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Research Article

Molecular taxonomic identification and species-level phylogeny of the narrow-mouthed frogs of the genus *Rhombophryne* (Anura: Microhylidae: Cophylinae) from Madagascar

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The study of diamond frogs (genus *Rhombophryne*, endemic to Madagascar) has been historically hampered by the paucity of available specimens, because of their low detectability in the field. Over the last 10 years, 13 new taxa have been described, and 20 named species are currently recognized. Nevertheless, undescribed diversity within the genus is probably large, calling for a revision of the taxonomic identification of published records and an update of the known distribution of each lineage. Here we generate DNA sequences of the mitochondrial 16S rRNA gene of all specimens available to us, revise the genetic data from public databases, and report all deeply divergent mitochondrial lineages of *Rhombophryne* identifiable from these data. We also generate a multi-locus dataset (including five mitochondrial and eight nuclear markers; 9844 bp) to infer a species-level phylogenetic hypothesis for the diversification of this genus and revise the distribution of each lineage. We recognize a total of 10 candidate species, two of which are identified here for the first time. The genus *Rhombophryne* is here proposed to be divided into six main species groups, and phylogenetic relationships among some of them are not fully resolved. These frogs are primarily distributed in northern Madagascar, and most species are known from only few localities. A previous record of this genus from the Tsingy de Bemaraha (western Madagascar) is interpreted as probably due to a mislabelling and should not be considered further unless confirmed by new data. By generating this phylogenetic hypothesis and providing an updated distribution of each lineage, our findings will facilitate future species descriptions, pave the way for evolutionary studies, and provide valuable information for the urgent conservation of diamond frogs.

Keywords: amphibians, candidate species, diamond frogs, mitochondrial lineages, northern Madagascar, species-identification, systematics

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Introduction

Madagascar is a worldwide hotspot of amphibian diversity (Koo et al., 2013), with 370 currently recognized native species (AmphibiaWeb, 2021). Native Malagasy amphibians belong to five major clades of independent origin: (1) the family Mantellidae Laurent, 1946; (2) the clade made up by the subfamilies Cophylinae Cope, 1889 and Scaphiophryninae Laurent, 1946 (family Microhylidae Günther, 1858); (3) the subfamily Dyscophinae Boulenger, 1882 (family Microhylidae); (4) the genus *Heterixalus* Laurent, 1944 (family Hyperoliidae Laurent, 1943); and (5) the family Ptychadenidae Dubois, 1987 with three mitochondrial lineages of *Ptychadena mascareniensis* (Duméril and Bibron, 1841) sensu lato (Glaw & Vences, 2007; Zimkus et al., 2017). With 114 currently described species (AmphibiaWeb, 2021), the subfamily Cophylinae is the second-largest amphibian radiation of Madagascar, after the mantellid frogs (Glaw & Vences, 2007), and it has been suggested that the clade comprising the Cophylinae and the Scaphiophryninae colonized the island ∼70 million years ago (Hime et al., 2021).

Cophylines exhibit a large variety of natural-history traits and show extreme variation in body size – including species that are among the smallest amphibians (e.g. *Stumpffia contumelia* Rakotoarison et al., 2017, adult snout–vent length (SVL) 8.0–8.9 mm; Rakotoarison et al., 2017) and others that are the largest microhylids in the world (e.g. *Plethodontohyla inguinalis* Boulenger, 1882, adult max SVL up to 100 mm; Glaw & Vences, 2007) – that evolved multiple times independently during their evolutionary history (Scherz et al., 2019). Studying cophylines has been historically difficult, although substantial systematic and taxonomic advances have been achieved over the last 15 years (e.g. Andreone et al., 2005; Scherz et al., 2019, 2016b; Wollenberg et al., 2008). In addition to the identification of several candidate species (Perl et al., 2014; Vieites et al., 2009), several new species of cophylines have been described over the last few years (e.g. Crottini et al., 2020; Rakotoarison et al., 2015, 2017; Rosa et al., 2014), numerous taxonomic changes with generic rearrangements have been proposed (e.g. Bellati et al., 2018; Frost et al., 2006; Wollenberg et al., 2008), two new genera have been named (Scherz et al., 2019, 2016b), and the general inter-generic relationships have been further elucidated (Andreone et al., 2005; Scherz et al., 2019, 2016b; Tu et al., 2018; Wollenberg et al., 2008). To date, the subfamily Cophylinae includes nine genera: *Anilany* Scherz et al., 2016b, *Anodonthyla* Müller, 1892, *Cophyla* Boettger, 1880, *Madecassophryne* Guibé, 1974, *Mini* Scherz et al., 2019, *Platypelis* Boulenger, 1882, *Plethodontohyla* Boulenger, 1882, *Rhombophryne* Boettger, 1880, and *Stumpffia* Boettger, 1881.

Diamond frogs (genus *Rhombophryne*) mostly inhabit rain forests from low to high elevations in northern Madagascar, which corresponds to their centre of diversity and endemism (Glaw & Vences, 2007; Wollenberg et al., 2008). Similar to most cophylines, *Rhombophryne* species are often micro-endemic, with distributions apparently restricted to a single site or a few geographically close localities (Glaw & Vences, 2007; Scherz et al., 2017; Wollenberg et al., 2008). Diamond frogs exhibit lifestyles from largely terrestrial (e.g. *R. coronata* (Vences and Glaw, 2003), *R. vaventy* Scherz et al., 2014, *R. regalis* Scherz et al., 2017) to partly fossorial (e.g. *R. testudo* Boettger, 1880, *R. matavy* D'Cruze et al., 2010), and only a few species show body characteristics that suggest a deviation from these habits (e.g. the rather long-legged and slender *R. longicrus* Scherz et al., 2015a and *R. minuta* (Guibé, 1975); Glaw & Vences, 2007; Scherz et al., 2015a).

