasslands) that are possibly more recent than the ancestor of pyxicephalids, we assigned taxa from these biomes to one of the two habitat states: (i) moist forest (coastal forest, lowland rainforest and montane forest) and (ii) open vegetation (dry forest, grasslands, savannah and fynbos). Our coding system for habitat was based on the IUCN (IUCN, 2014) habitat description. Breeding mode, coded according to place of egg deposition and larval habitat, comprises three states: (i) fully aquatic; (ii) semi-terrestrial; and (iii) and direct development. The state fully aquatic refers to eggs deposited in water and larvae developing in aquatic environment, whereas semi-terrestrial includes species that lay eggs out of water (e.g., wet moss, nest hanging on branches) but the larvae develop in water. Pereira et al. (2015) demonstrated that multiple reversals occur between habitat states (e.g., open area versus forest formations) in leptodactylids. Similarly, there is evidence that evolution of anuran breeding modes is not an ordered and gradual process towards terrestrial reproduction (Gomez-Mestre et al., 2012), including observation of reversals from terrestrial to aquatic larval development (Pereira et al., 2015). Hence, we considered transitions between states independent for both habitat and breeding mode.

Traits were mapped onto the ultrametric tree resulting from our BEAST analysis. The tree was pruned leaving only one representative of each genus, given that traits do not vary within genera. We applied two methods, parsimony and maximum likelihood (ML), using Mesquite v. 3.03 (Maddison and Maddison, 2015). ML reconstructions were performed using the Markov k-state 1-parameter model (Mk1; Lewis, 2001), which gives equal probability for changes between any two

character states. Similarly, parsimony analysis used Fitch (unordered or non-additive) optimisation, which gives equal cost to all character-state changes.

2.6. *Species delimitation*

We explored putative species boundaries within our samples of *Nothophryne* using a Bayesian implementation of the General Mixed Yule-Coalescent (bGMYC; Reid and Carstens, 2012) model implemented in R v. 2.13.0 (R Core Team, 2014) using 100 trees randomly selected from a BEAST analysis (same settings as before but without the divergence-time constraints). We set the bGMYC simulations to 50,000 generations, discarding the first 10% as burn-in and sampling every 100th generation. The upper threshold was set to 42 (number of tips on the tree), and a cut-off value was set as 0.8 in order to determine the lineages. Additionally, we calculated the pairwise distance between *16S* sequences for 41 species included in the small-scale analysis using MEGA6 (Tamura et al., 2013). *Tomopterna* cf. *tandyi* was excluded because it overlapped only with five other sequences. A final dataset of 508 bp was produced after eliminating all positions with less than 5% site coverage.

2.7. *Ecological niche model (ENM)*

Historically, *Nothophryne* has been reported only from two localities: Mount Mulanje, Malawi (type locality) and Mount Ribáuè in Mozambique (Blake, 1965; Poynton, 1963), both higher than 1200 m above sea level (asl). Our recent fieldwork expeditions revealed the occurrence of members of this taxon in three new sites (Fig. 1), including one that is 500 km away from the type locality and at considerably lower elevation (Taratibu, Mozambique, 450 m asl). In order to identify regions with similar environmental conditions to where *Nothophryne* has been found, we developed an

ENM using MaxEnt version 3.3.3k (Phillips et al., 2006). Given the small sample size (n = 8), we set the replication mode to cross-validation, which uses all the samples leaving one out in each run. All parameters were set as default.

Environmental variables were selected based on the authors understanding about the species habitat requirements. We assembled a total of seven environmental variables related to temperature (mean diurnal range, temperature seasonality and mean temperature of coldest quarter), precipitation (precipitation of driest quarter and precipitation of warmest quarter) and topography (digital elevation model and slope) on a 30 s grid (ca. 1 km^2 resolution). Climatic data were obtained from the WorldClim database (Hijmans et al., 2005) and the digital elevation model from HydroSHEDS (Lehner et al., 2006). Slope was calculated using the digital elevation model. All variables were treated using R packages "raster" (Hijmans, 2015) and "rgdal" (Bivand et al., 2015). In order to produce a binary map (presence-absence) of habitat suitability, we applied the minimum training presence (MTP) threshold, which uses the lowest predicted value associated with any of the observed presence records.

3. Results

3.1. *Phylogenetic analyses*

The large-scale ML analysis (Fig. 3) supported monophyly of Pyxicephalidae and of the two sub-families of this group (Cacosterninae and Pyxicephalinae) with high bootstrap values (99%, 96% and 100%, respectively), corroborating previous studies (e.g., Scott, 2005; van der Meijden et al., 2011). A clade comprising *Nothophryne broadleyi* and all *Tomopterna* Duméril and Bibron, 1841 was recovered with bootstrap support of 94%. Additionally, we found that the position of

