

# Phylogenetic relationships of the *Rhacophorus everetti*-group and implications for the evolution of reproductive modes in *Philautus* (Amphibia: Anura: Rhacophoridae)

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This study presents the first phylogenetic analysis of the enigmatic *Rhacophorus everetti* species group and the first description of its unique tadpole. A total sample of 95 12S and 16S mitochondrial rDNA sequences were compiled including new sequence data from 28 rhacophorid species. Based on 1332 and 1407 bp, respectively, and on the gap coding method applied, a new hypothesis about the phylogeny of rhacophorid tree frogs from Sundaland was obtained. While *Rhacophorus* was uncovered as a polyphyletic assemblage, the monophyly of the Bush Frogs of the genus *Philautus*, including the *Rhacophorus everetti*-group, is robustly supported. We, therefore, transfer the *everetti*-group to the genus *Philautus*. As a second step, we recognise *Philautus macroscelis* (comb. nov.) from Borneo and *P. everetti* (comb. nov.) from Palawan as distinct allopatric species. Molecular and morphological evidence clearly indicates that each is a distinct lineage with a unique ancestry and discrete evolutionary fate. Moreover, close phylogenetic relationships of several *Philautus* species from Borneo to taxa from outside Borneo were recovered; *P. everetti* and *P. macroscelis* being the only one example. These findings indicate a complex biogeographical history of Sundaland Bush Frogs, which can only be explained by repeated dispersal and vicariance events between the Asian mainland and the Sunda islands. Finally, a single tadpole discovered on Gunung Kinabalu was matched genetically to *P. macroscelis*. Features of its peculiar external morphology suggest that this larva is endotrophic and possibly nidicolous. A comparable reproductive biology was formerly unknown in rhacophorid tree frogs. The presence of a free-swimming tadpole in *Philautus* challenges the notion that terrestrial direct development represents an apomorphic character unambiguously shared by all members of this genus. The implications for the evolution of reproductive modes in Bush Frogs are discussed in a phylogenetic context.

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## Introduction

Rhacophorid tree frogs represent a monophyletic radiation of about 300 extant species, which are predominantly

distributed in southern and south-eastern Asia (Liem 1970; Channing 1989; Frost *et al.* 2006; Li *et al.* 2009). Recognition of this taxon as a separate family Rhacophori-

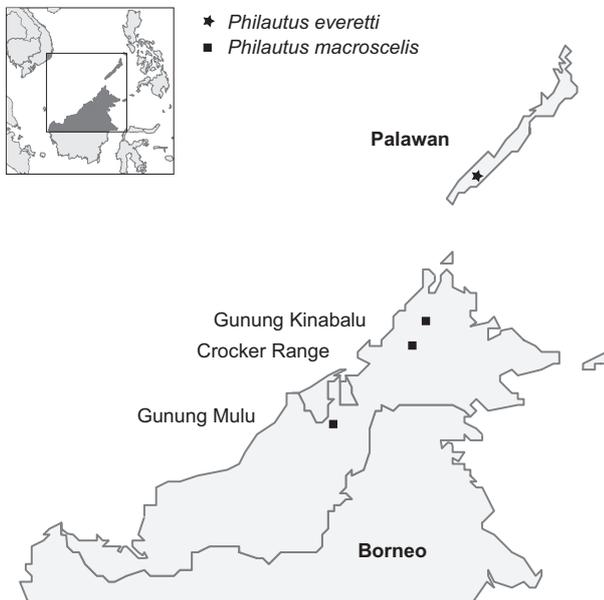
dae is now uncontroversial, as following recent comprehensive studies on anuran phylogeny (Frost *et al.* 2006; Pyron & Wiens 2011). The reproductive biology of this group encompasses a great diversity of reproductive modes, ranging from the production of foam nests and free-feeding tadpoles to lecithotrophy and direct development (Brown & Alcalá 1982, 1994; Grosjean *et al.* 2008). The evolution of these intriguingly diverse reproductive strategies in rhacophorids provides a strong impetus for studies of their phylogenetic relationships. Within the Rhacophoridae, two monophyletic major groups, Buergeriinae and Rhacophorinae, have been recognized (Channing 1989; Richards & Moore 1998; Wilkinson *et al.* 2002; Frost *et al.* 2006; Grosjean *et al.* 2008; Li *et al.* 2008; Yu *et al.* 2008). The systematics of especially the species-rich Rhacophorinae and the delimitation and definition of its 12 described genera are still a matter of debate (Liem 1970; Delorme *et al.* 2005; Frost *et al.* 2006; Yu *et al.* 2007, 2009; Biju *et al.* 2008; Grosjean *et al.* 2008; Li *et al.* 2008, 2009). This state of taxonomic instability is exacerbated by the fact that numerous genera described or revalidated in recent years, predominately based on genetic data, and even traditionally recognized genera lack clearly defined morphological synapomorphies (Ye *et al.* 1999; Frost *et al.* 2006; Biju *et al.* 2008; Grosjean *et al.* 2008; Li *et al.* 2008, 2009; Yu *et al.* 2009). Furthermore, the majority of original descriptions of recently discovered species do not discuss the available potential synapomorphic character states that have been proposed to define genera, for instance, by Liem (1970) for adult or by Grosjean *et al.* (2008) for some tadpole characters (but see Wilkinson & Drewes 2000).

The resulting uncertainty on phylogenetic relationships, in combination with the lack of knowledge of the effective reproductive behaviour in a number of species, obstructs the reconstruction of evolutionary changes in the reproductive strategies of these frogs. An example of this unsatisfying situation is the genus *Philautus*, as traditionally constituted including species from Sri Lanka and India as well as from South-east Asia (Frost 2010). While most species of rhacophorid genera have a free-swimming and free-feeding tadpole stage, the majority of species of *Philautus sensu lato* examined so far seem to lack such larval stages (Brown & Alcalá 1982; Dring 1987; Bahir *et al.* 2005). Consequentially, direct development into froglets has even been proposed as an important character to distinguish and delineate *Philautus* from related rhacophorids (Grosjean *et al.* 2008). However, Meegaskumbura *et al.* (2002) suspected an independent origin of direct development within *Philautus* in Sri Lanka and the Sunda Islands. Grosjean *et al.* (2008) did not find sufficient support in their molecular data for the monophyly of *Philautus* in the

traditional sense. Subsequently, this genus was considered a polyphyletic assemblage, and as a consequence, several species formerly assigned to *Philautus* have been transferred to *Feihyla*, *Liuixalus*, *Kurixalus*, *Pseudophilautus*, *Raorchestes* or *Theلودerma* so that *Philautus* currently consists exclusively of species from South-east Asia (Li *et al.* 2008, 2009; Yu *et al.* 2008; Biju *et al.* 2010).

Apart from India and Sri Lanka, South-east Asia (particularly Sundaland) is a centre of species richness in rhacophorid tree frogs, as evinced by the fact that many new species continue to be discovered in this region (Brown *et al.* 2008; Brown & Stuart 2011). A total of 40 rhacophorid species have been described from Borneo alone and are currently allocated to six genera (*Philautus*, *Polypedates*, *Rhacophorus*, *Theلودerma*, *Nyctixalus* and *Kurixalus*) (Frost 2010). The nearby Philippine archipelago contains 17 species in five genera (*Philautus*, *Polypedates*, *Rhacophorus*, and *Nyctixalus*) (Brown & Alcalá 1994; Brown 2007). However, only a few of them have been represented in previous phylogenetic analyses; hence, the taxonomic assignment of most species to genera has not been tested within a phylogenetic framework. Moreover, life history data for many species are deficient; for example, tadpole descriptions based on a reliable identification of the specimens are available for only 13 Bornean species of rhacophorid tree frogs belonging to *Polypedates* or *Rhacophorus* (Das & Haas 2006). In the majority of species from Sundaland assigned to *Philautus*, the effective breeding behaviour has never been confirmed by direct observations or genetic matching of semaphoronts.