Diamond frogs are most closely related to the often-miniaturized species of the genera *Anilany* and *Stumpffia* (Andreone et al., 2005; Peloso et al., 2016; Scherz et al., 2016b; Tu et al., 2018; Wollenberg et al., 2008). However, some *Rhombophryne* species are morphologically more similar to members of the genus *Plethodontohyla* (Andreone et al., 2005; Bellati et al., 2018; Glaw & Vences, 2007; Wollenberg et al., 2008), which has often hampered and confused research on them. In a molecular phylogeny of cophyline frogs, Andreone et al. (2005) was the first to identify the paraphyly of the then more inclusive *Plethodontohyla*. Several species assigned to this genus clustered with *Rhombophryne*, which was, at that time, a monotypic genus including only the type species *R. testudo* (Andreone et al., 2005). Following these results, Frost et al. (2006) assigned *R. alluaudi* (Mocquard, 1901), *R. coudreaui* (Angel, 1938), and *R. laevipes* (Mocquard, 1895) to the genus *Rhombophryne*. Glaw and Vences (2007) suggested four additional reassignments (*R. minuta*, *R. coronata*, *R. guentherpetersi* (Guibé, 1974) and *R. serratopalpebrosa* (Guibé, 1975)). These four reassignments were later confirmed by a new phylogeny of the subfamily Cophylinae (Wollenberg et al., 2008). More recently, *R. matavy* was transferred to *Plethodontohyla* (Peloso et al., 2016) based on mislabelled tissue samples, but it was returned to *Rhombophryne* shortly thereafter (Scherz et al., 2016b). Finally, Bellati et al. (2018) transferred *R. alluaudi* to *Plethodontohyla* after a clarification of the identity of the type material of that species.

In a large-scale assessment of Malagasy amphibians using molecular taxonomic identification, Vieites et al.

(2009) revealed the presence of 10 unnamed lineages within the genus *Rhombophryne*, later increased to 13 candidate species by Perl et al. (2014). Since 2009, 13 new species of *Rhombophryne* have been described (D'Cruze et al., 2010; Glaw et al., 2010; Lambert et al., 2017; Scherz, 2020; Scherz et al., 2016a, 2017, 2019, 2015a, 2014, 2015b) for a total number of 20 currently recognized species (AmphibiaWeb, 2021). This boost in taxonomic and systematic research is explained by the increased availability of specimens hosted in institutional collections, and by the application of an integrative taxonomic approach (Padial et al., 2010). This includes the use of osteological data (from X-ray microcomputed tomography), which has proven a powerful tool to identify diagnostic morphological characters for these species (e.g. Scherz et al., 2017, 2014).

Despite these substantial advances, the level of undescribed diversity in *Rhombophryne* is still high and worthy of investigation. Furthermore, the distribution of most lineages has been poorly documented, and the inter-specific phylogenetic relationships remain poorly understood. The aim of the present study is therefore to (1) generate a comprehensive dataset of 16S rRNA reference sequences for all specimens available to us; (2) reassess and revise the taxonomic assignment of all previously published molecular data belonging to the genus *Rhombophryne*; (3) generate a multi-locus, species-level phylogenetic hypothesis of the genus *Rhombophryne*; (4) identify candidate species and main species groups; and (5) provide a revised distribution for each lineage.

Materials and methods

Laboratory procedures

Genomic DNA was extracted from 37 tissue samples (Supplemental Table S1). following the proteinase K and saline solution protocol described by Bruford et al. (1992). We amplified DNA for 12 gene fragments comprising both mitochondrial and nuclear markers (Supplemental Table S2). Mitochondrial markers included the 3′ and 5′ termini of the 16S rRNA gene (16S3′ and 16S5′, respectively), cytochrome oxidase I (COI), 12S rRNA (12S), and Cytochrome b (Cytb). For the nuclear markers, we amplified seven fragments: brain-derived neurotrophic factor (BDNF), pro-opiomelanocortin (POMC), recombination activating gene 2 (RAG2), two non-overlapping portions of sacsin (SACS-A and SACS-B), leucine-rich repeat and WD repeat-containing protein (KIAA1239), and titin (TTN).

PCR amplifications were performed in a total volume of 25 µl: 12.5 µl Milli-Q water, 5 µl 5× Green GoTaq Flexi Buffer (Promega, Madison, US), 4 µl 25 mM $MgCl_2$ (Promega, Madison, USA), 1 µl of forward and reverse primers (10 pM) (Thermo Fisher Scientific, Waltham, USA), 0.4 µl dNTPs (10 mM) (Invitrogen, Waltham, USA), 0.1 µl 5 U/µl GoTaq Flexi DNA Polymerase (Promega, Madison, USA), and 1 µl of extracted DNA. Amplifications of the KIAA1239, TTN, SACS-A, SACS-B, and RAG2 fragments were performed with a nested PCR approach. The first amplification was performed in half of the standard volume (12.5 µl) described above, while the second amplification was executed in a volume of 25 µl including: 13.3 µl Milli-Q water, 5 µl 5× Green GoTaq Flexi Buffer, 4 µl 25 mM $MgCl_2$, 1 µl of forward and reverse primers (10 pM), 0.4 µl dNTPs (10 mM), 0.1 µl 5 U/µl GoTaq Flexi DNA Polymerase, and 0.2 µl of amplified DNA from the first reaction. See Supplemental Table S3 for information on thermal profile and primers sequences and references. The sequencing was performed on an ABI 3730XL automated sequencer at Macrogen Inc. (Spain). Chromatograms were examined, and sequences corrected where necessary, with BioEdit 7.2.6 (Hall, 1999). Newly generated sequences were deposited in GenBank (OL780539–OL780550; OL780555–OL780562; OL780587–OL780593; OL780564–OL780586; OL780594–OL780609; OL790123–OL790137; OL853686–OL853704; (Supplemental Tables S1 and S2).

Abbreviations used

ACP and MVTIS refer to sample extraction codes and field numbers, respectively. AMNH (American Museum of Natural History, New York City, USA), KU (Biodiversity Institute of the University of Kansas, Lawrence, USA); MRSN (Museo Regionale di Scienze Naturali, Torino, Italy), and ZSM (Zoologische Staatssammlung, Munich, Germany) refer to institutional catalogue abbreviations.

We refer to candidate species with the consecutive numbering system introduced by Vieites et al. (2009) for Malagasy frogs, with the refinement of Perl et al. (2014) in which each candidate species number is preceded by 'Ca'.

Molecular taxonomic identification

We first compiled a dataset for species-identification that included all individuals of *Rhombophryne* with associated molecular data, comprising both sequences deposited in GenBank and newly generated sequences (see Laboratory procedures section and Supplemental Table S1). This dataset included the three mitochondrial markers commonly used for molecular species

identification in Malagasy amphibians: 16S3′, 16S5′, and COI (Perl et al., 2014; Vences et al., 2003; Vieites et al., 2009) (Supplemental Table S1). We primarily used the 16S3′ gene for species identification, as this marker has the largest barcoding reference database for Malagasy amphibians (Vieites et al., 2009), and we used the numerous sequences of COI and 16S5' that we generated to refine specimen identifications when 16S3′ was not available.

