Molecular Phylogenetics and Evolution 127 (2018) 1010–1019

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Comprehensive multi-locus phylogeny of Old World tree frogs (Anura: Rhacophoridae) reveals taxonomic uncertainties and potential cases of over- and underestimation of species diversity

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ARTICLE INFO

Keywords:
ABGD
Species-delimitation
Taxonomy
Systematics
Molecular phylogenetics

ABSTRACT

The family Rhacophoridae is one of the most diverse amphibian families in Asia, for which taxonomic understanding is rapidly-expanding, with new species being described steadily, and at increasingly finer genetic resolution. Distance-based methods frequently have been used to justify or at least to bolster the recognition of new species, particularly in complexes of "cryptic" species where obvious morphological differentiation does not accompany speciation. However, there is no universally-accepted threshold to distinguish intra- from interspecific genetic divergence. Moreover, indiscriminant use of divergence thresholds to delimit species can result in over- or underestimation of species diversity. To explore the range of variation in application of divergence scales, and to provide a family-wide assessment of species-level diversity in Old-World treefrogs (family Rhacophoridae), we assembled the most comprehensive multi-locus phylogeny to date, including all 18 genera and approximately 247 described species (~60% coverage). We then used the Automatic Barcode Gap Discovery (ABGD) method to obtain different species-delimitation schemes over a range of prior intraspecific divergence limits to assess the consistency of divergence thresholds used to demarcate current species boundaries. The species-rich phylogeny was able to identify a number of taxonomic errors, namely the incorrect generic placement of *Chiromantis inexpectatus*, which we now move to the genus *Feihyla*, and the specific identity of *Rhacophorus bipunctatus* from Peninsular Malaysia, which we tentatively reassign to *R. rhodopus*. The ABGD analysis demonstrated overlap between intra- and interspecific divergence limits: genetic thresholds used in some studies to synonymize taxa have frequently been used in other studies to justify the recognition of new species. This analysis also highlighted numerous groups that could potentially be split or lumped, which we earmark for future examination. Our large-scale and *en bloc* approach to species-level phylogenetic systematics contributes to the resolution of taxonomic uncertainties, reveals possible new species, and identifies numerous groups that require critical examination. Overall, we demonstrate that the taxonomy and evolutionary history of Old-World tree frogs are far from resolved, stable or adequately characterized at the level of genus, species, and/or population.

1. Introduction

Molecular phylogenetic estimates have become an important historical scalar for elucidating a clade's evolutionary history, but can also be utilized to provide a framework for taxonomic assessment and species-delimitation. Many recent large-scale phylogenetic studies of amphibians have focused on resolving phylogenetic relationships in deep time to infer patterns of diversification and evolutionary traits (Chan and Brown, 2017; Hertwig et al., 2013; Li et al., 2013; Meegaskumbura et al., 2015; Oliver et al., 2015; Pan et al., 2017; Roelants et al., 2007; Van Bocxlaer et al., 2009; Wiens et al., 2009; Yuan et al., 2016), whereas shallow-scale studies are usually focused on smaller subclades and emphasize relationships at the species level for taxonomic revisions and the diagnosis of new species (Chan et al., 2014; Chen et al., 2017; Li et al., 2012; Poyarkov et al., 2015; Sivongxay et al., 2016; Smart et al., 2017; Wostl et al., 2017; Wu et al., 2016; Hertwig et al., 2012). However, when these approaches are combined, the ability to assess taxonomic uncertainty and to screen for potential new species can be scaled-

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https://doi.org/10.1016/j.ympev.2018.07.005
Received 10 April 2018; Received in revised form 10 July 2018; Accepted 10 July 2018
Available online 17 July 2018
1055-7903/ Published by Elsevier Inc.

up considerably to hundreds or even thousands of taxa (Frost et al., 2006; Li et al., 2008; Pyron and Wiens, 2011). Additionally, evaluating specific and supraspecific taxa within a broad and comprehensive phylogenetic context can provide insights into the consistency (or the lack thereof) of genetic divergence thresholds commonly used to delimit species boundaries. This is particularly important in recent years, as molecular data have become commonly used to delimit species at an increasingly finer genetic resolution—where distinguishing the boundaries between species may be challenging (Barley et al., 2013; Chan et al., 2017; Welton et al., 2017).

Old-World tree frogs of the family Rhacophoridae consist of 413 species that are widely distributed across Asia with a disjunct occurrence in Africa (Frost, 2017). Within the family, numerous studies have been conducted at species/genus-specific scales (Hertwig et al., 2013; Nguyen et al., 2014a, 2015; Poyarkov et al., 2015; Wostl et al., 2017; Yu et al., 2013) to genus/family-level studies at a global scale (Abraham et al., 2013; Li et al., 2013, 2009, 2008; Meegaskumbura et al., 2015; Pyron and Wiens, 2011; Wilkinson et al., 2002; Yu et al., 2009). However, due to the rapid rate of taxonomic revisions and new species discoveries, many of these phylogenies are now obsolete. For example, the most recent and comprehensive phylogenetic study of rhacophorids to date included 185 species (Abraham et al., 2013), which at this point, represents 45% of the family's diversity. Moreover, Malaysian, Indonesian, and Philippine taxa were severely underrepresented in all these studies, resulting in a critical lack of understanding regarding their taxonomic placement, biogeography, and evolutionary history. The exclusion of such a significant portion of diversity introduces substantial gaps within the larger Rhacophoridae phylogeny that can lead to misinformed systematic inferences. Therefore, in order to gain a better understanding of species-level relationships, we estimate a more comprehensive phylogeny of the family Rhacophoridae as a platform to evaluate the phylogenetic placements and taxonomic status of understudied taxa. Accordingly, the goals of this study are to (1) estimate a species-rich, multi-locus phylogeny of the family Rhacophoridae consisting of approximately 60% of the known species, representing all 18 genera and numerous taxa that have never before been sampled and included in molecular phylogenetic estimates and (2) use this phylogeny as a platform to survey comprehensively for divergent lineages (potential new species) while assessing the validity of presently described species towards the goal of determining if species diversity has been under- or overestimated.

