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MOLECULAR EVIDENCE FOR DIRECT DEVELOPMENT IN THE RHACOPHORID FROG, *PHILAUTUS ACUTUS* (RHACOPHORIDAE, ANURA) FROM BORNEO

Stefan T. Hertwig
Naturhistorisches Museum der Burgergemeinde Bern, Bernastrasse 15, CH-3005 Bern, Switzerland
Email: stefan.hertwig@nmbe.ch (Corresponding author)

Karin E. Lilje
Kistlerstrasse 18, CH-3065 Bolligen, Switzerland
Email: k.lilje@gmx.ch

Pui Yong Min
Institute of Biodiversity and Environmental Conservation, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia
Email: pui8783ibec@gmail.com

Alexander Haas
Zoologisches Museum Hamburg, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany
Email: alexander.haas@uni-hamburg.de

Indraneil Das
Institute of Biodiversity and Environmental Conservation, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia
Email: idas@ibec.unimas.my

ABSTRACT. — The tree frogs of the taxon Rhacophoridae are known for their impressive diversity of reproductive strategies. Direct development on land has been described in the Old World Bush Frogs belonging to the genera *Philautus*, *Pseudophilautus*, and *Raorchestes*. However, in numerous species especially within the Bornean *Philautus,* breeding behaviours remain unknown. In this paper, we match a clutch of eggs found on Gunung Mulu National Park, Sarawak, Malaysia (Borneo), using genetic barcoding to syntopically occurring adults of *Philautus acutus*. This species is known only from its type locality in the montane forests at high elevations on Gunung Mulu. The eggs were found on leaf litter of the forest floor and are characterised by a protective, compact, outer jelly capsule. The froglets inside the eggs were at advanced stages of development and showed a bifurcating dorsal pattern similar to adults of *P. acutus*. Beside the discovery of its breeding behaviour, we add a description of the habitat of this rare species. Furthermore, this account of aerial direct development in a *Philautus* species from Borneo contributes to our understanding of the evolution of reproductive strategies within the lineage. Finally, we present a review of observations of the breeding behaviour in Bornean *Philautus* species available in the literature.

KEY WORDS. — Rhacophoridae, *Philautus acutus*, ecology, reproduction, direct development

INTRODUCTION

The evolution of complex reproductive behaviour in tropical tree frogs belonging to the family Rhacophoridae has been the topic of numerous phylogenetic studies, using both morphological and molecular data (e.g., Liem, 1970; Ye et al., 1999; Delorme et al., 2005; Yu et al., 2007, 2008, 2009; Biju et al., 2008; Grosjean et al., 2008; Li et al., 2008, 2009; Hertwig et al., 2012). While a majority of rhacophorid tree frogs produce one of several different types of foam nests and have free-swimming, ectotrophic tadpoles, the numerous species of Bush Frogs from south and south-east Asia belonging to the genera *Philautus* Gistel, 1848, *Pseudophilautus* Laurent, 1943, and *Raorchestes* Biju et al., 2010, are notable exceptions for exhibiting direct development (Alcala & Brown, 1982; Brown & Alcala, 1983; Grosjean et al., 2008). In this mode of reproduction, a free-swimming larval stage is absent and the lecithotrophic larva completes its development and metamorphosis on land within the egg. Direct development is interpreted as an adaptation to habitats with few or no surface waterbodies that are typical breeding habitats for other amphibian lineages (see Alcala, 1962; Marmayou et al., 2000; Callery et al., 2001), presumably an effect of local climatic or geomorphological conditions. At higher elevations of tropical montane forests, amphibians from various lineages (including *Eleutherodactylus*, *Brachycephalus*, *Myersiella*, and *Platymantis*: see Wells, 2007) are direct developers (sensu Duellman & Trueb, 1986; Wake, 1989), as standing water bodies are scarce or absent.