We retrieved field collection information for all specimens and assigned each of them to a mitochondrial lineage using an inter-specific threshold of 3% genetic distance at the 16S3′ marker (following Vieites et al., 2009). To facilitate specimen identification and their assignment to mitochondrial lineages, we computed a Neighbour-joining tree based on individual uncorrected *p*-distances at the 16S3′ and produced a matrix of uncorrected *p*-distances averaged over conspecific individuals between the identified inter-specific lineages with MEGA X 10.0.5 (Kumar et al., 2018). Among the described species of the genus *Rhombophryne*, molecular data are unavailable only for *R. serratopalpebrosa* (Scherz et al., 2017, 2014; Supplemental Table S1).

Phylogenetic analyses

We compiled a species-level multi-locus matrix for the genus *Rhombophryne* including all mitochondrial lineages that have been previously identified (see Molecular taxonomic identification section; Supplemental Tables S1 and S2). Conspecific individuals were merged into single Operational Taxonomic Units, and we included 10 gene fragments in addition to the three markers used for species-identification, comprising both publicly available and newly generated sequences (Supplemental Table S2), for a total of 13 gene fragments and 31 ingroup taxa. We selected four outgroups from the genera *Anilany* and *Stumpffia*, which are the phylogenetically closest cophylines to *Rhombophryne* (Scherz et al., 2016b; Tu et al., 2018; Supplemental Table S2). Within the ingroup, we included three taxa that show less than 3% genetic distance to the closest lineage (*R.* sp. Ca03, *R.* cf. *vaventy* and *R.* cf. *nilevina*) because these taxa will need to be analysed morphologically to ascertain their taxonomic status (see Results section; Table 1).

The following steps were performed in R 4.0.2 using PipeLogeny (Muñoz-Pajares et al., 2019; R Core Team, 2020), a pipeline implementing some commonly used phylogenetic tools and facilitating the creation of input files for each of them. Sequences were aligned with MAFFT 7.310 (Katoh & Standley, 2013) with option L-INS-i. The best-fitting partitioning scheme was inferred on the concatenated alignment with PartitionFinder2 2.1.1 (Lanfear et al., 2017), implemented on the CIPRES Science Gateway (Miller et al., 2010), using the Bayesian Information Criterion and the greedy search algorithm. Phylogenetic inferences were performed with MrBayes 3.2.7 (Ronquist et al., 2012) on CIPRES. We executed two parallel runs of 80 million generations, each consisting of four Markov Chain Monte Carlo chains sampling at every 1000 generations, and we applied a 40% burn-in on the resulting trees and posterior distributions. The remaining posterior samples were retained to generate the 50% majority rule consensus tree that was visualized with FigTree 1.4.4 (Rambaut, 2009). *Stumpffia psologlossa* Boettger, 1881, the type species of the genus *Stumpffia*, was selected as outgroup to root the tree. We used Tracer 1.7.1 (Rambaut et al., 2018) to evaluate run convergence and posterior distributions for each prior, considering a standard minimum threshold of 200 for the Effective Sampling Size.

Results

Molecular taxonomic identification

Our revised taxonomic identification of 80 samples (Supplemental Table S1; Supplemental Fig. S1; the 16S3′ alignment is available in the online supplemental material) revealed the existence of 28 inter-specific mitochondrial lineages (Supplemental Table S2) showing more than 3% genetic distance among them (Table 1). Some of these lineages are shown in Fig. 1. This list of 28 lineages includes all currently recognized nominal species with available genetic data (19) and nine candidate species. Among the candidate species, three meet the criteria proposed by Vieites et al. (2009) to be recognized as confirmed candidate species (lineages characterized by a detectable genetic differentiation from all described species and with at least one additional line of evidence that supports their distinctness): *Rhombophryne* sp. Ca09, *R.* sp. Ca17, and *R.* sp. Ca19, all showing distinct morphological differences to all other nominal species of the genus *Rhombophryne*. Six are unconfirmed candidate species (lineages identified by a detectable genetic differentiation to all described species but with data deficiency in morphology, ecology, and distribution): *R.* sp. Ca01, *R.* sp. Ca07, *R.* sp. Ca10, *R.* sp. Ca13, *R.* sp. Ca15, and *R.* sp. Ca16. Candidate species numbers from Ca13 to Ca19 are newly established here. Among the candidate species, lineages *R.* sp. Ca15 and *R.* sp. Ca17 are identified here for the first time, while the remaining lineages had been identified previously, but were not referenced following