One of the most enigmatic rhacophorid taxa from Sundaland is the Mossy Tree Frog *Rhacophorus everetti* Boulenger 1894. As currently understood, *R. everetti* has only been reported from Palawan and few isolated localities on northern Borneo (Fig. 1) (Inger 1954, 1966; Brown & Alcalá 1970, 1994; Alcalá & Brown 1998). The phylogenetic relationships of this species have been controversial since its original description (Inger 1966; Liem 1970), and its taxonomic status has not been confirmed by molecular markers. *Rhacophorus everetti* is a mid-sized tree frog (SVL of males: 30–32 mm and females: 45–49 mm; Inger & Stuebing 1989; Alcalá & Brown 1998; personal observation), which is characterised on Borneo by a distinctive moss-bark mimic colouration and a rough skin with numerous tuberculate projections of differing size and shape (Fig. 2A). In contrast, the specimens from Palawan have a smooth skin and a pale colouration (Fig. 2B–D). The species is restricted to primary submontane and montane forests at elevations between 1100 and 1800 m asl at isolated mountains in western Sabah and north-eastern Sarawak (Inger 1966; Inger & Stuebing 2005; personal observation) but has been recorded at much lower eleva-



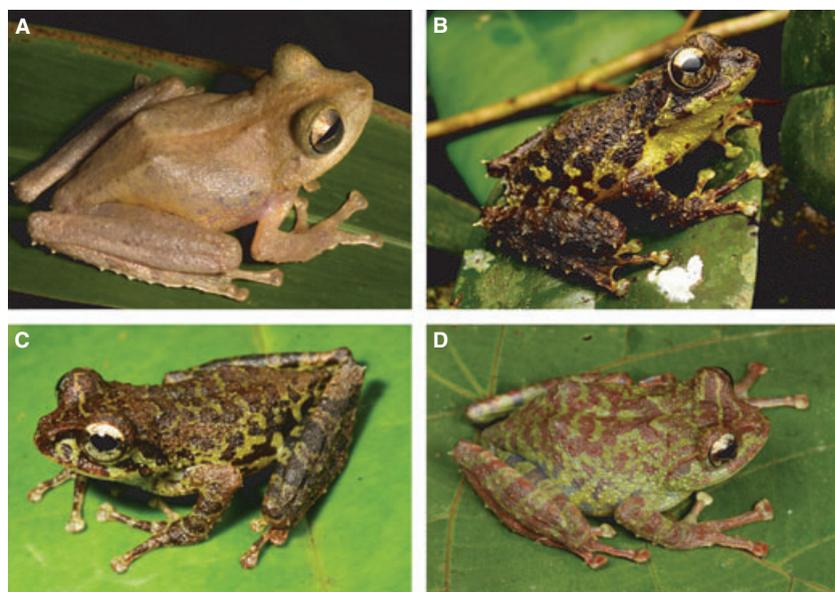
**Fig. 1** The known localities of the species of the *Philautus everetti*-group on Sundaland.

tions on Palawan (Inger 1954; Alcalá & Brown 1998; Brown & Alcalá 1994; RMB, personal observation). In summary, it must be stated that little well-corroborated information about the habitat requirements of this species is available, and consequentially, its reproductive biology is completely unknown (Noble 1927; Inger 1954; Brown & Alcalá 1982).

The name *R. everetti* Boulenger 1894; was originally applied to specimens from Palawan, in the Philippines

archipelago, while specimens from Gunung Kinabalu on Borneo were named *R. macroscelis* Boulenger, 1896. The latter taxon was treated subsequently as a subspecies of *R. everetti* (Inger 1966; Malkmus *et al.* 2002). A further rhacophorid taxon, *Philautus spiculatus*, was described by Smith (1931) from the Gunung Kinabalu area. It was assigned to the genus *Philautus* because of the absence of vomerine teeth, one of the few characters used in the past to define this genus. Later, Inger (1954, 1966) reported vomerine teeth in the holotype of *P. spiculatus*. Thus, he transferred it to the genus *Rhacophorus*, where such teeth are typically present, and identified *P. spiculatus* as a junior synonym of *R. everetti* (Inger 1954, 1966; Inger & Tan 1996). In general, the presence or absence of vomerine teeth as a phylogenetically informative character in rhacophorids has fallen into question because of the apparent variability even within some species (Inger 1954, 1966; Taylor 1962; Liem 1970). In his phenetic analysis of the phylogenetic relationships of the Rhacophoridae, Liem (1970), again, questioned the phylogenetic relationships of *R. everetti* and suspected a closer affiliation to *Theioderma*, because Bornean specimens differed in many morphological characters from the remaining members of *Rhacophorus*. He nevertheless proposed its provisional retention in *Rhacophorus*.

In this study, we examine the phylogenetic relationships of the taxon known as '*Rhacophorus everetti macroscelis*' from Borneo with particular reference to newly collected specimens from the type locality of *R. everetti everetti* on Palawan. We set out to test as to whether the genus *Rhacophorus*, including '*R. everetti*', represents a natural, monophyletic group. For that purpose, we collected and



**Fig. 2** *Philautus everetti*. Colouration in life of an unstressed, free-ranging male. Mt. Mantalingajan, Palawan, Philippines. —A. *Philautus everetti macroscelis*. Daytime colouration in life of specimens somewhat stressed by being handled for photography (more dull brown and less bright green/yellow than at night): male, Gunung Kinabalu Park, Sabah, Malaysia, Borneo —B; male, Gunung Mulu, Sarawak, Malaysia, Borneo —C; female, Gunung Mulu, Sarawak, Malaysia, Borneo —D.

analysed new molecular data on a number of rhacophorid taxa from Borneo and the Philippines and used a selection of sequences available from past studies. Based on our hypothesis of phylogenetic relationships of this enigmatic frog species, we propose a novel taxonomic assignment. Moreover, we present the first account on the larva of ‘*R. e. macroscelis*’ from Borneo using a genetic identification approach and provide a description of its unique morphology. The discovery of the larval stage of this species and the discussion of its developmental mode in a phylogenetic context challenge our understanding of life cycle evolution in rhacophorid tree frogs.

**Material and methods**

*Sampling and markers*

Adult specimens of Bornean and Philippine rhacophorid frogs, tadpole and tissue samples were collected between the years 2000–2010 during multiple field trips to different areas in Sarawak, Sabah (Borneo) and Palawan Island (Philippines). A single tadpole of *R. e. macroscelis* was collected from the headwaters of Sungei Silau Silau (N06.01; E116.30; ca. 1500 m asl), within Gunung Kinabalu Park, Ranau District, Sabah, East Malaysia (northern Borneo), on 19 September 2004. It was discovered at the edge of a quiet seepage feeding into the main body of a small stream (Sungei Silau Silau). Water depth at the point was ca. 5 cm, and the area was covered with macrophytes growing around rocks. Its species identity was later confirmed by DNA analyses.

The tadpole and frogs were photographed alive (Nikon D70, Canon EOS 350 D, 105 and 180 mm macro lenses, various flashes) as previously described (A. Haas & I. Das submitted). Specimens were anesthetized and euthanized in a ca. 2% aqueous Chlorobutanol solution (1,1,1-trichloro-2-methyl-2-propanol). Frogs were preserved in 4% neutrally buffered formalin and later transferred to 75% ethanol via 30% and 50% steps to avoid shrinkage. Tissue samples from liver or femoral muscle tissue of adult voucher specimens (Table 1) were extracted before preservation and stored in either RNALater buffer solution (Ambi-

on/Applied Biosystems, Austin, Texas, USA) or absolute ethanol. The sole tadpole of *R. e. macroscelis* collected was directly transferred and stored in absolute ethanol; tissue for molecular analyses was taken from the tail musculature.

We included new sequence data of 42 samples representing 28 species from Borneo and Palawan, most of them are analysed in this study in a phylogenetic context using molecular data for the first time. In species with a wide geographical distribution on Borneo, we used two samples from two different population, whenever available, to subdivide terminal branches. Additionally, to augment these new data and obtain robust taxon sampling, we added 53 sequences from Genbank to compile a total sample of 95 12S–16S ribosomal RNA mitochondrial gene sequences (Appendix 1). The final data matrix contains representative taxa of all phylogenetic lineages currently considered as genera. Two species of Buergeriinae (*Buergeria japonica*, *B. robusta*) were defined as outgroup following present phylogenetic hypotheses (Wilkinson *et al.* 2002; Grosjean *et al.* 2008; Li *et al.* 2009) for tree rooting during and posttree-search procedures.

*Larval morphology*

We followed terms for tadpole descriptions from standard sources (McDiarmid & Altig 1999; Anstis 2002; Grosjean 2005; Altig 2007). Tadpoles were staged according to Gosner (1960). Descriptions of colouration features were derived from on-screen representations (sRGB colour space) of digital images that had been taken in the field from the living specimens. We refer to web-accessible colour lists ([http://www.en.wikipedia.org/wiki/List\\_of\\_colours](http://www.en.wikipedia.org/wiki/List_of_colours)) for colour descriptions.

