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## Diversification in a biodiversity hotspot – The evolution of Southeast Asian rhacophorid tree frogs on Borneo (Amphibia: Anura: Rhacophoridae)

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## ABSTRACT

The tree-frog family Rhacophoridae is a major group contributing to the high species richness and reproductive diversity among vertebrates of Sundaland. Nonetheless, rhacophorid evolution, specially on Borneo, has not been studied within a phylogenetic context. In this study, we examine the phylogenetic relationships of 38 (out of 41) Bornean species of Rhacophoridae, in combination with data from previous phylogenetic studies. In the final super matrix of 91 species, we analyse sequence data from two mitochondrial and three nuclear genes. The resulting trees show the genus *Rhacophorus* as a paraphyletic assemblage. As a consequence, we transfer *Rhacophorus appendiculatus* and *R. kajau* to two other genera and propose the new phylogeny-based combinations- *Kurixalus appendiculatus* and *Feihyla kajau*, respectively. Furthermore, we use our phylogenetic hypotheses to reconstruct the evolution of reproductive modes in rhacophorid tree frogs. Direct development to the exclusion of a free larval stage evolved twice independently, once in an ancestor of the *Pseudophilautus* + *Raorchestes* clade in India and Sri Lanka, and once within *Philautus* in Southeast Asia. The deposition of egg clutches covered by a layer of jelly in *Feihyla* is also present in *F. kajau* and thus confirms our generic reassignment. The remarkably high diversity of rhacophorid tree frogs on Borneo is the outcome of a complex pattern of repeated vicariance and dispersal events caused by past changes in the climatic and geological history of the Sunda shelf. We identified geographic clades of closely related endemic species within *Rhacophorus* and *Philautus*, which result from local island radiations on Borneo.

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## 1. Introduction

The biogeographic region termed Sundaland, which consists of the Malay Peninsula, Borneo, Sumatra, Java, Bali, Palawan and some smaller islands, is a global biodiversity hotspot (Myers et al., 2000). The extraordinarily high species richness found in numerous groups of plants and animals in this region is probably the result of its complex geological and climatological history (Den Tex et al., 2010; Esselstyn et al., 2010; Hall, 1998, 2002; Heaney, 1986). The rhacophorid frogs in this area are a prime example of a highly diverse vertebrate group. Similar to India and Sri Lanka (Meegaskumbura et al., 2002), Southeast Asia and Sundaland, in particular, constitute one of the centres of high species richness of the taxon Rhacophoridae (Liem, 1970; Channing, 1989; Frost et al., 2006; Pyron and Wiens, 2011). The approximately 300 extant species exhibit a broad variety of ecological adaptations and micro-

habitat preferences, mirrored by impressive variation in body size, colouration and other aspects of external morphology. This diversity also extends to modes of reproduction, which are typically associated with habitat preferences and range from the production of foam nests for egg protection to direct development without free-swimming larval stages (Brown and Alcalá, 1983, 1994; Channing, 1989; Grosjean et al., 2008; Li et al., 2009, 2011; Liem, 1970). Our knowledge and understanding of the evolution of these diverse reproductive strategies in rhacophorid tree frogs, however, are relatively sketchy; our observations of their field ecology are anecdotal.

Within Rhacophoridae, the taxon Buergeriinae Channing, 1989 consists of only one genus of four described species (Frost, 2011). In contrast, the taxon Rhacophorinae Hoffmann, 1932, which constitutes the other major group, comprises about 300 described species. Although numerous studies have been published on the phylogenetic relationships and resulting systematics of Rhacophorinae, the phylogeny of many of its genera and species remains a matter of debate (Biju et al., 2008; Channing, 1989; Delorme et al., 2005; Frost et al., 2006; Grosjean et al., 2008; Hertwig et al., 2012; Li et al., 2008, 2009; Liem, 1970; Pyron and Wiens,

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2011; Richards and Moore, 1998; Wilkinson et al., 2002; Wilkinson and Drewes, 2000; Ye et al., 1999; Yu et al., 2007, 2008, 2009). The usage, delimitation and definition of the 13 described genera of the taxon Rhacophorinae continue to be inconsistent. As a result, many of the species belonging to this group have a confusing taxonomic history involving several changes of generic assignment. The enduring state of taxonomic instability is exacerbated by the fact that numerous genera lack clear morphological synapomorphies that might be deemed diagnostic characters (Biju et al., 2008; Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008, 2009, 2011; Ye et al., 1999; Yu et al., 2009). This applies not only to genera described or revalidated in recent years, predominantly on the basis of genetic data, but also to genera traditionally recognised as such whose monophyly has never actually been demonstrated using comprehensive taxon samples.

The genus *Philautus*, for example, formerly used as a collective genus for smaller rhacophorid species distinguished by direct development (observed or suspected), was recently recognised as a polyphyletic assemblage. As a consequence, several species formerly assigned to *Philautus* have been transferred to *Feihyla*, *Liuixalus*, *Chiromantis*, *Ghatixalus*, *Kurixalus*, *Pseudophilautus*, *Raorchestes* and *Theloderma* (Biju et al., 2010; Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008, 2009, 2011; Yu et al., 2008). Another genus whose phylogenetic position and species composition is uncertain is *Chiromantis*, the sole genus whose distribution includes both Asia and Africa. Several molecular genetic studies over the past years have failed to resolve congruently the phylogenetic relationships of the species in this genus (Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008, 2009; Pyron and Wiens, 2011; Wilkinson et al., 2002; Yu et al., 2008). The uncertainty surrounding the phylogenetic relationships between and within the various rhacophorid genera and species, combined with our lack of knowledge of their effective diversity and reproductive behaviour, is a stumbling block to evolutionary, ecological, and biogeographical studies and, ultimately, to conservation efforts.

Borneo is the largest island in Sundaland and one of the most important diversity hotspots for rhacophorid tree frogs, boasting 41 described species. Under current taxonomic conventions, these species are allocated to five genera: *Philautus*, *Polypedates*, *Rhacophorus*, *Nyctixalus* and *Theloderma*. Not all of them, however, have been included in phylogenetic analyses, implying that their generic assignment has not been tested within a phylogenetic framework (Hertwig et al., 2011). Some genera may, therefore, constitute paraphyletic or polyphyletic assemblages, whose current species composition reflects historical perceptions of taxonomic concepts rather than phylogeny. The Bornean species of the widespread genus *Rhacophorus*, for example, include both small frogs such as *R. kajau* (snout-vent length [SVL] of under 20 mm), and the large parachuting frog *R. nigropalmatus* (SVL of 100 mm) (Dring, 1983; Inger, 1966; Inger and Stuebing, 2005). Besides displaying obvious differences in external morphology, the natural-history data available indicate that the *Rhacophorus* species on Borneo also vary considerably in ecology and reproductive behaviour, with species-specific breeding sites ranging from fast running streams in *R. gauri* to tree holes in *R. harrisoni* (Inger, 1966; Inger and Stuebing, 2005). While most species produce foam nests to protect their eggs, others including *R. kajau* (Dring, 1983; Inger and Stuebing, 2005) lay jelly-coated clutches. Furthermore, there is no synapomorphy-based morphological diagnosis available for *Rhacophorus* that allows us to test the assignment of a species to the genus. As a result it remains unclear whether *Rhacophorus* in its current taxonomic usage really does represent a natural group. *Philautus everetti* and *P. macroscelis*, for example, formerly regarded as members of *Rhacophorus*, have been recently transferred to *Philautus* on the basis of the first phylogenetic study using molecular data (Hertwig et al., 2011).

In this study, we examine the phylogenetic relationships of 38 (of 41) Bornean species of Rhacophoridae using sequence data from two mitochondrial and three nuclear genes. Taking a supermatrix approach, we then combine our data with sequence data from previous phylogenetic studies on rhacophorid frogs to include representatives from all valid genera. On the basis of this comprehensive data set of 91 species, we test whether the current generic assignments of rhacophorid frogs from Borneo correspond to their inferred phylogeny. We then use our results to propose new taxonomic assignments for species whose current taxonomic allocations were not verified by the phylogenetic hypothesis obtained. On the basis of our broad taxon sample, we reconsider the evolution of the reproductive strategies of rhacophorid frogs. Finally, we discuss the influence of local island radiations and independent dispersal events, respectively, on the evolution of species richness in the Bornean Rhacophoridae. Our goal is to progress towards a rigorous, phylogeny-based taxonomy that can contribute to subsequent studies on the phylogeography, evolution and ecology of this highly diverse group of frogs.

## 2. Materials and methods

### 2.1. Sampling and markers

Adult specimens, tadpoles and tissue samples of Bornean rhacophorid frogs were collected between 2002 and 2012 during multiple field trips to different areas of Sarawak and Sabah, Malaysia. Tadpoles and frogs were photographed alive after capture (Nikon D70, Canon EOS 350 D, Canon EOS 5D Mark II, 100 mm, 105 mm and 180 mm macro lenses, various flashes) as described elsewhere (Haas and Das, 2011; Hertwig et al., 2012). Specimens were then anesthetized and euthanised in a ca. 2% aqueous Chlorobutanol solution (1,1,1-trichloro-2-methyl-2-propanol). Tissue samples were taken from liver or femoral muscles in adult frogs, and from the tail musculature in tadpoles, and stored in either RNALater buffer solution (Ambion/Applied Biosystems) or absolute ethanol. Voucher specimens were preserved in 4% neutral buffered formalin for at least two weeks, then washed in tap water and later transferred to 75% ethanol via 30% and 50% steps to avoid shrinkage.

