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Biological notes on an enigmatic microhylid, *Gastrophrynoides borneensis* (Anura, Microhylidae)

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Abstract. We report on biological notes of an enigmatic asterophryine *Gastrophrynoides borneensis* based on a male collected from western Sarawak. The male is immaculate brown in dorsal colour, without small white spots contrasting to previous descriptions. Whether this indicates intraspecific variation or specific differentiation requires further study. Mitochondrial DNA analysis revealed the species to be monophyletic with, but sufficiently diversified from, the continental *G. immaculatus*. The species emits two call types that are distinct from calls reported in Asterophryinae.

Key words. acoustics, Asterophryinae, body color, *Gastrophrynoides immaculatus*, mitochondrial phylogeny

INTRODUCTION

Narrow-mouthed frogs of the family Microhylidae Günther, 1858a (1843) are cosmopolitan and as many as ca. 600 species are split into 13 subfamilies (Frost, 2016), whose phylogenetic relationships are difficult to assess (Matsui et al., 2011; Kurabayashi et al., 2011; Peloso et al., 2016). Southeast Asia is one of the centres of microhylid diversity, and more than 100 species in four subfamilies have been recorded (Frost, 2016). Of these, the most enigmatic are two species in the genus *Gastrophrynoides* Noble, 1926 (Matsui et al., 2011; Kurabayashi et al., 2011). The genus was long known as monotypic, represented only by *G. borneensis* (Boulenger, 1897a) but the second species, *G. immaculatus* Chan, Grismer, Norhayati & Daicus, 2009, was added to the genus fairly recently (Chan et al., 2009). Using molecular phylogenetic techniques, Matsui et al. (2011) and Kurabayashi et al. (2011) clarified phylogenetic position of *G. immaculatus* among microhylids, in the subfamily Asterophryinae Günther, 1858, mainly occurring on Papua New Guinea and Australia. Kurabayashi et al. (2011) estimated divergence time of *G. immaculatus* from other two asterophryine species, and suspected the species to exhibit direct development.

In contrast, molecular information of the nominotypic species, *G. borneensis*, has been lacking. In order to ascertain its taxonomic relationship with *G. immaculatus*, and estimating the time of their divergence, molecular data of *G. borneensis* are indispensable. In addition, acoustic information of the genus has been totally lacking. All of these problems arise from the difficulty of encountering the species, whose record has been limited to at most five localities (Inger, 1966; Kueh & Sudin, 2008; Chan et al., 2009). Fortunately, we were able to record the voice and take a tissue sample of the one individual from Matang, suburbs of Kuching, Sarawak, western Borneo.

MATERIAL & METHODS

Fieldwork was conducted on the night of 18 July 2010 at Batu Kawa, Matang, Kuching District, western Sarawak (1°30′51″N, 110°18′53″E, < 50 m asl). We recorded calls of the frog in the field using a Canon Power shot camera (model: S3 IS) on video mode at 44.1 kHz/ 16 bit as uncompressed wave files and analysed them with Raven Lite 1.0 for Mac OS X (http://www.birds.cornell.edu/raven) on a Macintosh computer. Temporal data were obtained from the oscillogram and frequency information was obtained from the audiospectrograms using Fast Fourier transformation (1024 point Hanning window).

After recording calls, we collected the specimen, kept it in the laboratory for a week, then took tissues for subsequent molecular analysis, and fixed the specimen as a voucher. The specimen, fixed in 10% formalin and later preserved in 70% ethanol, is stored at the Molecular Ecology Laboratory, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS R21555).

