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Reconciling molecular phylogeny, morphological divergence and classification of Madagascan narrow-mouthed frogs (Amphibia: Microhylidae)

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ABSTRACT

A recent study clarified several aspects of microhylid phylogeny by combining DNA sequences from Sanger sequencing and anchored phylogenomics, although numerous aspects of tree topology proved highly susceptible to data partition and chosen model. Although the phylogenetic results of the study were in conflict with previous publications, the authors made several changes to the taxonomy of Madagascar's cophyline microhylids. We re-analyzed part of their data together with our own molecular and morphological data. Based on a supermatrix of 11 loci, we propose a new phylogeny of the Cophylinae, and discuss it in the context of a newly generated osteological dataset. We found several sample misidentifications, partially explaining their deviant results, and propose to resurrect the genera *Platypelis* and *Stumpffia* from the synonymy of *Cophyla* and *Rhombophryne*, respectively. We provide support for the previous genus-level taxonomy of this subfamily, and erect a new genus, *Anilany* gen. nov., in order to eliminate paraphyly of *Stumpffia* and to account for the osteological differences observed among these groups. Deep nodes in our phylogeny remain poorly supported, and future works will certainly refine our classification, but we are confident that these will not produce large-scale rearrangements.

1. Introduction

Narrow-mouthed frogs of the family Microhylidae are a species-rich and cosmopolitan group of anurans mainly distributed in the tropics. Many microhylids are characterized by their specialized hydrostatic tongue (Meyers et al., 2004), larval morphology (Wassersug, 1984, 1989; Wassersug and Pyburn, 1987; McDiarmid and Altig, 1999; Haas, 2003; Grosjean et al., 2007; Roelants et al., 2011), and osteology (e.g., Parker, 1934; Trueb et al., 2011). These specializations, especially the frequent reductions of skeletal elements, led to the description of a large number of supraspecific units in this family. In consequence, narrow-mouthed frogs have had fewer species per genus and more monotypic genera than does any other species-rich anuran clade (Van der Meijden et al., 2007). Species contents of many genera have increased over the last decade, with intensive taxonomic revisions leading to the description of numerous new species of microhylids. Currently, 582 species are distinguished, allocated to 60 genera (AmphibiaWeb, 2016, accessed February 2016), compared to 400 species in 64 genera in 2007 (with currently 14 vs. formerly 22 monotypic genera).

Monophyly of the Microhylidae and its placement among neobatrachian frogs have been established in multiple studies (Frost et al., 2006; Roelants et al., 2007; Pyron and Wiens, 2011; Irisarri et al., 2012; Zhang et al., 2013). Various major clades within microhylids, typically each restricted to a single continent or biogeographical region, are well supported. Yet, despite dense taxon sampling for mitochondrial and nuclear DNA sequences (Frost et al., 2006; Van Bocxlaer et al., 2006; Van der Meijden et al., 2007; Matsui et al., 2011; Pyron and Wiens, 2011; Kurabayashi

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et al., 2011; de Sá et al., 2012; Peloso et al., 2016), the relationships among deep intra-familial clades, including subfamilies and genera, have yet to be satisfactorily resolved.

One such subfamily that has, thus far, evaded full phylogenetic resolution is the Cophylinae, a Madagascar-endemic clade of currently 71 species subdivided in seven genera (AmphibiaWeb, 2016). Cophylines are characterized by nidicolous (endotrophic) tadpoles, procoelous vertebral columns, and by stereotyped long series of tonal advertisement calls in most species, but otherwise are ecomorphologically highly diverse (Glaw and Vences, 2007). They range from some of the smallest terrestrial vertebrates (snout-vent length [SVL] approximately 10 mm) to the largest microhylids in the world (SVL over 105 mm), occur from just above sea level to montane habitats above 2500 m a.s.l., and have adapted to terrestrial (*Madecassophryne*, *Stumpffia*, some *Rhombophryne*, and some *Plethodontohyla*), fossorial (some *Rhombophryne* and some *Plethodontohyla*), arboreal (*Anodonthyla*, *Cophyla*, *Platypelis* and some *Plethodontohyla*) and rupicolous (*Anodonthyla montana*) habits (Andreone et al., 2005; Glaw and Vences, 2007).

In a recent study, Peloso et al. (2016) assembled a comprehensive dataset from classical Sanger sequencing and complemented it with an anchored phylogenomic dataset (Lemmon et al., 2012) for a subset of selected taxa aimed at revisiting the phylogeny of narrow-mouthed frogs. They provided a substantial advance in the understanding of microhylid relationships, but made some controversial changes in supraspecific classification. Especially controversial were changes to the genus-level classification of the Cophylinae, conflicting with all previous studies (e.g., Blommers-Schlösser and Blanc, 1991; Andreone et al., 2005; Wollenberg et al., 2008). They suggested that *Platypelis* should be synonymized with *Cophyla*, and *Stumpffia* with *Rhombophryne*. These changes prompted us to revise their proposed classification of cophyline microhylids on the basis of improved Sanger sequence coverage and a newly obtained osteological dataset.

2. Materials and methods

We herein follow the traditional genus-level classification of cophylines (Glaw and Vences, 2007; AmphibiaWeb, 2016) rather than adopting the changes proposed by Peloso et al. (2016) [hereafter named PEL], anticipating our main conclusions. Our classification differs from that suggested by PEL in considering *Platypelis* a valid genus separate from *Cophyla*, and *Stumpffia* a valid genus separate from *Rhombophryne*.

Field numbers used in the main article text and supplemental materials refer to the zoological collections of Christopher Raxworthy (RAX), Frank Glaw (FGZC and FGMV), Miguel Vences (ZCMV, FGMV, and MVTIS), Franco Andreone (FAZC) Achille P. Raselimanana (APR) and Ylenia Chiari (YCHIA). For consistency with previous studies, we mention M. Kondermann DNA extraction numbers (MK). Institutional acronyms used in the main article text and in the supplemental material are as follows: American Museum of Natural History, – Amphibians (AMNH(-A)); Ambrose Monell Cryo Collection of the American Museum of Natural History (AMCC); Museum of Zoology of the University of Michigan (UMMZ); Senckenberg Museum of Natural History, Frankfurt (SMF); Museo Regionale di Scienze Naturali, Torino (MRSN); Universite d'Antananarivo, Département de Biologie Animale (UADBA); Zoologisches Forschungsmuseum Alexander Koenig, Bonn (ZFMK); Zoological Museum Amsterdam (ZMA); Zoologische Staatssammlung München (ZSM).