We used the set of mitochondrial data (12S, 16S rDNA) from Hertwig et al. (2011) and extended the data matrix to include seven additional species and sequences from the three nuclear genes encoding POMC (proopiomelanocortin), BDNF (brain-derived neurotrophic factor) and tyrosinase. A total of 49 samples from 38 Bornean species (Appendix) were examined. For *Rhacophorus baluensis*, *R. appendiculatus*, *R. cyanopunctatus*, *R. kajau*, *R. gadingensis*, *Philautus disgregus* and *P. kerangae*, it was the first time they had ever been analysed in a phylogenetic context using molecular data. The sample contained all the described species of rhacophorid frogs in Borneo except *Philautus erythropthalmus*, *P. saueri* and *Polypedates chlorophthalmus*, for which tissue samples were unavailable in international collections. In species with a wide geographical distribution on Borneo, we used samples from two different populations wherever available to subdivide terminal branches. In the next step, we combined our data with sequences from GenBank that had been published in earlier studies into the phylogeny of rhacophorid frogs (Biju et al., 2008; Li et al., 2008, 2009; Wilkinson et al., 2002) to form a matrix consisting of 87 samples. Finally, we added 17 terminal taxa and compiled a supermatrix comprising 104 terminal samples representing 91 species from all the phylogenetic lineages currently regarded as genera within Rhacophoridae.

In most cases we included the type species of the respective genus: *Chiromantis xerampelina* Peters, 1854; *Feihyla* Frost et al., 2006, including *F. palpebalis* (Smith, 1924), described as *Philautus*

*palpebralis*; *Ghatixalus* Biju, Roelants and Bossuyt, 2006, including *G. variabilis* (Jerdon, 1853), described as *Polypedates variabilis*; *Gracixalus* Delorme, Dubois, Grosjean and Ohler, 2005, including *G. gracilipes* (Bourret, 1937), described as *Philautus gracilipes*; *Kurixalus* Ye, Fei and Dubois in Fei, 1999, including *K. eiffingeri* (Boettger, 1895), described as *Rana eiffingeri*; *Liuxalus* Li, Che, Bain, Zhao and Zhang, 2008, including *L. romeri* (Smith, 1953), described as *Philautus romeri*; *Nyctixalus margaritifer* Boulenger, 1882; *Philautus* Gistel, 1848, including *P. aurifasciatus* (Schlegel, 1837) described as *Hyla aurifasciata*; *Polypedates* Tschudi, 1838, including *P. leucomystax* (Gravenhorst, 1829), described as *Hyla leucomystax*; *Pseudophilautus* Laurent, 1943, *Raorchestes* Biju, Shouche, Dubois, Dutta and Bossuyt, 2010, including *R. glandulosus* (Jerdon, 1854), described as *Ixalis glandulosa*; *Rhacophorus reinwardtii* Kuhl and van Hasselt, 1822, described as *Rhacophorus moschatus* (a subjective synonym of *Hyla reinwardtii* Schlegel, 1840). Genetic data were unavailable for *Theloderma leporosum* Tschudi, 1838 and the extinct *Pseudophilautus temporalis* (Günther, 1864) (Biju and Manamendra-Arachchi, 2004).

Some of the species in the final super matrix were represented by a low number of orthologous base pairs. For *Nyctixalus margaritifer*, *Philautus aurifasciatus* and *Raorchestes glandulosus*, only short stretches of the mitochondrial sequences were available, but as they pertained to type species of genera (see above), we included them in our analyses anyway. The comparison of separate analyses of the matrix consisting of the complete sample of 104 terminals with a reduced matrix of 87 samples (excluding all terminals with incomplete sequence data) allowed us to evaluate the influence of the missing data on the outcome of phylogenetic reconstructions. Surprisingly, in three sequences from GenBank (*Ghatixalus asterops*, *G. variabilis*, *Nyctixalus margaritifer*), the most variable regions of the ribosome had been removed by the authors before submission. The obviously missing bases were thus coded as missing data in the super matrix.

For the purposes of tree rooting, the two species of *Buergeria* Tschudi, 1838 representing the taxon Buergeriinae (*Buergeria japonica*, *B. oxycephala*), were defined as an outgroup in accordance with current phylogenetic hypotheses regarding the monophyly of Rhacophorinae (Channing, 1989; Grosjean et al., 2008; Li et al., 2009; Liem, 1970; Wilkinson et al., 2002).

## 2.2. Laboratory protocols

Total genomic DNA was extracted from macerated muscle or liver tissue using peqGold Tissue DNA Mini Kits (PEQLAB Biotechnologie GmbH), Wizard<sup>®</sup> SV Genomic DNA Purification System (Promega, Switzerland), or DNeasy<sup>®</sup> Blood and Tissue Kit (Qiagen, Switzerland) according to the manufacturer's protocols. Different primer combinations were used for the amplification of the five markers (Table 1). For 12S rDNA and 16S rDNA, we used 25 µl PCR reaction volume containing 1 µl DNA, 1 µl of each primer (20 pmol/µl (20 µM), 1.5 µl MgCl<sub>2</sub> Magnesiumchlorid, 12.5 µl peqGOLD PCR-Master-Mix Y (PEQLAB) or GoTaq<sup>®</sup> Hot Start Green Master Mix (Promega) and 8 µl ddH<sub>2</sub>O (PEQLAB, Promega). The cycling conditions for amplification were: denaturation at 94 °C for 2.0 min; 35 cycles at 94 °C for 0.5 min, 48 °C or 50 °C for 0.5 min, and 72 °C for 1.0 min; then one final extension cycle at 72 °C for 5.0 min, stop at 4 °C. For the nuclear markers, PCR volumes were 25 µl PCR containing 2 µl DNA, 2 µl of each primer (10 pmol/µl (10 µM), 12.5 µl peqGOLD PCR-Master-Mix Y or GoTaq<sup>®</sup> Hot Start Green Master Mix and 6.5 µl ddH<sub>2</sub>O. The nuclear markers were amplified via an initial denaturation at 94 °C for 2.0 min, followed by 35 cycles at 94 °C for 0.5 min, 51.5 °C (BDNF), 56 °C (POMC) or 52 °C (Tyrosinase) for 0.5 min, and 72 °C for 1.0 min; then one final extension cycle at 72 °C for 5.0 min followed by a stop at 4 °C. All PCRs were performed on a Techne TC-512 thermo-cycler. PCR

products were excised from agarose gels and cleaned using the Wizard<sup>®</sup> SV Gel and PCR Clean-UP System (Promega). For most samples, two 25 µl reactions were run, and excised bands were put together for cleaning to increase the concentration of PCR product for sequencing. Sequencing was done in both directions by Microsynth AG (Balgach, Switzerland), or Macrogen Inc. (Seoul, South Korea) using the same primers as for amplification. Sequence preparation, editing and management were performed using Bio-Edit 7.0.5.2 (Hall, 1999) and Geneious Pro 5.6 (Drummond et al., 2009). Chromas lite 2.01 (Technelysium Pty. Ltd., [www.technelysium.com](http://www.technelysium.com)) software was used to check the trace files of the sequencers.

## 2.3. Phylogenetic analyses

The five markers were aligned separately using the MAFFT algorithm (Katoh et al., 2002) implemented as a plug-in in Geneious Pro, with the E-INS-i mode and standard parameters set to obtain alignments with maximised sequence similarity (Morrison, 2009). After alignment, the five genes were each analysed separately using the PhyML plug-in in Geneious Pro to check for sequencing or other laboratory errors. Then, we concatenated the sequences of the two ribosomal genes and the three nuclear genes, respectively, to obtain a mitochondrial (MIT = mitochondrial, consisting of concatenated 12S, 16S rDNA sequences) and a nuclear (NUC = nuclear, consisting of concatenated BDNF, POMC, tyrosinase sequences) data partition using Geneious Pro. We then combined the nuclear and mitochondrial sequence data into a single matrix (NUCMIT = nuclear + mitochondrial, equalling all five genes). To incorporate phylogenetic information stemming from indels (e.g. Egan and Crandall, 2008; Giribet and Wheeler, 1999; Hertwig et al., 2004; Lutzoni et al., 2000; Müller 2005, 2006; Nagy et al., 2012; Saurabh et al., 2012; Simmons and Ochoterena, 2000; Simmons et al., 2001) into the phylogenetic reconstructions using ML and BI optimality criteria, we coded the gaps in the alignment using SeqState 1.4.1 (Müller, 2005, 2006), selected the simple indel coding (SIC) algorithm (Simmons and Ochoterena, 2000) and appended the coded indels as additional characters to the alignments. Phylogenetic information from indels was included because this approach yielded the most resolved trees in a previous study (Hertwig et al., 2011). We repeated all analyses without the separately encoded indels. In a first step NUC and MIT were each analysed separately. Following the total evidence approach (Kluge, 1989, 1999; Thompson et al., 2012) we used the concatenated matrices NUCMIT87 (87 terminal taxa) – and NUCMIT104 (104 terminal taxa) corresponding to our sampling strategy described above for the final phylogenetic reconstructions.

On the basis of the Akaike information criterion, as implemented in MrModeltest 2.3 (Nylander, 2004), we then selected the best-fitting model of sequence evolution for the concatenated mitochondrial genes and for the three concatenated nuclear genes separately. In phylogenetic analyses, we applied separate sequence evolution models to the MIT partition, the three codon positions of each of the three nuclear genes, and to the appended encoded indels. This produced a total of eleven data sections.

When performing Maximum Likelihood (ML) analyses, we used the web server-based version of RAXML 7.0.4 (Stamatakis et al., 2008), 100 rapid bootstrap inferences, and the Gamma model of rate heterogeneity. All free model parameters were estimated by the software, and the Maximum Likelihood search option was used to search for the best-scoring tree. An additional alignment-partition file was uploaded to cause the RAXML software to search for separate sequence evolution models for each of the data sections (mitochondrial rDNA genes, nuclear genes, their codon positions, encoded indels, respectively).