Among the members of the Rhacophoridae, direct development and the associated morphological traits have been discussed as characters to distinguish the direct-developing species as a separate genus *Philautus* from its relatives (Bossuyt & Dubois, 2001; Grosjean et al., 2008). In contrast, recent studies using molecular data showed that *Philautus* sensu lato is not monophyletic and have resulted, therefore, in the separation of new genera from *Philautus* (Li et al., 2009; Biju et al., 2010; Yu et al., 2010). *Pseudophilautus* and *Raorchestes* have been resurrected and described for the species-rich lineages from India and Sri Lanka (Li et al., 2009, 2011; Biju et al., 2010), while *Philautus* sensu stricto contains predominantly species distributed in south-east Asia.

Reliable records of direct development on land are available from species of the genera *Pseudophilautus* (Kanamadi et al., 1996: *P.* aff. *variabilis*; Gururaja & Ramachandra, 2006: *P.* aff. *leucorhinus*; Karunarathna & Amarasinghe, 2007: *P. regius*; Kerney et al., 2007: *P. silus*; Bahir et al., 2005: >10 spp.) as well as *Raorchestes* (Bossuyt et al., 2001: *R. bombayensis*; Biju, 2003, Krishnamurthy et al., 2002: *R. akroparallagi* [as *Philautus glandulosus*]; Biju & Bissuyt, 2009: *R. tinniens*; Biju & Bossuyt, 2005a: *R. nerostagona*; Biju & Bossuyt, 2005a: *R. graminirupes*; Biju & Bossuyt, 2005b: *R. resplendens*). Within *Philautus*, different modes of development are probably present; ranging from presumably nidicolous (Hertwig et al., 2012: *P. macroscelis*; Inger, 1966: *P. hosii*) or free-swimming lecithotrophic tadpoles (Dring, 1987: *P. kerangae*; Mjöberg in Smith, 1925: *P. mjobergi*) to direct development (Yong et al., 1988: *P. aurifasciatus*; Malkmus et al., 2002: *P. saueri)*. Aerial direct development has been recorded plausibly only in few species of *Philautus* (Yong et al., 1988: *P. aurifasciatus*; Malkmus et al., 2002: *P. saueri*). In the vast majority of species of *Philautus*, including most representatives from Borneo, however, the effective breeding behaviour has never been confirmed by direct observations, by captive breeding or via genetic matching (= bar coding) of semaphoronts to each other. This lack of basic knowledge hampers both the understanding of their ecology, as well as the reconstruction of the evolution of their reproductive strategies. In particular, it remains unclear if aerial direct development has evolved once or several times independently within different lineages of *Philautus* and its relatives (Meegaskumbura et al., 2002; Grosjean et al., 2008; Li et al., 2009).

Philautus acutus Dring, 1987 is one of several endemic species of *Philautus* with a restricted distribution on the island of Borneo. It is a small tree frog (SVL of males 23.4–27.1 mm; Dring, 1987, pers. obs.), characterised by a short, rounded snout and a smooth skin with few small tubercles on snout, upper eyelid and occipital region. The dorsum shows a broad dark brown bifurcate pattern on a pale tan to clay-brown ground colour (Fig. 1). *P. acutus* was originally described from higher elevations of Gunung Mulu National Park, in north-eastern Sarawak, Malaysia (Borneo), and inhabits primary upper montane forests on the steep slopes of Gunung Mulu (Fig. 2). This species is known only from its type locality at about 1,300 m asl, although Dring (1987) reported this species also from the adjacent Gunung Api summit area at an elevation of 1,200 m asl, based on unconfirmed call records.

In his original description, Dring (1987) speculated that *P. acutus* would likely have a direct mode of development and laid its eggs in the thick epiphytic or ground moss layer. However, he did not mention direct observations of the reproductive behaviour of *P. acutus*. To the best of our knowledge the natural history of this rare species has not been studied in detail, on account of its restricted distribution in a remote, steep, and protected area. Its reproductive biology remains obscure and females have yet to be discovered. Moreover, data on population status of this species or its habitat requirements are not available (www.amphibiaweb.org). Herein, we describe the discovery of a clutch of eggs with advanced froglets and identify it as *P. acutus,* using techniques of genetic barcoding. Furthermore, we discuss the published observations of the breeding behaviour in *Philautus* species from Borneo.