*Laboratory protocols*

Total genomic DNA was extracted from macerated muscle or liver tissue using peqGold Tissue DNA Mini Kits (PEQLAB Biotechnologie GmbH, Erlangen, Germany) or DNeasy® Blood & Tissue Kit (Qiagen, Valencia, California, USA), according to the manufacturer’s protocols. Amplification of ~860 bp of 16S rDNA (forward: 16SC 5’

**Table 1** Genetic distances within the *Philautus everetti*-group obtained with Geneious Pro and Tamura Nei model of sequence evolution (for details see text)

	CR	GKa	GKt	GM	GM	P3	P1
<i>P. macroscelis</i> Crocker Range (CR)		0.001	0.001	0.007	0.007	0.054	0.074
<i>P. macroscelis</i> Gunung Kinabalu adult (GKa)	0.001		0.000	0.006	0.006	0.054	0.077
<i>P. macroscelis</i> Gunung Kinabalu tadpole (GKt)	0.001	0.000		0.006	0.006	0.054	0.077
<i>P. macroscelis</i> Gunung Mulu (GM)	0.007	0.006	0.006		0.000	0.057	0.079
<i>P. macroscelis</i> Gunung Mulu (GM)	0.007	0.006	0.006	0.000		0.057	0.079
<i>P. everetti</i> Palawan (P3)	0.054	0.054	0.054	0.057	0.057		0.005
<i>P. everetti</i> Palawan (P1)	0.074	0.077	0.077	0.079	0.079	0.005	

GTRGGCCTAAAAGCAGCCAC-3', 16SA-L CGCCT GTTTATCAAAAACAT, 16SCH TCAAHTAAGGCA CAGCTTA; reverse: 16SD 5'-CTCCGGTCTGAACT CAGATCACGTAG-3', 16SB-H CCGGTCTGAACT CAGATCACGT, 16SD-DR 5'-ACAAGTGATTAYGCT ACCT-3' Miguel Vences, personal communication) and of ~410 bp of 12S rDNA (forward: 12SA-L AAAGT GGGATTAGATACCCCACTAT, reverse: 12SB-H GAGGGTGACGGGCGGTGTGT) was carried out with peqGOLD PCR-Master-Mix Y (PEQLAB Biotechnologie GmbH). The following were the cycling conditions for amplification: denaturation at 94 °C for 2 min; 35 cycles at 94 °C for 0:30 min, 48 °C or 50 °C for 0:30 min, and 72 °C for 1:00 min; then one final extension cycle at 72 °C for 5:00 min, stop at 4 °C. We used 25 µL of PCR reaction mixture containing 1 µL DNA, 1 µL of each primer (20 pmol/µL (20 µM), 1.5 µL magnesium chloride (MgCl<sub>2</sub>), 12.5 µL MasterMix Y, 8 µL ddH<sub>2</sub>O (Peqlab, Erlangen, Germany) following manufacturer's protocol and a TC-512 thermocycler (Techne, Stone, UK). PCR products were excised from agarose gels and cleaned using the Wizard® SV Gel and PCR Clean-UP System (Promega, Madison, Wisconsin, USA).

For most samples, two 25-µL reaction mixtures were run and excised bands were put together for cleaning to increase the concentration of PCR product for sequencing. Sequencing was carried out in both directions by Microsynth AG (Balgach, Switzerland), LGC Genomics (Berlin, Germany) or Macrogen Inc. (Seoul, Korea) using the same primers as for amplification. Sequence preparation, editing and management were carried out with BioEdit 7.0.5.2 (Hall 1999, <http://www.mbio.ncsu.edu/BioEdit/>), and Geneious Pro 5.1.7 (Drummond *et al.* 2009). Chromas lite 2.01 (Technelysium Pty. Ltd., <http://www.technelysium.com>) software was used for checking the trace files of the sequencers.

### Phylogenetic analyses

Alignment of the ribosomal 12S and 16S gene fragments was carried out separately using the MAFFT algorithm (Katoh *et al.* 2002) implemented as plug-in in Geneious Pro with the E-INS-i mode and standard parameters to obtain alignments with maximized sequence similarity (Morrison 2009). The resulting alignments of both genes were then concatenated using Geneious Pro resulting in a single combined data matrix. We utilized this final alignment to choose the best-fitting model of sequence evolution based on the Akaike information criterion, as implemented in jModelTest 0.1.1 (Posada 2008) and MrModeltest 2.3 (Nylander 2004).

To incorporate phylogenetic information of indels into the phylogenetic reconstructions based on maximum likelihood (ML) and Bayesian inference (BI) optimality criteria,

we coded the gaps in the alignment using SeqState 1.4.1 (Müller 2006). We selected the simple indel coding (SIC) algorithm (Simmons & Ochoterena 2000) and appended the coded indels as additional characters block to the alignment and analysed the resulting two matrices separately. In maximum parsimony (MP) analyses, we tested the impact of the phylogenetic information of gaps in parallel analyses by coding them alternatively as fifth character state (FCS), using the simple indel coding (SIC) approach and additionally by coding them following the modified complex indel coding (MCIC) method in SeqState (Simmons & Ochoterena 2000; Simmons *et al.* 2001; Müller 2005).

Maximum likelihood phylogeny estimation was conducted with the web server-based version of RaxML 7.0.4 (Stamatakis *et al.* 2008) with 100 rapid bootstrap inferences with Gamma model of rate heterogeneity with all free model parameters estimated by the software and Maximum likelihood search option for searching for best-scoring tree after the bootstraps in effect. For the analysis of the SIC matrix that included the coded gaps, we provided an additional alignment partition file to force the RaxML software to search for a separate evolution model for the appended indel coding part of the data matrix.

Bayesian analyses were performed using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). We applied a one-model approach during tree search based on the FCS data matrix and a two-model approach using the SIC data matrix by forcing the software to treat the appended characters block coding for the indels as binary characters. We carried out 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 & Drummond 2007) and then discarded the first 25% of sampled trees as burn-in (25 000 trees). We inspected that the average standard deviation of split frequencies converged towards zero and compared likelihoods and posterior probabilities of all splits to assess convergence among the two independent runs using AWTY (Wilgenbusch *et al.* 2004; Nylander *et al.* 2008).

The MP analysis was performed with PAUP 4.0b10 software (Swofford 2003). The shortest trees were sought by the heuristic search method (options in effect: equally weighted parsimony, 10 000 random addition replicates, TBR branch swapping, MulTree, random sequence addition, steepest descent options in effect). Bootstrap analyses were performed with 2000 pseudo-replicates (heuristic search parameters, TBR, 10 random additions, respectively).

Genetic distances were obtained with Geneious Pro using the implemented tree builder and Tamura Nei model of sequence evolution. Geneious Pro and Inkscape (<http://www.inkscape.org>) were used for tree graphics.

## Results

### Phylogeny

The final concatenated alignment comprised 1261 bp. When gaps were coded separately and appended to the alignment, the complete data matrix then consisted of 1332 (MCIC approach) and 1407 characters (SIC algorithm), respectively. MrModeltest identified GTR + I + G as the best-scoring model, which was therefore selected for BI analysis. In RaxML, only the GTR model is implemented, because all other models can be regarded as simplified variants (see manual). Nodes in the resulting topologies were considered robustly supported when BI posterior probability values were greater than 90%, MP and ML bootstrapping outputs were greater than 70% (see Fig. 3 for details).

