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Molecular phylogeny and diversification of Malagasy bright-eyed tree frogs (Mantellidae: *Boophis*)

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ABSTRACT

We investigate the molecular phylogeny of *Boophis,* a group of arboreal frogs from the Malagasy-Comoroan family Mantellidae. Based on newly acquired DNA sequences of five mitochondrial and five nuclear markers (7444 base pairs), we infer a phylogeny of *Boophis* with complete species-level taxon sampling. We reconstruct the phylogeny using Bayesian inference and maximum likelihood and estimate divergence dates for the major clades of the genus. The phylogenetic analyses together support the monophyly of the two subgenera (*Sahona* and *Boophis*), and provide strong support for most previously identified species groups, except that the *B. ulftunni* group is nested within the *B. majori* group. We also erect a new species group related to the *B. mandraka* group, the *B. blommersae* group, composed of small-sized, brown stream-breeding frogs previously included within the *B. majori* group. Finally, we use the resulting phylogeny to illustrate striking examples of repeated evolution of coloration and ventral transparency and address the biogeographic history and broad pattern of species diversification in the genus. Ancestral area reconstructions provide evidence that *Boophis* diversified within the Eastern highland forests of Madagascar, and we suggest that adaptation to these highland areas was important in their diversification.

1. Introduction

Madagascar harbors among the highest rates of amphibian endemism in the world, with exceptionally high species richness relative to land area (Myers et al., 2000). Most species belong to the family Mantellidae, and the genus *Boophis* Tschudi, 1838 is the most species-rich genus in the family (AmphibiaWeb, 2017). The genus contains mostly arboreal frogs (Cadle, 2003), with 77 currently recognized species (AmphibiaWeb, 2017) and > 30 additional "candidate" species, which are genetically divergent lineages that require formal taxonomic revision (Vieites et al., 2009; Perl et al., 2014). The relatively recent integration of DNA barcoding and bioacoustic analyses with *Boophis* systematics has revealed numerous such genetically divergent lineages, many of which are morphologically cryptic. This led to the descriptions of 34 new species since 2001 (e. g. Glaw et al., 2001; Glaw and Vences, 2002; Vences and Glaw, 2002; Köhler et al., 2007, 2008; Wollenberg et al., 2008a,b; Glaw et al., 2010; Vences et al., 2012; Penny et al., 2014; Hutter et al., 2015). Despite this recent taxonomic progress, a well-sampled and multi-locus nuclear phylogeny is not yet available for this genus, hindering many potential comparative and biogeographic analyses.

Boophis have previously been divided into two sub-generic monophyletic groups: *Sahona* Glaw and Vences, 2006, which includes species that breed in ponds, and *Boophis* Tschudi, 1838, which are predominantly stream-breeding specialists (Glaw and Vences, 2006). *Sahona* species are distributed mainly in the lowland rainforests and also in the arid regions of western and southern Madagascar, while species in the subgenus *Boophis* are generally found in rainforests or montane habitats in eastern and northern Madagascar (Glaw and Vences, 2006; Glaw et al., 2006). In some early phylogenetic analyses, Vences et al. (2002) suggested that species now placed in *Sahona* may not form a clade; however, the addition of more taxa and molecular markers in later studies strongly supported *Sahona* as monophyletic (Glaw and Vences, 2006; Glaw et al., 2006). The subgenus *Boophis* has greater species richness than *Sahona* (68 vs. 9 species), and contains eight named species groups. Within mantellids, *Boophis* are characterized by a conserved external morphology of adults (Wollenberg Valero et al., 2017) and rather uniform karyotypes (Aprea et al., 2004), whereas the

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larvae of these frogs have evolved a remarkable ecomorphological diversity (e.g., Blommers-Schlösser, 1979; Blommers-Schlösser and Blanc, 1991; Altig and McDiarmid, 2006; Randrianiaina et al., 2009, 2012; Grosjean et al., 2011; Wollenberg Valero et al., 2017).

While early researchers placed *Boophis* in the Asian genus *Rhacophorus* (e.g., Guibé, 1978), its species were recognized as distinct by Blommers-Schlösser (1979) mostly by their reproductive traits, in particular the absence of foam nesting. This author diagnosed seven species groups within the genus based on morphology and bioacoustics. Several of these groups (*B. opisthodon* group, *B. pauliani* group, and *B. rhodoscelis* group) were later abandoned based on morphology (Blommers-Schlösser and Blanc, 1991), and on molecular phylogenetic evidence (Glaw and Vences, 2006). Additionally, Glaw and Vences (2006) newly defined the *B. mandraka* group and *B. albipunctatus* group, and transferred the *B. tephraeomystax* group to the subgenus *Sahona* using molecular phylogenetic evidence. The current eight species groups of *Boophis* are: *Boophis albilabris* group, *B. albipunctatus* group, *B. goudotii* group, *B. luteus* group, *B. majori* group, *B. mandraka* group, *B. microtympanum* group (Glaw and Vences, 2006), and the recently proposed *B. ulftunni* group (Wollenberg et al., 2008a,b; Köhler et al., 2008). Despite the numerous works that contributed to understanding the molecular phylogeny of *Boophis* (e.g., Richards et al., 2000; Vences and Glaw, 2001; Vences et al., 2002; Glaw and Vences, 2006; Frost et al., 2006; Kurabayashi et al., 2008; Wollenberg et al., 2008a,b, 2011; Pyron and Wiens, 2011), many facets of their evolutionary relationships remain insufficiently supported. More reliably resolving the deep and shallow relationships among species of *Boophis* is a prerequisite for understanding the origin of morphological adaptations in adults and tadpoles (Wollenberg Valero et al., 2017), as well as evolution of color pattern (Wollenberg et al., 2008a,b) and advertisement calls (Hutter et al., 2015).