Fig. 1. Habitat of *Philautus acutus* at type locality at Camp 3, Summit Trail to Gunung Mulu, Sarawak, Malaysia (Borneo).

Fig. 2. Colouration in life of *Philautus acutus*, from Gunung Mulu,Sarawak, Malaysia (Borneo). Daytime colouration of specimen somewhat stressed during photography.

MATERIAL AND METHODS

Three egg capsules were found incidental to a survey of amphibians in the area on 28 Mar.2006, on the forest floor within the montane forest below Camp Three, along the Summit Trail in Gunung Mulu National Park, Sarawak, Malaysia (north-eastern Borneo) (N 04'002.275'92, E 114'053.285'92, 1,360 m asl). One egg capsule was empty and the other two contained one froglet each. The clutch of eggs was discovered during the focused search for *Kalophrynus nubicola*, a ground dwelling microhylid endemic to the higher elevations on Gunung Mulu. During this and subsequent field trips, a total of eight adult specimens of *P. acutus* were also collected at the same locality. The eggs and frogs were photographed (Canon EOS 350 D, 60 mm macro lens, double flashes) as previously described (Haas & Das, 2011; the adults were subsequently anesthetised and euthanized in an ca. 2% aqueous chlorobutanol solution (1,1,1-trichloro-2-methyl-2-propanol). Tissue samples from liver or femoral muscle tissue, respectively, of the adult voucher specimens were taken and stored in RNALater® (Ambion/Applied Biosystems). Subsequently, specimens were fixed and preserved in 4% neutrally buffered formalin and later transferred to 75% ethanol via 30% and 50% steps to avoid shrinkage. The froglets were removed from the egg capsules and the vitelline layer, and fixed and preserved directly in molecular grade absolute ethanol (Appendix).

Total genomic DNA was extracted from macerated muscle or liver tissue using peqGold Tissue DNA Mini Kits (PEQLAB Biotechnologie GmbH) or DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocols. Stretches of ~860 bp of 16S rDNA (foreward: 16SC 5'-GTRGGCCTAAAAGCAGCCAC-3', 16SA-L CGCCTGTTTATCAAAAACAT, 16SCH TCAAHTAAGGCACAGCTTA; reverse: 16SD 5'-CTCCGGTCTGAACTCAGATCACGTAG-3', 16SB-H CCGGTCTGAACTCAGATCACGT (Vences et al., 2005; Hertwig et al., 2012; Rafe Brown, pers. comm.) and of ~410 bp of 12S rDNA (foreward: 12SA-L AAACTGGGATTAGATACCCCACTAT; reverse: 12SB-H GAGGGTGACGGGCGGTGTGT) were amplified. The cycling conditions for amplification were: 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. PCR products were purified using a Quiagen gel extraction kit. We used 25 µl PCR reactions containing 1 µl DNA, 1 µl of each primer (20 pmol μl^{-1} (20 µM), 1.5 µl $MgCl_2$, 12.5 µl MasterMix Y, 8 µl ddH_2O (Peqlab) following manufacturer's protocol and a TC-512 thermo-cycler (Techne). PCR products were excised from agarose gels and cleaned using the Wizard® SV Gel and PCR Clean-UP System (Promega). To increase concentration of PCR product for sequencing, typically two 25 µl reactions were run for each sample and excised bands were put together for cleaning. Sequencing was done in both directions by Microsynth AG (Balgach, Switzerland), LGC Genomics (Berlin, Germany) and Macrogen Inc. (Seoul, South Korea) using the same primers as for amplification. Sequence editing and management was done with BioEdit 7.0.5.2 (Hall, 1999, www.mbio.ncsu.edu/BioEdit/), Chromas lite 2.01 (Technelysium Pty. Ltd., www.technelysium.com), and Geneious Pro 5.1.7 (Drummond et al., 2009) software.