2. Materials and methods

2.1. Genetic sampling and taxonomy

We obtained molecular data from 327 samples representing approximately 247 ingroup species and all 18 genera of rhacophorids. Sequences from two mitochondrial (16S and Cytochrome-b) and two nuclear genes (RAG-1 and Tyrosinase) were compiled from Genbank, including 28 new samples from Malaysia that were sequenced at the 16S rRNA mitochondrial gene (Table S1). DNA for these samples was extracted with the Promega Maxwell© RSC Instrument using the Maxwell© RSC Tissue DNA Kit and subsequently sequenced at Genewiz, Frederick, MD. We used 16S primers from Evans et al. (2003) and followed the sequencing protocol from McLeod (2010). Sequences were assembled, aligned (MUSCLE algorithm, 8 iterations), and concatenated in Geneious Pro version 5.3 (Kearse et al., 2012). Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.07.005.

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We followed taxonomic nomenclature adopted by AmphibiaWeb and the Amphibian Species of the World 6.0, an Online Reference (AmphibiaWeb, 2018; Frost, 2017) in recognizing the genera *Buergeria, Beddomixalus, Chiromantis, Feihyla, Ghatixalus, Gracixalus, Kurixalus, Liuixalus, Mercurana, Nasutixalus, Nyctixalus, Philautus, Polypedates, Pseudophilautus, Raorchestes, Rhacophorus, Taruga,* and *Theloderma*. For consistency, all systematic comparisons and changes that we propose are predicated on the taxonomy used by these databases.

2.2. Phylogenetic analyses

The concatenated sequence matrix was partitioned by gene, and phylogenies were estimated using maximum likelihood (ML) and Bayesian methods. A ML phylogeny was estimated using W-IQ-TREE (Trifinopoulos et al., 2016) employing the model-testing function to infer the best-fit substitution model for each gene partition under the Bayesian information criterion. To evaluate alternative topological hypotheses, we implemented a Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT; Guindon et al. 2010), and branch support was assessed using Ultrafast Bootstrap Approximation (UFB; Hoang et al. 2017) using 1000 bootstrap replicates for each method. The Bayesian phylogeny was constructed using BEAST v2.4.8 (Bouckaert et al., 2014) through the CIPRES portal (Miller et al., 2010). Substitution models were averaged across each partition using bModelTest (Bouckaert and Drummond, 2017), and a relaxed log-normal clock and Yule model were selected for the molecular clock and tree priors. We executed two independent MCMC chains at 100,000,000 generations each, sampling at every 10,000 iterations for a total of 20,000 sampled trees. Convergence of parameters was assessed using Tracer v.1.6 (Rambaut et al., 2014) to ensure that trees were sampled from the posterior distribution. Sampled trees from both MCMC chains were subsequently combined at a lower frequency with a burn-in of 10% using the BEAST plug-in *logcombiner*, and a final maximum clade credibility tree was constructed using the plug-in *treeannotator*.

2.3. Assessing species boundaries

For rapid and large-scale assessment of species limits, we implemented the Automatic Barcode Gap Discovery (ABGD) method, which has been shown to perform well for large datasets (Puillandre et al., 2012) and unequal sampling regimes (Blair and Bryson, 2017). We implemented this method on the 16S gene (320 out of 327 samples), a universal DNA barcoding marker in amphibians that is also widely-used to delimit species (Vences et al., 2005b; Vieites et al., 2009). Due to the high number of species, we were unable, in many cases to include more than one sequence per species in our dataset. Although introducing singletons could potentially bias the analyses, studies have shown that the ABGD method performs well with singletons (Kekkonen et al., 2015). This quantitative approach sorts sequences into hypothetical species based on the "barcode gap," which is observed whenever intraspecific divergence is smaller than interspecific divergence. A range of prior intraspecific divergences are inferred from the data with a model-based one-sided confidence limit for intraspecific divergence. The ABGD then detects the barcode gap as the first significant gap beyond this limit and uses it to partition the data (i.e. the first candidate species). Inferences of the limit and gap detection are then recursively applied to obtain finer partitions until no further gaps can be detected (Puillandre et al., 2012). Prior to the analysis, pairwise genetic distances were calculated using the Kimura (K80) model with a transition/transversion rate of 1.5. Average transition/transversion rate was calculated from the alignment using a custom R script. For the range of prior intraspecific divergences (P), we sampled 100 values from $P_{min} = 0.001$ to $P_{max} = 0.1$. To obtain a wide range of partitions from conservative to liberal species-delimitation schemes, we used a small relative gap width of $X = 0.1$. This analysis was performed on the ABGD webserver platform available at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html.