2.1. Phylogenetic inference

We compiled a supermatrix of available DNA sequences for 11 loci and a total of 189 terminals (species and undescribed candidate species). Data were largely retrieved from our own work published over the past 10 years (e.g., Andreone et al., 2005; Wollenberg et al., 2008; Crottini et al., 2012; Rakotoarison et al., 2012, 2015) and include data from all 38 taxa of Madagascan origin that were included in PEL. These comprised segments of mitochondrial genes: *12S rRNA* (*12S*, 84 sequences), the 3′ and the 5′ ends of the mitochondrial *16S rRNA* (*16S_I*, 183 sequences; *16S_II*, 97 sequences), cytochrome *b* (*cytb*, 75 sequences), cytochrome oxidase subunit 1 (*cox1*, 127 sequences); and segments of six protein-coding nuclear genes: recombination-activating genes 1 and 2 (*rag1*, 57 sequences; *rag2*, 12 sequences), brain-derived neurotrophic factor (*bdnf*, 13 sequences), sacsin (*sacs*, 14 sequences), titin (*ttn*, 15 sequences), and leucine-rich repeat and WD repeat-containing protein (*kiaa1239*, 18 sequences). A set of 52 sequences was newly obtained using published primers and wet-lab protocols (Van der Meijden et al., 2007; Vieites et al., 2009; Rakotoarison et al., 2012, 2015; Perl et al., 2014). Newly determined sequences were submitted to GenBank (KU937772–KU937817, KX033507–KX033512). The total matrix of gene segments used for analysis, their GenBank accession numbers, and the alignment, have been submitted to Dryad (doi:10.5061/dryad.1b2k5).

Terminals in our genetic analysis include (*i*) samples of all nominal species of cophylines and scaphiophrynines, including type species of most genera (exceptions: *Madecassophryne truebae*, the sole member of a monotypic genus for which no DNA sequence data are so far available; *Platypelis cowanii*, the type species of *Platypelis*; *Rhombophryne serratopalpebrosa*; the recently described *Rhombophryne longicrus*—the sister species of *R. minuta*, see Scherz et al., 2015a; and *Stumpffia kibomena*, closely related to *S. grandis*, see Glaw et al., 2015), (*ii*) undescribed deep genetic lineages probably representing new species, named according to the standardized scheme proposed by Padial et al. (2010) with numbers of previously known candidate species following Vieites et al. (2009) and Perl et al. (2014), and (*iii*) cophylines and scaphiophrynines from the PEL dataset for two gene segments (*cox1* and *16S*) that overlapped the segments we sequenced. It is worth noting that, without considering PEL terminals, our matrix included a dense taxon sampling for all mitochondrial gene segments and for the nuclear *rag1*. For the other nuclear genes (*rag2*, *bdnf*, *sacs*, *ttn*, *kiaa1239*) at least one species per genus was used, with the exception of the *bdnf* gene fragment, where sequence of *Cophyla* is missing, and of the *rag2* gene fragment, where sequences of *Cophyla* and *Anilany* **gen. nov.** (described herein) are missing.

As cryptic diversity is high in cophylines, we preferred taxonomically unambiguous samples for the construction of the tree shown in Fig. 1. Of the 66 described species used as ingroup terminals (cophylines) in Fig. 1, 25 (38%) were sampled from type specimens of the respective species, and other 20 (30%) from topotypical specimens. The remaining 21 described taxa (32%) of our terminals were diagnosed to species level based on morphology. Terminals from PEL in our study were included largely for comparative purpose, i.e., to assign them to species based on their clustering with sequences determined by ourselves.

Homologous sequences of *Kaloula pulchra* and of all representatives of the subfamily Dyscophinae were used for outgroup rooting. The outgroup and the representatives of the Scaphiophryninae (which are not the focus of this paper) were excluded from the tree in Fig. 1 for graphical purposes, but are shown in the complete tree in the Supplementary Information (Appendix C, Fig. C.1). Phylogenetic analyses were run for each separate gene segment to detect possible contaminants or wrongly labelled sequences.

Chromatograms of newly determined sequences were checked and sequences manually corrected, if necessary, in CodonCode Aligner 3.5.6 (CodonCode Corporation). We used MEGA 6 (Tamura et al., 2013) to align sequences using the MUSCLE algorithm under