We compared the obtained sequence data of one of the eggs with sequences of syntopic and/or closely related *Philautus* species (following phylogenetic hypothesis of Hertwig et al., 2012) from our sequence database of Bornean rhacophorids. Alignment of the concatenated sequence data was performed using MAFFT (Katoh et al., 2002) using the plugin of Geneious Pro 5.1.7 (Drummond et al., 2009) with the E-INS-i algorhithm and standard parameters. Genetic distances were obtained and visualised with the Geneious Pro tree builder with a neighbour joining algorithm and the Tamura-Nei model of sequence evolution. A neighbour joining bootstrapping analysis with 1000 pseudoreplicates was used with the same software to infer node support. Inkscape (www.inkscape.org) were used for preparing the final tree graphics.

RESULTS AND DISCUSSION

The habitat at the type locality of *Philautus acutus* was described by Dring (1987) in his original description of *P. ingeri*. We found the site unaltered since Dring's visit during the Gunung Mulu expedition of the Royal Geographic Society in 1978. The area is situated on a small plateau on the western ridge of Gunung Mulu, just below a permanent shelter, referred to as Camp Three. The dense vegetation corresponds to the tall facies of the mossy or upper montane forest (Dring, 1987; Hazebroek & Abang Morshidi, 2001). The stems of the trees are covered by mosses, vines and epiphytes (Fig. 1). The forest soil is moist and peaty or loamy and covered in a thick layer of leaf litter, logs, roots, mosses and other low vegetation. An ephemeral stream with small pools with loamy or pebbly bottom drains the forest.

P. acutus is the most common rhacophorid species in the dense montane forest around Camp Three. Over a total of three field trips to this locality, numerous adult males were observed or heard at night, calling from the vegetation. Males of *Philautus acutus* (Fig. 2) call in groups or widely dispersed from shrubs or small trees (0.5–4 m above ground). Calls are a series of shrill "gree" notes. For a detailed description and comparison of the calls of *P. acutus* and related species from Borneo see Dring (1987). Females of *P. acutus* have not been found by us and have not yet been described until now. We found the following frog species during several field trips from 2006–2009 sympatrically with *P. acutus*: *Meristogenys* cf. *kinabaluenesis* (Ranidae), *Limnonectes* cf. *kuhli, L. palavanensis* (Dicroglossidae), *Philautus macroscelis*, *Philautus* cf. *petersi*, *P. ingeri*, *P. mjobergi*, at least two further unidentified *Philautus* species (Rhacophoridae), *Kalophrynus nubicola* (Microhylidae) and *Ansonia longidigita* and *A. platysoma* (Bufonidae). *P. acutus* was the most common species of tree frogs during our survey at this locality. Despite the protected status of the distribution area of *P. acutus* within the Gunung Mulu National Park as an approved World Heritage Site, there is an urgent need for targeted ecological surveys. Determination

Table 1. Genetic distances among the *Philautus*-samples obtained with Geneious Pro and Tamura Nei model of sequence evolution.

	P. mj.	*P. pe.*	*P. in.*	*P. te.*	*P. bu.*	*P. ab.*	*P. au.*	*P. ac.*	*P. ac.*	*P. ac.*	*P. ac.*
P. mjobergi		0.130	0.158	0.133	0.159	0.160	0.160	0.158	0.158	0.158	0.159
P. petersi	0.130		0.129	0.103	0.129	0.130	0.130	0.128	0.128	0.128	0.129
P. ingeri	0.158	0.129		0.131	0.157	0.158	0.158	0.156	0.156	0.156	0.157
P. tectus	0.133	0.103	0.131		0.132	0.133	0.133	0.131	0.131	0.131	0.132
P. bunitus	0.159	0.129	0.157	0.132		0.071	0.071	0.069	0.069	0.069	0.070
P. abditus	0.160	0.130	0.158	0.133	0.071		0.072	0.070	0.070	0.070	0.070
P. aurantium	0.160	0.130	0.158	0.133	0.071	0.072		0.037	0.037	0.037	0.037
P. acutus NMBE 1056429	0.158	0.128	0.156	0.131	0.069	0.070	0.037		0.000	0.000	0.001
P. acutus NMBE 1056430	0.158	0.128	0.156	0.131	0.069	0.070	0.037	0.000		0.000	0.001
P. acutus NMBE 1056431	0.158	0.128	0.156	0.131	0.069	0.070	0.037	0.000	0.000		0.001
P. acutus ZMH A10836	0.159	0.129	0.157	0.132	0.070	0.070	0.037	0.001	0.001	0.001	