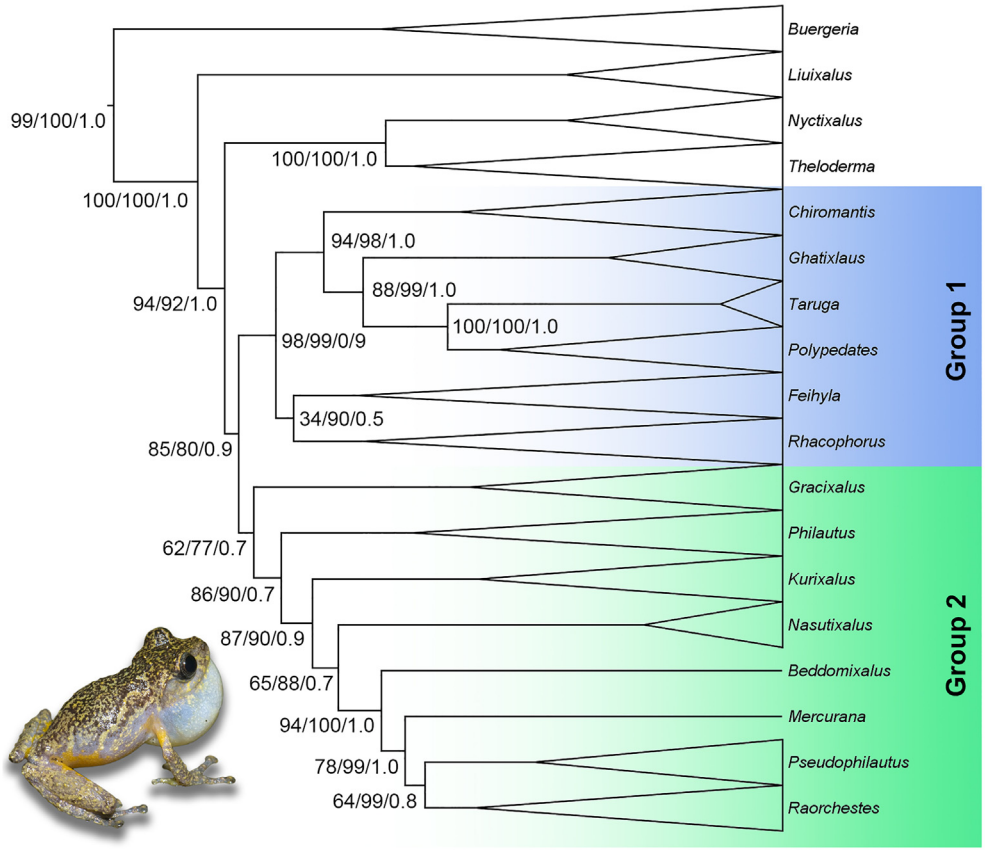

Fig. 1. Bayesian phylogeny at the generic level. Node support is denoted as: IQ-TREE SH-aLRT bootstrap support/Ultrafast bootstrap support/Bayesian posterior probability. Inset picture: *Philautus vermiculatus*.

3. Results

3.1. Intergeneric phylogenetic relationships

At the generic level, both ML and Bayesian phylogenies produced similar topologies with relatively high support at most major nodes (Fig. 1). However, the intergeneric relationships that we inferred were notably different from the most recent large-scale rhacophorid phylogenies (Abraham et al., 2013; Li et al., 2013; Pyron and Wiens, 2011) in a number of areas, namely within Groups 1 and 2 (Fig. 1). In the study by Pyron and Wiens (2011), the intergeneric relationships inferred for Group 1 were (*Ghatixalus*, (*Chiromantis*, (*Rhacophorus*, (*Feihyla*, (*Taruga*, *Polypedates*))))), whereas Li et al. (2013) inferred a different topology for the same clade: (*Chiromantis*, (*Rhacophorus*, (*Feihyla*, (*Ghatixalus*, (*Taruga*, *Polypedates*))))). Abraham et al. (2013) recovered yet another different topology (*Rhacophorus*, (*Chiromantis*, (*Feihyla*, (*Ghatixalus*, (*Taruga*, *Polypedates*))))). For Group 2, Pyron and Wiens (2011) recovered the relationships ((*Gracixalus*, *Philautus*), (*Kurixalus*, (*Pseudophilautus*, *Raorchestes*))), whereas Li et al. (2013) inferred ((*Gracixalus*, *Philautus*), (*Pseudophilautus*, (*Raorchestes*, *Kurixalus*))) and Abraham et al. (2013) inferred *Gracixalus* as the sister lineage to Group 1 and not part of Group 2. However, most of these relationships were weakly supported in those studies. The placement of the most recently described genus *Nasutixalus* also differs from previous studies that inferred the topology (*Nasutixalus*, (*Kurixalus*, (*Beddomixalus*, (*Mercurana*, (*Raorchestes*, *Pseudophilautus*))))) (Biju et al., 2016; Jiang et al., 2016), albeit with low support (SH-aLRT = 64.6; UFB = 88.0; BPP = 0.72).

3.2. ABGD analysis

The ABGD analysis recovered a total of nine partitions with prior maximal intraspecific distances (P) ranging from 0.001 to 0.06 and number of delimited species ranging from 1 to 278 (Fig. S1; Table 1). The recursive approach recovered a slightly higher number of species compared to the initial run except for partition six that delimited 271 species in both the initial and recursive runs. Partitions 1–3 were considered implausible as they overlumped large numbers of well-established species, and in some cases, multiple genera as a single species. Furthermore, prior maximal intraspecific distances among these partitions were high (0.2154–0.06) and more consistent with interspecific distances (Vieites et al., 2009). Partitions four and five delimited similar numbers of species during the initial run (2 4 6) and differed only in the recursive run by two species (from the *Rhacophorus bipunctatus* complex). However, the small difference in the number of delimited species was accompanied by a significant difference in prior maximal intraspecific distance between these partitions (0.0077–0.0129), indicating that these partitions produced consistent delimitation schemes

Table 1
Partitions, number of species (initial run followed by recursive run in parenthesis), and corresponding prior maximal distance from the ABGD analysis using a relative gap width, X = 0.1.

	Number of species	Prior maximal distance
Partition 1	271(278)	0.001
Partition 2	271(273)	0.001668
Partition 3	271(272)	0.002783
Partition 4	271(271)	0.004642
Partition 5	246(250)	0.007743
Partition 6	246(248)	0.012915
Partition 7	186(188)	0.021544
Partition 8	70(74)	0.035938
Partition 9	1(1)	0.059948

across a relatively large range of intraspecific distances (Tables 1 and S2). Moreover, these partitions also correctly lumped (arriving at the same conclusion as experienced empirical anuran taxonomists) multiple geographic samples of *Philautus larutensis* (Wostl et al., 2017) and confirmed the synonymy of *Liuixalus catbaensis* with *L. calcarius* (Nguyen et al., 2014b); *Theloderma chuyangsinense* with *T. palliatum; T. bambusicolum* with *T. laeve* (Poyarkov et al., 2015); *Kurixalus hainanus* with *K. bisacculus* (Yu et al., 2010); *Rhacophorus gongshanensis* with *R. burmanus;* and *R. pingbianensis with R. duboisi* (Orlov et al., 2002). Partitions 6–9 recovered the same number of species from the initial run (2 7 1) and mainly differed from the previous partitions by further splitting subgroups within the genera *Raorchestes* and *Pseudophilautus*, and separated species within *Theloderma albopunctatum*, *Rhacophorus rhodopus,* and the *Rhacophorus* complexes from southern China and northern Indochina (Fig. S1; Table S2). Because the differences in prior maximal intraspecific distances between these partitions were the smallest, we interpret these partitions as being the most accurate. In subsequent sections, we select the most conservative partition five for detailed discussion.