**Table 1**  
Primers used for the amplification of the five genes analysed in this study.

Marker	Primer name	Primer sequence (5'–3')
16S rDNA	16SC	GTRGGCCTAAAAGCAGCCAC
	16SA-L	CGCCTGTTTATCAAAAACAT
	16SCH	TCAAHTAAGGCACAGCTTA
	16SD	CTCCGGTCTGAACCTCAGATCACGTAG
	16SB-H	CCGGTCTGAACCTCAGATCACGT
	16SD-DR	ACAAGTGATTAYGCTACCT
12S rDNA	12SA-L	AAACTGGGATTAGATACCCCACTAT
	12SB-H	GAGGGTGACGGGCGGTGTGT
BDNF	BDNF F1	ACCATCCTTTTCTKACTATGG
	BDNF F	GCCCCATGAAAGAAGCCAGTGT
	BDNF R1	CTATCTTCCCCTTTTAATGGTC
	BDNF R	TCCTTATAAAGCGCCAGCCAACTTT
POMC	POMC DRV F1	ATATGTCATGASCCAYTTYCGCTGGAA
	POMC F	GGGGGCTACAAGCGGGAGGA
	POMC DRV R1	GGCRTTYTTGAAWAGAGTATTAGWGG
	POMC R	TGGTGTCTGGCTTCTCTCTGGRG
Tyrosinase	Tyr 1a	AGGTCTCTTRAGCAAGGAATG
	TyrFL	TGTGGAGAAGCCTCAGGCAGA
	TyrFS	CTCAGGCATTGATGACCGAGAAAAC
	Tyr 1g	TGCTGGGCRTCTCTCCARTCCA
	Tyr R	TCTCTCCAGTCCCAGAAGGGRATAGT

Bayesian Inference (BI) was performed using MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The indels in the appended character block were treated as binary characters. We made two independent runs of Metropolis-coupled Markov chain Monte Carlo analyses, each consisting of one cold chain and three heated chains with a default temperature of 0.2. The chains were run for 50 million generations, with sampling every 100 generations. We qualitatively checked whether the chains reached stationarity after this period using Tracer 1.5 (Rambaut and Drummond, 2007) and then discarded the first 10% of sampled trees as burn-in (10,000 trees). We checked that the average standard deviation of split frequencies converged towards zero and compared the likelihoods and posterior probabilities of all the splits to assess convergence between the two independent runs using AWTY (Wilgenbusch et al., 2004; Nylander et al., 2008).

Character evolution was reconstructed using Mesquite 2.75 (Maddison and Maddison, 2011). We selected the Markov k-state 1-parameter model (Mk1) to infer ancestral character states following the implemented Markov model approach (Lewis, 2001). Geneious Pro and Inkscape ([www.inkscape.org](http://www.inkscape.org)) were used to prepare the tree graphics.

### 3. Results

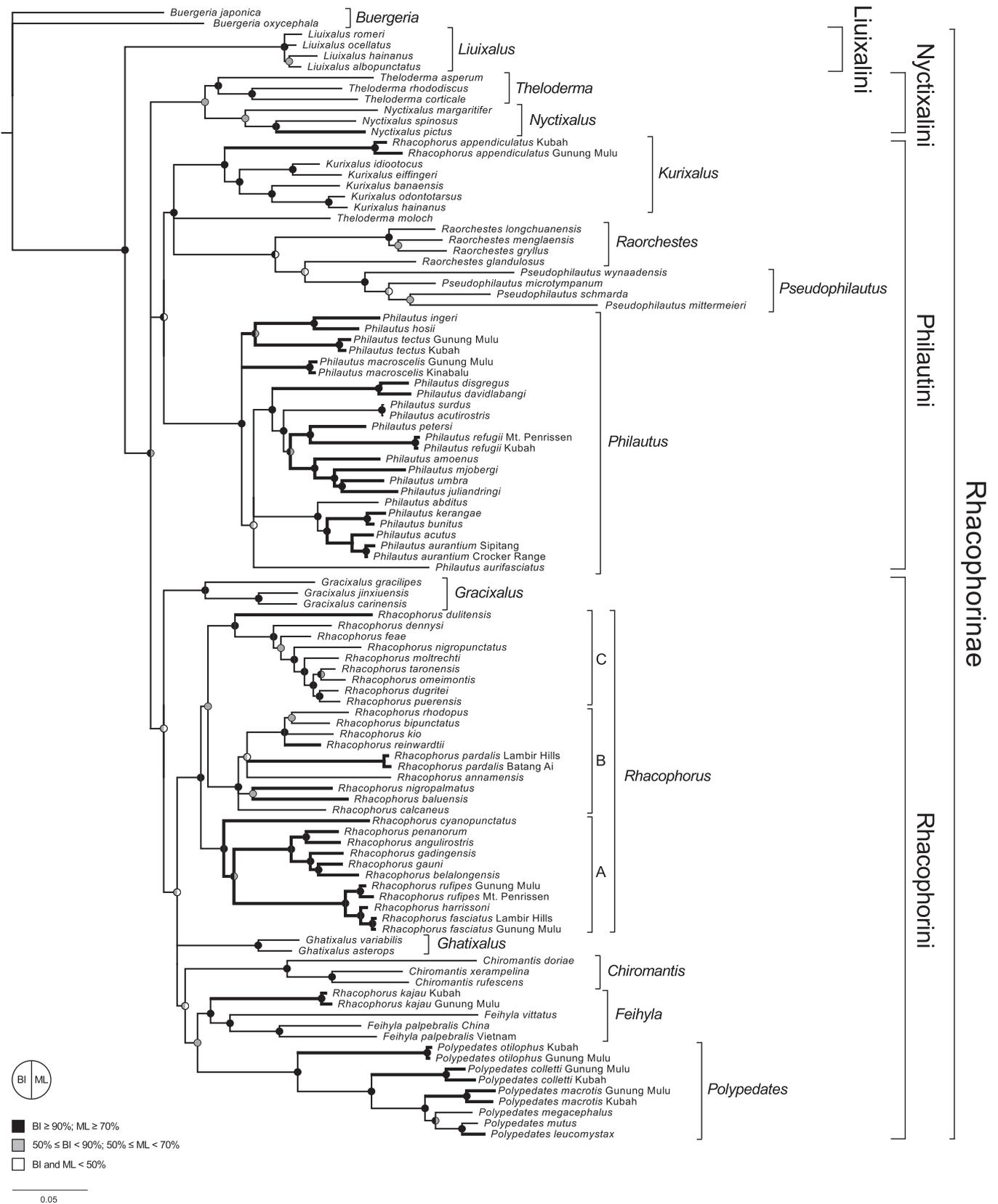
The final concatenated alignments based on 87 samples NUC-MIT87 consisted of 2954 bp (NUC = 1706 + MIT = 1248); with appended indels, the data matrix consisted of 3108 bp (NUC = 1715 + MIT = 1392). The super matrix of 104 samples NUC-MIT104 comprised 2960 bp and, when the indels were appended, 3133 positions.

Analysing mitochondrial and nuclear sequences separately (NUC87 and MIT87 data partitions) produced conflicting results with low support values for the relationships among genera. In contrast, the topologies of the eight trees resulting from the concatenated matrices (ML and BI, NUCMIT104 and NUCMIT87, with or without separate indel encoding, respectively) do not differ significantly from each other except for the positions of a few taxa (Figs. 1 and 2, Supplementary material). The support values of several nodes, however, differ between the analyses. Certain nodes are robustly supported only in the analyses of the reduced NUCMIT87

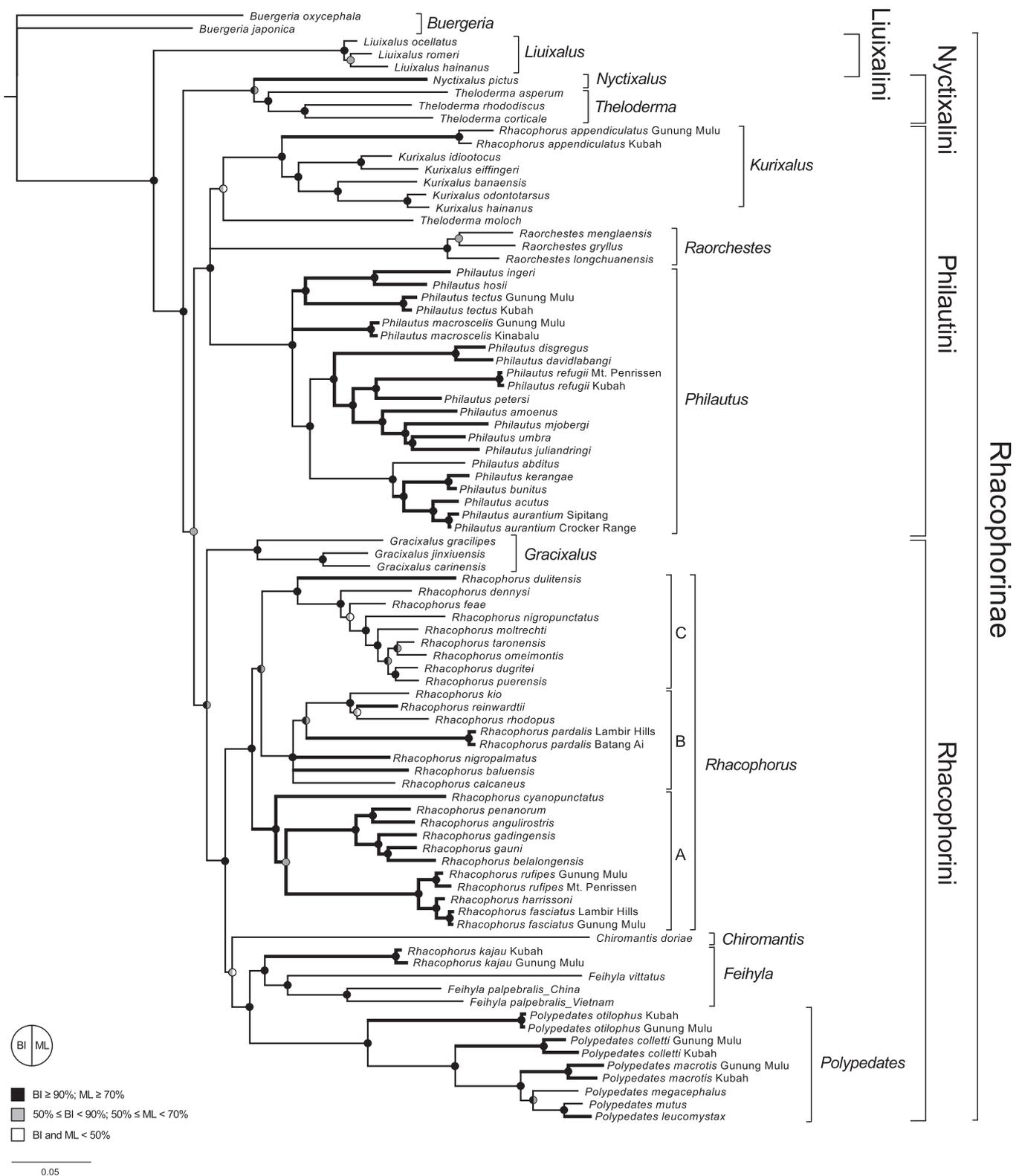
matrix or receive robust support only in BI and not in ML analyses (Figs. 1 and 2, Supplementary material). The BI 50% majority rule consensus trees resulting from the analyses including separately encoded indels tend to have a slightly higher resolution in comparison to the results of the matrices without appended encoded indels (Supplementary material). Nodes were considered robustly supported when either BI posterior probability values were greater than 90% or when ML bootstrapping outputs were greater than 70%. Moderately supported nodes received 50% to 89% posterior probability and/or 50% to 69% bootstrapping support.