and monitoring of the status of the only known population of this species would contribute to ensuring its long-term survival in its restricted range.

The clutch of eggs was discovered on the forest floor at about 10 am, during cool and cloudy weather conditions, and were deposited between wet, rotting leaf litter, beneath a dead branch of a tree. The diameter of the egg capsules was 13 mm. The outer jelly capsule was compact, yellowish opaque, with some adherent soil particles (Fig. 3a, b). The vitelline membrane was tough. One froglet was measured in the lab at the headquarters of Gunung Mulu Nationalpark before preservation in ethanol and had a SVL of 8.4 mm (Fig. 3b). Both specimens were in advanced developmental stages. The tail was in the process of reduction (approx. 60% of snout-vent length) and the tail fin was almost fully reduced, leaving a thin muscular part of the tail, with visible blood vessels (Fig. 3c). The angle of the mouth had reached to the posterior margin of the eye. The state of tail and mouth corresponded to Gosner stages 44 or 45 (Fig. 3c; Gosner, 1960). The dorsal side of head and dorsum showed a dark brown colour with a broad bifurcating or X-shaped dark dorsal pattern (Fig. 3c), which is also present in adults (Fig. 2). This somewhat indistinct pattern was overlaid by scattered golden and bluish iridophores. It is silhouetted by fewer iridophores from the surrounding area showing a high number of golden, and particularly bluish, iridophores, creating the impression of a grey ground colour (Fig. 3c). The posterior part of the back has dark, fuzzy mottling and numerous gold and few bluish iridophores. The upper side of the head, legs and arms showed a similar pattern of broad dark bands. The underside at throat and breast was dark brown also with few bluish iridophores, on the abdomen and on flanks translucent, such that the gut filled with a large mass of yolk, was visible (Fig. 3d). The iris was golden brown (Fig. 3b).

The final concatenated alignment of the 16S and 12S rDNA sequences comprised 1196 bp. The resulting consensus dendrogram of the neighbour joining analysis illustrates the unambiguous matching of one of the froglets (ZMH A10836) with three samples of adult *P. acutus* from the same locality (NMBE 105643, NMBE 1056430, NMBE 1056429) in comparison to the most closely related or syntopic *Philautus* species (Fig. 4). The egg sample differed in only one A to G transition from the adult samples within the 1196 bp leading to an uncorrected genetic distance of <0.1% (Table 1). The presence of froglets at the end of metamorphosis in a terrestrial gelatinous egg and their genetic matching demonstrate direct development in *P. acutus* unequivocally.

The evidence of direct development on land in *P. acutus* contributes to our knowledge of the natural history of this rare species endemic to Gunung Mulu National Park, and beyond that, to our understanding of the evolution of reproductive strategies in the genus *Philautus*. In the remaining Bornean *Philautus* species reliable information about their breeding behaviour is scarce. Dring (1987) published data on clutch sizes based on dissected females that had been collected during his expedition to Gunung Mulu. Aerial direct development has been described plausibly only in *P. saueri* (Malkmus et al., 2002). The authors found clutches of 9 to 17 eggs with gelatinous capsules (diameter 11–15 mm) within dead and leaking pitchers of *Nepenthes villosa*, which served probably as shelter providing constant high humidity. A photo shows an early froglet with a broad, vascularised tail (Malkmus et al., 2002: 193 – Fig. 189). The thin muscular part of the tail with large blood vessels possibly indicate its function as breathing organ within eggs capsules, comparably to our *P. acutus* sample (see Fig. 3c, d).