3.3. Systematics

3.3.1. Liuixalus, Nyctixalus, Theloderma (Fig. 2)

The genus *Liuixalus* was inferred as the sister taxon to the clade comprising the remaining members of the subfamiliy Rhacophorinae, consistent with previous studies (Biju et al., 2016; Jiang et al., 2016; Li et al., 2013; Pyron and Wiens, 2011). Aside from the deep divergence of *L. hainanus*, interspecific relationships among other congeners were relatively shallow, especially for *L. feii*, *L. romeri*, and *L. jinxiuensis*—all of which were proposed to constitute a single species in our ABGD analysis. The genera *Nyctixalus* and *Theloderma* were reciprocally monophyletic with high support, in contrast to Poyarkov et al. (2015), but consistent with the latest study of these groups (Sivongxay et al., 2016), which also discussed the discrepant results produced by Poyarkov et al. (2015). The ABGD species-delimitation scheme for *Nyctixalus* and *Theloderma* was mostly consistent with current taxonomy except for a sample of *N. pictus* from Borneo that was estimated as distinct from Peninsular Malaysia and Burmese samples. Substantial genetic structure was also detected within the *T. albopunctatum* group where the ABGD analysis delimited multiple distinct species-level groups (Table S2).

3.3.2. Chiromantis, Ghatixalus, Taruga, Polypedates, Feihyla, Rhacophorus (Figs. 3 and 4)

In comparison to other studies, the phylogenetic placement of the genus *Feihyla* varied most substantially. Our analyses estimated *Feihyla* as the sister lineage to the genus *Rhacophorus* with moderate bootstrap support (BS = 0.9) but low SH-aLRT (34.9) and BPP support (0.5;

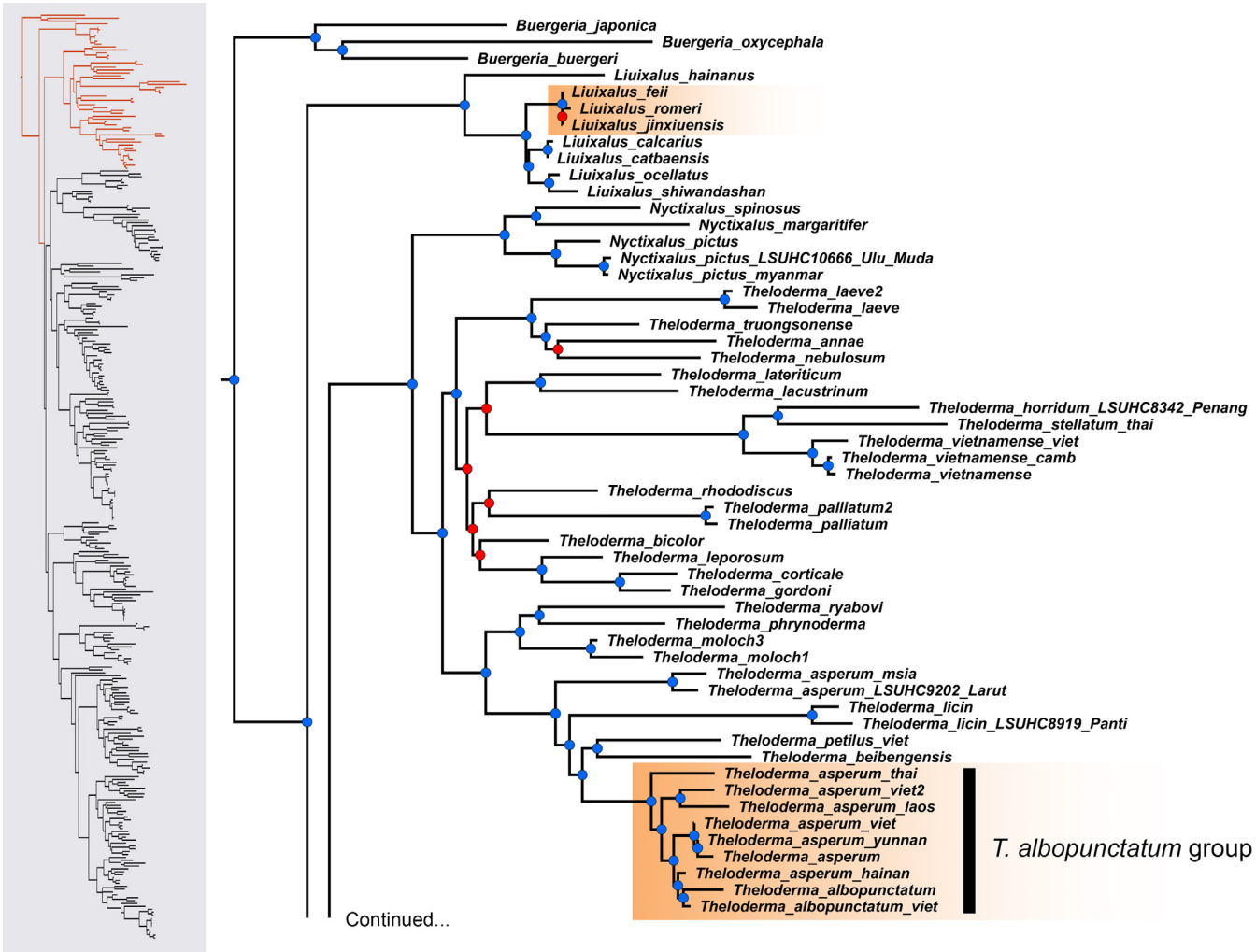

Fig. 2. Maximum likelihood phylogeny of the family Rhacophoridae estimated using 3483 bp of sequence data from four genes. A skeleton of the entire phylogeny is depicted in the left panel. Selected portions of the tree are highlighted in red and shown in detail on the right. Circles at nodes denote ultrafast bootstrap support values from the IQ-TREE analysis (blue = BS > 90; red = BS < 90). Clades that contain potential undescribed species or multiple species that could be lumped are highlighted in orange.