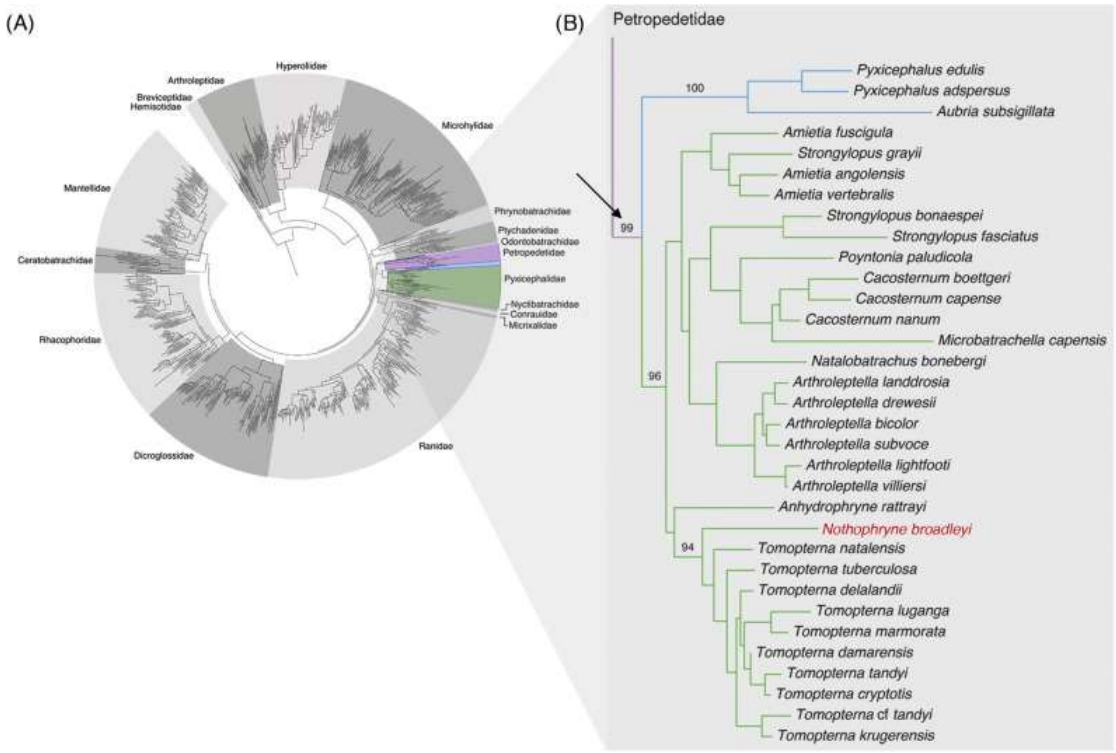


Figure 3. Phylogenetic relationships of *Nothophryne broadleyi.* A) ML tree from the large-scale phylogeny of Ranoidea. B) Close up view of the clade Pyxicephalidae and the position of *Nothophryne broadleyi*. Arrow indicates the high bootstrap support for Pyxicephalidae.

Tomopterna natalensis was unstable, and that pruning this taxon from the bootstrap trees increased the support of the association of *Nothophryne* and the remaining *Tomopterna* to 100%.

The focused small-scale analysis with ML and BI also recovered *Nothophryne* as the sister taxon of *Tomopterna* with maximum support values for both non-parametric bootstrap and posterior probability (Fig. 4). AU tests do not distinguish between the ML and Bayesian trees, whereas both trees displaying Scott's (2005) placement of *Nothophryne* were rejected as having a significantly worse fit to the data (p-values < 0.005, see Table 1). This corroborates our phylogenetic placement of *Nothophryne* obtained in both our Bayesian and ML analyses. The grouping of *Strongylopus grayii*

with *Amietia* is potentially due to sequence misassignment (see comments in Frost, 2015).