The usage of living pitchers of pitcher plants (*Nepenthes* species) as breeding facilities has been supposed as a further reproductive strategy in *Philautus*. The eggs are laid in the liquid within the pitchers, the lecithotrophic tadpoles hatch from the eggs and finish their development in these phytothelmes. Dring (1987) even speculated that monticolous species such as *P. acutus* could be forced to lay their eggs in the epiphytic or ground moss layer, because *Nepenthes* are rare in their habitat and the small pitchers of those *Nepenthes* growing in the montane forests would not be appropriate as breeding facilities. In its original description of *Philautus kerangae*, Dring (1987) mentioned details of the breeding biology of this species. He observed clutches and calling males in older pitchers of *Nepenthes bicalcarata* and described the development of lecithotrophic tadpoles. At the type locality of *P. kerangae* we found also frog eggs and tadpoles in pitchers of *N. bicalcarata* and *N. ampullaria* during our field work within the Kerangas forest in the Gunung Mulu National Park at an elevation of about 200 m asl.. However, these eggs have been assigned genetically and unequivocally to the microhylid *Microhyla borneensis* sensu Matsui, 2011 (see also Das & Haas, 2010: [as *M. nepenthicola*]). In *P. mjobergi* Mjöberg (in Smith, 1925) stated deposition of eggs in pitchers of pitcher plants (*Nepenthes*) and suspected that development was carried out on land. It remains unclear, however, if Mjöberg found the clutches in dead, empty or in living, water-filled pitchers. Malkmus et al. (2002) reported a female *P. mjobergi* that deposited a clutch of seven eggs on a dead leaf in a terrarium. In summary, we conclude that true nepenthiphilious breeding behaviour has not been confirmed undoubtedly in a *Philautus* species from Borneo.

There have been additional anecdotal reports of clutches of eggs found in different stages of development, that have been assigned to different *Philautus* species (Hoffmann, 1998: *P. petersi*; Malkmus, 1994: *P.* cf. *mjobergi* and *P. amoenus*; Malkmus et al., 2002: *P. mjobergi*, *P. petersi*, *P. amoenus*; Dring, 1987: *P. mjobergi*, *P. tectus*) or left unallocated to species (Malkmus, 1994). However, these observations