Table 1. Uncorrected *p*-distances (16S rRNA 3′ terminus) of the analysed lineages.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	*R. botabota* (6)	**1.2**																														
2	*R. coronata* (2)	11.0	**0.0**																													
3	*R. coudreaui* (4)	9.9	13.3	**0.5**																												
4	*R. diadema* (2)	10.2	9.5	11.6	**0.0**																											
5	*R. ellae* (1)	7.8	13.0	12.8	11.3	**NA**																										
6	*R. guentherpetersi* (2)	7.9	8.6	11.6	6.0	9.4	**0.8**																									
7	*R. laevipes* (2)	6.4	11.5	12.2	10.0	8.9	9.7	**0.0**																								
8	*R. longicrus* (2)	8.9	12.2	11.1	11.0	10.0	8.6	9.7	**0.0**																							
9	*R. mangabensis* (6)	10.3	14.5	14.7	14.3	13.3	13.5	12.2	13.3	**0.1**																						
10	*R. matavy* (2)	11.4	13.8	15.2	13.9	12.3	13.9	10.9	11.5	14.9	**0.0**																					
11	*R. minuta* (2)	8.7	12.1	12.7	9.5	12.7	10.3	10.4	7.2	12.1	12.9	**0.2**																				
12	*R. nilevina* (1)	3.8	10.5	10.4	10.1	7.8	8.3	6.9	9.4	11.2	11.5	10.7	**NA**																			
13	*R. ornata* (3)	8.7	10.7	13.4	6.1	9.2	4.2	10.1	10.3	13.9	12.4	9.3	9.5	**0.1**																		
14	*R. proportionalis* (2)	8.2	10.6	11.6	10.2	10.0	7.4	9.3	10.9	13.0	11.5	11.6	8.4	9.3	**0.0**																	
15	*R. regalis* (1)	8.9	9.2	12.7	6.7	12.5	7.0	10.3	10.4	13.5	13.9	11.1	9.2	7.5	9.3	**NA**																
16	*R. savaka* (2)	9.5	12.4	13.8	13.2	12.0	11.9	11.6	13.6	8.8	14.6	11.8	9.9	12.4	12.1	13.2	**0.0**															
17	*R. tany* (1)	8.2	8.8	12.2	5.8	10.3	5.5	10.7	10.3	13.4	12.9	9.7	8.0	5.3	9.3	7.6	12.1	**NA**														
18	*R. testudo* (2)	9.0	13.2	14.4	12.1	9.9	11.6	9.0	11.7	11.9	9.4	12.2	9.7	11.0	11.6	12.7	11.2	11.3	**1.6**													
19	*R. vaventy* (1)	8.8	10.6	12.7	6.7	11.1	6.0	9.1	10.6	14.2	13.5	11.1	9.2	5.3	10.3	7.4	13.5	7.5	12.5	**NA**												
20	*R.* sp. Ca01 (3)	4.9	10.5	10.2	10.7	9.0	8.1	7.9	8.6	11.0	11.0	9.7	4.7	11.2	7.7	9.3	10.6	9.5	9.4	10.1	**0.0**											
21	*R.* sp. Ca03 (4)	3.9	10.2	10.0	10.0	7.9	8.1	6.7	9.5	11.0	11.6	9.2	1.8	9.8	8.1	8.7	9.8	7.7	9.3	8.3	4.7	**0.1**										
22	*R.* sp. Ca07 (5)	8.0	10.4	12.5	11.0	6.3	9.3	7.7	10.1	13.3	11.9	11.3	8.0	9.3	9.9	10.4	13.5	10.4	11.0	9.3	9.0	8.4	**0.3**									
23	*R.* sp. Ca09 (3)	9.6	10.5	10.0	12.7	12.5	12.4	11.7	15.0	12.8	14.4	13.1	9.2	13.7	12.4	13.3	11.0	11.5	12.5	12.3	9.8	10.0	12.0	**0.5**								
24	*R.* sp. Ca10 (2)	7.8	11.6	12.8	10.6	9.1	8.9	9.2	10.9	14.2	12.3	12.2	8.1	9.3	9.4	10.5	12.9	9.8	11.5	10.0	8.8	8.1	9.3	12.6	**1.6**							
25	*R.* sp. Ca13 (1)	10.8	3.4	13.5	9.8	12.0	8.8	9.8	12.3	14.3	13.0	12.3	10.1	10.5	9.8	8.5	13.5	9.2	12.3	8.7	10.1	10.3	11.0	12.0	11.4	**NA**						
26	*R.* sp. Ca15 (1)	8.5	11.7	13.1	11.1	9.9	9.5	10.6	12.2	14.2	13.5	13.0	7.6	9.7	9.8	10.8	12.6	10.3	12.9	10.8	9.6	8.1	9.6	13.5	3.6	12.2	**NA**					
27	*R.* sp. Ca16 (1)	8.2	12.6	12.4	13.1	10.3	11.5	10.6	13.2	8.0	12.1	11.9	9.3	12.5	11.6	12.4	9.5	10.5	10.7	12.9	9.5	9.5	11.1	9.5	12.5	12.8	12.7	**NA**				
28	*R.* sp. Ca17 (1)	11.3	14.1	12.0	15.0	13.5	13.3	12.5	14.6	10.3	15.1	13.7	11.2	14.9	11.8	14.4	10.9	13.2	12.4	14.2	11.1	11.0	14.4	11.8	15.4	14.7	15.1	9.3	**NA**			
29	*R.* cf. *vaventy* (1)	7.8	10.1	11.4	7.2	10.0	5.2	8.7	9.3	13.6	12.5	10.6	8.0	4.9	9.5	7.0	12.4	7.0	11.5	1.5	9.0	7.5	8.6	11.8	10.0	8.7	10.5	12.1	13.1	**NA**		
30	*R.* sp. Ca19 (5)	3.6	10.1	10.2	9.7	8.3	8.0	7.0	8.8	10.5	11.0	9.4	4.0	9.3	8.5	8.8	9.8	8.7	8.7	8.5	3.8	4.8	7.6	8.3	8.3	9.7	9.4	8.3	11.5	7.5	**1.3**	
31	*R.* cf. *nilevina* (2)	3.6	9.6	10.7	10.2	8.0	8.4	6.6	9.9	10.5	12.2	10.0	1.4	9.8	8.8	8.9	10.9	8.0	10.1	8.4	5.1	2.0	8.3	10.4	7.7	9.8	8.0	9.4	11.5	7.8	4.4	**0.0**

Values are expressed in percentage and were computed between (below the diagonal) and within (in bold along the diagonal) lineages averaging over conspecific individuals (number shown beside species name). When only one individual of a lineage was available, within *p*-distances were not computed (NA). In grey are highlighted values of between-lineage distances that have an inter-specific genetic distance lower than 3%. See Supplemental Table S1 for more information on the analysed individuals.