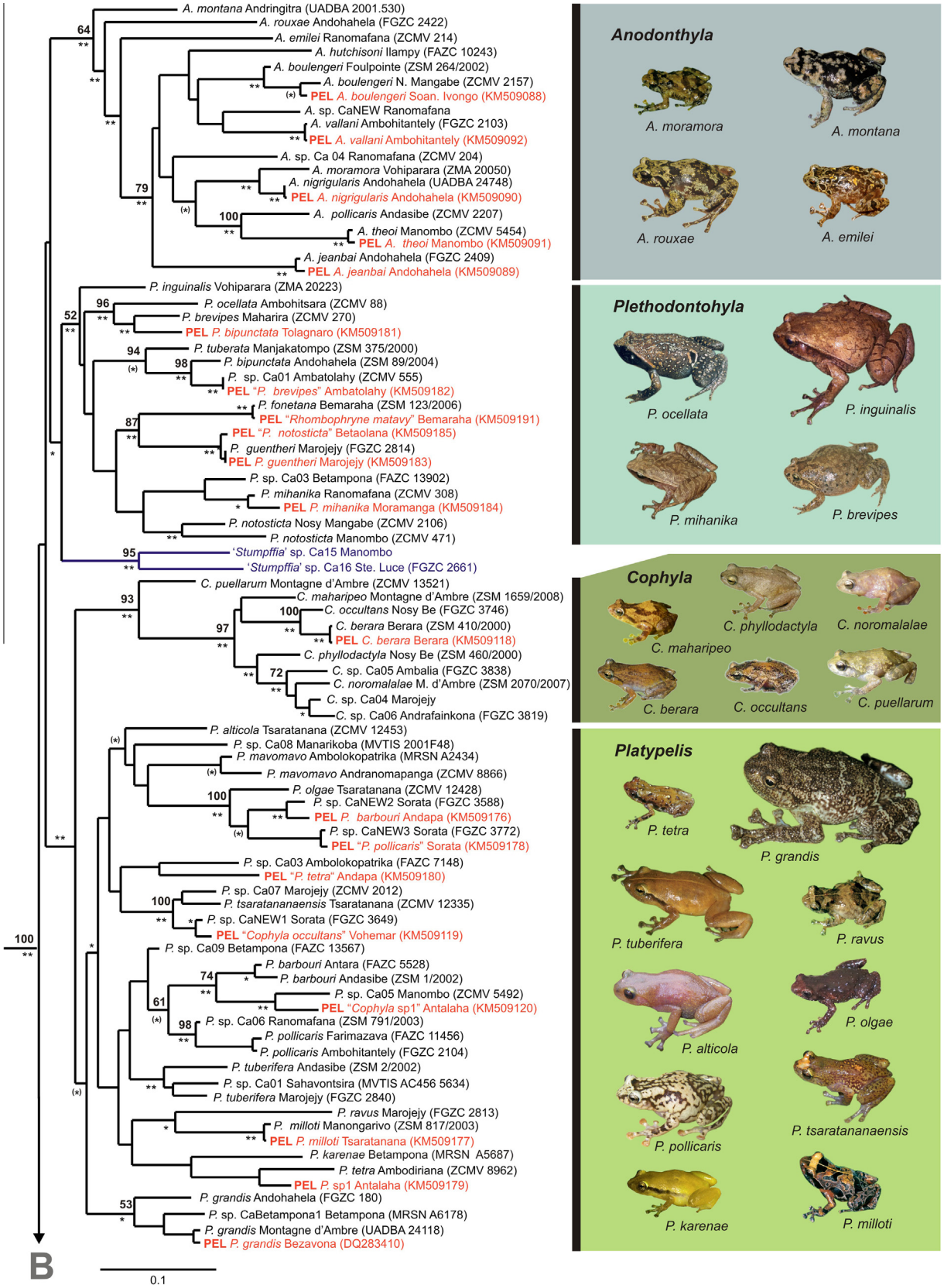

Fig. 1. Phylogram (50% majority rule consensus tree) from a Bayesian Inference analysis of the Cophylinae based on our supermatrix of data combined with the PEL Sanger sequences of the *16S_I* and *cox1* fragments. Terminals from the PEL study are indicated by the bold prefix PEL and are in red, with species names in quotation marks indicating misidentified samples. Asterisks mark posterior probabilities: (*) 0.85–0.94, * 0.95–0.98, ** 0.99–1. Numbers at nodes are MP bootstrap values (2000 replicates), shown only if >50%. In blue are the three deep clades that have been preliminarily assigned to *Stumpffia* sensu lato (two of which consist of undescribed species only, the third is herein described as genus *Anilany*). Name after the species refers to the sampling locality of the sample that was sequenced for the *16S_I* gene fragment. In brackets is the field number of the voucher specimens or the GenBank accession number of the *16S_I* gene fragment sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

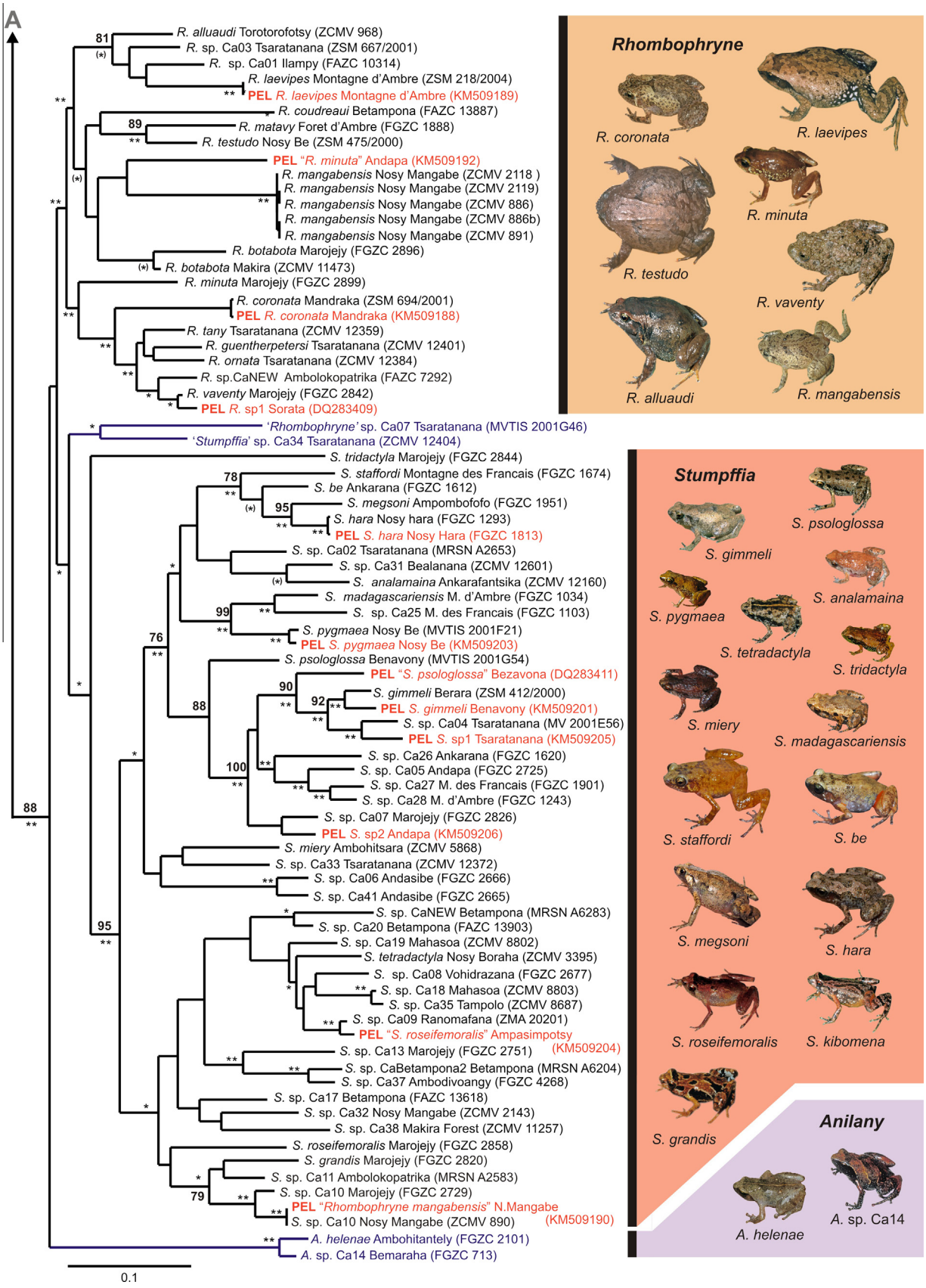

Fig. 1 (*continued*)