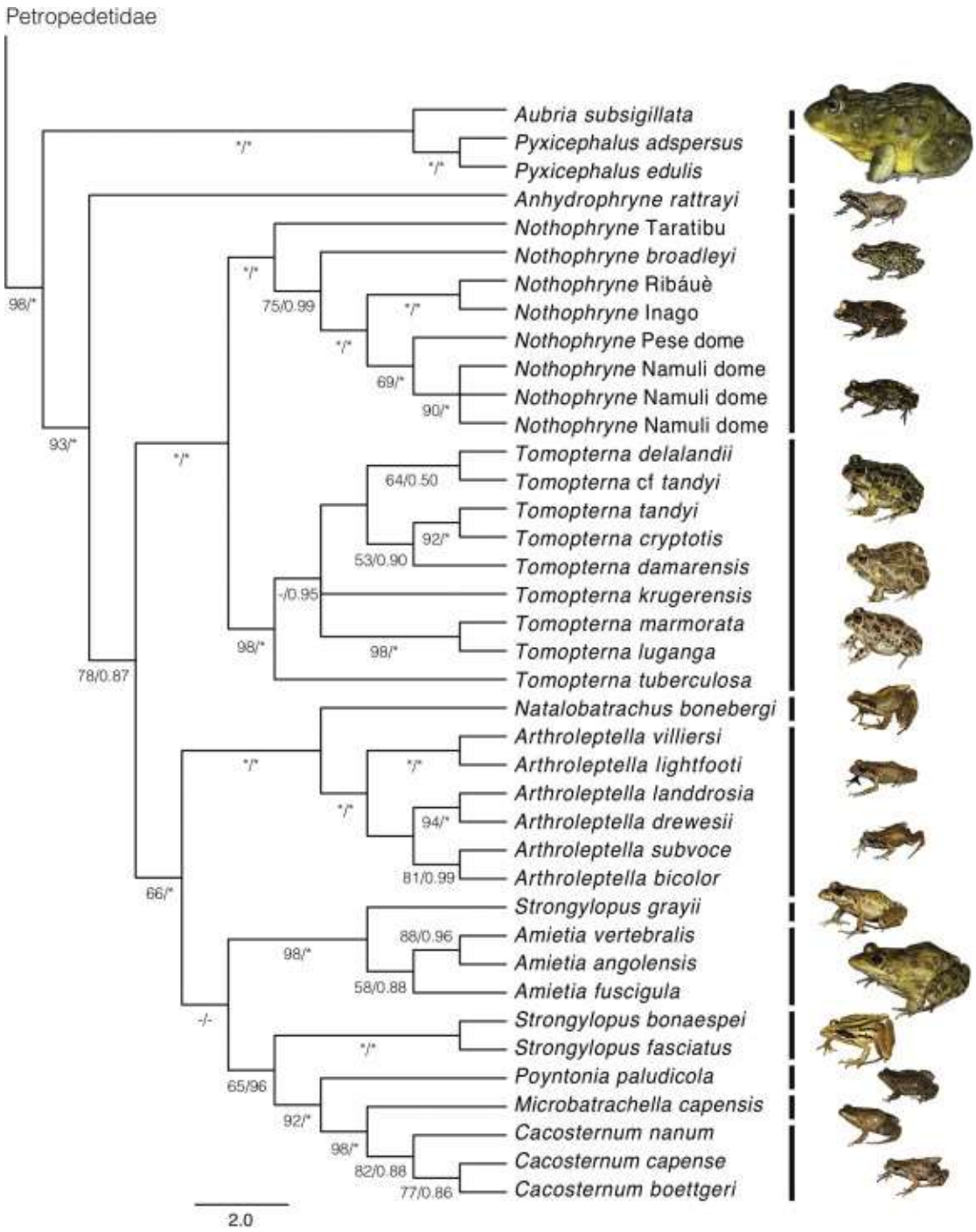


Figure 4. Phylogenetic relationships of Pyxicephalidae. Consensus tree (ML and BI) with branch support values corresponding to non-parametric bootstraps (left) and posterior probabilities (right). Maximum support values are represented by "*" and values equal or below 50/0.50 are denoted by "-".

Table 1. Hypothesis-testing results. Values shown refer to the Approximately Unbiased test (AU test) from CONSEL. Scott (2005) hypothesis represents the full set of relationships for that area of the tree proposed in Scott's work. Scott (2005)*Nothophryne* hypothesis represents only the phylogenetic relationship for *Nothophryne* in Scott's work.

Rank	Item	AU test
1	Present work, Bayesian tree	0.507
2	Present work, small-scale ML tree	0.500
3	Scott (2005) hypothesis	9e−05
4	Scott (2005)*Nothophryne* hypothesis	6e−05

3.2. *Divergence-time estimation*

Based on secondary calibration points and the assumption that Pyxicephalidae originated around 70 mya, we inferred that the split between *Nothophryne* and *Tomopterna* occurred approximately 36 mya (Fig. 5). Within *Nothophryne* there are substantial divergences between the lineage from Taratibu and the rest (estimated ca. 22 mya, Fig. 5).

3.3. *Ancestral-state reconstruction*

The result for the ancestral habitat reconstruction using parsimony reveals a forest ancestor for pyxicephalids (Fig. 6). However, the ML method shows equivocal results regarding the ancestral habitat of Pyxicephalidae. Multiple transitions between habitat types occurred in the evolution of the family. Reconstructions of ancestral breeding mode using both methods show equivocal results. The parsimony method suggests either a fully or semi-aquatic ancestor of pyxicephalids.