Maximum parsimony analyses of the FCS, SIC and MCIC data matrices resulted in 60, 157 and 483 equally parsimonious trees of a length of 6289, 6058 and 6050 steps based on 675, 723 and 687 parsimony-informative characters, respectively. The strict consensus trees using SIC and MCIC data matrices show a reduced resolution compared to the gap treatment as fifth character state. The consensus tree topologies of the ML and BI analyses of the SIC and FCS matrices were nearly identical, and only few well-supported nodes differed between the trees. The BI consensus trees are nearly resolved; many deeper nodes are not supported by the ML analyses. The MP strict consensus trees and the 50% majority rule consensus trees of bootstrapping tests show even a lower resolution. The phylogenetic relationships of *Feibyla palpebralis* and *Chiromantis vittatus*, the monophyly of *Theلودerma*, the branching patterns between the different genera as well as among species groups within *Philautus* and *Rhacophorus* are not congruently resolved in ML, BI and MP analyses. Certain nodes, e.g., *Kurixalus* + *Raochestes* + *Philautus* or *Rhacophorus* Clade B + *Rhacophorus* Clade C, and the phylogenetic relationships of *Feibyla palpebralis*, *Chiromantis vittatus* and *Philautus tectus* within the genus *Philautus* were susceptible to the application of different gap coding schemes, result-

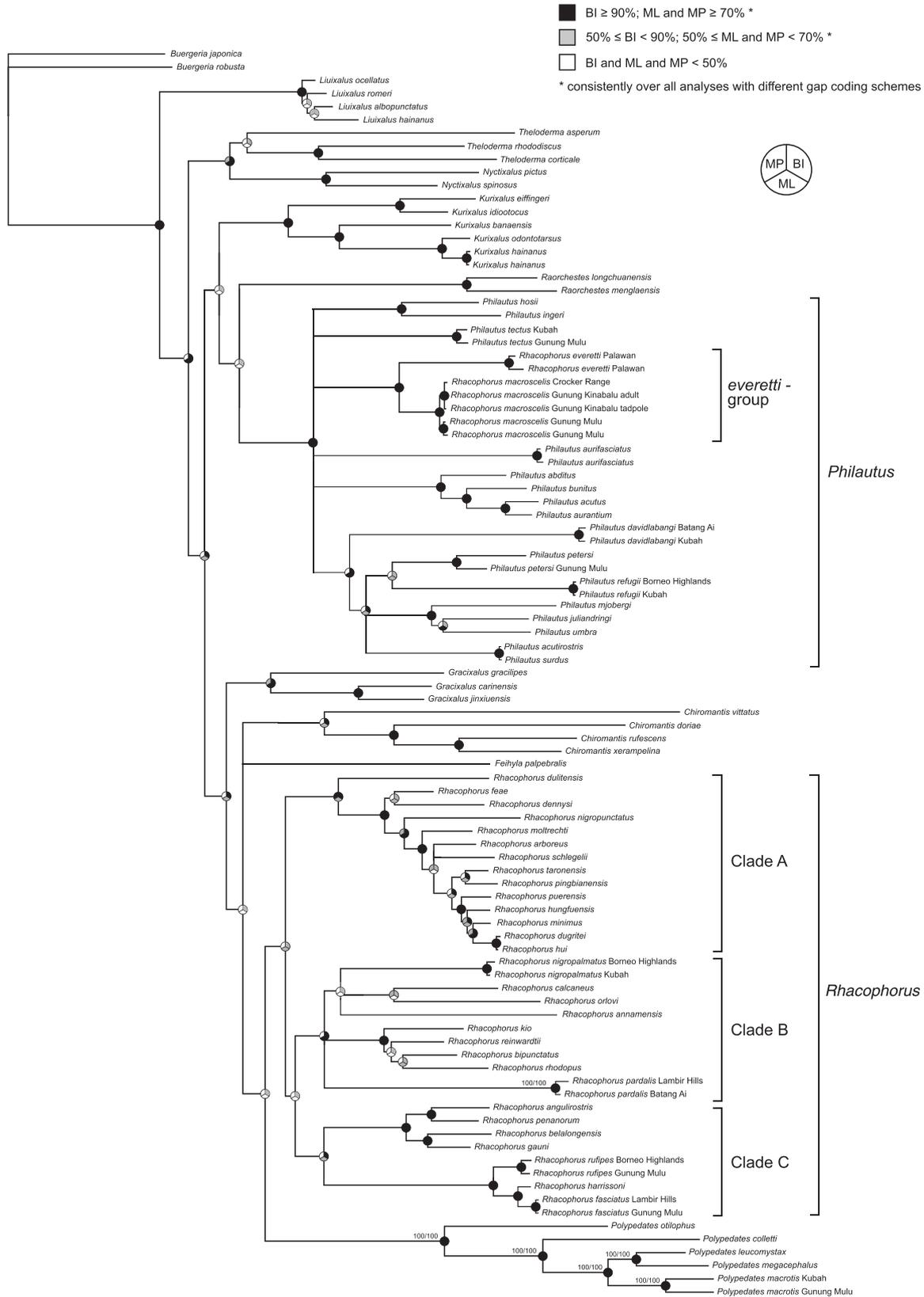
ing in incongruent sister-group relationships or strongly differing support values (see Supporting Information).

Although the results of MP, ML and BI analyses differ clearly from each other regarding the resolution of the resulting consensus topologies and of the support values for the nodes, the following phylogenetic hypotheses are consistently uncovered (Fig. 3): The ingroup representing Rhacophorinae is monophyletic with robust support and is the sister taxon to Buergeriinae (*Buergeria japonica* + *B. robusta*). Within Rhacophorinae, the sister-group relationship of the genus *Liuixalus* to the remaining taxa is robustly supported in BI and ML analyses. Further robustly supported monophyletic entities are the taxon Nyctixalini consisting of *Nyctixalus* + *Theلودerma* and each of the following genera: *Chiromantis* (*C. doriae*, *rufescens*, *xerampelina*, but excluding *C. vittatus*), *Gracixalus* (*G. carinensis*, *gracilipes*, *jinxiuensis*), *Raochestes* (*sensu* Biju et al. 2010) (*R. longchuanensis*, *menglaensis*), *Kurixalus* (*sensu* Li et al. 2008) (*K. banaensis*, *eiffingeri*, *bainanus*, *idiotocus*, *odontotarsus*), *Liuixalus* (*L. albopunctatus*, *bainanus*, *ocellatus*, *romeri*), *Nyctixalus* (*N. pictus*, *spinosum*), *Polypedates* (*P. colletti*, *leucomystax*, *macrotis*, *megacephalus*, *otilophus*). The genus *Aquixalus* (*sensu* Grosjean et al. 2008) is revealed to be polyphyletic (*Gracixalus gracilipes* + *G. carinensis*, but also *Kurixalus odontotarsus*).

The species-rich genus *Rhacophorus* is polyphyletic. The *R. everetti*-group (*R. everetti* + *R. macroscelis*) is unambiguously placed within the genus *Philautus*, which forms a robustly supported monophyletic clade with the inclusion of these taxa (Fig. 3). We consider *R. everetti* and *macroscelis* as separate species based on arguments given in the next section and conclude that both taxa were erroneously placed in *Rhacophorus* in traditional classification and transfer them, therefore, into the genus *Philautus*. In the following sections, we use the new combinations *Philautus everetti* comb. nov. and *Philautus macroscelis* comb. nov. We redefine the genus *Philautus* in a phylogenetic framework as the most inclusive clade including *P. tectus*, *P. bosii* + *P. ingeri*, *P. everetti* + *P. macroscelis* and *P. aurifasciatus*, their most recent common ancestor, and all of its descendants.

The ML and BI topologies within the monophyletic taxon *Philautus* (i.e. including *P. everetti* and *P. macroscelis*) show long terminal branches (Fig. 3, Supporting Information).

**Fig. 3** Phylogenetic hypothesis for South-east Asian Rhacophoridae based on the tree of BI using simple indel coding (SIC). Conflicting nodes in comparison with the BI results using NOG indel coding were removed and drawn as polytomies. The node support obtained with Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) analyses were plotted using three classes of support values: robust support: BI  $\geq$  90%; ML and MP  $\geq$  70% (black); weak support: 50%  $\leq$  BI < 90%; 50%  $\leq$  ML and MP < 70% (grey); not supported: BI and ML and MP < 50% (white). The respective class was coded only if all analyses using the different gap coding approaches resulted consistently in the same level of node support. The separate consensus trees of BI, ML and MP analyses are available as Supporting Information.



The sister-group relationships of *P. ingeri* + *P. bosii* and of *P. acutirostris* + *P. surdus* as well as a clade which contains *P. abditus* from Vietnam are robustly supported. The latter clade is the sister taxon of a cluster of three Bornean species (*P. acutus*, *aurantium*, *bunitus*). Within the monophyletic *P. everetti* clade, the two samples from Palawan, Philippines, representing the topotypic *P. everetti*, are the sister-group to a clade including all *P. macroscelis* from Borneo comprising samples from Gunung Mulu, Crocker Range and Gunung Kinabalu. The 12S and 16S sequences of the tadpole and the adult sample from the Gunung Kinabalu population are identical. The remaining *Philautus* species form a clade with robust support in BI and ML analyses including species from Borneo (*P. davidlabangi*, *juliandringi*, *mjoebergi*, *petersi*, *refugii*, *umbra*) as well as from the Philippines (*P. acutirostris*, *surdus*). Within this assemblage, only a clade of three Bornean species (*P. juliandringi*, *mjoebergi*, *umbra*) gains robust and congruent support. The internal phylogenetic relationships of species and species groups in *Philautus*, such as of *P. aurifasciatus*, *P. tectus*, *P. everetti* + *P. macroscelis* and the *P. ingeri* + *P. bosii* clade, remain as yet unresolved because results of ML, BI and MP analyses are not fully congruent (Fig. 3, Supporting Information).