The taxon Rhacophorinae was corroborated as monophyletic, receiving robust support. The monophyly of the following genera is consistently supported: *Chironomantis* (sensu Fei et al., 2010), *Feihyla* (sensu Fei et al., 2010), *Ghatixalus*, *Gracixalus*, *Kurixalus* (sensu Li et al., 2008), *Liuxalus*, *Philautus* (sensu Hertwig et al., 2011), *Polypedates* and *Pseudophilautus*. *Raorchestes* (sensu Biju et al., 2010) is not unambiguously revealed to be a monophyletic entity when *R. glandulosus* is included, but receives robust support in the analyses of the reduced NUCMIT87 matrix. The species considered to belong to the genus *Theloderma* are not supported as a monophyletic group. Although *Theloderma asperum*, *T. corticale* and *T. rhododiscus* cluster together with robust support, *T. moloch* constitutes an isolated lineage within Rhacophorinae.

Likewise, the genus *Rhacophorus* is polyphyletic. The sister-group relationship between *R. appendiculatus* and the genus *Kurixalus* is robustly supported. *Rhacophorus kajau* forms the sister taxon to the genus *Feihyla*. The remaining species of the genus *Rhacophorus* (excluding *R. appendiculatus* and *R. kajau*) constitute a robustly supported monophyletic group corroborating previous studies (Hertwig et al., 2011; Li et al., 2008, 2012). Within Clade A (Figs. 1 and 2), *R. cyanopunctatus*, a species with a wide distribution on mainland southeast Asia and Borneo, is revealed to be the sister taxon to a group of exclusively Bornean species. This Bornean group can in turn be divided into two subclades, the *angulirostris* group (*R. angulirostris*, *belalongensis*, *gadingensis*, *gaumi*, *penanorum*) and the *fasciatus* group (*R. fasciatus*, *harrissoni*, *rufipes*). Clade B consists of the Bornean flying frogs (*R. baluensis*, *pardalis*, *nigropalmatus*, *reinwardtii*) and related mainland species. Clade C predominantly contains species from the Southeast Asian mainland, except *R. dulitensis*, which is from Borneo. *R. dulitensis* is the sister taxon to the remaining members of Clade C (Figs. 1 and 2).



**Fig. 1.** Total evidence phylogenetic hypothesis for the Rhacophoridae of Sundaland, based on the 50% majority rule consensus tree of BI using the super matrix which includes three nuclear and two mitochondrial gene fragments of 104 samples (NUCMIT104). The node support obtained from BI and RAxML analyses was plotted using three classes of support values: robust support: BI ≥ 90%; ML ≥ 70% (black); moderate support: 50% ≤ BI < 90%; 50% ≤ ML < 70% (grey); not supported: BI and ML < 50% (white). Bold branches indicate occurrence on Borneo.



**Fig. 2.** Total evidence phylogenetic hypothesis for the Rhacophoridae of Sundaland, based on the 50% majority rule consensus tree of BI using the NUCMIT87 matrix of five concatenated genes. The node support obtained from BI and RAxML analyses was plotted using three classes of support values: robust support: BI ≥ 90%; ML ≥ 70% (black); moderate support: 50% ≤ BI < 90%; 50% ≤ ML < 70% (grey); not supported: BI and ML < 50% (white). Bold branches indicate occurrence on Borneo.

Within *Philautus*, most nodes corroborate our previous study (Hertwig et al., 2011). However, we found a signal for a sister-group relationship between *P. tectus* and the clade consisting of

*P. ingeri* and *P. hosii*. These three species are endemic on Borneo. The position of *P. macroscelis* within *Philautus* remains unresolved. The remaining species of *Philautus* are robustly supported as a

clade when *P. aurifasciatus* is excluded from the analyses (NUCMIT87) (Figs. 1 and 2).

Within the Rhacophorinae several deeper nodes that correspond to phylogenetic relationships between the genera are not congruently supported in ML and BI analyses of NUCMIT104 and NUCMIT87 matrices. The genus *Liuixalus* represents the sister group to the remaining taxa of the Rhacophorinae. The sister-group relationship between *Nyctixalus* + *Theلودerma*, which together form the taxon Nyctixalini, is only moderately supported. The group consisting of *R. appendiculatus* and *Kurixalus*, *T. moloch*, *Raorchestes*, *Pseudophilautus* and *Philautus* form one of two major clades within Rhacophorinae (Philautini). However, this clade was not supported in the ML analysis of NUCMIT104 (Supplementary material). Within Philautini *Philautus* is revealed as the sister-group of the remaining lineages with high support values when NUCMIT104 was analysed. The relationships within Philautini remain unresolved in the analyses of NUCMIT87. The second major clade (Rhacophorini) comprises the genera *Feihyla*, *Gracixalus*, *Rhacophorus*, *Chiro-mantis* and *Polypedates*, but it is not unequivocally supported (Figs. 1 and 2, Supplementary material). Within Rhacophorini, the sister-group relationship of (*R. kajau* + *Feihyla*) and *Polypedates* is only moderately supported in the analyses of NUCMIT104 but receives high support values in NUCMIT87 analyses.

## 4. Discussion

### 4.1. Phylogenetic signal

The phylogeny of rhacophorid tree frogs is far from being resolved, despite decades of intense work on this group. Several monophyletic groups are now accepted unequivocally, but a significant portion of the deep nodes within Rhacophorinae (mostly inter-generic relationships) have not yet been resolved with confidence. The low support values in a number of deeper nodes and the conflicting placement of certain taxa in the trees of the present study were also reported in earlier studies that used different combinations of molecular markers and optimality criteria (Biju et al., 2010; Grosjean et al., 2008; Hertwig et al., 2011; Li et al., 2008, 2009; Pyron and Wiens, 2011; Wilkinson et al., 2002; Yu et al., 2009). We can only speculate that this taxon underwent an episode of rapid radiation at some point in the past that left a weak phylogenetic signal in the genes currently used as markers. Our super matrix included 91 of about 300 extant species of rhacophorid tree frogs and contained representatives of all the taxa currently accepted as valid genera. Several taxa from Borneo were analysed here for the first time in a phylogenetic framework based on a combined analysis of nuclear and mitochondrial markers. The known total diversity of the group, thus, was well-mirrored in our sample, and we suggest that incomplete taxon sampling cannot be the major cause of the weak support obtained for certain conflicting nodes.

On the other hand, the large portion of missing data in our NUCMIT104 super matrix may well have influenced the outcome of the ML and BI analyses in some taxa. The uncertain position of *Raorchestes glandulosus* within the *Raorchestes/Pseudophilautus* clade is probably due to the paltry 17.6% of orthologous mitochondrial sequence data available. In *Philautus aurifasciatus* only 17.9% of orthologous base pairs were available, which is likely the reason for its unresolved position within *Philautus*. By lowering the available number of potentially informative characters relative to the number of terminal taxa, the missing data are likely to have reduced support values for certain nodes. In most cases, however, the missing data did not influence significantly the topology of the consensus trees obtained.

When the mitochondrial and nuclear data partitions were analysed separately, we detected weak and conflicting phylogenetic signals for several clades. In contrast, when the data partitions were concatenated and analysed simultaneously, mainly congruent topologies were obtained, regardless of whether 104 or 87 samples were included in the data matrix. We take this as evidence of hidden support for those nodes in the data partitions corresponding to nuclear and mitochondrial genes (Thompson et al., 2012). In this sense, the outcome of the analysis of the combined data set currently constitutes the phylogenetic hypothesis with the highest degree of corroboration (total evidence; Kluge, 1989, 1999).