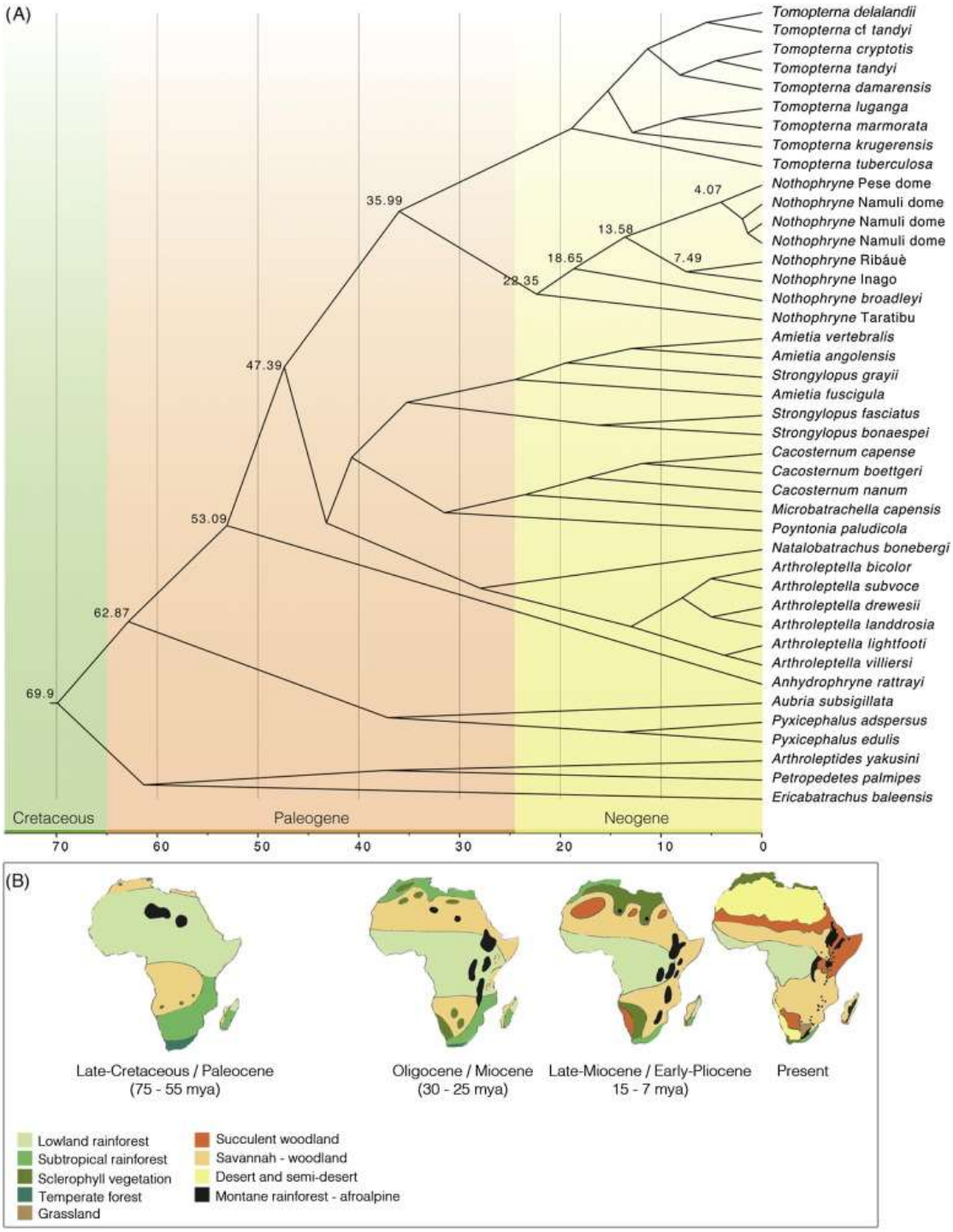


Figure 5. Divergence-time estimation for pyxicephalids and vegetation map for Africa. (A) Calibrated tree based on secondary-calibration derived from van der Meijden et al. (2011) estimations. (B) Schematic vegetation map of Africa adapted from Axelrod and Raven (1978).

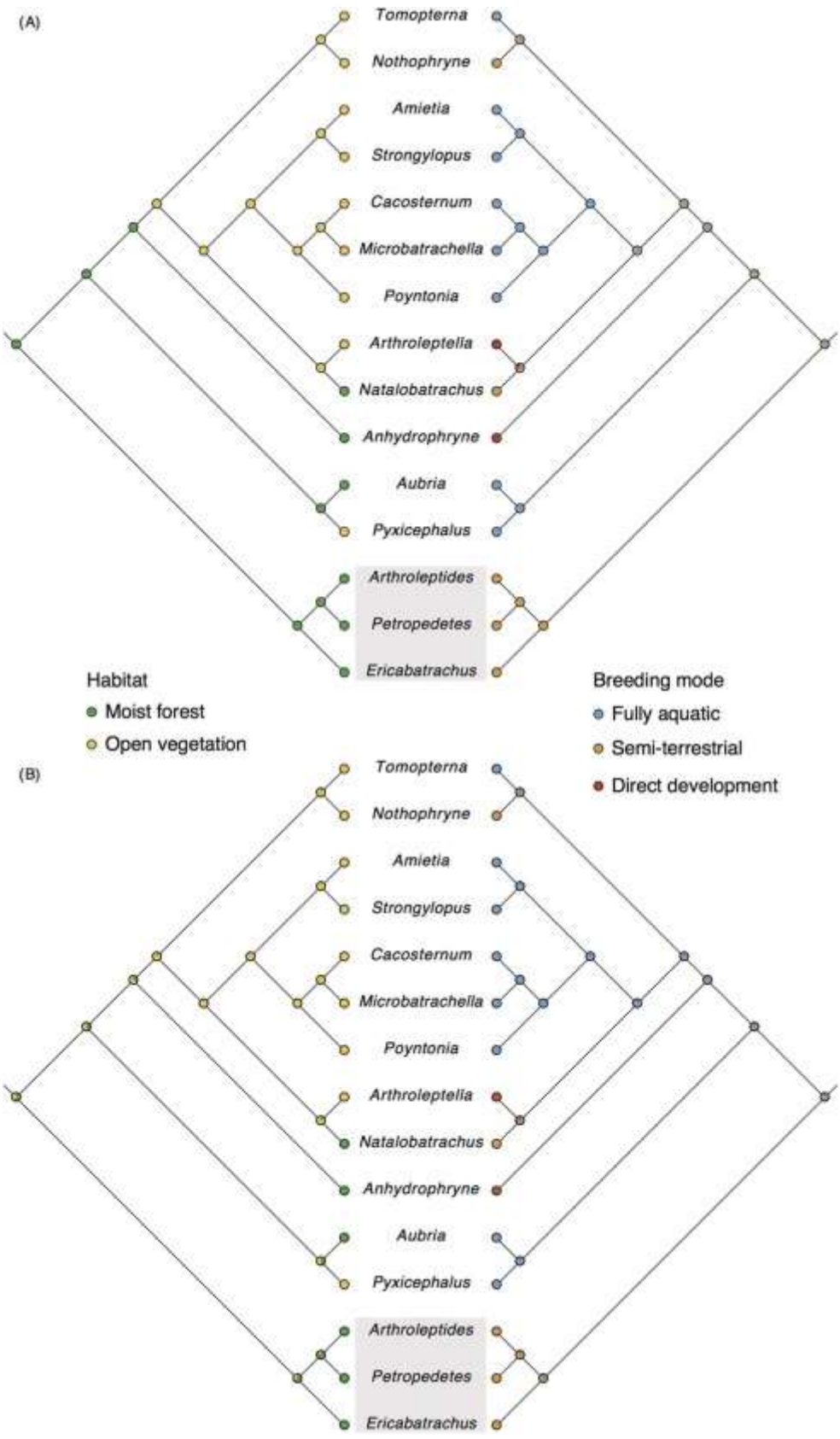


Figure 6. Reconstructed ancestral habitat and breeding mode of pyxicephalids. (A) Parsimony method and (B) Maximum likelihood method. Grey box indicates outgroup.