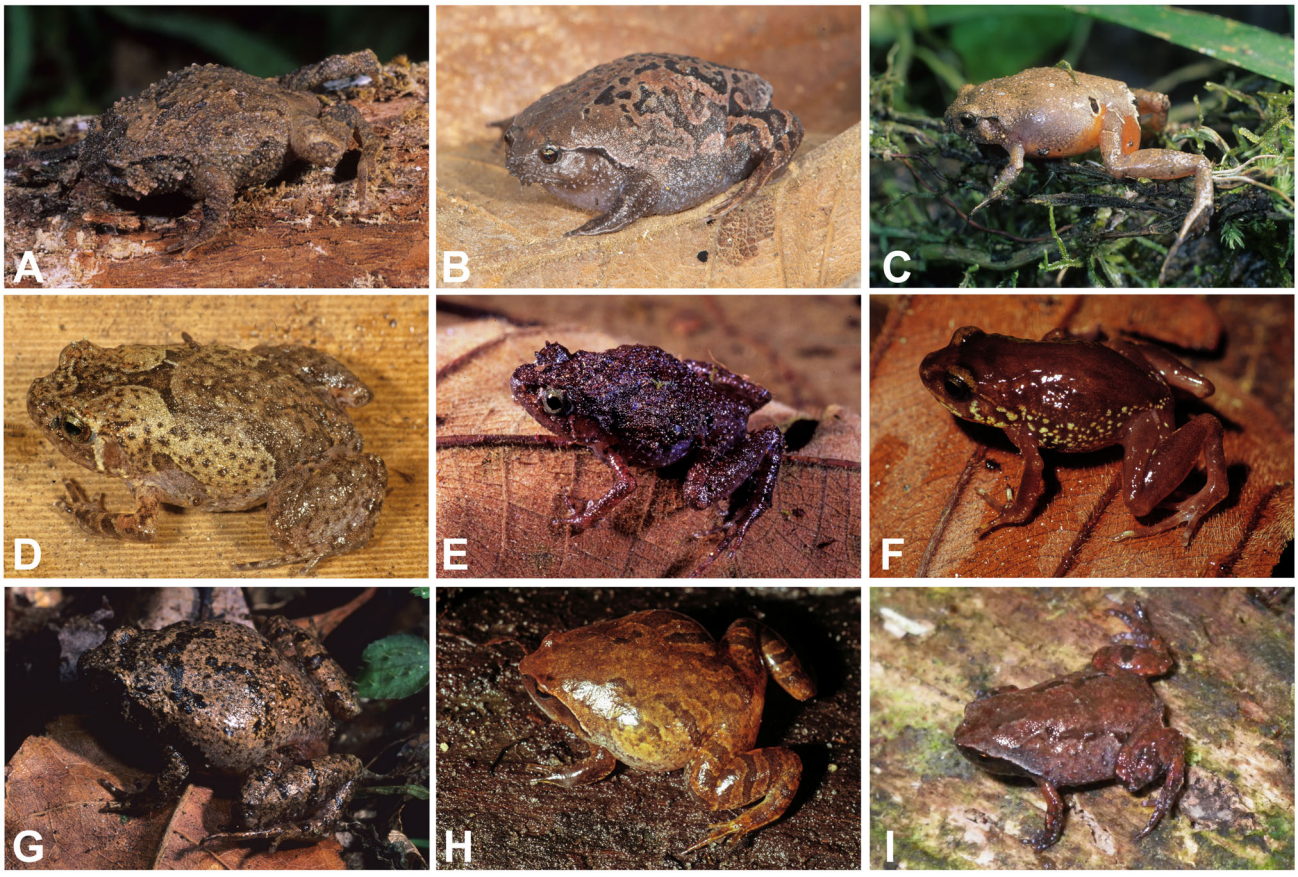

Fig. 1. An overview of the morphological diversity of *Rhombophryne* species: (A) *Rhombophryne* sp. Ca09, Masoala; (B) *Rhombophryne testudo*, Nosy Be (FGZC 5620); (C) *Rhombophryne* sp. Ca07, Tsaratanana; (D) *Rhombophryne coronata*, Ankeniheny (ZFMK 57459); (E) *Rhombophryne regalis*, Ambolokopatrika; (F) *Rhombophryne minuta*, Marojejy; (G) *Rhombophryne* sp. Ca19, Tsararano (MRSN A2620); (H) *Rhombophryne botabota*, Marojejy (ZSM 358/2005); (I) *Rhombophryne proportionalis*, Tsaratanana (ZSM 1826/2010). (Photograph credits: A, C, E, G: F. Andreone; B: M. D. Scherz; D, F, H: F. Glaw and M. Vences; I: M. Vences).

the consecutive numbering system proposed by Vieites et al. (2009).

We identified three lineages (*R.* sp. Ca03, *R.* cf. *vaventy*, and *R.* cf. *nilevina*) with less than 3% genetic distance from any other lineage (Table 1). We preferred to treat them separately for the following reasons: (1) *Rhombophryne* sp. Ca03 was established as a candidate species by Vieites et al. (2009). This lineage has a 16S3′ genetic distance of 1.8% to *R. nilevina* Lambert et al., 2017, and of 2.0% to *R.* cf. *nilevina* (Table 1). However, morphologically *R.* sp. Ca03 shows some differences to *R. nilevina*, especially in colouration, where *R.* sp. Ca03 has white ocelli in its inguinal region and on its posterior thighs, and this colouration is lacking from *R. nilevina*. We emphasize that it may be recognized in the future as a deep conspecific lineage (sensu Vieites et al., 2009) of *R. nilevina*; (2) *R.* cf. *nilevina* has an uncorrected *p*-distance of 1.4% from *R. nilevina* (Table 1). Specimens of *R.* cf. *nilevina* conform with *R. nilevina* morphologically, but their sampling localities are separated by ∼800 km; (3) *R.* cf. *vaventy* has an uncorrected *p*-distance of 1.5% from *R. vaventy* (Table 1). Because we could not examine the specimen AMNH A167315, it was impossible to assess its morphological identity with respect to *R. vaventy*. The status of each of these three lineages should be thoroughly investigated in the future.

The analyses of some published records revealed a few inconsistencies:

(1) the 16S3′ accession FJ559293, associated with field number MVTIS 2001E50, a juvenile frog assigned in the field to '[*Rhombophryne*] cf. *alluaudi*' from Manarikoba (Tsaratanana), was assigned to *Plethodontohyla* sp. 2 by Vieites et al. (2009). However, the ∼13% uncorrected *p*-distance between FJ559293 and a 16S3′ sequence newly generated from the same specimen (ACP3231; GenBank accession number OL780572) suggests that sampling information for FJ559293 was probably confused with another specimen. FJ559293 is almost identical (∼0.3% uncorrected

p-distance) with two accession numbers assigned to individuals of *P. brevipes* Boulenger, 1882 (AY594113; EU341063) and should therefore be assigned to this species. Our newly generated sequence of MVTIS 2001E50 is almost identical to the sequences generated from the specimens MRSN A2631 (AY594107; 99.8% similarity) and ZSM 667/2001 (FJ559296; 99.75%), both collected from Antsahamanara (Tsaratanana), and is here assigned to *R.* sp. Ca03.

(2) Andreone and Randrianirina (2008) reported the presence of *Rhombophryne* from Andamozavaky (Tsingy de Bemaraha), the only record of the genus from western Madagascar. Specimen MRSN A5524 was identified as *R. coudreaui* based on morphological similarities with this species (Andreone & Randrianirina, 2008). We generated a 16S3′ sequence of this specimen (MRSN A5524; OL780579), which is identical to the 16S3′ sequence of MRSN A2115 (AY594110) and MRSN A2497 (OL780578) assigned to *R.* sp. Ca09. This candidate species is known only from the region of Masoala (Ambatoledama and Amparihy), in the northeast (Fig. 2), ∼670 km away from Tsingy de Bemaraha. In addition, a specimen morphologically assignable to the *Plethodontohyla ocellata* Noble and Parker, 1926 species complex (MRSN A5476; OL780586; 16S3') was also allegedly collected in the same expedition to Tsingy de Bemaraha. The sequence of this specimen was found to be almost identical to the sequences of several other specimens assigned to *Plethodontohyla ocellata* from multiple localities of the Masoala Peninsula. We have inspected the field book of the expedition to Tsingy the Bemaraha, and after failing to find notes on the sampling of these two specimens, together with the fact that Tsingy de Bemaraha and Masoala were surveyed in two consecutive years by the same team, we believe that during long-term storage of this material, some specimens collected in Masoala have been erroneously placed into the jar containing the specimens of Tsingy de Bemaraha, generating confusion with regard to the sampling site of this material (MRSN A5524 and MRSN A5476). The geographic occurrence of *R.* sp. Ca09 from Tsingy de Bemaraha is, therefore, considered erroneous and excluded from the distribution maps of this lineage (Fig. 2).