### 4.2. Phylogenetic relationships and taxonomic consequences

In general terms, our findings are in agreement with previous studies with regard to the composition of most genera, the early separation of *Liuixalus* from other rhacophorids and the sister-group relationships between *Nyctixalus* and *Theلودerma* and between *Kurixalus* and the *Raorchestes/Pseudophilautus* clade (Biju et al., 2008; Channing, 1989; Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008, 2009, 2012; Pyron and Wiens, 2011; Wilkinson et al., 2002; Ye et al., 1999; Yu et al., 2009). Below, we present phylogenetic hypotheses for a number of species analysed here for the first time, and their implications for the evolution of some genera within the Rhacophorinae.

The Bornean species currently subsumed under *Rhacophorus* were not supported as a natural group. *Rhacophorus appendiculatus* (Günther, 1858), originally described as *Polypedates appendiculatus* (see Brown and Alcalá, 1994; Inger, 1966), receives a high level of support as the sister taxon to *Kurixalus*. As far as is currently known, *R. appendiculatus* is distributed over the Southeast Asian mainland, Sumatra, Borneo and the Philippines, but is probably a species complex (B. Stuart, pers. comm.). On the basis of the new evidence, we transfer *R. appendiculatus* to the genus *Kurixalus* Ye, Fei, and Dubois, 1999 and propose the new combination *Kurixalus appendiculatus* comb. nov.

The validity of the genus *Kurixalus* was questioned by Wilkinson et al. (2002) and Matsui and Orlov (2004). However, subsequent phylogenetic analyses using molecular data provided evidence in support of its monophyly, although they did lead to a reassessment of its species composition, with some species transferred to *Aquixalus* and *Gracixalus* and vice versa (Frost et al., 2006; Grosjean, 2008; Li et al., 2008). The phylogenetic relationships between *Kurixalus* and other genera of rhacophorid tree frogs have been a matter of controversy since the genus was first described. Yu et al. (2009) considered *Kurixalus* to be the sister taxon of *Philautus*. In contrast, Pyron and Wiens (2011) found moderate support for a closer relationship between *Kurixalus* and a clade consisting of *Raorchestes* and *Pseudophilautus*. As a result of the inclusion of *K. appendiculatus* in our analyses, we found a strong signal in favour of a clade comprising *Theلودerma moloch*, *Kurixalus sensu nova*, *Raorchestes* and *Pseudophilautus*. *Theلودerma molch* is probably an isolated lineage within the Rhacophorinae, and its assignment to the genus *Theلودerma* may not be correct (Li et al., 2008).

A sister-group relationship between *Philautus* and *Raorchestes*, as suggested by Grosjean et al. (2008), was not corroborated by our data. Within *Philautus*, the new data presented herein confirm the relationships indicated by our previous phylogenetic analysis with regard to the *P. everetti* species group (Hertwig et al., 2011) and provide a first hypothesis concerning the relationships of *P. kerangae* and *P. disgregus*. *P. kerangae* is placed within the *aurantium* group and is closely related to *P. bunitus*. Together, they constitute the sister group to the species pair containing *P. acutus* and *P. aurantium*. *Philautus disgregus*, known only from Sabah, is the sister species to *P. davidlabangi*, a recently described species from wes-

tern Sarawak. We found no convincing evidence in favour of the taxon *Gorhixalus* Dubois 1987, formerly used as a subgenus for *P. ingeri* and *P. hosii* (see the discussion in Hertwig et al., 2011), as the remaining *Philautus* were not supported as a natural group.

Species of the genus *Chiromantis* Peters, 1854 (type species, the African *C. xerampelina*) occur in both Asia and Africa (Channing and Howell, 2006; Frost et al., 2006). The Asian species were formerly assigned to the genus *Chirixalus*, which was later synonymised with *Chiromantis* (Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008, 2009, 2011; Pyron and Wiens, 2011; Richards and Moore, 1998; Wiens et al., 2009; Wilkinson et al., 2001; Yu et al., 2008). *Feihyla palpebralis* (Smith, 1924) is one of those species of rhacophorid frogs with a particularly turbulent taxonomic history. After its description as *Philautus palpebralis*, the taxon was subsequently transferred by different authors to the genera *Rhacophorus*, *Chirixalus*, *Chiromantis* and *Aquixalus*, respectively. Finally, Frost et al. (2006) erected the monotypic genus *Feihyla* for this enigmatic taxon.

*Feihyla vittatus* is another species formerly regarded as belonging to *Chiromantis* whose phylogenetic relationships have long been contentious. Various studies using molecular data have failed to resolve its position within Rhacophorinae congruently (Grosjean et al., 2008; Li et al., 2008, 2009; Pyron and Wiens, 2011; Richards and Moore, 1998; Wiens et al., 2009; Wilkinson et al., 2001; Yu et al., 2008). Most recently, Fei et al. (2010) moved this species into *Feihyla*, but without compelling evidence (see also Li, 2011). In contrast to those of Pyron and Wiens (2011) and Yu et al. (2008), our results confirm the close relationship between *F. vittatus* and *F. palpebralis*, as proposed by Fei et al. (2010). Moreover, our findings for *Rhacophorus kajau*, included in a data set for the first time here, robustly support it as the sister taxon to *Feihyla palpebralis* + *F. vittatus*. We propose, therefore, that *Rhacophorus kajau* be transferred to the genus *Feihyla* and suggest the new combination *Feihyla kajau* comb. nov.. The genus *Feihyla*, thus, currently contains four species: *F. fuhua*, a recently described species from China (Fei et al., 2010), *F. kajau*, *F. palpebralis* (type species) and *F. vittatus*.

The taxonomic rearrangements concerning *Kurixalus appendiculatus* and *Feihyla kajau* proposed herein are justified by the obtained phylogenetic hypothesis. The removal of *K. appendiculatus* and *F. kajau* renders *Rhacophorus* a monophyletic entity. Furthermore, with the incorporation of *K. appendiculatus* and *F. kajau* into established genera, we express our opinion that expanding existing genera on phylogenetic grounds is preferable to creating monotypic genera for each of the two species. Although we found high support values in favour of the nodes relevant to the new position of *K. appendiculatus* and *F. kajau*, the terminal branches of both taxa are relatively long. This may indicate either an isolated evolutionary history or simply point to the fact that taxon sampling is as yet incomplete. In general we understand a genus as a monophyletic group of species that is diagnosable by synapomorphic characters. This premise implies that a genus consists of at least two sister species whose relationship is evidenced by morphological and/or molecular synapomorphies. Assigning a species to a genus expresses information about its closest relatives. In contrast, monotypic genera are established on the basis of an unjustified weighting of autapomorphic characters and are not informative from a phylogenetic point of view (Schweizer et al., 2013). Furthermore, the concept of monophyly at the species level is controversial (Rieppel, 2010), which further calls into question the use of monotypic genera.

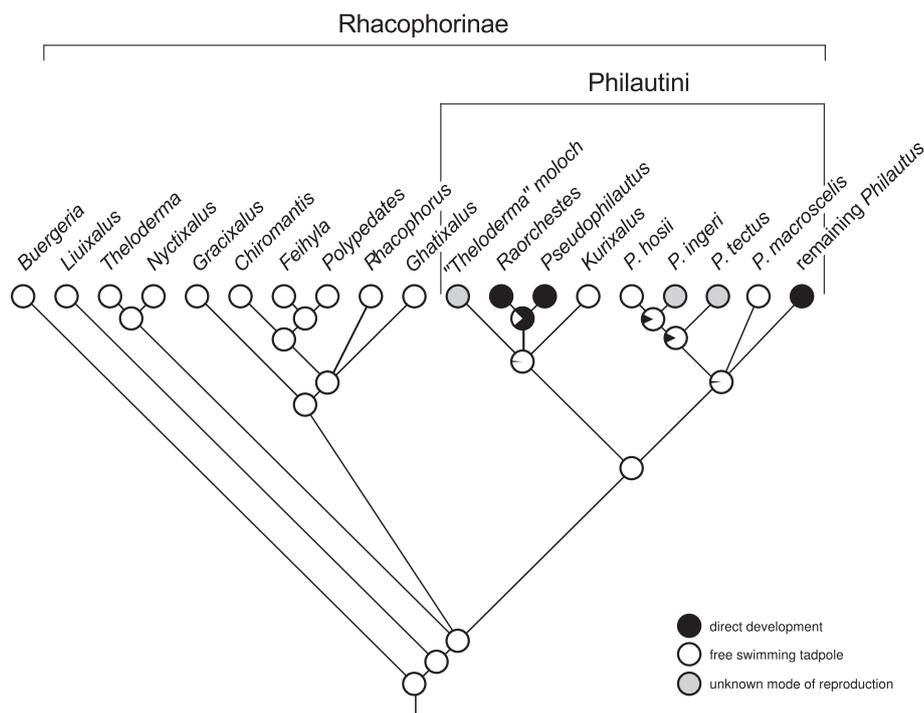
Our results regarding the phylogenetic relationships within *Rhacophorus sensu nov.* are in agreement with Li et al. (2012). *R. gadingensis*, a species recently described from Borneo (Das and Haas, 2005), is placed within Clade A as the sister taxon of *R. gauini* + *R. belalongensis*. The enigmatic montane species from Borneo, *R. baluensis*, which is likewise analysed in a phylogenetic frame-

work for the first time in this study, is part of Clade B together with the large flying frogs from Sundaland and the Asian mainland.