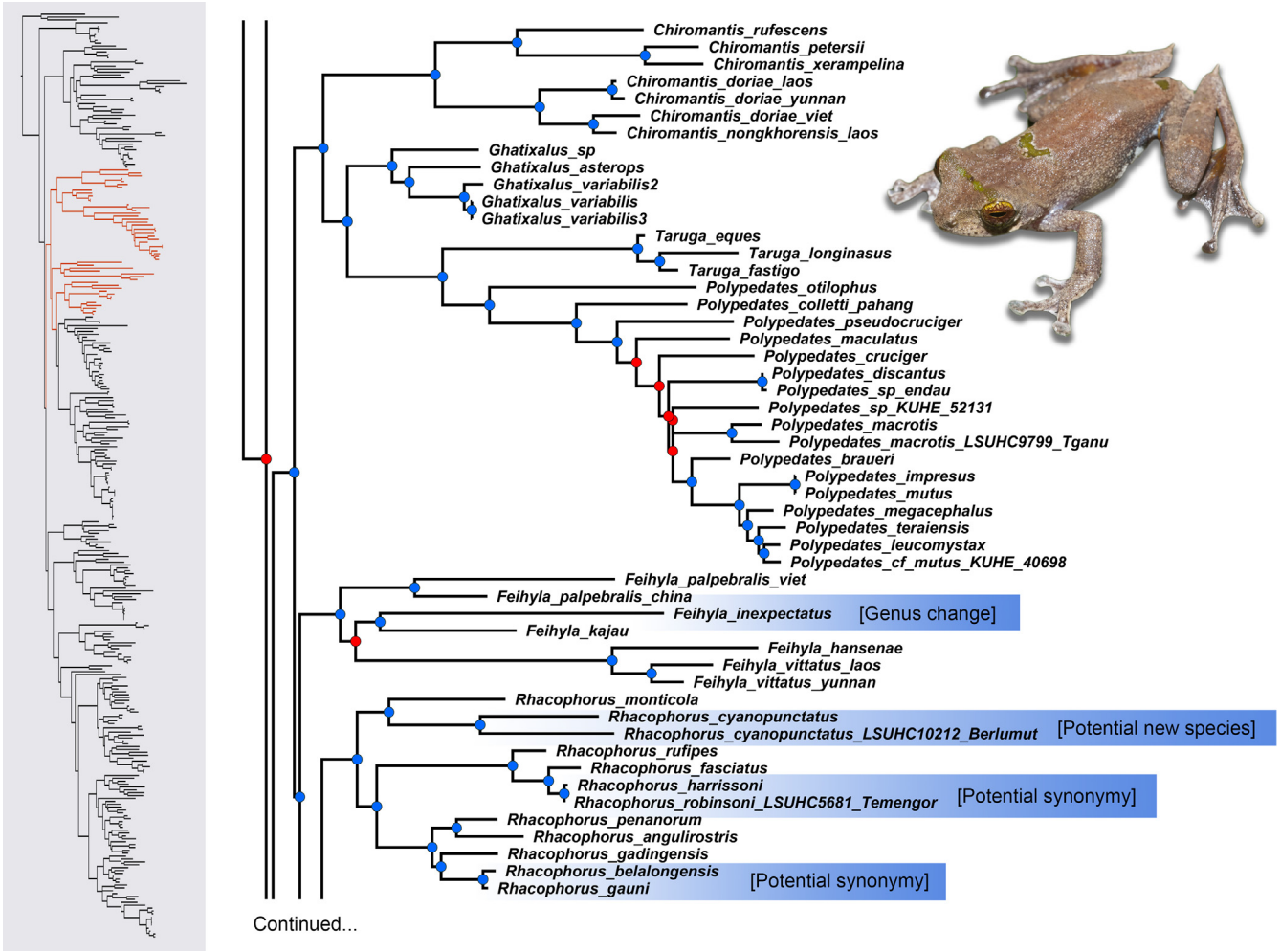

Fig. 3. Large-scale maximum likelihood phylogeny of the family Rhacophoridae estimated using 3483 bp of sequence data from four genes. A skeleton of the entire phylogeny is depicted in the left panel. Selected portions of the tree are highlighted in red and shown in detail on the right. Circles at nodes denote ultrafast bootstrap support values from the IQ-TREE analysis (blue = BS > 90; red = BS < 90). Taxa that require taxonomic revision are highlighted in blue. The inset picture is a specimen of *Rhacophorus gauni* from Lanjak Entimau, Sarawak.

Fig. 1), whereas previous studies recovered *Feihyla* as the sister lineage to the *Taruga* and *Polypedates* clade (Li et al., 2013; Pyron and Wiens, 2011). The species *Chiromantis inexpectatus* from Borneo (Matsui et al., 2014) was inferred as the sister species to *Feihyla kajau*, also from Borneo, indicating that *C. inexpetatus* should be placed within the genus *Feihyla*. *Polypedates impresus* and *P. mutus* were lumped as a single species, but the identity of the genetic samples requires confirmation. *Rhacophorus pardalis* and *R. dulitensis* were proposed as a single species, but we suspect that this most likely represents a misidentification of Genbank samples due to the unmistakable morphological differences between these species. The level of genetic divergence between Bornean and Peninsular Malaysian *Rhacophorus cyanopunctatus* was high and consistent with interspecific divergences among other rhacophorid taxa. This separation was shown in even the most conservative ABGD partition (Partition 8), indicating that these lineages are distinct enough to be evaluated as separate putative species. Conversely, genetic divergences between *R. harrissoni*, and *R. robinsonii* and between *R. belalongensis* and *R. gauni* were low and more consistent with population-level variation. The merging of these taxa was inferred across all analyses, and even the most liberal ABGD partition schemes failed to split them into separate species. The ABGD analysis also indicated numerous other potential synonymizations within the genus *Rhacophorus*, many of which will require further validation. These include the proposed synonymization of *R. calcaneus* with *R. orlovi*; *R. dugritei* with *R. puerensis*; *R. omeimontis* with *R. duboisi*; and *R. hui* with *R. wui, R. hongchibanesis*, and *R. minimus*. For the *R. bipunctatus* complex, our analyses also suggested that Peninsular Malaysian *R. bipunctatus* are genetically more similar to *R. rhodopus* than to the true *R. bipunctatus* from Myanmar (Bordoloi et al., 2007; Li et al., 2012). However, the ABGD analysis delimited Peninsular Malaysian samples as distinct from the true *R. rhodopus* from Yunnan, China. Within the *R. rhodopus* group, ABGD identified multiple putatively distinct taxa, indicating that the species may represent a complex of multiple undescribed species requiring further study (Table S2).