default settings. GBLOCKS 0.91b (Castresana, 2000) with default parameters was used to identify and to exclude nucleotide positions of unreliable alignment in the *12S* and *16S* gene fragments from the analyses. After exclusion of these positions, the alignment consisted of a total of 7646 bp.

PartitionFinder 1.1.1 software (Lanfear et al., 2012) was used to infer the best-fitting models of molecular evolution and partition scheme on the basis of the AICc criterion, using an input configuration file with 27 partitions, corresponding to individual codon positions for the eight protein-coding gene fragments and one partition each for every rRNA gene fragment. This represents the most finely partitioned scheme possible for our dataset. We used the 'greedy' algorithm (heuristic search) with branch lengths estimated as 'unlinked'. A total of 703 *a priori* schemes with varying degrees of complexity and the best-fit and the worst-fit schemes were statistically compared using AIC in PartitionFinder. The partition strategy including only two partitions (1st partition including *12S* + *16S_I* + *16S_II* + 3rd codon position of *cox1* and *cytb*; 2nd partition including all remaining positions) yielded the lowest score and was therefore identified as the optimal partitioning scheme for our analyses. The GTR + I + G model was determined within PartitionFinder as the best-fitting model of substitution for the two suggested partitions. *Dyscophus insularis* was used *a priori* as outgroup in all MrBayes analyses. Partitioned Bayesian Inference analyses (BI) were performed in the MPI version of MrBayes 3.2 for Unix Clusters (Ronquist et al., 2012) and in the CIPRES portal (Miller et al., 2010) using MrBayes 3.2.6 running on XSEDE. We performed four runs of 100 million generations (starting with random trees) and four incrementally heated Markov chains (using default heating values), sampling the Markov chains at intervals of 1000 generations. Stabilization and convergence of likelihood values were assessed in Tracer 1.4 (Rambaut and Drummond, 2007) and occurred after about 35–40 million generations. Therefore, sixty million trees were retained post burn-in and used to generate the 50% majority-rule consensus tree.

Maximum Parsimony (MP) analysis was performed with TNT (Goloboff et al., 2008) after removal of unreliable alignment positions as identified by GBLOCKS. We used the multi command with 100 random sequence-addition replicates, followed by branch-swapping with tree-bisection and reconnection (command break tbr), with up to 1000 trees specified to be retained. We also executed 2000 MP bootstrap replicates in TNT (provided in the Supplementary Information, Appendix C, Fig. C.2).

2.2. Morphological examination and morphometrics

Specimen examination followed standard protocols for cophyline microhylids, taking note of characters that have been most informative for taxonomic work in this subfamily (for full details, see recent species descriptions, e.g. Köhler et al., 2010; Klages et al., 2013; Rakotoarison et al., 2012, 2015; Glaw et al., 2015; Scherz et al., 2015a,b). Overall, we have examined over 500 specimens collected by ourselves in the field.

Non-metric multidimensional scaling (NMDS) was performed in Past 3.07 (Hammer et al., 2001) using characters that showed potential diagnostic value in *Rhombophryne* and *Stumpffia*. 46 *Stumpffia* and 70 *Rhombophryne* specimens were examined to generate a morphological character matrix for this analysis. Measurements were taken using a digital calliper to 0.01 mm under a binocular dissecting microscope, rounded to 0.1 mm, and averaged across all specimens of each species. Measurements selected for inclusion in our analysis were: snout-vent length (SVL), head width (HW), head length (HL), eye-nostril distance (END), nostril-snout distance (NSD), forelimb length (given by the sum of hand length, lower arm length, and upper arm length), hindlimb length (given by the sum of foot length, tarsus length, tibia length and thigh length).

For conclusions on the taxonomy of *Stumpffia*, *Rhombophryne*, *Plethodontohyla*, and *Stumpffia*-like genera, a total of 174 specimens belonging to these genera were examined. Specimens used by PEL (except those housed at the ZSM) were not examined. As outlined above, conclusions on these specimens are based on their genetic affinities.

2.3. Osteological analyses

High resolution micro-Computed Tomography (micro-CT) scans were produced on a phoenix nanotom m cone-beam micro-CT scanner (GE Measurement & Control, Wunstorf, Germany). Large specimens (>20 mm) were scanned using a standard target, with typical settings of 140 kV and 80 mA, using a timing of 500 ms for 2440 projections. Small specimens (<20 mm, especially *Stumpffia*) were scanned using a molybdenum element, at 70 kV and 160 mA, using a timing of 750 ms for 2440 projections. Models were prepared and examined in VG Studio Max 2.2 (Volume Graphics GMBH, Heidelberg, Germany). Selected specimens were processed into PDF-embedded 3D models using Amira 5.4.5 or 6.0 (FEI Visualization Sciences Group, Burlington MA, USA), and are provided in the Supplementary Information (Appendix C, Figs. C.3–C.5). Osteological terminology follows Trueb (1968, 1973). Osteological examination was conducted based on both volume and surface renders. Because its X-ray absorption is low, cartilage is not rendered in normal micro-CT scans; cartilages are therefore omitted from our discussion here, and from figures.