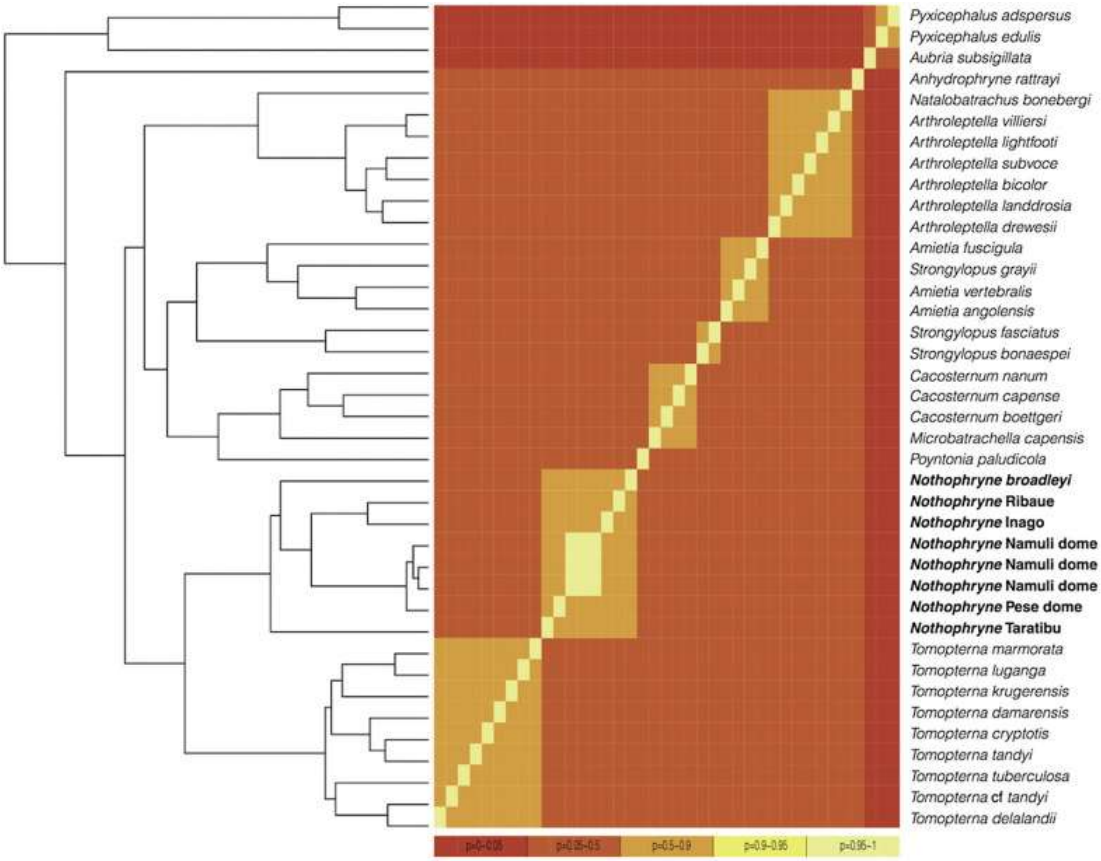


Figure 7. Species delimitation using bGMYC. Heat map shows six putative species of *Nothophryne* (in bold).

3.4. *Species delimitation*

The bGMYC analysis recovered six putative species of *Nothophryne*, one from Malawi (*N. broadleyi*) and five from Mozambique (Fig. 7). Two separate putative species of *Nothophryne* were identified from Mount Namuli (one from Namuli dome and another from Pese dome). However, these findings should be interpreted with caution given that bGMYC is prone to over-splitting lineages (Carstens et al., 2013). Hence, addition of morphological and acoustic data would be crucial to confirm the hypothesis of multiple species on the Namuli massif. The pairwise distance matrix shows that the differences within our *Nothophryne* samples

(excepting the samples from the Namuli massif) are equivalent to the differences observed within species from other pyxicephalid genera (Table S1 - Appendix C).

3.5. *Ecological niche models*

The ecological niche model shows a number of areas outside the known distribution that might be suitable for *Nothophryne* (Fig. 8). Of note is that slope (46.2%) and elevation (34.9%) contributed highly to the total variation in the model, with temperature seasonality (13%) and precipitation of the driest quarter (5.8%) also important. Because *Nothophryne* is typically found in moss and shrub on high granite domes on inselbergs, slope and elevation might be expected to have a strong contribution.

Regardless, some areas identified as suitable by the model are actually sloped areas (e.g., edges of plateaus and densely forested mountains), which when overlapped with satellite images do not appear to be suitable *Nothophryne* habitat, suggesting that the model over-predicts based on this suite of environmental variables. It is important to note that our aim with this model is merely to identify areas with habitats similar to that where *Nothophryne* has been found in order to propose biogeographical scenarios, and to guide future surveys for this group. For example, Mount Mabu is one of the areas predicted as suitable for *Nothophryne*, and in November 2014 several individuals of *Nothophryne* were heard calling on the granite dome summit but were not captured (Bittencourt-Silva, Conradie, Loader, Pers. Obs.).