The support of *Rhacophorus* excluding the *R. everetti*-group is weak (Fig. 3). The *Rhacophorus* species in this study form three clades: Clade A, which corresponds to cluster K2 in Li *et al.* (2008), consists of mostly mainland species with the Bornean *R. dulitensis* as sister taxon to the remaining members of this clade. Clade B, corresponding to cluster K1 in Li *et al.* (2008), contains the Bornean flying frogs (*R. pardalis*, *nigropalmatus*, *reinwardtii*) and relatives. The Bornean flying frogs are not revealed as each others' closest relatives. Clade C consists of two clades of endemic species from Borneo with low genetic differentiation among each other (Fig. 3). We refer to them here as the *angulirostris*-group (*R. angulirostris*, *belalongensis*, *gauni*, *penanorum*) and the *fasciatus*-group (*R. fasciatus*, *harrissoni*, *rufipes*), respectively. Within the genus *Polypedates*, the Bornean species do not form a monophyletic cluster, because the Bornean *P. otlophus* shows a basal position within this genus while the Bornean *P. macrotis* is the sister species of a clade consisting of the mainland species *P. leucomystax* and *P. megacephalus* (Fig. 3).

### Taxonomic reallocation

Examination of type material of *Rhacophorus everetti* Boulenger 1894 (BMNH 94.6.3.126; syntype) and *R. macroscelis* Boulenger, 1896 (BMNH 1895.11.7.82; holotype), as well as of fresh topotypic material (see Appendix 1), confirms Inger's (1966:298) observations concerning morphological differences between the two allopatric groups. Head and

dorsum shagreened in the material from Palawan (the holotype of *R. everetti* and newly collected specimens, deposited at Kansas University Collection, Lawrence and Philippines National Museum, Manila), without marked tuberculate structures (Fig. 2A). Forehead and dorsum of Bornean specimens (the type of *R. macroscelis* in addition to recent material examined) show distinct tuberculate structures, which are more prominent in specimens from the Gunung Kinabalu population (Fig. 2B). Forehead patternless in *R. everetti*, with a conspicuous dark brown pattern in male *R. macroscelis* consisting of spots and transverse bars on the upper surface, a preocular stripe from tip of snout to eye just below canthus, and three spots or broad bars laterally below eye (pre-, sub-, and postocular, respectively), and a postocular stripe just below the supratympanic fold. Tympanum in *R. everetti* patternless; in *R. macroscelis*, tympanum with a dark brown patch, connected to the postocular stripe, and an anteroventral light margin. Iris with silvery to pale golden ground colour, unicoloured in *R. everetti* with faint dark reticulations; solid dark brown patches forming a dark mottling or a broad transverse bar in *R. macroscelis*. Dorsum in *R. everetti* uniform tan or yellowish-brown; in *R. macroscelis*, unstressed individuals are bright pale green or yellow-green, with contrasting dark brown or reddish brown patches forming a bark- or lichen-like pattern; stressed individuals have a more brown dorsum, but darker patches remain distinct. Upper surfaces of limbs in *R. everetti* unbanded; with dark brown bands in *R. macroscelis*. In females pattern elements on dorsum, head, and limbs are pale or reddish brown and less contrasting (Fig. 2D). Additional features (primarily body proportions and colour pattern) are listed in Inger (1966).

The diagnosable nature of individuals from these two allopatric populations, in both morphology and genetic differences, as well as their reciprocal monophyly (Fig. 3), unambiguously indicates their unique ancestry and historical fate and hence their status as separate evolutionary lineages. Given our findings, we here recognise these two discrete populations as distinct species following the Phylogenetic Species Concept (Nixon & Wheeler 1990; Cracraft 1992), the Evolutionary Species Concept (Wiley 1978), and the Unified Species Concept (De Queiroz 2007). Hereafter, the species epithet *everetti* will be applied to the population from Palawan, *macroscelis* for populations from Borneo. Smith's (1931) *Philautus spiculatus* is considered a junior subjective synonym of *Rhacophorus macroscelis* Boulenger (1896) (see Bossuyt & Dubois 2001); this taxon had, in fact, been originally allocated correctly to the genus *Philautus*.

The genetic distances based on 491 bp of mitochondrial 16S rDNA of individual one of *P. everetti* to *P. macroscelis* range from 7.4% (Crocker Range) to 7.9% (Gunung

Mulu. In individual two of *P. everetti*, 472 bp were available and showed a distance between 5.4% (Crocker Range) and 7.9% (Gunung Mulu) (Table 1). The difference in genetic distances between these two individuals of *P. everetti* is caused by the fact that the sequences were obtained using alternative primer combinations resulting in overlapping but not fully orthologous sequences of the 16S rDNA gene.

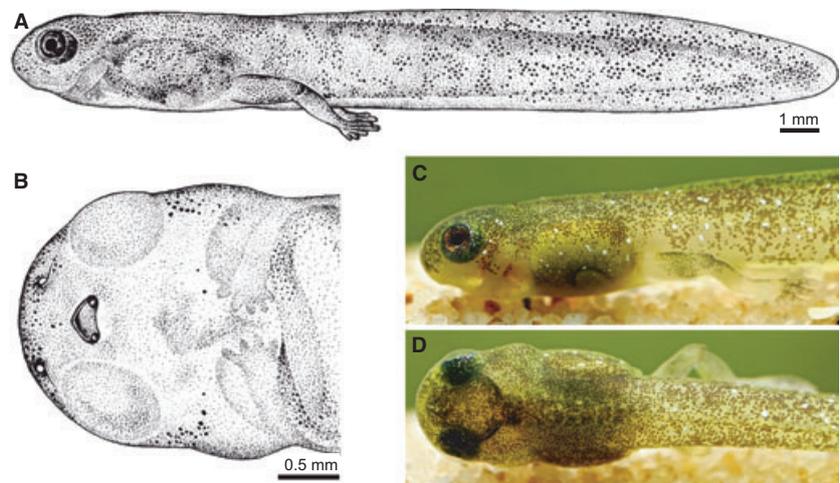
#### Larval morphology

The 12S and 16S rDNA sequences of the tadpole and the adult samples from the Gunung Kinabalu population of *Philautus macroscelis* comb. nov. are identical and provide sufficient reason to assign this larva to this taxon. As only one tadpole could be collected, any morphological description faces limitations, particularly because this unique specimen was preserved directly in absolute ethanol and underwent shrinkage.

The stage 36 larva (Fig. 4) measures 18.5 mm in total length (TL); body length (BL = head and trunk): 4.5 mm from snout to the point where the axis of the tail myotomes contacts the body wall; and tail length (TAL = TL–BL): 14 mm. Tail long, relative tail length 75% of total length (Fig. 4A). Body moderately depressed, longer than wide and only slightly wider than high, approximating a cylindrical body shape (Fig. 4C,D). Tail mostly formed by its muscular part, upper and lower tail fins low. Dorsal tail fin rises posterior to the body–tail junction. Tail fins approximately parallel in the proximal half of the tail, not profoundly different in height in any transverse plane along the length of the tail, maximum height at about 60% of the tail length. Fins softly converge in the posterior 25% of the tail. Tail tip rounded. Limbs well developed (Fig. 4A,C), front limbs still under operculum in this stage, but visible through the skin

(Fig. 4B). In dorsal view, developing front limbs bulge the contour line of the body (Fig. 4D). Snout very short, letting the eyes appear relatively large (ED 11.7% of BL). Eyes lateral. Head length (neurocranium and hyobranchium) very short, position of the forelimb immediately behind hyobranchium. Nares on the ventral side of the snout, closer to the snout than to the eyes. Mouth very small with crescentic orifice (Fig. 4B). Oral disc almost completely reduced, except for lateral papillae, one per side. Keratinized structures absent. Two bends of the gut clearly visible through the skin (Fig. 4C). Intestine thick, yellowish in colour (interpreted as rich load of yolk). Condition of the gut, narrow (in dorsal and lateral views) gill region and reduced mouthparts suggest an endotrophic mode of development.