3. Results and discussion

3.1. Misidentification of taxa in PEL

PEL produced a matrix of 66 homologous loci for 48 representative species generated with an anchored phylogenomic approach, plus Sanger sequencing of seven loci for 142 species. Five species of cophylines were included in the phylogenomic dataset (identified by the authors as: *Anodonthyla nigrigularis*, *Cophyla occultans*, *Platypelis pollicaris*, *Rhombophryne mangabensis*, and *Stumpffia roseifemoralis*). We applied a molecular taxonomic identification of these five taxa by comparing PEL *16S* and *cox1* Sanger sequences against homologous sequences of all of Madagascar's cophylines as described above. We can confirm the identity of their *Anodonthyla nigrigularis* sequences. On the other hand, (*i*) their '*Platypelis pollicaris*' is an undescribed species of the genus *Platypelis* so far known only from the Sorata Mountains in northern Madagascar; (*ii*) their '*Cophyla occultans*' is another undescribed species of the genus *Platypelis* so far known only from the Sorata Mountains (see below for more details); (*iii*) their '*Stumpffia roseifemoralis*' is an undescribed species of the genus *Stumpffia* (*Stumpffia* sp. Ca9 Ranomafana); and (*iv*) their '*Rhombophryne mangabensis*' is an undescribed species of the genus *Stumpffia* similar to *Stumpffia grandis*. Thus, the phylogenomic dataset of PEL contains one *Anodonthyla*, two *Platypelis*, and two *Stumpffia*, and lacks genomic information for the genera *Madecassophryne*, *Plethodontohyla*, *Cophyla*, and *Rhombophryne* (*Anilany* **gen. nov.**, which we describe below, is also not included in their dataset).

Using the molecular taxonomic identification approach outlined above, we identified a number of sample misidentifications in PEL, and partly traced the origins of these (see Supplementary Information, Appendix A for details). Four of these errors affect phylogenetic and classificatory conclusions: (*i*) a sample from the holotype of *Plethodontohyla fonetana* was mistakenly supplied by our second author's institution (TU Braunschweig) as *Rhombophryne matavy*

and included in the PEL dataset as such, despite the respective *16S* sequence being identical to a sequence of the same specimen, available in GenBank under the correct taxon name (EU341058) and despite the sampling locality being the type locality of *Plethodontohyla fonetana*, a site far away from the type locality of *R. matavy*; (*ii*) a sample of an undescribed species of *Stumpffia* was mistakenly supplied as *Rhombophryne mangabensis*, and included in the PEL dataset despite the respective *16S* sequence being strongly divergent from a homologous sequence of the *R. mangabensis* holotype (KF611588); (*iii*) a sample named '*Cophyla* sp.' in the PEL dataset (specimen RAN 42521 from Antalaha) does not agree or cluster with topotypical specimens of any of the nominal *Cophyla* species by its *16S* or *cox1* sequences, and instead probably belongs to an undescribed species of *Platypelis*; (*iv*) a sample named '*Cophyla occultans*' in the PEL dataset (AMNH-A 167233/AMCC 103335) is very similar in *16S* and *cox1* (and forms a highly supported clade) to a sample of an undescribed species of *Platypelis* from the Sorata Mountains, both of which are within a well-supported clade also including *P. tsaratananaensis* and a further undescribed species of *Platypelis*, all of which superficially resemble *C. occultans*. Numerous additional inconsistencies, unjustified emendations and misspellings in PEL do not impact their phylogenetic conclusions; these are therefore corrected in Supplementary Information, Appendix A but not further discussed here.

3.2. Revised phylogeny of cophylinae

Bayesian Inference (BI) analysis of our supermatrix of 11 gene segments yielded a phylogenetic tree (Fig. 1) largely in agreement with those published by Andreone et al. (2005) and Wollenberg et al. (2008). The Cophylinae received maximum support as a monophyletic group, but deep nodes within the subfamily were basically unresolved. Of the six cophyline genera included and not considering the misidentifications of the PEL terminals, *Anodonthyla*, *Rhombophryne*, *Plethodonthyla* and *Cophyla* received high support (posterior probabilities $\geqslant 0.99$), and the clade comprising *Platypelis* species received moderate support (PP = 0.91). A single named species assigned to the genus *Stumpffia* (*S. helenae*), along with an undescribed candidate species, was the sister taxon to a clade comprising *Rhombophryne* and *Stumpffia*, thus rendering *Stumpffia* paraphyletic. We discuss this in more detail below, where we also erect a new genus for these *Stumpffia*-like frogs. Excluding these morphologically and genetically divergent frogs renders *Stumpffia* monophyletic with relatively high support (PP = 0.97), but as we explain, further divisions may be necessary in the future. *Cophyla* and *Platypelis* were sister clades, with high support for *Cophyla* (PP > 0.999).

Analyses of our matrix under MP yielded largely similar results. A total of 144 equally most parsimonious trees were obtained (tree length 17,840 steps). A strict consensus of these (Supplementary Information, Appendix C, Fig. C.2) closely matches the BI tree. Considering nominal species only, all traditional genera were recovered as monophyletic, although bootstrap support for these clades was usually low, and for some genera below 50% (Fig. 1). Differences to the BI tree were mostly apparent in the basal nodes of the *Stumpffia* clade: *S. tridactyla* was placed with a clade containing *S. helenae* and two samples of undescribed miniaturized frogs from Tsaratanana and Tsingy de Bemaraha, and the two samples of undescribed miniaturized frogs from the Tsaratanana massif (clustering as the sister clade of *Stumpffia* sensu strictu in the BI analysis) were placed with the *S. helenae* clade and within *Rhombophryne*, respectively.

Regarding the phylogenetic topology with sample identification amended, two major discordances between the MP tree of PEL and our BI phylogeny exist: (*i*) one unambiguous species of *Cophyla* (*C. berara*) is phylogenetically nested within *Platypelis* in the PEL tree, while this sequence is identical to our *C. berara* sample and clearly within the *Cophyla* clade in our tree (also supported by our MP analysis); (*ii*) the four species of *Rhombophryne* included (*R. coronata*, *R. laevipes*, '*R. minuta*', *R.* sp.) do not form a monophyletic group in the PEL tree, although all four terminals are included in the strongly supported *Rhombophryne* clade in our BI tree.

Based on our new phylogenetic analysis (Fig. 1), the reidentification of deviant PEL sequences, and newly obtained osteological data from CT scans, we discuss the evidence for separate generic classification of *Platypelis* vs. *Cophyla*, *Stumpffia* vs. *Rhombophryne*, and for recognizing new genus-level taxa within *Stumpffia* sensu lato. We formalize our suggestions on the basis of the taxon-naming criteria (TNCs) proposed by Vences et al. (2013). We focus on the nominal species in each clade because only fragmentary data are available for the many undescribed candidate species of cophylines included in our phylogenetic tree; where applicable, these fragmentary data do, however, support our conclusions.