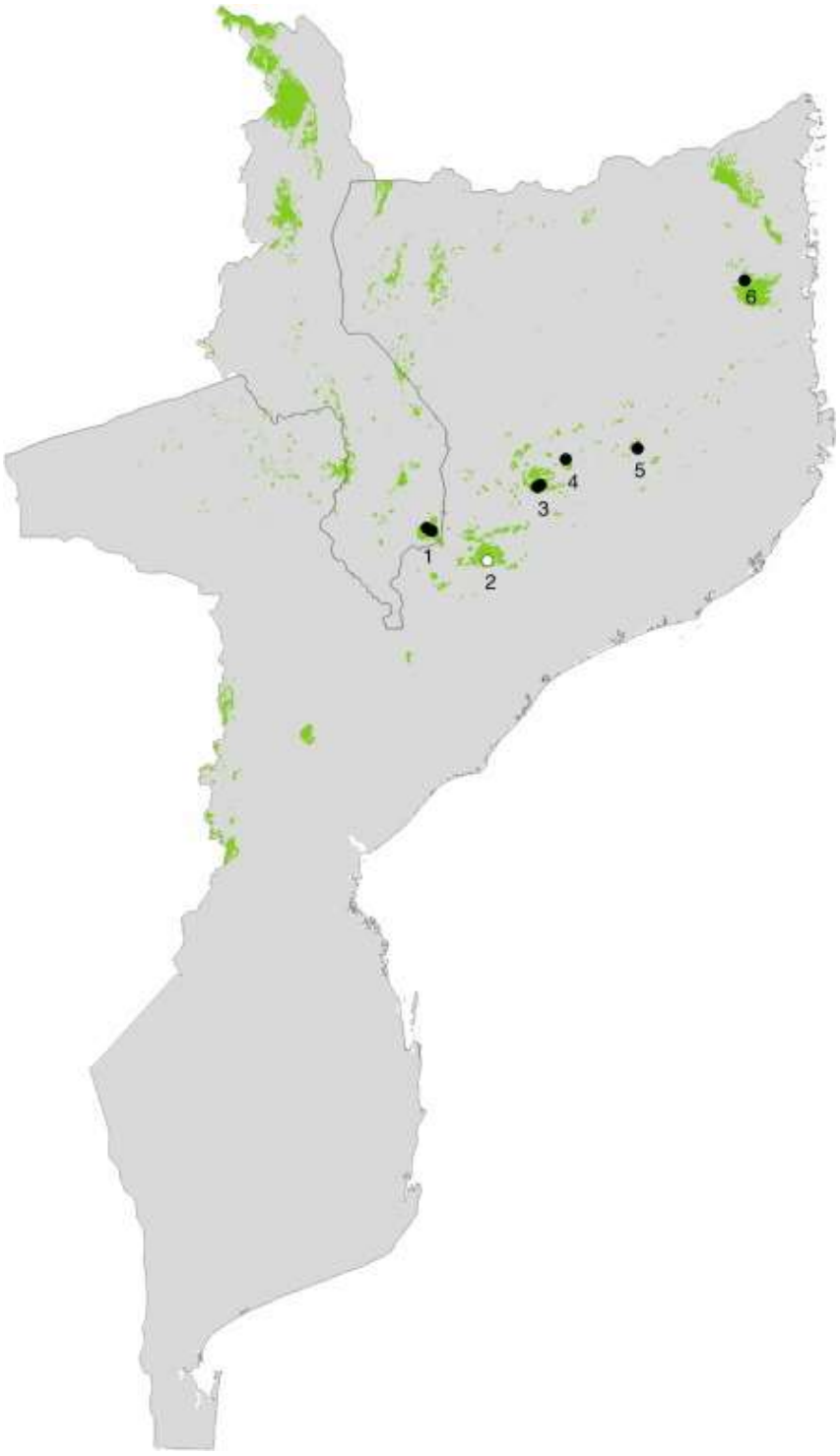


Figure 8. Modelled distribution of *Nothophryne broadleyi* using Maxent. Green areas represent habitat suitability when applying the minimum presence threshold. Squares show the data points used to build the model. Data points used to generate the model are shown as black circles. The white circle represents the locality where *Nothophryne* was heard but not collected. (1) Mount Mulanje, (2) Mount Mabu, (3) Mount Namuli, (4) Mount Inago, (5) Mount Ribáuè and (6) Taratibu.

4. Discussion

4.1. *Phylogeny*

Based on our multi-locus data the genus *Nothophryne* is shown to be placed within the subfamily Cacosteninae of the African family Pyxicephalidae, and is the sister taxon to the genus *Tomopterna.* This conclusion contrasts with the previous morphological-based hypothesis that *Nothophryne* is the sister group to *Cacosternum* and *Microbatrachella* (Scott, 2005) and the relationship with *Tomopterna* was previously not suspected. Despite our recovered relationship, there are no known unique morphological synapomorphies (though many non-unique ones) that unite *Nothophryne* and *Tomopterna* (Scott, 2005), but constrained trees including the grouping of *Nothophryne, Cacosternum* and *Microbatrachella* have significantly worse fits to the molecular data. Thus we agree with Scott (2005) that the placement of *Nothophryne* in her study might be an artefact caused by the lack of molecular data.

With the molecular sampling of *Nothophryne,* our study is the first to include representatives of all pyxicephalid genera. The addition of genetic data from *Nothophryne* is effective not only in the placement of this taxon but also provides an alternative phylogenetic hypothesis amongst other pyxicephalids. In previous studies (Bossuyt et al., 2006; Frost et al., 2006; van der Meijden et al., 2011, 2005) *Tomopterna* is the sister taxon to all other members of Cacosterninae, whereas in the present study and in Pyron and Wiens' (2011) phylogeny, *Anhydrophryne* Hewitt, 1919 is placed in this position. However, this part of the pyxicephalid tree is relatively poorly supported and will require further sampling of genes and species to resolve the precise positions of genera within Cacosterninae with confidence.