(3) In the description of *R. nilevina*, Lambert et al. (2017) report both institutional collection codes KU 340893 and KU 340897 as the holotype, and associated the only sequence generated for this species (KY288475; 16S3′) to KU 340893. After consulting with the authors, we confirm that the code KU 340897 corresponds to the specimen number of the holotype of *R. nilevina* and that the accession KY288475 was generated from this individual, whereas the code KU 340893 is not associated with any specimens of this species.

Phylogenetic analyses

The species-level multi-locus matrix included 9844 bp (Supplemental Table S2; the concatenated alignment with MrBayes data block and the original file of the 50% majority rule consensus tree are available in the online supplemental material). The best-fitting partition scheme comprised eight partitions (Supplemental Table S4). In interpreting the results of the phylogenetic analyses, we define as strong, moderate, and weak support the following values of Posterior Probability (PP): > 0.98 PP, > 0.95–0.97 PP, and 0.90–0.94 PP, respectively.

In this study, we propose the establishment of six species groups that can be morphologically identified (only the *R. serratopalpebrosa* species group has been previously formally defined (Scherz et al., 2015b)). Five of these species groups form clades with maximal statistical support (PP = 1.00) (Fig. 3): (1) the *R. testudo* species group including *R. testudo*, *R. coudreaui*, *R. mangabensis* Glaw et al., 2010, *R. matavy*, *R. savaka* Scherz et al., 2016a, *R.* sp. Ca09, *R.* sp. Ca16, and *R.* sp. Ca17; (2) the *R. ellae* species group comprising *R. ellae* Scherz, 2020 and *R.* sp. Ca07; (3) the *R. serratopalpebrosa* species group comprising *R. guentherpetersi*, *R. coronata*, *R. diadema* Scherz et al., 2017, *R. ornata* Scherz et al., 2015b, *R. regalis*, *R. tany* Scherz et al., 2015b, *R. vaventy*, *R.* cf. *vaventy*, and *R.* sp. Ca13; (4) the *R. minuta* species group comprising *R. minuta* and *R. longicrus*; (5) the *R. laevipes* species group, which contains the largest number of candidate species, and comprises *R. laevipes*, *R. botabota* Scherz et al., 2016a, *R. nilevina*, *R.* cf. *nilevina*, *R.* sp. Ca01, *R.* sp. Ca03, *R.* sp. Ca10, *R.* sp. Ca15, and *R.* sp. Ca19. The sixth species group (*R. proportionalis* species group) includes only *R. proportionalis* Scherz et al., 2019, whose phylogenetic relationships remained very poorly resolved (PP = 0.84 supports the sister relationship of *R. proportionalis* with the *R. laevipes* species group; Fig. 3).

The *R. ellae* species group was retrieved (without statistical support; PP = 0.84) as sister to the clade composed of the *R. minuta* and *R. serratopalpebrosa* species groups (Fig. 3). The sister relationship between the *R. minuta* and the *R. serratopalpebrosa* species groups received full support (PP = 1.00), as in previous works (e.g. Scherz et al., 2017). In the *R. testudo* species group, *R.* sp. Ca09 was found to be the sister species of *R. coudreaui* (PP = 1.00). Together, they are sister to a weakly supported clade (PP = 0.90),