3.3. *Cophyla* and *Platypelis*: morphologically similar sister genera

The *Cophyla/Platypelis* clade consists of unambiguously arboreal cophylines. The relationships within this clade have recently been discussed by Rakotoarison et al. (2015) and can be summarized as follows: (*i*) *Cophyla* and *Platypelis* are probably both monophyletic (as also supported by our tree; Fig. 1); (*ii*) *Cophyla* species are restricted to northern Madagascar, whereas *Platypelis* occurs across the entire humid biome of the island; (*iii*) no reliable external morphological characters exist to diagnose these two clades; (*iv*) all but one species of *Cophyla* lack a clavicle, while this element is present in all *Platypelis* species examined; (*v*) all species of *Cophyla* examined have medially fused vomers, whereas this element is either centrally divided or reduced in *Platypelis*.

The data available so far thus characterize *Cophyla* and *Platypelis* as monophyletic groups that can be diagnosed by a combination of osteological characters. Distinguishing the two genera is also informative from a biogeographic perspective. It is true, however, that the two clades together form a monophyletic group (satisfying the Stability of Monophyly TNC; Vences et al., 2013) and might together become more easily diagnosable in external morphology (which would satisfy the Diagnosibility TNC). Nevertheless, we here maintain both genera as valid taxa. This proposal is formalized in Supplementary Information, Appendix A. However, we emphasise that a comprehensive revision of osteological variation of *Platypelis* is pending, and that this should be combined with additional phylogenetic and phylogenomic data of both genera, *Cophyla* and *Platypelis*, for a definitive outcome.

3.4. *Stumpffia* and *Rhombophryne*: ecomorphologically distinct sister genera

Our BI phylogenetic tree recovered *Rhombophryne* as a monophyletic group within a paraphyletic *Stumpffia* sensu lato, with full support. The paraphyly of *Stumpffia* is caused by the highly divergent '*S.*' *helenae* clade, composed of '*S.*' *helenae* and '*S*' sp. Ca14. This clade is morphologically and osteologically distinct, in addition to its strong genetic differentiation from all other *Stumpffia*, and therefore warrants recognition as a new genus (erected below). This change renders *Stumpffia* sensu stricto a monophyletic genus and sister taxon to *Rhombophryne*, albeit with poor support ($P < 0.95$). Support increased ($P = 0.97$) for a clade of all species except for *S. tridactyla*. We emphasize that for several of the miniaturized species at the base of the tree, only a small number of genes have so far been sequenced, while for others a large number of sequences are available. This imbalance, along with very deep divergences, might add to the instability of basal nodes in the *Rhombophryne* + *Stumpffia* clade.

PEL did not recover *Stumpffia* and *Rhombophryne* as sister clades, and they are sister taxa in our analysis only after transferring part of *Stumpffia* to a separate genus. In such a situation, the Stability of Monophyly TNC (Vences et al., 2013) would suggest, other things being equal, a classification with both groups subsumed in a single genus with unequivocal support. However, the two clades are ecomorphologically highly distinct, and it is therefore worth exploring whether grouping them in a single genus (PEL) has advantages over a two-genera classification.

The differences between *Rhombophryne* and *Stumpffia* are exemplified by the two type species (neither of which was included in the PEL dataset), the miniaturized leaf-litter dweller *S. psologlossa* and the stout, fossorial *R. testudo* (3D models of the skeletons of the type specimens of these species are provided in Supplementary Information, Appendix C, Figs. C.3–C.4). These differences hold for most species in both clades, but some *Rhombophryne* are smaller and have more elongated bodies (e.g. *R. minuta*: max. SVL 22 mm; *R. longicrus*: max. SVL 28 mm), and some *Stumpffia* are relatively large (e.g. *S. staffordi*: max. 28 mm; Köhler et al., 2010). These exceptional species are deeply nested within their respective major clades (Fig. 1), suggesting extensive morphological homoplasy.

Defining morphological synapomorphies for *Rhombophryne* and *Stumpffia* is challenging because data are unavailable for many cophyline genera, and basal nodes in the subfamily are unresolved (Fig. 1). Still, it is possible to diagnose all or most species of the two genera by morphology; a NMDS analysis based on discrete coding of characters fully distinguished the two genera (Fig. 2). In more concrete terms, *Rhombophryne* are characterized (vs. *Stumpffia*) by the presence of vomerine teeth in all but one species (vs. absence), presence of maxillary teeth in all but one species (vs. absence), relatively wider heads in all species, larger body sizes in most species, a smaller braincase-width to head-width ratio, and (if present) clavicles curved along the antero-posterior axis (vs. straight) (see Fig. 3 and compare Supplementary Information, Appendix C, Figs. C.4, C.5). Given that both genera are monophyletic (after the transfer of '*S.*' *helenae* to a new genus, see below), all members of the two genera are morphologically

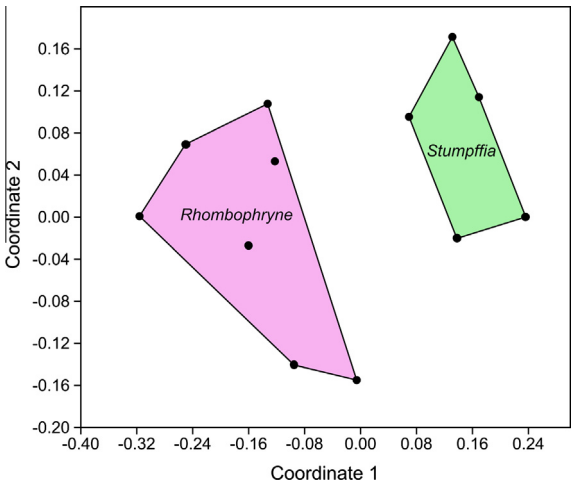

Fig. 2. Non-metric multidimensional scaling (NMDS) scatterplot of *Rhombophryne* and *Stumpffia* based on morphological characters (see Supplementary Information, Appendix B, Tables B.1–B.2). Convex hulls are drawn around each genus, showing clear distinction of genera based on our coding of their morphology.

diagnosable against each other, and with a few exceptions they occupy non-overlapping adaptive zones (leaf litter vs. soil): their synonymy is untenable. Thus, we strongly advocate continued recognition of *Stumpffia* as a valid genus distinct from *Rhombophryne*. This proposal is formalized in Supplementary Information, Appendix A, in which we provide a new and effective key to identify specimens to *Stumpffia*, *Stumpffia*-like genus-level clades (see below), *Rhombophryne*, and *Plethodontohyla*.