3.3.3. *Gracixalus, Philautus, Kurixalus, Pseudophilautus, Raorchestes* (*Figs. 5 and 6*)

Interspecific genetic divergences within the genera *Gracixalus*, *Philautus*, and *Kurixalus* were relatively high, and the ABGD delimitation scheme was mostly consistent with current taxonomy except for our analyses' proposed splitting of Bornean and Peninsular Malaysian *K. appendiculatus* (Lv et al., 2018). The ABGD analysis lumped numerous taxa within the genera *Pseudophilautus* and *Raorchestes*. These include the lumping of *P. hankeni* with *P. dilmah* and *P. schmarda*; *P. papillosus* with *P. reticulatus*; *P. amboli* with *P. leucorhinus*; *R. manohari* with *R. uthamani*; *R. flaviocularis* with *R. chalazodes* and *R. ochlandrae*; and *R. tinniens* with *R. montanus* and *R. primarrumfi* (Table S2). Two samples of *R. parvulus* from northern Thailand are genetically similar to

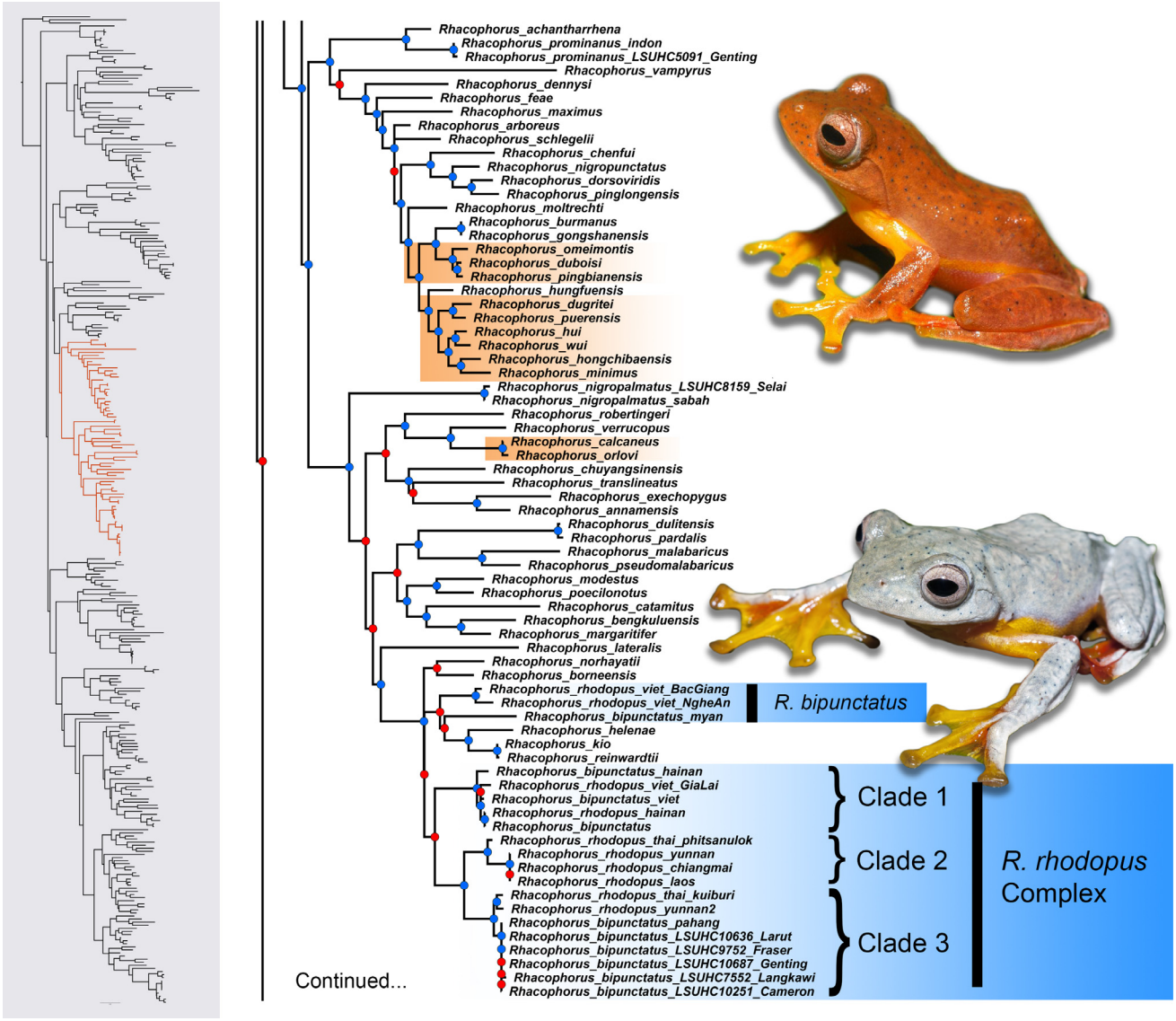

Fig. 4. Maximum likelihood phylogeny of the family Rhacophoridae estimated using 3483 bp of sequence data from four genes. A skeleton of the entire phylogeny is depicted in the left panel. Selected portions of the tree are highlighted in red and shown in detail on the right. Circles at nodes denote ultrafast bootstrap support values from the IQ-TREE analysis (blue = BS > 90; red = BS < 90). Clades that contain potential undescribed species or multiple species that could be lumped are highlighted in orange. Taxa that require taxonomic revision are highlighted in blue. The inset pictures are *Rhacophorus rhodopus* from Langkawi Island (top) and *R. rhodopus* from Cameron Highlands, Malaysia.

R. menglaensis from Yunnan, China. Because *R. menglaensis* has been confirmed only from its type locality in Yunnan, the Thai samples most likely represent a new species, or an extension in the distribution range of *R. menglaensis*.