#### 4.3. Implications for the evolution of reproductive behaviour in the Rhacophorinae

Previous definitions and delimitations of the genera of rhacophorid treefrogs were based on single traits of their reproductive biology and failed to reflect the complex evolution and phylogeny of this taxon (Frost, 2011). All small-sized species suspected of direct, aerial development which lack a free-swimming larval stage, for instance, have traditionally been merged together as the genus *Philautus*, implying that this mode of reproduction evolved only once in the taxon Rhacophorinae (Bossuyt and Dubois, 2001; Dring, 1979). Moreover, the actual mode of reproduction of the species in question has been confirmed only in a few cases, either by direct observation of the complete reproductive cycle or by the unambiguous genetic matching of semaphoronts (Hertwig et al., 2012). The presence of larvae in *Chirixalus* species, on the other hand, has been used to separate this genus from *Philautus* (Frost, 2011). However, several studies based on molecular data have failed to reveal convincing evidence in favour of the monophyly of *Philautus* and actually revealed this taxon to be a polyphyletic assemblage (Biju et al., 2010; Grosjean et al., 2008; Li et al., 2009; Pyron and Wiens, 2011; Yu et al., 2008). As a consequence, species that exhibit direct development have recently been assigned to three different genera: *Philautus* Gistel, 1848, *Pseudophilautus* Laurent, 1943 and *Raorchestes* Biju et al., 2010 (Bahir et al., 2005; Biju et al., 2010; Li et al., 2009, 2011). The genus *Pseudophilautus* was resurrected by Li et al. (2009) from synonymy with *Philautus* to distinguish the independent, monophyletic radiation on the Indian subcontinent from the remaining *Philautus* species on the Sunda shelf. Subsequently, Biju et al. (2010) erected the genus *Raorchestes* for the clade of species from the Asian mainland (India to China), while the clade that is endemic to Sri Lanka remained in the genus *Pseudophilautus*. What these authors failed to do, however, was to provide a morphological diagnosis based on synapomorphies or any kind of differential diagnosis for *Raorchestes* as opposed to *Pseudophilautus*.

The present study did not resolve the phylogenetic relationships within Philautini consistently with robust support in all analyses (Figs. 1 and 2). However, a signal in favour of a sister-group relationship between *Philautus* and the *Pseudophilautus*/*Raorchestes* clade was not found. While the reproductive behaviour of *T. moloch* has never been described, *Kurixalus* species are known to have free-swimming tadpoles (Kuramoto and Wang, 1987; Lin and Kam, 2008; Lin et al., 2008; pers. obs. in *K. appendiculatus*). *Philautus macroscelis* breeds via endotrophic, free-swimming tadpoles (Hertwig et al., 2011). A comparable tadpole adapted to an abbreviated, or even missing, free-swimming stage was described by Inger (1966) in *P. hosii* (as *Rhacophorus hosii*). According to our reconstruction of ancestral character states using the combined consensus tree obtained using NUCMIT104 matrix (Fig. 1), the last common ancestor of the extant *Philautus* species most likely possessed a free larva too (Fig. 3). Direct development, therefore, probably evolved twice within Philautini: first in the common ancestor of *Pseudophilautus* and *Raorchestes* in the western part of the Asian distribution of the Rhacophorinae, and second within *Philautus* in Southeast Asia (Fig. 3). We regard the alternative scenario of independent reversals to free-swimming tadpoles in *Philautus hosii* and *P. macroscelis* within *Philautus* as less likely. The finding that direct development evolved within *Philautus* from ancestors with endotrophic larvae would contradict the results obtained by Gomez-Mestre et al. (2012), who tested patterns of evolution in the reproductive modes of frogs and found transitions to direct development only from exotrophic tadpoles.



**Fig. 3.** Ancestral “character state” reconstruction for the evolution of direct development within the Sundaland Rhacophoridae, obtained using Mesquite and based on the topology of the 50% majority rule consensus tree of BI using the NUCMIT104 matrix. Mk1 model parameters: rate: 1.30506007,  $-\log L$ : 7.09879278. Reporting likelihoods as proportional likelihoods; threshold when decisions made: 2.0; calculation by “maximum likelihood” reconstruction. For discussion see text.

The second outstanding trait in the reproductive biology of rhacophorid tree frogs is the production of foam nests to protect the eggs from desiccation and predation. Foam nests occur in *Chiromantis*, *Ghatixalus*, *Polypedates* and *Rhacophorus* (Biju et al., 2008; Inger and Stuebing, 2005; Li et al., 2009), but not in *Feihyla* and *Gracixalus* (Li et al., 2009, 2010; Rowley et al., 2011; van Dijk et al., 2004). Previous authors have argued that foam nesting evolved just once within the Rhacophoridae (Grosjean et al., 2008; Li et al., 2009; Wilkinson et al., 2002). Unfortunately, we could not resolve the phylogenetic relationships within the Rhacophorini unambiguously (Fig. 1 and 2, Supplementary material), and, thus, we do not provide an ancestral character state reconstruction of foam nesting in this study. Our data set did not reveal the foam-nest producing taxa to form a clade, but we detected a moderately or even robustly supported signal for the sister-group relationship between *Feihyla* and *Polypedates* (Figs. 1 and 2) as repeatedly indicated by previous molecular studies (Grosjean et al., 2008; Li et al., 2009; Pyron and Wiens, 2011).

*Feihyla* species cover their eggs with a layer of jelly containing air bubbles (Li et al., 2009, 2010) in contrast to the foam-nest producing species of *Polypedates*. According to Dring (1983) and Das (1994) and our observations made during field work in Malaysia, *F. kajau* do not produce foam nests. The egg clutches of this species are attached directly to the undersides of leaves overhanging small streams in primary lowland dipterocarp forest. The unpigmented eggs have a thick, crystal-clear outer jelly capsule and are covered by a thin layer of jelly without bubbles (Das, 1994). The egg clutches that we observed were well developed, so any bubbles that might have been present in the jelly after deposition of the eggs may already have dissipated. We consider oviposition in *F. kajau*, *F. palpebralis* and *F. vittatus* to be homologous and interpret this reproductive behaviour as an argument for our taxonomic assignment of *F. kajau* to *Feihyla*. Furthermore, a secondary loss of foam nesting in *Feihyla* could be interpreted as a derived character state in the sense of a behavioural synapomorphy, if

the sister-group relationship of *Feihyla* and *Polypedates* and the hypothesis of a single origin of foam nesting in the most recent ancestor of *Chiromantis*, *Feihyla*, *Ghatixalus*, *Polypedates* and *Rhacophorus* are accepted (Li et al., 2009, 2010).

#### 4.4. Biogeography

The unique diversity of rhacophorid frogs on Borneo (currently comprising 40 described species) is the result of a complicated biogeographical history. The incongruence of most of the identified phylogenetic clades with geographical limits indicates that several lineages of rhacophorid frogs colonised Borneo independently from other parts of Sundaland and adjacent regions of Southeast Asia. The observed patterns of recent distribution shown by rhacophorid frogs are probably explained by repeated dispersal and vicariance events in the past, but whatever the cause, the rhacophorid fauna on Borneo is a composite that includes species with a wider distribution in Southeast Asia, such as *Kurixalus appendiculatus*, *Nyctixalus pictus* and *Rhacophorus cyanopunctatus*, as well as numerous endemics. We identified some clades consisting of relatively recent, closely related species that presumably originated on Borneo as a result of local radiations.

Species that occur also in other parts of Sundaland or on the Philippines as well may have reached or left Borneo fairly recently, whereas the ancestors of local endemic radiations must have colonised Borneo earlier. Comparable biogeographic patterns have been found in other groups of amphibians and in other vertebrates in Southeast Asia (Arifin et al., 2011; Blackburn et al., 2010; Brown and Guttman, 2002; Brown et al., 2009; Esselstyn et al., 2004, 2009, 2010; Evans et al., 2003; Matsui et al., 2010; Oliveros and Moyle, 2010; Sanguila et al., 2011; Schweizer et al., 2012). Plate movements and collisions, the emergence of land and subsidence below sea level and changes in climate and ocean circulation have all influenced the distributions and compositions of the faunas and floras of Southeast Asia (Den Tex et al., 2010; Hall, 1998, 2002;

Appendix A

Materials examined. FMNH Field Museum Chicago, US; NMBE, Naturhistorisches Museum Bern; Switzerland; UNIMAS, Museum of Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia; ZMB Museum für Naturkunde Berlin; ZMH, Zoological Museum of Hamburg, Germany.