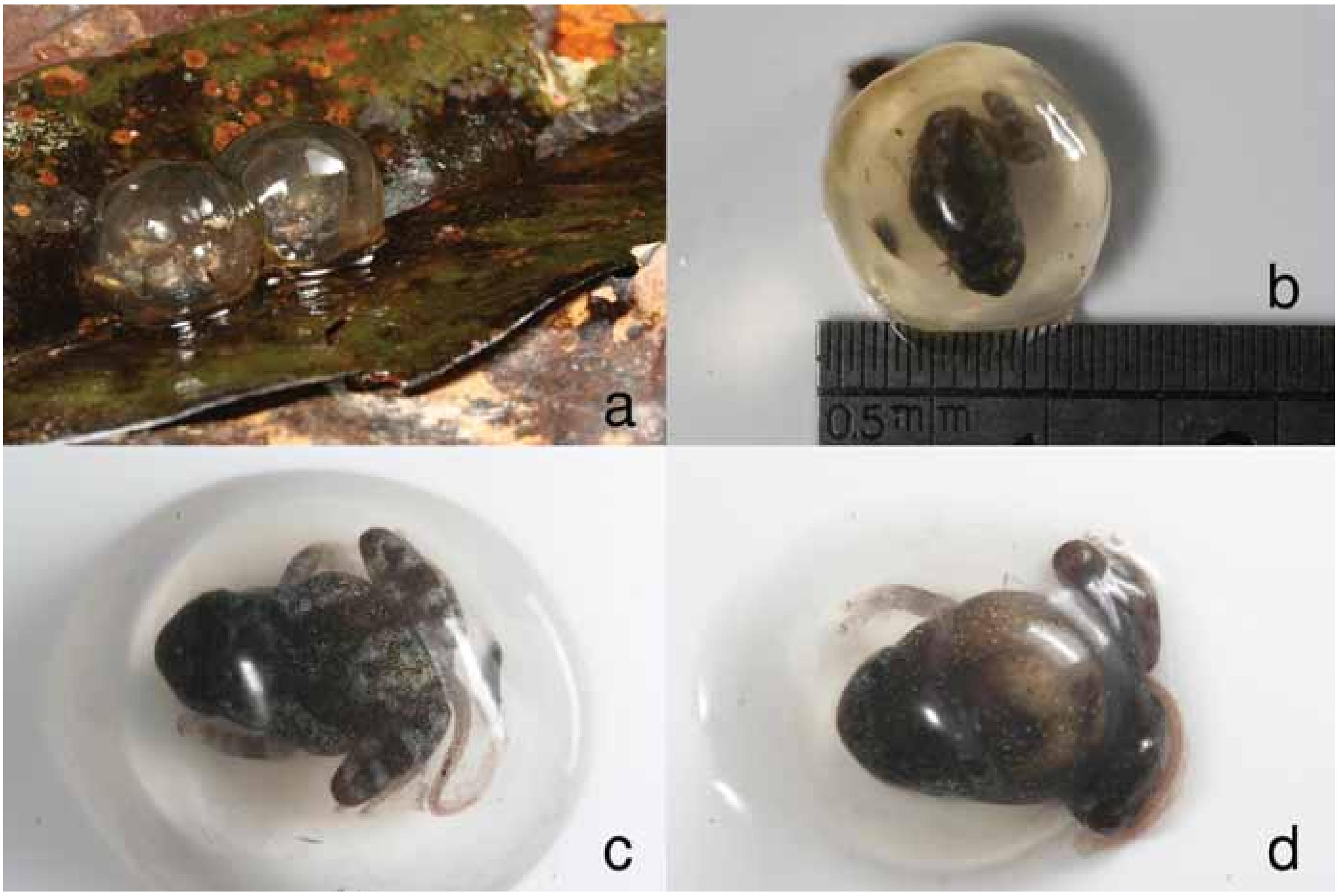

Fig. 3. Eggs with froglets assigned to *Philautus acutus*, from Gunung Mulu, Sarawak, Malaysia (Borneo): a, close-up of two eggs, containing froglets; b, close-up of a single egg. Note the bright iris colouration of the froglet and the opaque yellow outer jelly capsule of the eggs; c, dorsal view of froglet in the vitelline layer, after compact outer jelly capsule was removed. Note X-shaped pattern on dorsum and reduced tail with blood vessels. d, ventral view of froglet in vitelline layer, with compact outer jelly capsule removed. Note gut filled with yolk visible through skin and scattered iridophores.

remain unconfirmed, because the complete reproductive cycle of the frogs was not observed and a confirmed assignment of semaphoronts was not possible.

Furthermore, Inger (1966) described the morphology and development of lecithotrophic larvae within eggs in *Philautus hosii*, and assumed an abbreviated or even lacking free-swimming larval stage. This observation could be interpreted as indication for a nidicolous lecithotrophic tadpole in *P. hosii*. Hertwig et al. (2012) described a presumably nidicolous tadpole in *Philautus macroscelis*, based on genetic matching and transferred this species, from the genus *Rhacophorus* to *Philautus*, as a result of a phylogenetic analysis. *P. hosii* and *P. macroscelis* species represent probably basal lineages within *Philautus*, thus the presence of a tadpole could be the plesiomorphic state in the genus (Hertwig et al., 2012). However, the reconstruction of the complex evolutionary pattern of reproductive strategies in *Philautus*, and hence the understanding of the evolution of direct development within the Rhacophoridae, requires further studies combining field and laboratory work.