3.5. Convergent miniaturization: multiple *Stumpffia*-like genus-level clades

Non-monophyly of *Stumpffia* has been previously inferred (e.g., Wollenberg et al., 2008) but is complicated by the fact that many of the species involved are taxonomically undescribed. Three deep

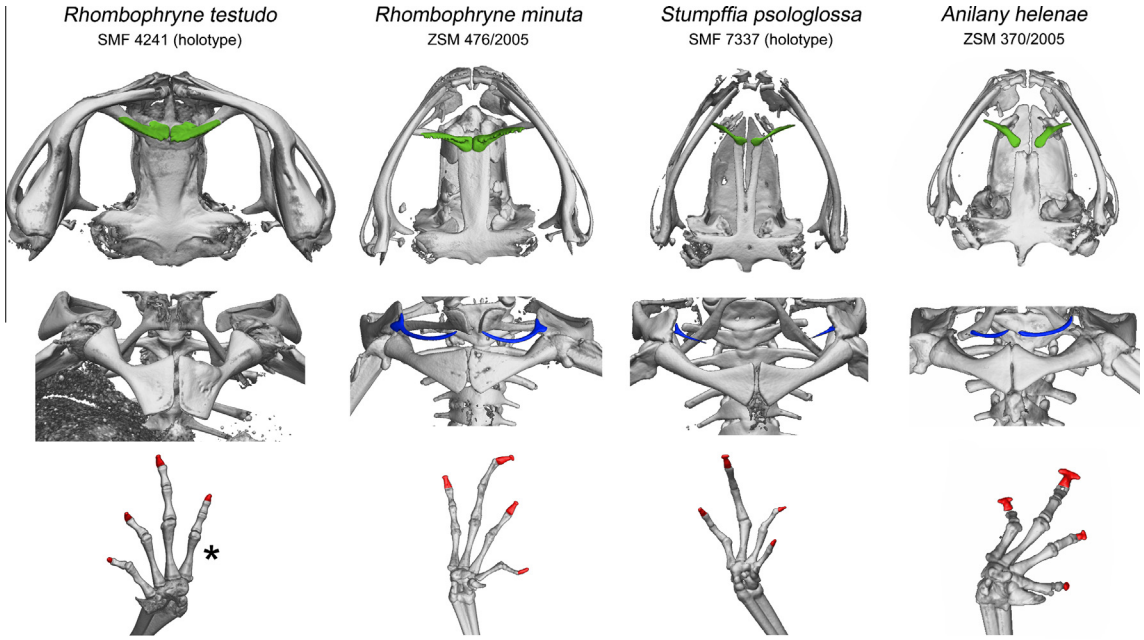

Fig. 3. Comparative micro-CT images of the heads, pectoral girdles, and hand bones of representative members of the genera *Rhombophryne*, *Stumpffia*, and *Anilany*. Postchoanal vomers/neopalatines are colored in green, clavicles in blue, and terminal phalanges in red. * The hands of SMF 4241 were poorly oriented in the micro-CT scan, so a hand of its sister species, *R. matavy* (ZSM 1628/2008), is shown in its stead. The hand morphology of these two species is highly similar. Not to scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Representatives of *Stumpffia* and *Stumpffia*-like clades. (A) *S. tetradactyla* (Nosy Boraha; holotype, ZFMK 52547); (B) *Anilany helenae* (Ambohitantely; ZSM 370/2005); (C) '*Stumpffia*' sp. Ca34 (Tsaratanana; ZSM 636/2014); (D) '*Stumpffia*' sp. Ca15 (Manombo; ZMA 20172).

clades have been assigned to *Stumpffia*, mainly by their small body sizes (<16 mm), but probably do not belong to this genus: (*i*) '*S.*' *helenae* and an undescribed candidate species ('*S.*' sp. Ca14; discussed briefly above and in more detail in the next paragraph); (*ii*) two small-sized candidate species from south-eastern Madagascar ('*S.*' spp. Ca15 and Ca16; see Fig. 4) which, interestingly, have been recovered as more closely related to *Plethodontohyla* than to the *Stumpffia-Rhombophryne* clade, supporting the hypothesis of Wollenberg et al. (2008) that they probably constitute a new genus (see Fig. 1); (*iii*) two divergent lineages from Tsaratanana ('*R.*' sp. Ca7 and '*S.*' sp. Ca34), which fall within *Stumpffia* sensu stricto in the BI analysis (Fig. 1) but are within *Rhombophryne* or the sister taxon to '*S.*' *helenae* under MP; these lineages are morphologically distinct, being miniaturized microhylids of different body shape than all other *Stumpffia* (Fig. 4), possessing both maxillary and vomerine teeth (absent in *Stumpffia* sensu stricto), and lacking clavicles (present in *Stumpffia* sensu stricto).

The second and third clades of these *Stumpffia*-like frogs contain only taxonomically undescribed lineages, and currently their existence does not challenge the taxonomic definition of *Stumpffia*. We anticipate that these clades merit distinction as separate genera, which will be described together with the species in forthcoming revisions. The first clade, however, contains the nominal '*S.*' *helenae* (and its sister lineage, the undescribed '*S.*' sp. Ca14, depicted in Fig. 4) and thus requires taxonomic discussion at this time. Wollenberg et al. (2008) revealed '*S.*' *helenae* to be the sister taxon to all other *Stumpffia*, although without statistical support; herein it is the sister taxon to the *Rhombophryne* + *Stumpffia* clade. This species has long been known to be outstanding among *Stumpffia* in that it possesses dilated terminal disks of fingers and toes (Vallan, 2000). Micro-CT scans revealed that it also possesses T-shaped terminal phalanges of the fingers and toes (Fig. 3 and Supplementary Information, Appendix C, Fig. C.5). In *Stumpffia*, terminal phalanges are typically weakly distally expanded, knobbed, or unornamented, but a group of large-sized cave-adapted *Stumpffia* (*S. be*, *S. hara*, *S. megsoni*, *S. staffordi*) also possess expanded terminal phalanges and discs. '*Stumpffia*' *helenae* is the only nominal species to combine small size and expanded finger

and toe tips, but less ambiguously it also has a broad, flattened vomer that tapers slowly distally, rather than the vomer consisting of a thin rod of bone with a rounded proximal base as in *Stumpffia*, clavicles curved along the anteroposterior axis (straight in eight examined *Stumpffia* species), and the only male available for examination has a broad *crista ventralis* and *crista lateralis* of the humerus, as well as a strong prepollex, not known from any other *Stumpffia* (see Supplementary Information, Appendix C, Fig. C.5). Based on these morphological differences and the phylogenetic position apart from other *Stumpffia*, we transfer '*S.*' *helenae* to a new genus:

***Anilany* gen. nov.**

This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The LSID (Life Science Identifier) for this publication is: urn:lsid:zoobank.org:act:DF982DCE-5CB9-4B01-9ED7-4D992170C603. The electronic edition of this work was published in a journal with an ISSN, and will be archived and made available from the following digital repository: http://www.zenodo.org/.

Type species: *Stumpffia helenae* Vallan, 2000

Diagnosis: Miniaturized terrestrial cophyline microhylids (14–16 mm adult SVL). Dilated terminal discs on fingers and toes. T-shaped terminal phalanges of fingers and toes, the distal tip broader than the base. Vomer broad, flattened, and not tapering strongly. Clavicles curved along the anteroposterior axis. Males with broad *crista ventralis* and *crista lateralis* of the humerus, and strongly developed, broad prepollex almost equal in length to the first metacarpal (not observed in females of *A.* sp. Ca14, possibly reflecting sexual dimorphism; males of this candidate species are unknown).

The new genus is morphologically distinguished from *Stumpffia* by the combination of small size (14–16 mm SVL) and dilated terminal discs of the fingers and toes, with T-shaped terminal phalanges of these digits with tips broader than their bases (found only in large-bodied *Stumpffia* species), curved clavicles (straight in all examined *Stumpffia* species), and broader vomer. Genetically, representatives of the new genus are strongly divergent from all nominal species of *Stumpffia*, and phylogenetically *Anilany* might be the sister genus of *Stumpffia* or of the *Rhombophryne* + *Stumpffia* clade.

A 3D model of the skeleton of a specimen of *A. helenae* is provided in Supplementary Information, Appendix C, Fig. C.5.

Included species: *Anilany helenae* (Vallan, 2000) and one undescribed candidate species (*Anilany* sp. Ca14).

Distribution: Central and western Madagascar. Known from rainforest in Ambohitantely Special Reserve (*Anilany helenae*) and dry forests in karstic limestone formations in Tsingy de Bemaraha National Park (*Anilany* sp. Ca14).

Etymology: *Anilany* is derived from the Malagasy contraction anilan'ny, meaning 'at the side of', as in the statement '*A. helenae* mianjera teo anilan'ny *Rhombophryne* + *Stumpffia*' (*S. helenae* falls at the side of *Rhombophryne* + *Stumpffia*), in reference to the phylogenetic position of this genus beside the *Rhombophryne* + *Stumpffia* clade. It is to be treated as an invariable feminine noun.

4. Conclusions

It is almost unavoidable that large-scale phylogenetic analyses with dense sampling will contain some errors in species identity of samples, especially because most organismal groups require alpha-taxonomic revision. This is particularly true for taxa from highly biodiverse developing countries where the lack of both well-maintained in-country natural history collections and a sufficiently high number of expert taxonomists precludes efficient cataloguing of biodiversity (Paknia et al., 2015). Sample misidentification is insidious; at higher taxonomic levels, the impact of individual misidentified samples is generally low, as their effect on deep tree topology is weak, especially when taxon sampling is dense. Investigating higher taxonomy using dense taxon sampling almost inevitably leads to conclusions being made also at lower taxonomic levels. Here, the impact of these misidentifications is much higher, and can mislead conclusions and cause taxonomic errors. Therefore, even when the main focus of a study is a higher taxonomic question, proofing procedures (e.g. performing blast searches of barcode sequences) should be implemented to ensure maximum possible confidence in sample identity.

Our re-analysis of the cophyline members of the PEL dataset revealed several misidentified taxa, partly caused by upstream sample confusion (for which the authors are not to blame, although precautionary procedures would have revealed these), and partly by incorrect taxonomic identification. While these misidentifications do not undermine the central conclusions of PEL—having likely had little impact on deep tree topology—we have here shown that they caused erroneous genus-level changes within the Cophylinae.

Based on our densely sampled phylogeny, together with morphological and osteological data, we have shown that (i) *Cophyla* and *Platypelis* are highly similar but monophyletic, diagnosable genera (although a thorough revision of their taxonomy will be necessary in future); (ii) *Rhombophryne* and *Stumpffia* sensu stricto are ecomorphologically distinct, monophyletic, diagnosable genera; (iii) two species formerly considered as *Stumpffia* fall outside the *Rhombophryne* + *Stumpffia* clade, and are transferred to the new genus *Anilany*, which is diagnosable in osteology; and (iv) two further clades of *Stumpffia*-like frogs may warrant recognition as new genera.

As discussed elsewhere (Vences et al., 2013), genus names are particularly relevant for the end users of taxonomies, such as conservation biologists and lawmakers. We therefore suggest that a general principle of economy of change in these names should apply. Making changes based on weakly supported clades can necessitate repeated revision before reaching a stable and unambiguous taxonomy (see Supplementary Information, Appendix A.6 for a brief discussion of the new microhylid subfamily Chaperininae erected by PEL). Deep nodes in our phylogeny remain poorly resolved, and our revised classification is not free of ambiguities in diagnosing genera by morphology alone. Future revisions will doubtless refine this classification, as osteological datasets expand, candidate species are described, and genetic coverage improves. We are confident, though, that this will entail few large-scale rearrangements.

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Appendices A–C. Supplementary material

Appendix A. Comments and corrections on classificatory changes (A.1–A.6); Appendix B. Supplementary Tables (Table B.1–B.2); Appendix C. Supplementary Figures (Figure C.1–C.5). Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.04.019.

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