We here construct a multi-locus phylogeny using Bayesian and maximum likelihood approaches, sampling a total of five mitochondrial and five nuclear genes across all nominal *Boophis* species and 35 candidate species. We use the resulting phylogeny to revisit prior species-group definitions and to test their monophyly. We also discuss subgeneric classification, illustrate repeated evolution of dorsal and ventral coloration, and address the biogeographic history and broad patterns of species diversification in the genus.

2. Materials and methods

2.1. Taxon and marker sampling

We collected data for 10 genetic markers from 77 nominal *Boophis* species and 35 candidate species through new sequencing and previously published sequences from GenBank (112 total terminals; Table S1). We obtained 365 new sequences and added 338 sequences from GenBank, doubling the amount of molecular data for *Boophis*. We increased the molecular sampling from 10 to 82 species compared to the (mitochondrial + nuclear) multi-locus dataset of Wollenberg et al. (2011) and added four additional nuclear markers. Whenever possible, we used a single individual for all markers. In some species, we combined sequences from multiple individuals, but only in situations where sequences of the *16S* rRNA gene were available for all individuals and they were identical or very similar (< 1% uncorrected p-distance) in this marker (Table S1). Each marker had varying levels of completeness, and the mean marker completeness (species sampled per marker) was 74% for recognized species (decreasing to 58% when including all candidate species; see Table 1 for complete summary statistics). All markers had 75% species sampling or greater for recognized species, except rhodopsin, which did not show much genetic variation, so we only included data from past studies. Additionally, we added genetic data for outgroup taxa available in GenBank (Table S1), choosing the species from each genus with the most relevant genetic data available. We included 26 outgroup species from all other genera in Mantellidae and five species from Rhacophoridae. All alignments and trees generated were deposited on the Open Science Framework (osf.io/5uqm2).

Table 1
Summary of sampling for genetic markers for *Boophis*. Includes all 77 described species and 35 candidate species (112 total *Boophis* terminals).

Marker	Length (bp)	Number of (ingroup) species sampled	Parsimony-informative sites (with outgroups)	Parsimony-informative sites (without outgroups)
12S	618	69 (63%)	296	250
16S (part 1)	792	76 (69%)	373	288
16S (part 2)	634	107 (97%)	252	206
CO1	625	86 (78%)	272	256
Cyt-b	535	75 (68%)	328	295
ND1	1151	61 (55%)	634	590
DNAH3	909	41 (37%)	90	90
POMC	512	45 (54%)	123	64
RAG1	726	61 (41%)	171	105
RAG2	626	64 (58%)	220	129
Rhod	316	18 (16%)	46	16
Summary	7444 bp	Mean = 58%	2805	2289

2.2. DNA sequencing

We extracted genomic DNA from ethanol-preserved tissues using proteinase K (final concentration 20 mg/ml) and a standard phenol-chloroform protocol. We amplified 10 mitochondrial and nuclear markers (Table 1) using polymerase chain reaction (PCR; markers and primers are included in Table S2).

PCR reactions were performed using the following procedure: 1–2 µl genomic DNA, 0.2 µM of each primer, 200 µM of dNTPs, 1.25 U of OneTaq® DNA polymerase, and 5 µl OneTaq® Standard Reaction Buffer (20 mM Tris-HCl, 22 mM NH_4Cl, 22 mM KCl, 1.8 mM $MgCl_2$, 0.06% IGEPAL® CA-630, and 0.05% Tween® 20). The final volume was brought to 25 µl with nuclease-free water. We visualized amplification success using 2 µl of PCR product on 1.5× agarose gel.

PCR products were purified of excess dNTPs and primers using Shrimp Alkaline Phosphatase (1 U/µl) and Exonuclease I (20 U/µl). We used the remaining 22 µl of PCR product and added 0.025 µl Exonuclease I, 0.250 µl Shrimp Alkaline Phosphatase and 9.725 µl nuclease-free water for a final volume of 32 µl per reaction. Next, we incubated the samples at 37 °C for 30 min and then 95 °C for 5 min in a thermocycler. For cycle sequencing, we conducted two separate reactions for the target 5′ and 3′ end DNA strands per purified PCR product. We prepared a 7 µl sequencing reaction that included 2 µl of the purified PCR product, 1.5 µl of 5X sequencing buffer, 0.5 µl of the forward or reverse primer (separately), 0.5 µl of BigDye Terminator® (version 3.1 sequencing standard, Applied Biosystems) and 2.5 µl of water. The sequence reaction began with 1 cycle at 95 °C for 60 s, and 30 cycles of 95 °C for 15 s, 50 °C for 15 s, and 60 °C for 240 s. In preparation for sequencing, we purified the reaction product of BigDye Terminator® by filtering the product through Sephadex® G-50 in a spin column centrifuged at 850 rcf for 5 min. We collected and visualized sequence data on an Applied Biosystems 3130 automated sequencer. DNA sequences were then manually edited in Geneious R9 (Biomatters, 2016) to trim poor-quality stretches, correct obvious base-calling errors, and identify heterozygous positions.

2.3. Phylogenetic analyses and divergence dating

DNA sequences were aligned in Geneious R9 (Biomatters, 2016) using MAFFT v7 (Katoh and Standley, 2013) and alignments manually inspected for accuracy. The *12S* and *16S* rRNA data were aligned using the Q-INS-I algorithm in MAFFT that considers RNA secondary structure in alignment. We did not remove hyper-variable regions in the *12S*