The previously unsuspected grouping of *Nothophryne* with *Tomopterna* has interesting implications in terms of shifts in niches, evolution of breeding strategies,

morphological parallelism, and biogeography of Pyxicephalidae. The genus *Tomopterna* comprises 15 species of medium-sized frogs, and is widespread throughout sub-Saharan Africa. *Tomopterna* lives in both moist and arid savannah, whereas *Nothophryne* is a relatively small frog that occurs in isolated patches of Afromontane environments, more precisely in areas with exposed granitic rocks. It has been hypothesised that pyxicephalids originated in Southern Africa where medium to large sized ancestors resembling some extant genera (i.e. *Pyxicephalus* and *Tomopterna*) occupied savannah and lowland forests (van der Meijden et al., 2011, 2005). Our habitat-reconstruction analyses (Fig. 6) suggest that the ancestor of pyxicephalids may have inhabited moist forested habitats similar to those currently restricted to montane environments. This conclusion remains speculative, however, because there are theoretical and practical shortcomings when reconstructing ancestral habitats for species (Hardy, 2006). One caveat is that our findings are based on only 32 of the 77 species currently recognised in Pyxicephalidae. It is known that taxon sampling can affect ancestral state reconstruction (see Hardy, 2006), and hence the addition of the remaining taxa would be crucial to test hypotheses of the type of habitat occupied by the ancestor of pyxicephalids.

The evolution of terrestrialised breeding forms in pyxicephalids (i.e. eggs laid out of water) was first investigated by van der Meijden et al. (2011), and they inferred that direct development evolved independently twice in this group (*Arthroleptella* and *Anhydrophryne*). However, no comparative approaches were applied to examine these transitions. Our ancestral-state reconstruction corroborates independent terrestrialisation of breeding in *Arthroleptella* and *Anhydrophryne*. Additionally, semi-terrestrial breeding modes also evolved independently during the evolution of this family (i.e. *Nothophryne* and *Natalobatrachus*). The diversity of habitats and

reproductive modes exhibited by pyxicephalids and their evolutionary lability might be the result of changes driven by geography. Climatic and geological changes across the African continent have been of crucial importance in explaining evolution in many groups (e.g., Ceccarelli et al., 2014; Couvreur et al., 2008; Loader et al. 2014; Matthee et al., 2004). Wider taxonomic sampling within Pyxicephalidae and a comprehensive understanding of their habitats are required before any firm conclusions can be made on the correlated evolution of breeding biology and geographic distribution.

Phylogenetic relationships of pyxicephalids provide an interesting insight into biogeographic patterns in sub-Saharan Africa, and in particular, connections between specific regions across large distances. The population of *Nothophryne* in coastal forest (Taratibu) inselbergs in northeastern Mozambique is a geographic outlier, nearly 600 km from Mount Mulanje and more than 200 km from the nearest isolate (Mount Ribáuè). Our discovery of this population suggests that there may have been a connection between the coastal forest and the Afromontane isolates from southern Malawi and north/central Mozambique (e.g., Mulanje, Mabu, Namuli) that no longer exists. Indeed, it has been suggested that subtropical forest was once widespread along the eastern margin of Africa, from Kenya to South Africa, during the Oligocene-Miocene (e.g., Axelrod and Raven, 1978). The aridification of East Africa, triggered by the formation of the East African Rift System, retracted the forested areas, which became confined to higher elevations (i.e. mountains and inselbergs) or coastal areas. Such changes might have been important in driving the isolation of populations of *Nothophryne*. Other taxa have similar distribution scattered across montane inselbergs and East African coastal forest, including frogs in the genus *Mertensophryne* (Poynton, 1991), caecilians in *Scolecomorphus* (Farooq and Conradie, 2015) and chameleons in the genus *Rhampholeon* (Branch et al., 2014).

Similar evidence from multiple taxa may strengthen biogeographic hypotheses regarding connections between these inselbergs and the timing of diversification events.

The phylogenetic position of *Tomopterna* and its grouping with the morphologically dissimilar *Nothophryne* provides an interesting example of divergence in form, also highlighting the problems of understanding evolutionary relationships based largely on morphology (Scott, 2005). In fact, the genus *Tomopterna* was previously thought to have disjunct distribution in continental Africa, Madagascar and Asia. *Laliostoma labrosum* (Cope, 1868), from Madagascar and *Sphaerotheca breviceps* (Schneider, 1799), from Sri Lanka, were previously included in *Tomopterna* based on their shared characters involved in burrowing (see Bossuyt and Milinkovitch, 2000 and references therein, e.g., Glaw et al., 1998). This example shows how parallelism in morphological traits can cause taxonomic confusion. In addition, *Nothophryne* provides yet another example of morphological parallelism — with similar tadpoles to other rock-dwellers with semi-terrestrial larvae in the family Petropedetidae). Other species of rock-dwellers with semi-terrestrial larvae, such as *Nothophryne*, are also found in Asia (*Nannophrys* Günther, 1869) and South America (*Cycloramphus* Tschudi, 1838).

The substantial genetic divergence observed between populations of *Nothophryne* could be predicted given their limited dispersal capabilities (considering their specialised habitat and breeding). It is also interesting that the basal divergences within *Nothophryne* split the lineage at the edge of the distribution, at Taratibu, from all others. This might reflect a formerly more extensive distribution of subtropical forest across the region that became increasingly isolated and restricted to moist areas (i.e Afromontane and coastal forest) over time (Axelrod and Raven, 1978; Brenan,

1978). The biogeographic interpretations of these patterns merits further investigation, particularly if additional populations are discovered on other inselbergs.

4.2. *Taxonomy and conservation*

Our molecular-based analyses confirm that *Nothophryne* is not a monotypic genus and that there are likely multiple species distributed among isolated inselbergs as shown by large genetic differences between populations. In addition to the strong genetic differentiation, there are some obvious morphological differences (Fig. 9) that support the hypothesis that each mountain block has a distinct species. For example, the populations from Taratibu are slightly flattened dorso-ventrally and have smooth skin on the dorsum, whereas the other populations have warty (Malawi) or spiky (Namuli, Inago and Ribáuè) dorsal skin. More comprehensive studies of these populations are required to document their morphological distinctiveness.