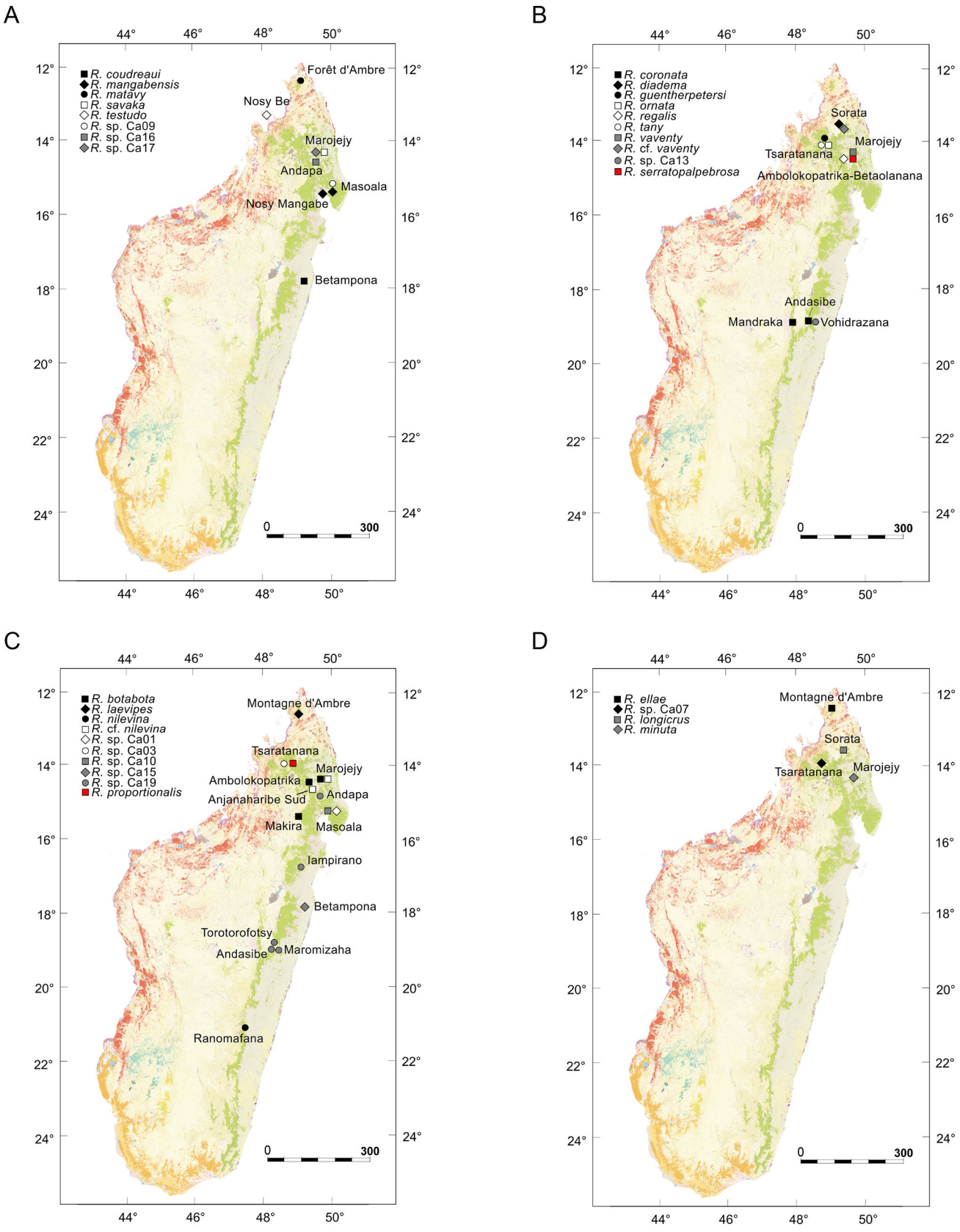

Fig. 2. Distribution maps of each of the analysed lineages of *Rhombophryne* sorted by species groups: (A) *R. testudo* species group; (B) *R. serratopalpebrosa* species group; (C) *R. laevipes* and *R. proportionalis* species groups; (D) *R. ellae* and *R. minuta* species groups. The background map displays remaining primary vegetation (Moat & Smith, 2007): rain forest (green), deciduous dry forest (reddish), arid spiny forest (orange), western sub-humid forest (blueish), and tapia forest (yellowish). Coordinates are provided in Supplemental Table S1. Scale bar represents 300 km.

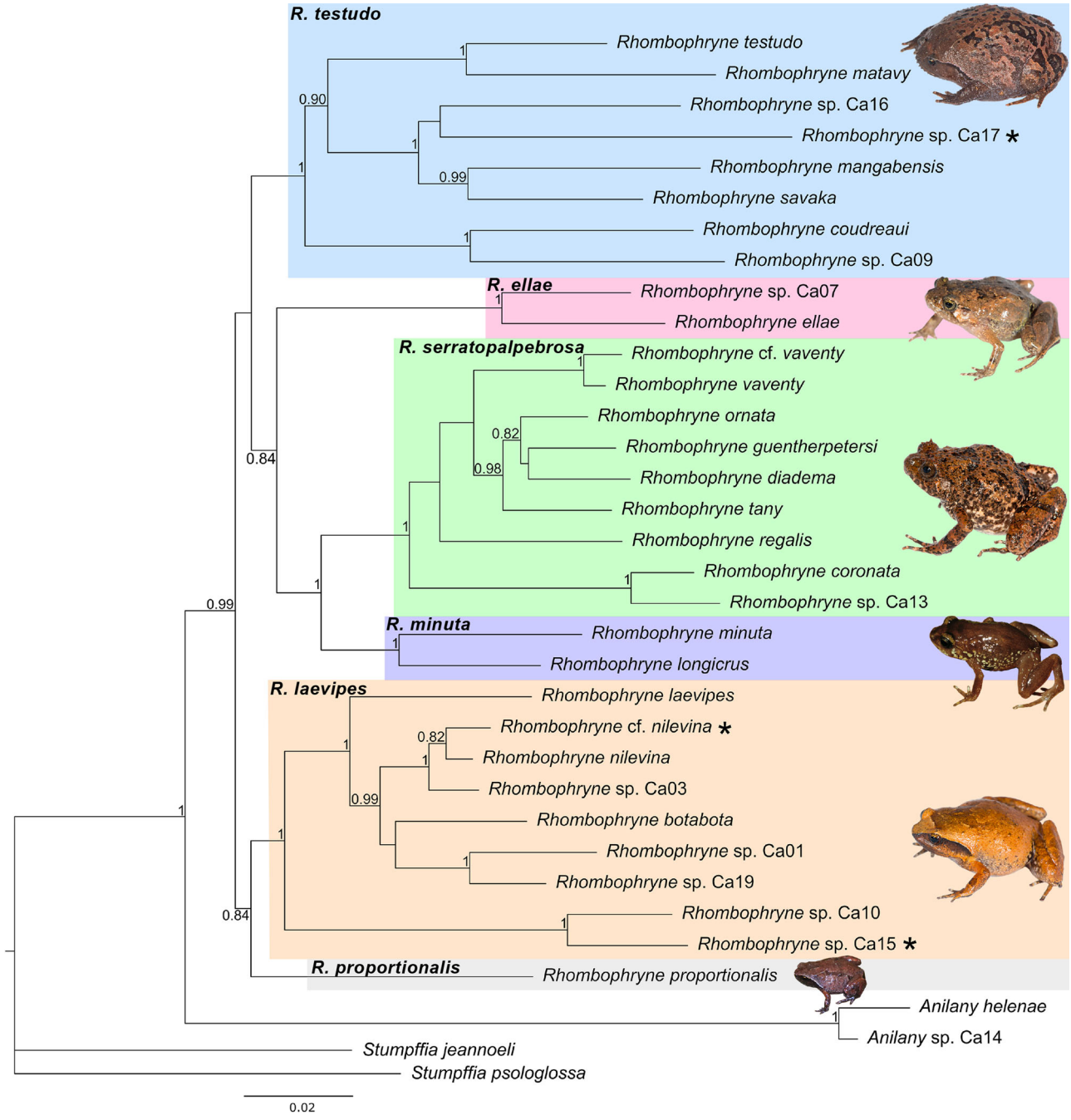

Fig. 3. Concatenated multigene (50% majority rule consensus) phylogenetic tree of the genus *Rhombophryne* inferred using MrBayes based on 9844 bp from mitochondrial and nuclear gene fragments. Bayesian posterior probability values are shown at corresponding nodes and values below 0.80 are not displayed. The two newly discovered candidate species and *R.* cf. *nilevina*, which were molecularly characterized for the first time in this study, are marked with an asterisk. Names of the six species groups are in bold in the top left corner of each colour-coded clade. Photographs show one representative for each defined species group (in descending order): *R. testudo*, *R. ellae*, *R. vaventy*, *R. minuta*, *R. laevipes*, and *R. proportionalis*. Supplemental Table S2 provides all dataset information, including accession numbers and sample codes for each gene and analysed lineage. Photographs of *R. proportionalis* and *R. minuta* by M. Vences and F. Glaw; photographs of *R. testudo*, *R. ellae*, *R. vaventy*, and *R. laevipes* by M.D. Scherz.