Species	Author	87er	Collection Nr.	Locality	Genbank Accession 16S	12S	BDNF	POMC	Tyrosinase
<i>Buergeria japonica</i>	(Hallowell, 1861)	✓			AF458123	AF458123	GQ285691	GQ285722	GQ285801
<i>Buergeria oxycephala</i>	(Boulenger, 1900)	✓			EU215524	EU215524	GQ285695	GQ285726	EU215585
<i>Chiromantis doriae</i>	(Boulenger, 1893)	✓			EU215527	EU215527	GQ285716	GQ285747	EU215584
<i>Chiromantis rufescens</i>	(Günther, 1869)				AF458126	AF458126	-	-	-
<i>Chiromantis xerampelina</i>	Peters, 1854				AF458132	AF458132	-	-	-
<i>Feihyla kajau</i>	Dring, 1984	✓	NMBE 1057090	Malaysia: Sarawak: Kubah Natl. Park: Summit Road	KC961088	KC961241	KC961110	KC961180	KC961234
<i>Feihyla kajau</i>		✓	NMBE 1056500	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 1	JN377362	KC961240	KC961109	KC961179	KC961233
<i>Feihyla palpebralis China</i>	(Smith, 1924)	✓			EU215546	EU215546	GQ285710	GQ285741	EU215606
<i>Feihyla palpebralis Vietnam</i>		✓			GQ285681	GQ285681	GQ285709	GQ285740	GQ285810
<i>Feihyla vittatus</i>	(Boulenger, 1887)	✓			AF458131	AF458131	GQ285711	GQ285742	GQ285811
<i>Ghatixalus asterops</i>	Biju, Roelants & Bossuyt, 2008				EU178098	EU178098	EU178091	-	-
<i>Ghatixalus variabilis</i>	(Jerdon, 1854)				EU178099	EU178099	EU178092	-	-
<i>Gracixalus carinensis</i>	(Boulenger, 1893)	✓			GQ285670	GQ285670	GQ285699	GQ285730	GQ285806
<i>Gracixalus gracilipes</i>	(Bourret, 1937)	✓			DQ283051	DQ283051	GQ285701	GQ285732	GQ285807
<i>Gracixalus jinxiuensis</i>	(Hu, 1978)	✓			EU215525	EU215525	GQ285700	GQ285731	EU215587
<i>Kurixalus appendiculatus</i>	(Günther, 1858)	✓	NMBE 1056476	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 5	KC961091	KC961248	KC961139	KC961189	KC961232
<i>Kurixalus appendiculatus</i>		✓	ZMH A10416	Malaysia: Sarawak: Kubah Natl. Park	KC961090	KC961247	KC961138	KC961188	KC961231
<i>Kurixalus banaensis</i>	(Bourret, 1939)	✓			GQ285667	GQ285667	GQ285689	GQ285720	GQ285799
<i>Kurixalus effingeri</i>	(Boettger, 1895)	✓			DQ283122	DQ283122	-	-	DQ282931
<i>Kurixalus idiootocus</i>	(Kuramoto & Wang, 1987)	✓			AF458129	AF458129	GQ285688	GQ285719	EU215607
<i>Kurixalus hainanus</i>	Zhao, Wang & Shi, 2005	✓			EU215548	EU215548	GQ285686	GQ285717	-
<i>Kurixalus odontotarsus</i>	(Ye & Fei, 1993)	✓			EU215549	EU215549	GQ285687	GQ285718	EU215609
<i>Liuxalus albopunctatus</i>	(Liu & Hu, 1962)	✓			EU215526	EU215526	-	-	-
<i>Liuxalus hainanus</i>	(Liu & Wu, 2004)	✓			GQ285671	GQ285671	GQ285694	GQ285725	GQ285803
<i>Liuxalus ocellatus</i>	Liu & Hu, 1973	✓			GQ285672	GQ285672	GQ285692	GQ285723	GQ285802
<i>Liuxalus romeri</i>	(Smith, 1953)	✓			EU215528	EU215528	GQ285693	GQ285724	EU215589
<i>Nyctixalus margaritifera</i>	Boulenger, 1882				EU178094	EU178087	-	-	-
<i>Nyctixalus pictus</i>	(Peters, 1871)	✓	NMBE 1056413	Malaysia: Sarawak: Batang Ai Natl. Park	JN377342	<b>JN705355</b>	KC961092	KC961141	KC961190
<i>Nyctixalus spinosus</i>	(Taylor, 1920)				DQ283114	DQ283114	-	-	-
<i>Philautus abditus</i>	Inger, Orlov & Darevsky, 1999				GQ285673	GQ285673	GQ285712	GQ285743	GQ285812
<i>Philautus acutirostris</i>	(Peters, 1867)				AY326059	AY326059	-	-	-
<i>Philautus acutus</i>	Dring, 1987	✓	NMBE 1056431	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	<b>JN705366</b>	<b>JN705337</b>	KC961117	KC961174	KC961191
<i>Philautus amoenus</i>	Smith, 1931		UNIMAS 8052	Malaysia: Sabah: Gunung Kinabalu Park: Laban Rata Trail	KC961076	KC961236	KC961140	KC961159	KC961196
<i>Philautus aurantium</i>	Inger, 1989	✓	UNIMAS 8666	Malaysia: Sabah: Crocker Range Park: 16th mile	<b>JN705367</b>	<b>JN705338</b>	KC961118	KC961175	KC961192
<i>Philautus aurantium</i>		✓	FM233226	Malaysia: Sabah: Sipitang District	KC961078	KC961237	KC961119	KC961178	KC961193
<i>Philautus aurifasciatus</i>	(Schlegel, 1837)				-	AY141805.1	-	-	-
<i>Philautus burnitus</i>	Inger, Stuebing & Tan, 1995	✓	UNIMAS 9045	Malaysia: Sabah: Gunung Kinabalu Park: Sayap	JN705368	JN705339	KC961120	KC961176	KC961194
<i>Philautus davidlabangi</i>	Matsui, 2009	✓	NMBE 1056444	Malaysia: Sarawak: Batang Ai Natl. Park: Hilton Lighthouse Resort	JN705386	JN705356	KC961130	KC961166	KC961207
<i>Philautus disgregus</i>	Inger, 1989	✓	FM231141	Malaysia: Sabah: Lahad Datu District	KC961077	KC961250	KC961137	KC961167	-
<i>Philautus hosii</i>	(Boulenger, 1895)	✓	NMBE 1057287	Malaysia: Sarawak: Usun Apau Natl. Park	JN705384	JN705353	KC961113	KC961172	KC961205
<i>Philautus ingeri</i>	Dring, 1987	✓	NMBE 1056435	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JN705385	JN705354	KC961114	KC961173	KC961206
<i>Philautus juliaedrangi</i>	Dehling, 2010	✓	NMBE 1056439	Malaysia: Sarawak: Gunung Mulu Natl. Park: Gunung Api	JN705378	JN705347	KC961131	KC961160	KC961199

<i>Philautus kerangae</i>	Dring, 1987	✓	NMBE 1056437	Malaysia: Sarawak: Gunung Mulu Natl. Park: Mentawai Ranger Station	KC961079	KC961238	KC961121	KC961177	KC961195
<i>Philautus macroscelis</i>	(Boulenger, 1896)	✓	UNIMAS 8158	Malaysia: Sabah: Gunung Kinabalu Ntl. Park	JN705372	JN705343	KC961111	KC961169	KC961202
<i>Philautus macroscelis</i>		✓	NMBE 1056486	Malaysia: Sarawak: Gunung Mulu Natl. Park: Sungai Tapin	JN705375	JN705346	KC961112	KC961168	KC961203
<i>Philautus mjoebergi</i>	Smith, 1925	✓	NMBE 1056434	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JN705380	JN705349	KC961132	KC961162	KC961197
<i>Philautus petersi</i>	(Boulenger, 1900)	✓	NMBE 1056443	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JN705381	JN705350	KC961134	KC961163	KC961204
<i>Philautus refugii</i>	Inger & Stuebing, 1996	✓	NMBE 1057544	Malaysia: Sarawak: Borneo Highlands Resort	JN705382	JN705351	KC961135	KC961164	KC961200
<i>Philautus refugii</i>		✓	ZMH A10415	Malaysia: Sarawak: Kubah Natl. Park: Summit Road	JN705383	JN705352	KC961136	KC961165	KC961201
<i>Philautus surdus</i>	(Peters, 1863)	✓	NMBE 1056451	Malaysia: Sarawak: Gunung Mulu Natl. Park: Deer Cave Trail	AF458138	AF458138	-	-	-
<i>Philautus tectus</i>	Dring, 1987	✓	NMBE 1056451	Malaysia: Sarawak: Gunung Mulu Natl. Park: Deer Cave Trail	JN705370	JN705341	KC961115	KC961170	KC961208
<i>Philautus tectus</i>		✓	NMBE 1057080	Malaysia: Sarawak: Kubah Natl. Park: Summit Road	JN705369	JN705340	KC961116	KC961171	KC961209
<i>Philautus umbra</i>	Dring, 1987	✓	NMBE 1056454	Malaysia: Sarawak: Gunung Mulu Natl. Park: Gunung Api	JN705379	JN705348	KC961133	KC961161	KC961198
<i>Polypedates colletti</i>	(Boulenger, 1890)	✓	NMBE 1057165	Malaysia: Sarawak: Matang Wildlife Center: Sungai Rayu	KC961080	KC961243	KC961123	KC961181	KC961214
<i>Polypedates colletti</i>		✓	NMBE 1056463	Malaysia: Sarawak: Gunung Mulu Natl. Park: Mentawai Ranger Station	KC961081	KC961244	KC961124	KC961182	KC961215
<i>Polypedates leucomystax</i>	(Gravenhorst, 1829)	✓	NMBE 1057524	Malaysia: Sarawak: Borneo Highlands Resort	KC961082	KC961245	KC961127	KC961183	KC961216
<i>Polypedates macrotis</i>	(Boulenger, 1891)	✓	NMBE 1056471	Malaysia: Sarawak: Gunung Mulu Natl. Park	JN377343	JN705360	KC961125	KC961184	KC961217
<i>Polypedates macrotis</i>		✓	UNIMAS 8638	Malaysia: Sarawak: Matang Wildlife Center: Sungai Rayu	JN377345	JN705359	KC961126	KC961185	KC961218
<i>Polypedates megacephalus</i>	Hallowell, 1861	✓			AF458141	AF458141	GQ285708	GQ285739	EU215612
<i>Polypedates mutus</i>	(Smith, 1940)	✓			EU215551	EU215551	GQ285707	GQ285738	EU215611
<i>Polypedates ottilophus</i>	(Boulenger, 1893)	✓	NMBE 1056473	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 5	KC961083	KC961246	KC961128	KC961186	KC961219
<i>Polypedates ottilophus</i>		✓	ZMH A10465	Malaysia: Sarawak: Kubah Natl. Park: Frog Pond	JN377346	JN705361	KC961129	KC961187	KC961220
<i>Pseudophilautus microtympanum</i>	(Günther, 1858)				GQ204739	GQ204678	GQ204441	-	AF249189
<i>Pseudophilautus mittermeieri</i>	(Meegaskumbura & Manamendra-Arachchi, 2005)				GQ204741	GQ204681	GQ204443	-	-
<i>Pseudophilautus schmarda</i>	(Kelaart, 1854)				AY141780	AY141826	GQ204435	-	-
<i>Pseudophilautus wynaadensis</i>	(Jerdon, 1854)				DQ346966	JN644773	-	-	AF249190
<i>Raorchestes glandulosus</i>	(Jerdon, 1854)				-	EU450020	-	-	-
<i>Raorchestes gryllus</i>	(Smith, 1924)	✓			GQ285674	GQ285674	GQ285714	GQ285745	GQ285814
<i>Raorchestes longchuanensis</i>	(Yang & Li, 1979)	✓			GQ285675	GQ285675	GQ285713	GQ285744	GQ285813
<i>Raorchestes menglaensis</i>	Kou, 1990	✓			GQ285676	GQ285676	GQ285715	GQ285746	GQ285815
<i>Rhacophorus angulirostris</i>	Ahl, 1927	✓	UNIMAS 8681	Malaysia: Sarawak: Gunung Kinabalu Natl. Park: Silau Silau	JN377348	JN705322	KC961099	KC961142	KC961221
<i>Rhacophorus annamensis</i>	Smith, 1924	✓			DQ283047	DQ283047	-	-	-
<i>Rhacophorus baluensis</i>	Inger, 1954	✓	FM235958	Malaysia: Sabah: Tambunan District	KC961089	KC961239	KC961093	KC961153	-
<i>Rhacophorus belalongensis</i>	Dehling & Grafe, 2008	✓	ZMB70378	Brunei Darus-salam: Temburong district: Kuala Belalong Field Studies Centre	JN377352	JN705324	KC961101	KC961144	-
<i>Rhacophorus bipunctatus</i>	Ahl, 1927				AF458144	AF458144	-	-	EU924574
<i>Rhacophorus calcaneus</i>	Smith, 1924	✓			DQ283380	DQ283380	-	-	DQ282991
<i>Rhacophorus cyanopunctatus</i>	Manthey & Steiof, 1998	✓	NMBE 1056480	Malaysia: Sarawak: Gunung Mulu Natl. Park: Dear Cave Trail: Sungai Melinau Paku	KC961084	KC961249	KC961098	KC961152	KC961230
<i>Rhacophorus dennysi</i>	Blanford, 1881				EU215545	EU215545	-	-	EU215605
<i>Rhacophorus dugritei</i>	(David, 1872)	✓			EU215541	EU215541	-	GQ285736	EU215601