The finding of other populations is also likely across this region given the paucity of surveys across many isolated inselbergs. For example, based on recent fieldwork in Mount Mabu, Mozambique, we suspect that *Nothophryne* is present there based on call records. Furthermore, this record could potentially confirm the ENM predictions about the suitability of that area for *Nothophryne*. The suggestion of multiple species of *Nothophryne* on Mount Namuli is not unprecedented; Poynton and Broadley (1985, p. 172) suggested the possible presence of two species on Mount Mulanje, based on a morphologically distinct specimen collected by Stevens at a much lower elevation and in a different situation to those from the type locality and adjacent plateau. Overall, our work suggests that the distribution of *Nothophryne* is not yet well known. Future targeted fieldwork should explore these areas of predicted occurrence, providing additional tests for these predictions.

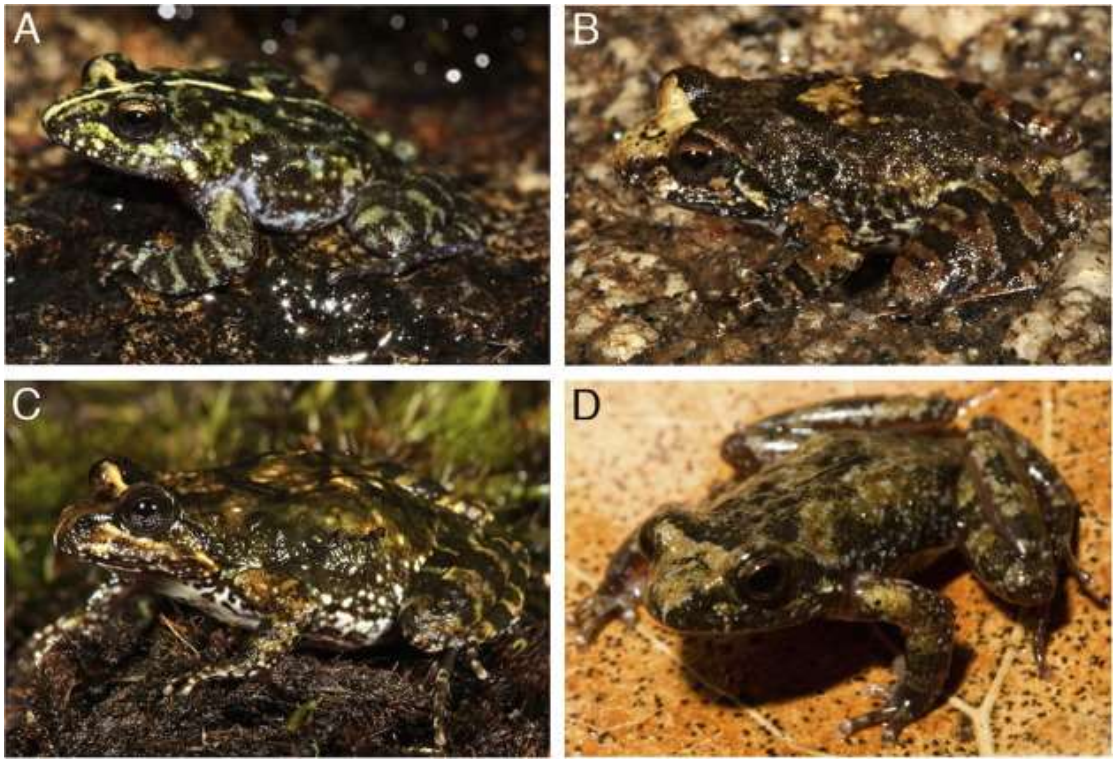


Figure 9. *Nothophryne* populations from Malawi and Mozambique. (A) Mount Mulanje, (B) Mount Ribáuè, (C) Mount Namuli and (D) Taratibu. Photos A, B and C by W Conradie, and photo D by HM Farooq.

Recognition of more than one species of *Nothophryne* has conservation implications. The narrow ranges of these putative species — so far only known from a single mountain block each — draw our attention to their susceptibility. Furthermore, their specific habitat requirements and breeding biology means that they are likely to be susceptible to any changes in habitat quality. With the continuing practice of slash-and-burn agriculture on forests in Mozambique (Temudo and Silva, 2012), and on-going clearing due to population pressure, these species face serious risk of disappearing. Targeted surveys and studies are crucial to understand population trends and their precise distribution and habitat requirements.

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Appendix A

Table S1. Specimens data and GenBank accession numbers for the *Nothophryne* sequences generated in the present study. Mozambique (MZ); Malawi (MW); Mount (Mt). Collections: (HF) Universidade Lúrio; (WC and PEM) Port Elisabeth Museum; (QQ) South African Institute of Biodiversity, (NA) not available.

Table S2. Primers used in this study.

Table S3. PCR protocols.

Appendix B

GenBank accession numbers for the species included in the phylogenetic analysis of *Nothophryne broadleyi*. Sequences added to the original alignment form Siu-Ting et al. (2014) are marked in bold. Previous names are those used in Siu-Ting et al. (2014).

Appendix C

Table S1. Estimates of evolutionary divergence between 16S rDNA sequences (uncorrected p-distance). Numbers shown indicate nucleotide differences per site between sequences. Values in bold (colour-coded per genus) show intra-specific differences and values in blue indicate inter-generic differences. Numbers in red represent the two populations from Mount Namuli, considered here as one species.