Colour in life (Fig. 4C,D): Overall appearance olive, pigmentation moderate. Melanocytes predominantly on dorsal and lateral body faces. Ventral side mostly unpigmented and translucent. Heart, fore limbs, and intestine visible through ventral body skin. Dorsal side with concentrations of melanocytes and darker colourations between the eyes (brain cavity), along spine, and in the dorsal parts of the abdominal cavity. Pigmentation of body continues onto the muscular part of tail and dorsal tail fin. Dorsal and ventral fins mostly clear at their margins. Dorsal fin bears more melanophores than ventral fin. Conspicuous platelets of silver-blue iridophores start posterior to the eye and are scattered along flanks and tail (mostly its muscular part, few on dorsal fin). Sclera of eye black in background colour with silver-blue iridocytes. Iridocytes dense ventrally and scattered dorsally on the eye ball. Iris background colour black dusted with dense coppery spots, which partially fuse along the pupil. A conspicuous bright coppery-red pupil ring (present in tadpoles of many other Bornean species) not present.



**Fig. 4** Tadpole of *Philautus macroscelis* (ZMH A10833) in lateral —A, C, ventral —B and dorsal —D views. C and D show the colouration in life.

Colour in ethanol: Overall olive tinge transforms to brown, iridescent silver-blue spots on body and scleral colouration disappeared; eye is black.

## Discussion

### *Phylogenetic signal*

The inconsistent support of nodes in the different analyses using BI, ML and MP algorithms as well as different indel coding schemes indicates a heterogeneous phylogenetic signal in the ribosomal mtDNA sequences used in this study. While the deep node corresponding to the Rhacophorinae and most phylogenetic relationships between clusters of closely related species were robustly and congruently supported, several nodes remained unresolved or were only weakly supported (Fig. 3). Conflicting topologies obtained with different optimality criteria and low support for certain clades were also reported from previous phylogenetic studies on rhacophorid tree frogs that used different combinations of mitochondrial and nuclear markers (Wilkinson *et al.* 2002; Grosjean *et al.* 2008; Li *et al.* 2008, 2009; Yu *et al.* 2009; Biju *et al.* 2010). It must be emphasized that the phylogeny of rhacophorid tree frogs is still far from resolved despite decades of intense work on this group. This study, however, contributes new insights into several rhacophorid taxa from Sundaland based on new and substantial taxon sampling. Our findings regarding the composition of genera, the sister-group relationship of *Rhacophorus* and *Polypedates*, and the basal position of *Liuixalus* are in agreement with previous studies (Channing 1989; Ye *et al.* 1999; Wilkinson *et al.* 2002; Frost *et al.* 2006; Biju *et al.* 2008; Grosjean *et al.* 2008; Li *et al.* 2008, 2009; Yu *et al.* 2009). In contrast, Biju *et al.* (2010) presented a tree that depicted Sundaland *Philautus* as sister clade to the remaining Rhacophorinae instead of Nyctixalini and the genus *Rhacophorus* as polyphyletic assemblage. The study of Biju *et al.* (2010) however, did not consider *Liuixalus* which could partly explain the differences to our results. In summary, we consider the signal in our mitochondrial sequence data as a robust basis for establishing the hypothesis on the phylogeny of Bush Frogs of the genus *Philautus* as discussed later.

### *Phylogenetic relationships of Sundaland Bush Frogs*

The unequivocal matching of the tadpole and adult 12S and 16S sequences of *P. macroscelis* allows the reliable identification of its larvae in future studies. The assignment of this species together with *P. everetti* to the *Philautus* clade is robustly supported and provides the basis for their formal transfer into the genus *Philautus* and a redefinition of this genus from a phylogenetic perspective. Moreover, we conclude from the molecular and morphological evidence and the current distribution that *P. macroscelis* from Borneo

and *P. everetti* from Palawan are separate allopatric species following modern species concepts. The genetic distances observed between the sequences of *P. everetti* and *P. macroscelis* in our sample range from 5.4% to 7.7%, depending on sequence and population. These values lie well within the range of distances between closely related but widely accepted species of the genus *Philautus*: we found 4% genetic distance between *P. acutus*/*P. aurantium*, 6.6% for *P. acutus*/*P. bunitus* and 8.5% for *P. bosii*/*P. ingeri*, respectively. In the light of this considerable divergence observed in the 16S rDNA sequences, more data from natural history, bioacoustics, and reproductive behaviour of *P. everetti* would be desirable to compare it with its Bornean sister species, *P. macroscelis*, to corroborate the species status of both taxonomic units.

Within Bornean *Philautus*, Dring (1987) proposed four or five species groups based on external morphology as well as bioacoustic and ecological characters. The first of these species group, the *tectus*-group, consists only of the widespread lowland species *P. tectus*, which is characterised by the possession of a suite of derived and plesiomorphic characters (Dring 1987). In our analyses, it is placed in an unresolved basal position within *Philautus* (Fig. 3). A second species group is the *bosii*-group, which distinguishes *P. bosii* and *P. ingeri*, the latter described by Dring in the same paper, from the remaining *Philautus*. At the same time, Dubois (1987) proposed a separate subgenus, *Gorbixalus* Dubois 1987; exclusively for *P. bosii* (Boulenger 1895).

Our data support the sister-group relationship of *P. bosii* and *P. ingeri* and their placement within the genus *Philautus*. We propose *Gorbixalus* as a junior synonym of *Philautus*, because *Philautus* is not monophyletic in relation to *Gorbixalus*. Following our results on the phylogenetic relationships of *P. everetti* with *P. macroscelis*, we propose the *everetti*-group as a further monophyletic entity within this genus. However, a more comprehensive phylogenetic and taxonomic analysis is needed to resolve the early genealogical branching pattern within this genus with confidence. *Philautus acutus* is the sole representative of the *vermiculatus*-group (Dring 1987) in our sample. It is part of a well-supported clade including *P. abditus*, *P. aurantium* and *P. bunitus* (Fig. 3). The monophyly of the *aurifasciatus*-group (*sensu* Dring 1987) is not supported by our data, because the position of *P. aurifasciatus* (the type species for *Philautus*) remains unresolved. The genetic sequences of this taxon available from GenBank (vouchers ZRC.1.5266 and ZRC.1.5267) were short, and more data are needed to address this issue. *P. petersi* (Borneo) and *P. acutirostris* (Mindanao, Philippines), both regarded as members of the *aurifasciatus*-group by Dring (1987), are part of a monophyletic clade including also *P. davidlabangi*, *P. juliandringsi*,

*P. mjobergi*, *P. petersi*, *P. refugii* and *P. umbra* from Borneo, as well as *P. surdus* from the Philippines (Fig. 3).

### Biogeography

An intriguing outcome of our study is the close phylogenetic relationships of different species or clades of species of Bush Frogs from Borneo to species from outside of Borneo: *P. abditus* (Vietnam) + (*P. bunitus*, *P. acutus*, *P. aurantium*) (Borneo); *P. everetti* (Palawan) + *P. macroscelis* (Borneo); the clade *P. acutirostris* + *P. surdus* (Philippines) within a large cluster of Bornean species (Fig. 3). Similar distribution patterns between Philippines and Borneo have been reported in the fanged frogs of the genus *Limnonectes*, Dicroglossidae (Evans *et al.* 2003), slender toads of the genus *Ansonia* (Matsui *et al.* 2010; Sanguila *et al.* 2011) litter toads of the genus *Leptobrachium* (Brown *et al.* 2009), and splash frogs of the genus *Staurois* (Arifin *et al.* 2011). These findings indicate a complex biogeographical history of the amphibian fauna of northern Sundaland and suggest repeated dispersal and/or vicariance events between the mainland and the different islands. The complicated patterns of dispersal and colonization of islands on the edge of the Sunda Shelf have resulted in geographical limits of taxa being incongruent with phylogenetic clades in the Philippines and Borneo (review: Esselstyn *et al.* 2010).