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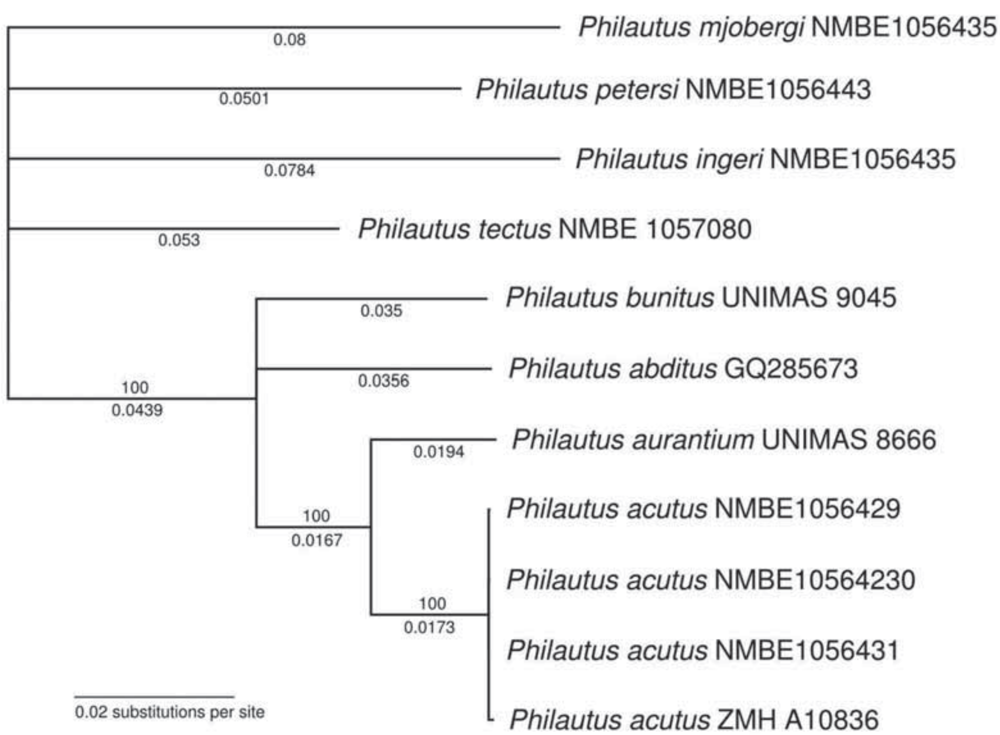

Fig. 4. Consensus dendrogram of neighbour joining bootstrapping analysis illustrating distances of 12S and 16S rDNA sequences. The four *Philautus acutus* specimens are conspecific.

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Appendix 1. Materials examined. NMBE, Naturhistorisches Museum Bern, Switzerland; UNIMAS, Museum of the Institute of Biodiversity and Environmental Conservation, Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia; ZMH, Zoological Museum of Hamburg, Germany.

Species	Collection Nr.	Locality	Genbank Accession	
			12S	16S
Philautus abditus			GQ285673	
Philautus acutus	NMBE 1056431	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JN705366	JN705337
Philautus acutus	NMBE 1056430	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JX091305	JX091302
Philautus acutus	NMBE 1056429	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JX091304	JX091301
Philautus acutus	ZMH A10836	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JX091306	JX091303
Philautus aurantium	UNIMAS 8666	Malaysia: Sabah: Crocker Range Park: 16th mile	JN705367	JN705338
Philautus bunitus	UNIMAS 9045	Malaysia: Sabah: Gunung Kinabalu Park: Sayap	JN705368	JN705339
Philautus ingeri	NMBE 1056435	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JN705385	JN705354
Philautus petersi	NMBE 1056443	Malaysia: Sarawak: Gunung Mulu Natl. Park: Camp 3	JN705381	JN705350
Philautus tectus	NMBE 1057080	Malaysia: Sarawak: Kubah Natl. Park: Summit Road	JN705369	JN705340