including *R. testudo* and *R. matavy* (found to be sister species; PP = 1.00), and *R. mangabensis*, *R. savaka*, *R.* sp. Ca16, and *R.* sp. Ca17 (PP = 1.00). In the latter clade the sister relationship between *R. mangabensis* and *R. savaka* is strongly supported (PP = 0.99). Within the *R. serratopalpebrosa* species group, *R. coronata* is

the sister species of *R.* sp. Ca13 (PP = 1.00) and together they are sister to all other members of this group. *Rhombophryne vaventy* is sister of *R.* cf. *vaventy* (PP = 1.00). *Rhombophryne tany*, *R. ornata*, *R. diadema*, and *R. guentherpetersi* form a strongly supported clade (PP = 0.98), whose internal relationships remain unresolved (Fig. 3). In the *R. laevipes* species group, *R.* sp. Ca10, and *R.* sp. Ca15 form a clade (PP = 1.00), which is sister to a clade containing all the other species of this group (PP = 1.00) and where *R. laevipes* is sister to *R.* cf. *nilevina*, *R. nilevina*, *R.* sp. Ca03, *R. botabota*, *R.* sp. Ca01, and *R.* sp. Ca19 (PP = 0.99). In this clade, *R.* sp. Ca01 is found to be the sister species of *R.* sp. Ca19 with full support (PP = 1.00), and *R. nilevina*, *R.* cf. *nilevina* and *R.* sp. Ca03 form another strongly supported clade (PP = 1.00) (Fig. 3).

Distribution

The list of distributional records for all identified lineages is presented in Supplemental Table S1. Species records in the genus *Rhombophryne* are predominantly limited to a single locality (Fig. 2). Northern Madagascar is the distributional centre of the genus, both in terms of number of taxa and absolute number of occurrences, as previously highlighted by Wollenberg et al. (2008) for a smaller geographic dataset. Some species are restricted to the central east (*R. coudreaui*, *R. coronata*, *R. nilevina,* *R.* sp. Ca10, *R.* sp. Ca13, and *R.* sp. Ca15). *Rhombophryne nilevina* represents the southernmost record for the genus and, if *R.* sp. Ca03 and *R.* cf. *nilevina* are confirmed to be conspecific, this species will be the one with the largest distributional range.

Discussion

Knowledge of the genus *Rhombophryne* improved remarkably over the last 15 years (D'Cruze et al., 2010; Frost et al., 2006; Glaw et al., 2010; Glaw & Vences, 2007; Lambert et al., 2017; Scherz, 2020; Scherz et al., 2016a, 2017, 2019, 2015a, 2014, 2015b; Wollenberg et al., 2008). The secretive habits of these animals and, consequently, their low detectability in the field, have historically resulted in a paucity of available specimens (Supplemental Table S1) and a limited knowledge of their natural history (including bioacoustics; Glaw et al., 2010; Glaw & Vences, 2007; Scherz, 2020), which has, in turn, hampered systematic and taxonomic research of this genus. Indeed, it is not uncommon to find new species in relatively well-surveyed areas, as exemplified by the recent description of *R. ellae* from Montagne d'Ambre (Scherz, 2020), *R. nilevina* from Ranomafana (Lambert et al., 2017), and by the identification of *R.* sp. Ca15 in Betampona and *R.* sp. Ca17 in Marojejy; all these sites have been the subject of extensive herpetological surveys over the last 20 years (e.g. Rosa et al., 2012; Vieites et al., 2009). Interestingly, the only adult known individual of *R. ellae* was found during intense rains associated with a cyclone, similar to the conditions under which *R. nilevina* was found in Ranomafana, suggesting that these animals might be active on the surface for a limited amount of time and under unpredictable and exceptional climatic conditions (Lambert et al., 2017; Scherz, 2020). This might be because they are extremely secretive and leave their refugia (e.g. holes under roots, under thick leaf litter or underneath rotten logs) only under certain conditions, or because their refugia may get filled with water and they are forced to leave them during heavy rain. They may also be highly philopatric, always returning to their exact refuge, further decreasing their detection probability, although there is no direct evidence for any of these hypotheses.

By increasing the number of available sequences for all lineages of the genus *Rhombophryne* (populating the existing multi-locus matrix of available sequences for this group; Andreone et al., 2005; Peloso et al., 2016; Scherz et al., 2016b; Tu et al., 2018; Wollenberg et al., 2008), we have here provided a new hypothesis for the interspecific phylogenetic relationships of this genus which included all described and candidate species with available molecular data that have been identified until now (Fig. 3). By revising the taxonomic identification of all genetic records, we have provided a curated reference dataset that will facilitate the identification of new candidate species of the genus. Our study revealed that, with 20 described species (AmphibiaWeb, 2021), one third of the diversity of *Rhombophryne* is still scientifically unnamed. Among the candidate species, several show clear morphological differences to all other currently described species and will be formally named and described in the near future.

Considering the generally restricted distributions and micro-endemism of the species within this genus (Fig. 2), it is possible that the evaluation of the conservation status of the candidate species against IUCN Red listing criteria will determine an assignment of these lineages within the threatened categories (Vulnerable, Endangered, and Critically Endangered). Formally describing these taxa will be an important step towards granting them formal protection.

Although testing hypotheses on the diversification of the genus within a statistical framework is still hampered by the lack of statistical support for several nodes (Fig. 3; Huelsenbeck et al., 2001; Rabosky, 2015), this limitation can potentially be overcome in the future by applying target-enriched DNA sequencing. A more

robust phylogenetic hypothesis, if coupled with a targeted fieldwork aimed at collecting information on their natural history, will help disentangle their evolution and explore the association between their diversification and the evolution of their ecomorphological adaptations (Glaw & Vences, 2007). For instance, it might be possible to test if the evolution of a terrestrial lifestyle, which seems to be shared by most lineages of the *R. serratopalpebrosa* species group (Scherz et al., 2017, 2014; Vences and Glaw, 2003), could be related to an increase in diversification rate in this relatively species-rich clade. Alternatively, the evolution of a long-legged and slender phenotype in the *R. minuta* species group (Glaw & Vences, 2007; Scherz et al., 2015a) could correlate to decreasing diversification rates in this relatively species-poor clade. In addition, the newly revised distributional patterns identified for this genus could be tested in a phylogenetic framework to investigate a possible influence of the large environmental variability of northern Madagascar (where most lineages are found; Fig. 2) on the evolution of the pattern of microendemism observed in *Rhombophryne*.

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Disclosure statement

The authors have no conflict of interest to declare.

Supplemental material

Supplemental material for this article can be accessed here: http://dx.doi.org/10.1080/14772000.2022.2039320.

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