The Palawan group of islands (comprising Palawan, Balabac and the Calamianes archipelago) has previously been considered part of Sundaland. Some components of the fauna of Palawan are more similar to that of Borneo than to the rest of the Philippines Archipelago (Huxley 1868; Hall 1998; Voris 2000; Esselstyn *et al.* 2004). However, a wide variety of new evidence, comprising both phylogenetic studies and geological data, have called that interpretation into question (Brown & Diesmos 2009; Blackburn *et al.* 2010; Esselstyn *et al.* 2010). Multiple invasions from Borneo to the southern islands of the Philippines have been proposed (Brown & Guttman 2002; Arifin *et al.* 2011), which could explain the complex relationships between the *Philautus* species from both areas in our study. Because the recent geological connections of Palawan and Borneo during periods of lowered sea levels have not been reconstructed unambiguously (Voris 2000), strongly postulating hypotheses regarding the temporal pattern of colonization in either direction between Sundaland and the Philippines for *Philautus* would currently be too speculative. Furthermore, the landmasses of Palawan and Borneo were reported to harbour similar floral assemblages, taken as evidence of refugia of species belonging to both the Tertiary moist and Quaternary dry forests (Meijer 1982; Tan 1998). Nonetheless, the high degree of endemism in Palawan's amphibian fauna (Alcala & Brown 1998; Brown *et al.* 2008; Brown & Diesmos 2009) may

have resulted from long isolation from larger landmasses (Inger 1954, 1999; Brown & Alcala 1970; Gascoyne *et al.* 1979), followed by a recent (~5 Ma) uplift of southern Palawan (Taylor & Hayes 1980; Holloway 1982; Heaney 1986; Esselstyn *et al.* 2004). Alternatively, if portions of Palawan were above water before the collision of the Palawan Microcontinent Block with the northern edge of the Sunda Shelf (north Borneo; Yumul *et al.* 2003), a longer period of isolation on fragments of what is now Palawan may have given rise to high levels of diversity and ancient phylogenetic divergences seen in many of its endemic vertebrates today (Blackburn *et al.* 2010; Esselstyn *et al.* 2010).

### Evolution of reproductive biology

Rhacophorid frogs possess an impressive diversity in their reproductive biology. The deposition of a relatively high number of eggs and the presence of free-feeding tadpoles must be regarded as the plesiomorphic condition in this group (Grosjean *et al.* 2008). In members of several genera within the Rhacophorinae, different types of foam nests are produced that represents a derived form of parental care. In *Philautus* and *Raorchestes*, terrestrial direct development evolved independently, i.e., the lecithotrophic tadpole develops in the egg and transforms into a hatching froglet (Bossuyt & Dubois 2001; Bahir *et al.* 2005; Grosjean *et al.* 2008; Li *et al.* 2009; Biju *et al.* 2010).

However, only a few reliable reports on the reproductive biology of South-east Asian *Philautus* species are available, and the reproductive modes of most Bornean species remain unknown. Inger (1966) described the development and tadpole of *Philautus bosii* (as *Rhacophorus bosii*) based on a clutch of eggs raised in captivity. He interpreted the absence of the larval oral apparatus, gills and operculum at early limb bud stages as adaptations to an abbreviated, or even missing, free-swimming stage. *Philautus kerangkae* breeds with endotrophic tadpoles in *Nepenthes bicalcarata* pitchers (Dring 1987), while *P. aurifasciatus* lays eggs in tree holes and probably also undergoes direct development (Iskandar 2004). In *P. acutus*, direct development and hatching of froglets at a stage approximately equivalent to stage 44 or 45 in biphasic species was confirmed by genetic matching of semaphoronts collected at the type locality (S. T. Hertwig, K. E. Lilje, I. Das & A. Haas, submitted). Unfortunately, data on the developmental mode in *P. tectus*, representing one of the basal branches within *Philautus*, are not available. Dring (1987) reported only unidentified terrestrial clutches of eggs in the original description of *P. tectus*.

The tadpole of *Philautus macroscelis* identified in this study is conspicuous in body shape, short snout, pigmentation and mouthpart reduction (Fig. 4). The latter and the

yellow, thick intestine suggest an endotrophic mode of life. Tadpoles perplexingly similar to the larva of *P. macroscelis* have been described for *Limnonectes laticeps* (Leong 2004; DNA samples of free-swimming and feeding larvae for *L. laticeps* have been confirmed for Borneo, however, A. Haas and I. Das, unpublished data) and some species of *Gephyromantis* (Randrianiaina et al. 2011). The latter have been identified to be nidicolous, i.e., hatching from the egg jelly but staying in the terrestrial nest situation for some time. The identification of the endotrophic larva of *L. laticeps* was concluded by Leong (2004) from the presence of *L. laticeps* adults at the collection site and on raising one larvae up to stage 41 (Leong 2004); however, genetic matching was not performed. The similarity of *P. macroscelis* to larvae of members of *Gephyromantis*, for which details of nesting and development are known, suggests a similar reproductive and developmental mode in *P. macroscelis* (i.e. endotrophic larva developing first in egg jelly in a terrestrial site and enters the stream at advanced stages). However, in the latter species, no egg-deposition sites have ever been reported. A secretive, mostly nidicolous mode of development might explain why only one larva could be collected during multiple field trips to the site at various times of the year.

The presence of a free-swimming and possibly nidicolous tadpole in *P. macroscelis* suggests that endotrophy may be plesiomorphic for *Philautus*. However, this observation challenges the assumption that terrestrial direct development represents an apomorphic character of *Philautus sensu* Biju et al. (2010) (Dring 1979; Bossuyt & Dubois 2001; Grosjean et al. 2008; Li et al. 2009). Two alternative hypotheses need to be tested: (i) direct development was already present in the last common ancestor of all *Philautus* species; the presence of a tadpole in the *everetti*-group was a secondary reversal and a potential autapomorphy of *P. macroscelis* or a potential synapomorphy of the *everetti*-group (larvae unknown in *everetti*) and (ii) direct development evolved independently in *P. tectus* and the large clade consisting of the remaining Bornean and Philippine Bush frogs. Both alternatives are currently possible because, first, crucial branches in the phylogenetic topologies within *Philautus* are as yet unresolved and, second, the lack of information about the reproductive biology in important species, in particular in *P. tectus*.

Despite this uncertainty about the reconstruction of the evolutionary history of reproductive behaviour in *Philautus*, we interpret the remarkable nidicolous tadpole in *P. macroscelis* as an evolutionary step in getting independent from bodies of water. *Philautus macroscelis* have been found exclusively along small, semipermanent streams or headwaters in montane forests (personal observation), so that its larvae are probably specifically adapted to a habitat

bordering such streams. Following this hypothesis, the driving force for leaving the main water body in *P. macroscelis* is avoidance of competition with tadpoles of syntopic frogs (*Leptobranchium montanum*, *Ansonia hanitschi*, *Rhacophorus pennanorum*, *Meristogenys* sp., *Xenophrys dringi*), particularly during temporally reduced water levels depending on amounts of precipitation. Syntopic *Philautus* species have probably a direct mode of development as confirmed in *P. acutus* (S. T. Hertwig, K. E. Lilje, I. Das & A Haas, submitted).

### Conflict of interest

The authors declare that there is no conflict of interest.

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**Appendix Materials examined**