(continued on next page)

## Appendix A (continued)

Species	Author	87er	Collection Nr.	Locality	Genbank Accession 16S	12S	BDNF	POMC	Tyrosinase
<i>Rhacophorus dulitensis</i>	Boulenger, 1892	✓	NMBE 1056482	Malaysia: Sarawak: Gunung Mulu Natl. Park: HQ	JN377355	JN705326	KC961122	KC961158	KC961235
<i>Rhacophorus fasciatus</i>	Boulenger, 1895	✓	NMBE 1056492	Malaysia: Sarawak: Gunung Mulu Natl. Park: HQ	JN377357	JN705331	KC961104	KC961148	KC961226
<i>Rhacophorus fasciatus</i>		✓	NMBE 1057405	Malaysia: Sarawak: Lambir Hills Natl. Park: HQ	KC961085	JN705330	KC961105	KC961147	KC961225
<i>Rhacophorus feae</i>	Boulenger, 1893	✓			EU215544	EU215544			EU215604
<i>Rhacophorus gadingensis</i>	Das & Haas, 2005	✓	NMBE 1057173	Malaysia: Sarawak: Kubah Natl. Park: Waterfall trail	KC961087	KC961242	KC961102	KC961145	KC961223
<i>Rhacophorus gauni</i>	(Inger, 1966)	✓	NMBE 1056493	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 5	JN377351	JN705325	KC961103	KC961146	KC961224
<i>Rhacophorus harrissoni</i>	Inger & Haile, 1959	✓	NMBE 1056497	Malaysia: Sarawak: Gunung Mulu Natl. Park: Sungai Melinau Paku	JN377359	JN705332	KC961107	KC961149	KC961227
<i>Rhacophorus kio</i>	Ohler & Delorme, 2006	✓			EU215532	EU215532	GQ285703	GQ285734	EU215592
<i>Rhacophorus moltrechti</i>	Boulenger, 1908	✓			AF458145	AF458145	-	-	EU215603
<i>Rhacophorus nigropalmatus</i>	Boulenger, 1895	✓	ZMH A10414	Malaysia: Sarawak: Kubah Natl. Park: Main Trail	JN377363	JN705328	KC961094	KC961154	KC961210
<i>Rhacophorus nigropunctatus</i>	Liu, Hu & Yang, 1962	✓			EU215533	EU215533	GQ285704	GQ285735	EU215593
<i>Rhacophorus omeimontis</i>	(Stejneger, 1924)	✓			EU215535	EU215535			EU215595
<i>Rhacophorus orlovi</i>	Ziegler & Köhler, 2001	✓			DQ283049	DQ283049	-	-	-
<i>Rhacophorus pardalis</i>	Günther, 1858	✓	NMBE 1056514	Malaysia: Sarawak: Batang Ai Natl. Park: Bebyong Trail	JN377369	JN705335	KC961095	KC961156	KC961212
<i>Rhacophorus pardalis</i>		✓	ZMH A10834	Malaysia: Sarawak: Lambir Hills Natl. Park: Latak Waterfall Trail	-	JN705336	KC961096	KC961157	KC961213
<i>Rhacophorus penanorum</i>	Dehling, 2008	✓	ZMH A10168	Malaysia: Sarawak: Gunung Mulu Natl. Park: Sungai Tapin	JN377349	JN705323	KC961100	KC961143	KC961222
<i>Rhacophorus puerensis</i>	(He, 1999)	✓			EU215542	EU215542			EU215602
<i>Rhacophorus reinwardtii</i>	(Schlegel, 1840)	✓	NMBE 1056517	Malaysia: Sarawak: Batang Ai Natl. Park: Bebyong Trail	JN377366	JN705329	KC961097	KC961155	KC961211
<i>Rhacophorus rhodopus</i>	Liu & Hu, 1960	✓			EU215531	EU215531			EU215591
<i>Rhacophorus rufipes</i>	Inger, 1966	✓	NMBE 1056519	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 5	JN377360	JN705334	KC961106	KC961150	KC961228
<i>Rhacophorus rufipes</i>		✓	NMBE 1057529	Malaysia: Sarawak: Borneo Highlands Resort	KC961086	JN705333	KC961108	KC961151	KC961229
<i>Rhacophorus taronensis</i>	Smith, 1940	✓			EU215537	EU215537	-	-	EU215597
<i>Theloderma asperum</i>	(Boulenger, 1886)	✓			GQ285678	GQ285678	GQ285697	GQ285728	GQ285804
<i>Theloderma corticale</i>	(Boulenger, 1903)	✓			DQ283050	DQ283050	-	-	DQ282904
<i>Theloderma moloch</i>	(Annandale, 1912)	✓			GQ285679	GQ285679	GQ285690	GQ285721	GQ285800
<i>Theloderma rhododiscus</i>		✓	(Liu and Hu, 1962)		EU215530	EU215530	GQ285696	GQ285727	EU215586

Heaney, 1986). The complex paleogeology and climatology of the Sunda shelf has caused repeated episodes of isolation and connection of land masses through fluctuating sea levels. As a result, periodic faunal exchanges between the Southeast Asian mainland, Sundaland and the Philippines have occurred (Chuan Lim et al., 2010; Esselstyn et al., 2009; Inger and Voris, 2001; Moss and Wilson, 1998; Outlaw and Voelker, 2008; Taylor and Hayes, 1980; Voris, 2000; Woodruff, 2010). The discovery of the phylogenetic relationships of *Feihyla kajau* introduces an Indo-Chinese element into the fauna of Borneo, because the other known species of *Feihyla* are from northern Vietnam, northern India and southern China.

Monophyletic groups of closely related Bornean species within *Rhacophorus* and *Philautus* result from local adaptive and non-adaptive radiations of ancestral lineages. Within *Philautus*, such clades include the *bunitus* group (*P. acutus*, *P. aurantium*, *P. bunitus*, *P. kerangae*) and the *petersi* group (*P. amoenus*, *P. juliandringi*, *P. mjobergi*, *P. refugii*, *P. umbra*). The members of the *angulirostris* group within *Rhacophorus* (*R. angulirostris*, *belalongensis*, *gauni*, *gad-ingensis*, *penanorum*) are also endemic to Borneo. These species share an ecological affinity to fast running streams as breeding sites and possess rheophilic tadpoles (Haas et al., 2012; Leong, 2004). A further endemic radiation of closely related species on Borneo comprises *R. harrisoni*, *R. fasciatus* and *R. rufipes*. These species have typical pond-type tadpoles but breed either in ponds (*R. fasciatus*, *R. rufipes*) or tree holes (*R. harrisoni*). The use of different breeding sites within the *fasciatus* group might be interpretable as a case of adaptive radiation.

It remains to be investigated, however, whether all of the closely related Bornean species described so far can actually be confirmed as valid species in studies based on a broader sampling strategy, e.g. including populations from their entire distributional range. Whether or not phylogenetically recent and allopatrically distributed species are accepted as valid species depends on the species concept applied. Nevertheless, our current phylogenetic hypotheses and biogeographic interpretations underpin the uniqueness of the impressive diversity of rhacophorid treefrogs on Borneo, showing it to include radiations of endemic species and phylogenetically isolated lineages. Finally, our results affirm the importance of long-term conservation efforts of the amphibian fauna of Sundaland as a significant element of this biodiversity hotspot with an extraordinary biogeographical history.

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## Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2013.04.001>.

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