4. Discussion

4.1. Intra- vs interspecific divergence thresholds

Using a divergence-based species-delimitation approach in conjunction with a comprehensive species-level phylogeny, we were able to provide a large-scale, preliminary, relative assessment of species diversity in the family Rhacophoridae. Our results demonstrate the existence of substantial overlap between intra- and interspecific divergence thresholds as applied in many recent studies. Although this phenomenon has been shown in previous studies (Vences et al., 2005b, 2005a; Vieites et al., 2009) and does not necessarily reflect inaccuracies in species-delimitation, our results nevertheless highlight specific taxonomic groups that should be earmarked for future re-examination. Our findings are particularly relevant for rhacophorids because numerous named taxa exhibiting low genetic divergences (consistent with intraspecific population-divergence levels based on our ABGD analysis) have been synonymized recently (Nguyen et al., 2014b; Poyarkov et al., 2015; Yu et al., 2013), indicating that species diversity in other genera included here also may be overestimated. Conversely, we recognize that interspecies genetic divergences can be low (Wickramasinghe et al., 2015), thereby underscoring one of the major limitations of distance-based species delimitation and the importance of incorporating multiple lines of evidence within an integrative species-delimitation framework (Barley et al., 2013; Chan et al., 2017; Welton et al., 2017)

4.2. Systematics

Our results demonstrate that *Chiromantis inexpectatus* belongs to the genus *Feihyla*. This was partly implicated in the original description of the species (Matsui et al., 2014), which inferred *C. inexpectatus* as the

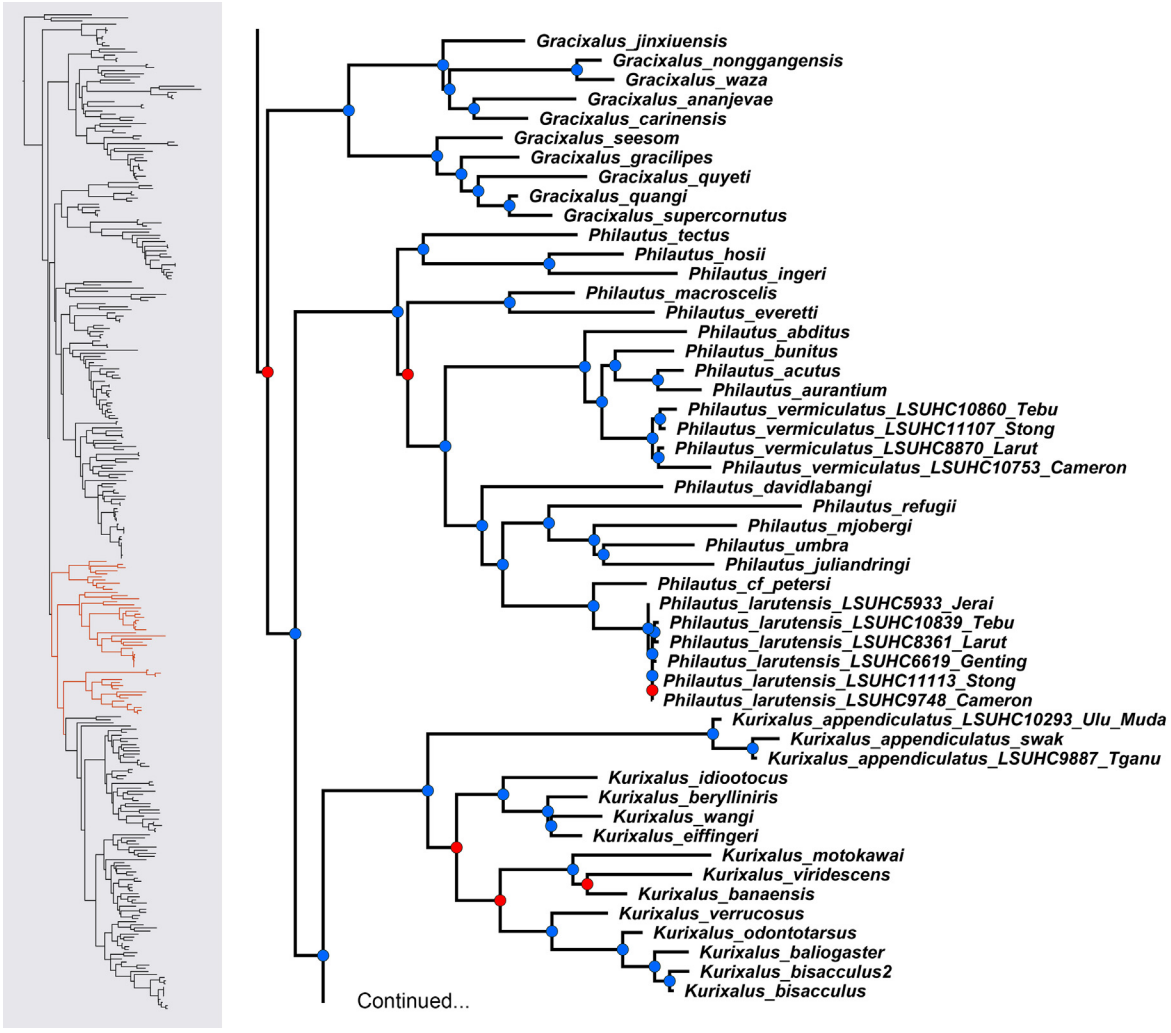

Fig. 5. Large-scale maximum likelihood phylogeny of the family Rhacophoridae estimated using 3483 bp of sequence data from four genes. A skeleton of the entire phylogeny is depicted in the left panel. Selected portions of the tree are highlighted in red and shown in detail on the right. Circles at nodes denote ultrafast bootstrap support values from the IQ-TREE analysis (blue = BS > 90; red = BS < 90).

sister lineage to *F. hansenae* and *F. vittatus*, both of which were considered *Chiromantis* at that time. The same study also included a sample of *F. kajau* that was not recovered as part of the *F. hansenae* and *F. vittatus*, *sensu stricto* clade, but instead was the sister lineage to the genus *Rhacophorus*, albeit with low support. We consider the phylogenetic placements of *F. kajau*, *F. hansenae,* and *F. vittatus* inferred in Matsui et al. (2014) to be in error, as multiple studies (including ours) have corroborated the placement of *F. kajau*, *F. hansenae*, *F. vittatus*, and *F. palpebralis* within the monophyletic genus *Feihyla* (Hertwig et al., 2013; Li et al., 2013). Biogeographically, the sister-species relationship of *F. kajau* and *F. inexpectatus* is not unexpected because both species are endemic to Borneo, whereas *Chiromantis* occurs only on mainland Asia and Africa (Frost, 2017). Therefore, we formally reassign *C. inexpectatus* to the genus *Feihyla*.