Species	Collection Nr.	Locality	Genbank Accession	
			16S	12S
<i>Buergeria japonica</i>				AF458123
<i>Buergeria robusta</i>				AF458125
<i>Chiromantis doriae</i>				EU215527
<i>Chiromantis rufescens</i>				AF458126
<i>Chiromantis vittatus</i>				AF458131
<i>Chiromantis xerampelina</i>				AF458132
<i>Feihyla palpebralis</i>				EU215546
<i>Gracixalus carinensis</i>				GQ285670
<i>Gracixalus gracilipes</i>				DQ283051
<i>Gracixalus jinxiuensis</i>				EU215525
<i>Kurixalus banaensis</i>				GQ285667
<i>Kurixalus eiffingeri</i>				DQ283122
<i>Kurixalus idiotocus</i>				AF458129
<i>Kurixalus hainanus</i>				EU215548
<i>Kurixalus hainanus</i>				GU227273
<i>Kurixalus odontotarsus</i>				EU215549
<i>Kirtixalus longchuanensis</i>				GQ285675
<i>Kirtixalus menglaensis</i>				GQ285676
<i>Liuixalus albopunctatus</i>				EU215526
<i>Liuixalus hainanus</i>				GQ285671
<i>Liuixalus ocellatus</i>				GQ285672
<i>Liuixalus romeri</i>				EU215528
<i>Nyctixalus pictus</i>	NMBE 1056413	Malaysia: Sarawak: Batang Ai Natl. Park	JN377342	JN705355
<i>Nyctixalus spinosus</i>				DQ283114
<i>Philautus abditus</i>				GQ285673
<i>Philautus acutirostris</i>				AY326059
<i>Philautus acutus</i>	NMBE 1056431	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JN705366	JN705337
<i>Philautus aurantium</i>	UNIMAS 8666	Malaysia: Sabah: Crocker Range Park: 16th mile	JN705367	JN705338
<i>Philautus aurifasciatus</i>			–	AY141805.1
<i>Philautus aurifasciatus</i>			–	AY141804.1
<i>Philautus bunitus</i>	UNIMAS 9045	Malaysia: Sabah: Gunung Kinabalu Park: Sayap	JN705368	JN705339
<i>Philautus davidlabangi</i>	NMBE 1056444	Malaysia: Sarawak: Batang Ai Natl. Park: Hilton Lighthouse Resort	JN705386	JN705356
<i>Philautus davidlabangi</i>	ZMH A10429	Malaysia: Sarawak: Kubah Natl. Park: Belian Trail	JN705387	JN705357
<i>Philautus hosii</i>	NMBE 1057287	Malaysia: Sarawak: Usun Apau Natl. Park	JN705384	JN705353
<i>Philautus ingeri</i>	NMBE 1056435	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JN705385	JN705354
<i>Philautus juliandringi</i>	NMBE 1056439	Malaysia: Sarawak: Gunung Mulu Natl. Park: Gunung Api	JN705378	JN705347
<i>Philautus everetti</i>	KU 309610	Philippines: Palawan: Municipality of Brooke's Point: Boundary of Barangay Samarina and Saubg: Mt. Mantalingahan: Area Pitang	JN705377	–
<i>Philautus everetti</i>	KU 309611	Philippines: Palawan: Municipality of Brooke's Point: Boundary of Barangay Samarina and Saubg: Mt. Mantalingahan: Area Pitang	JN705376	–
<i>Philautus macroscelis</i>	UNIMAS 8664	Malaysia: Sabah: Crocker Range Park: 16th mile	JN705371	JN705342
<i>Philautus macroscelis</i>	UNIMAS 8158	Malaysia: Sabah: Gunung Kinabalu Ntl. Park	JN705372	JN705343
<i>Philautus macroscelis</i>	ZMH A10833	Malaysia: Sabah: Gunung Kinabalu Ntl. Park: Silau-Silau trail	JN705373	JN705344
<i>Philautus macroscelis</i>	NMBE 1056486	Malaysia: Sarawak: Gunung Mulu Natl. Park: Sungai Tapin	JN705375	JN705346
<i>Philautus macroscelis</i>	NMBE 1056488	Malaysia: Sarawak: Gunung Mulu Natl. Park: Sungai Tapin	JN705374	JN705345
<i>Philautus mjoebergi</i>	NMBE 1056434	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JN705380	JN705349
<i>Philautus petersi</i>			AF026349	AF026366
<i>Philautus petersi</i>	NMBE 1056443	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JN705381	JN705350

## Appendix Continued

Species	Collection Nr.	Locality	Genbank Accession	
			16S	12S
<i>Philautus refugii</i>	NMBE 1057544	Malaysia: Sarawak: Borneo Highlands Resort	JN705382	JN705351
<i>Philautus refugii</i>	ZMH A10415	Malaysia: Sarawak: Kubah Natl. Park: Summit Road	JN705383	JN705352
<i>Philautus surdus</i>			AF458138	
<i>Philautus tectus</i>	NMBE 1056451	Malaysia: Sarawak: Gunung Mulu Natl. Park: Deer Cave Trail	JN705370	JN705341
<i>Philautus tectus</i>	NMBE 1057080	Malaysia: Sarawak: Kubah Natl. Park: Summit Road	JN705369	JN705340
<i>Philautus umbra</i>	NMBE 1056454	Malaysia: Sarawak: Gunung Mulu Natl. Park: Gunung Api	JN705379	JN705348
<i>Polypedates colletti</i>	ZRC 1.11912	Malaysia: Sarawak: Logan Bunut Natl. Park	EF566973	JN705358
<i>Polypedates leucomystax</i>			DQ283048	
<i>Polypedates macrotis</i>	NMBE 1056471	Malaysia: Sarawak: Gunung Mulu Natl. Park	JN377343	JN705360
<i>Polypedates macrotis</i>	UNIMAS 8638	Malaysia: Sarawak: Matang Wildlife Center: Sungai Rayu	JN377345	JN705359
<i>Polypedates megacephalus</i>			AF458141	
<i>Polypedates otilophus</i>	ZMH A10465	Malaysia: Sarawak: Kubah Natl. Park: Frog Pond	JN377346	JN705361
<i>Rhacophorus angulirostris</i>	UNIMAS 8681	Malaysia: Sarawak: Gunung Kinabalu Natl. Park: Silau Silau	JN377348	JN705322
<i>Rhacophorus annamensis</i>			DQ283047	
<i>Rhacophorus arboreus</i>			AF458142	
<i>Rhacophorus belalongensis</i>	ZMB70378	Brunei Darus-salam: Temburong district: Kuala Belalong Field Studies Centre	JN377352	JN705324
<i>Rhacophorus bipunctatus</i>			AF458144	
<i>Rhacophorus calcaneus</i>			DQ283380	
<i>Rhacophorus dennysi</i>			EU215545	
<i>Rhacophorus dugritei</i>			EU215541	
<i>Rhacophorus dulitensis</i>	NMBE 1056482	Malaysia: Sarawak: Gunung Mulu Natl. Park: HQ	JN377355	JN705326
<i>Rhacophorus fasciatus</i>	NMBE 1056492	Malaysia: Sarawak: Gunung Mulu Natl. Park: HQ	JN377357	JN705331
<i>Rhacophorus fasciatus</i>	NMBE 1057405	Malaysia: Sarawak: Lambir Hills Natl. Park: HQ	JN705363	JN705330
<i>Rhacophorus feae</i>			EU215544	
<i>Rhacophorus gauni</i>	NMBE 1056493	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 5	JN377351	JN705325
<i>Rhacophorus harrissoni</i>	NMBE 1056497	Malaysia: Sarawak: Gunung Mulu Natl. Park: Sungai Melinau Paku	JN377359	JN705332
<i>Rhacophorus hui</i>			EU215540	
<i>Rhacophorus hungfungensis</i>			EU215538	
<i>Rhacophorus kio</i>			EU215532	
<i>Rhacophorus minimus</i>			EU215539	
<i>Rhacophorus moltrechti</i>			AF458145	
<i>Rhacophorus nigropalmatus</i>	ZMH A10414	Malaysia: Sarawak: Kubah Natl. Park: Main Trail	JN377363	JN705328
<i>Rhacophorus nigropalmatus</i>	ZMH A10835	Malaysia: Sarawak: Borneo Highlands Resort: 400 m downhill from Kalimantan Viewpoint	JN705362	JN705327
<i>Rhacophorus nigropunctatus</i>			EU215533	
<i>Rhacophorus orlovi</i>			DQ283049	
<i>Rhacophorus pardalis</i>	NMBE 1056514	Malaysia: Sarawak: Batang Ai Natl. Park: Bebyong Trail	JN377369	JN705335
<i>Rhacophorus pardalis</i>	ZMH A10834	Malaysia: Sarawak: Lambir Hills Natl. Park: Latak Waterfall Trail	JN705365	JN705336
<i>Rhacophorus penanorum</i>	ZMH A10168	Malaysia: Sarawak: Gunung Mulu Natl. Park: Sungai Tapin	JN377349	JN705323
<i>Rhacophorus pingbianensis</i>			EU215536	
<i>Rhacophorus puerensis</i>			EU215542	
<i>Rhacophorus reinwardtii</i>	NMBE 1056517	Malaysia: Sarawak: Batang Ai Natl. Park: Bebyong Trail	JN377366	JN705329
<i>Rhacophorus rhodopus</i>			EU215531	
<i>Rhacophorus rufipes</i>	NMBE 1056519	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 5	JN377360	JN705334
<i>Rhacophorus rufipes</i>	NMBE 1057529	Malaysia: Sarawak: Borneo Highlands Resort	JN705364	JN705333
<i>Rhacophorus schlegelii</i>			AB202078	
<i>Rhacophorus taronensis</i>			EU215537	
<i>Theلودerma asperum</i>			GQ285678	
<i>Theلودerma corticale</i>			DQ283050	
<i>Theلودerma rhododiscus</i>			EU215530	

KU, Kansas University Collection, Lawrence, 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; ZRC, Zoological Reference Collection, Raffles Museum of Biodiversity Research Singapore.

### **Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1** Majority rule consensus tree for south-east Asian Rhacophoridae using the Bayesian Inference with MrBayes.

**Fig. S2** Phylogenetic hypothesis within south-east Asian Rhacophoridae obtained using RaxML.

**Fig. S3** Combined strict consensus tree for south-east Asian Rhacophoridae when applying maximum parsimony heuristic search algorithm with PAUP.

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