We earmark a number of taxa that could potentially be synonymized based on low genetic and morphological differentiation. Because no specimens were examined and the identity of Genbank genetic material could not be positively verified, we hold the formal taxonomic action in abeyance pending further examination of genetic material and evaluation of name-bearing type specimens.

Rhacophorus robinsonii and *R. harrissoni* are morphologically similar species that occur in southern Thailand and Peninsular Malaysia (*R. robinsonii*) and Borneo (*R. harrissoni*) (Berry, 1975; Chan et al., 2010; Inger et al., 2017). There are no morphological characters that discretely distinguish these species (Berry, 1975; Chan and Ahmad, 2009; Inger et al., 2017), and our analyses show that they also are genetically similar, even when applying the most liberal splitting scheme. Another closely related and morphologically similar species, *R. fasciatus,* potentially could be conspecific with *R. robinsonii* as well. Similarly, we earmark *R. belalongensis* and *R. gauni* for potential synonymization based on the non-diagnosability of genetic and morphological data, and overlap in the species' geographical range. The morphological characters that distinguish these species include blue blotches in the groin region for *R. belalongensis* (rich golden yellow in *R. gauni*) and reported presence, versus absence, of a dermal tubercle on the eyelid of *R. gauni* (Inger et al., 2017). However, these characters do not appear to be exclusive to either species; Inger et al. (2017) noted that the eyelid tubercle in *R. gauni* is only "usually" present. Additionally, we have photographic evidence of a specimen from Lanjak Entimau, Sarawak lacking this eyelid tubercle, and both blue blotches and a rich golden yellow patch in the groin region (Fig. 3 inset). These two named species were lumped in all ABGD partitions indicating high genetic similarity, and an absence of a substantive genetic divergence between the two "species." Another genetically and morphologically similar species, *R. gadingensis,* could also potentially be conspecific and requires further investigation. Based on published descriptions, calls of *R. belalongensis*, *R. gauni*, and *R. gadingensis* also appear to be similar (Inger et al., 2017).

Fig. 6. Large-scale maximum likelihood phylogeny of the family Rhacophoridae estimated using 3483 bp of sequence data from four genes. A skeleton of the entire phylogeny is depicted in the left panel. Selected portions of the tree are highlighted in red and shown in detail on the right. Circles at nodes denote ultrafast bootstrap support values from the IQ-TREE analysis (blue = BS > 90; red = BS < 90). Clades that contain potential undescribed species or multiple species that could be lumped are highlighted in orange.

The *Rhacophorus bipunctatus* complex has been a source of taxonomic confusion for many decades due to its morphological variability and similarity to *R. rhodopus* (Bordoloi et al., 2007; Dubois, 1987; Inger, 1966; Li et al., 2012; Wilkinson et al., 2005). Once thought to be widespread from northeastern India, southern China, and most of Southeast Asia, the distribution of *R. bipunctatus* has now been restricted to northeastern India and Myanmar, whereas *R. rhodopus* is known from northern Thailand, Laos, Vietnam, southern China, and occurs sympatrically with *R. bipunctatus* in northeastern India and Myanmar (Bordoloi et al., 2007; Li et al., 2012). This leaves populations from Peninsular Malaysia (currently referred to *R. bipunctatus*) without a name (Bordoloi et al., 2007). Bordoloi et al. (2007) defined *R. bipunctatus* as having green dorsal coloration and 1–3 ink-black spots on the flanks, (versus reddish-brown or brown dorsal coloration and usually one spot on the flank in *R. rhodopus*). In Peninsular Malaysia, both green and brown forms are present, including a population on Langkawi Island that has reddish-brown dorsal colouration and no flank spots (Berry, 1975; Grismer et al., 2006; Fig. 4 inset). Our analyses showed that all Peninsular Malaysian samples (both green and brown forms, including the population from Langkawi Island) are conspecific and genetically most similar to *R. rhodopus*. However, the ABGD analysis did not lump Peninsular Malaysian samples with *R. rhodopus*, indicating that the former could represent an undescribed lineage. For now, we consider Peninsular Malaysian populations to be *R. rhodopus*, pending examination of true *R. rhodopus* specimens and the name-bearing types. Our phylogeny and ABGD analysis reveal considerable genetic structure within *R. rhodopus*, which warrants further investigation, particularly in cases of populations from Laos, northern Thailand, Yunnan (Fig. 4, Clade 2), Hainan, and Vietnam (Clade 1). We note that dorsal coloration and flank spots are not reliable diagnostic characters for distinguishing *R. bipunctatus* from *R. rhodopus*. In fact, specimens vary from green to brown within seconds when handled or stressed (*personal observations*). The significant genetic structuring and overlap in distribution and morphology among *R. bipunctatus* and *R. rhodopus* populations begs the question of whether gene flow/hybridization occurs between these two species.

5. Conclusions

In this study, we have shown that the taxonomy and evolutionary history of the family Rhacophoridae is far from stable or clearly understood; we report potential inconsistencies between taxonomy and phylogeny at the level of genus, species, and population. Phylogenetic relationships among numerous genera have yet to be resolved, and species are still being synonymized and described at a high rate. Using a comprehensive phylogeny, in conjunction with distance-based species-delimitation methods, we were able to screen the family for taxonomic inconsistencies and potential unrecognized species. Our results demonstrate the large and overlapping variance in genetic divergence levels underlying currently accepted taxonomy. As more and more cryptic and recently diverged lineages are described as new species, the use of integrative species-delimitation approaches, employing multiple data streams, are unambiguously preferred—but might also be required if we are to move beyond the largely arbitrary and subjective taxonomic practices of the last several centuries.

Acknowledgements

RMB's work on Asian rhacophorids has been supported by grants from the U.S. National Science Foundation (DEB 0344430, 0640737, 0743491, 1654388 and EF 0334952). CKO's work was supported by U.S. National Science Foundation (DEB 1702036) and the National Geographic Society (9722-15).

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