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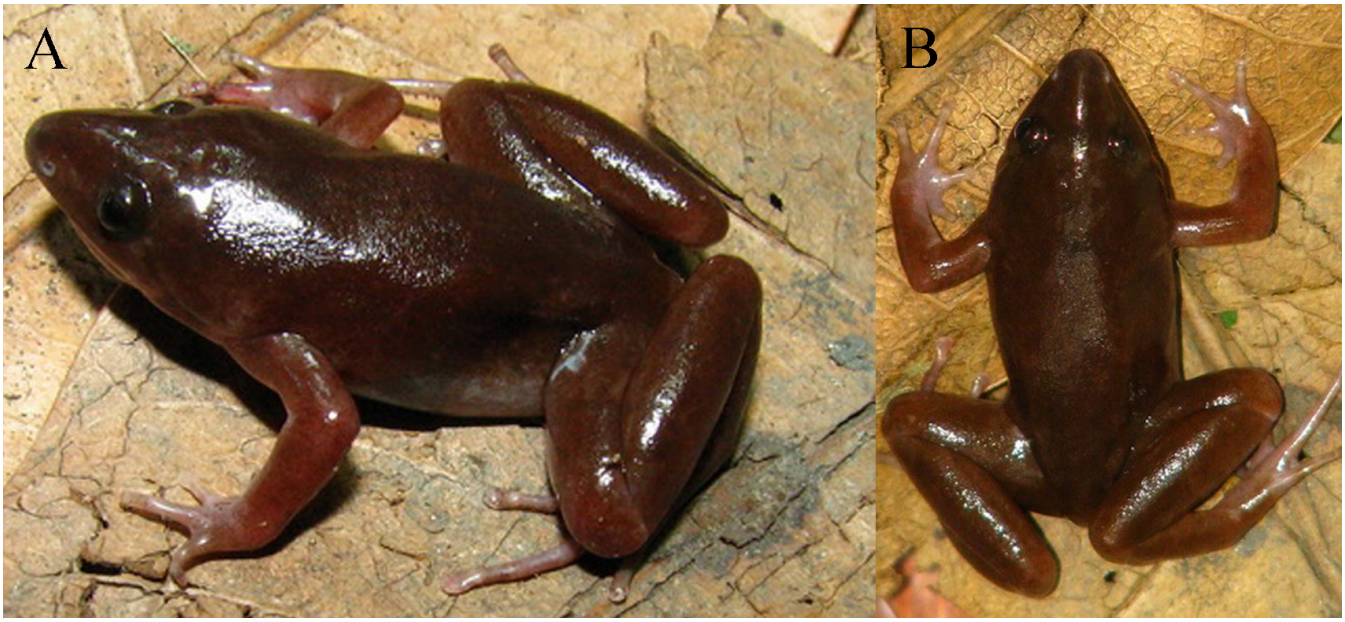

Fig. 1. Dorsolateral (A) and dorsal (B) views of *Gastrophrynoides borneensis* in life from Batu Kawa, western Sarawak (UNIMAS R21555).

The following 18 body measurements were taken off the specimen to the nearest 0.1 mm, following Matsui (1984): 1) snout-vent length (SVL); 2) head length (HL); 3) nostril-eyelid length (N-EL); 4) snout length (SL); 5) eye length (EL, including eyelid); 6) eye diameter (ED); 7) head width (HW); 8) internarial distance (IND); 9) interorbital distance (IOD); 10) upper eyelid width (UEW); 11) forelimb length (FLL); 12) lower arm and hand length (LAL); 13) outer palmar tubercle length (OPTL); 14) inner palmar tubercle length (IPTL); 15) hindlimb length (HLL); 16) tibia length (TL); 17) foot length (FL); and 18) inner metatarsal tubercle length (IMTL). Additionally, we recorded coloration and markings on the dorsum that are also useful in differentiating the two species of *Gastrophrynoides* (Chan et al., 2009). We compared the Batu Kawa sample with a specimen of *G. borneensis* collected in Tawau Hills, Sabah (Kueh & Sudin, 2008), stored at the Institute for Tropical Biology and Conservation, University Malaysia Sabah (TBT025, examined on 2 August 2007).

DNA sequence data were obtained from the muscle tissue preserved in 99% ethanol. We followed Matsui et al. (2011) for methods for DNA extraction, and amplification and sequencing of the mtDNA fragments. The resultant sequences (1540 base pairs of partial sequences of mitochondrial 12S rRNA and 16S rRNA genes) are deposited at GenBank (Accession number: LC208814).

For comparisons, DNA sequences (12S rRNA, 16S rRNA) already reported by Matsui et al. (2011) were obtained from GenBank for 13 microhylid taxa: *Gastrophrynoides immaculatus*; *Oreophryne monticola* (Boulenger, 1897b); *Scaphiophryne gottlebei* Busse & Böhme, 1992; *Gastrophryne olivacea* (Hallowell, 1856); *Phrynomantis bifasciatus* (Smith, 1847); *Micryletta steinegeri* (Boulenger, 1909); *Micryletta inornata* (Boulenger, 1890); *Metaphrynella pollicaris* (Boulenger, 1890); *Metaphrynella sundana* (Peters, 1867); *Microhyla malang* Matsui, 2011; *Microhyla borneensis* Parker, 1928; *Microhyla petrigena* Inger & Frogner, 1979; and *Microhyla perparva* Inger & Frogner, 1979. As an outgroup species, we used sequences of a rhacophorid, *Rhacophorus schlegelii* (Günther, 1858b), also from GenBank (Table 1). We followed Matsui et al. (2011) for tree construction and calculation of genetic distances (uncorrected p-distance).

RESULTS

Natural history. The habitat where we collected the male *G. borneensis* specimen was a fruit garden with a few durian and rambutan trees, and there were bamboos growing five metres away from the site. Previously, for several years, we had encountered this species there, but could not capture any individuals because they were highly cautious and quickly escaped into nearby burrows. On the night of 18 July 2010 around 1930 hours, there was heavy rain and a male was heard calling very weakly from a hidden site in curled leaves above the ground. We managed to locate that specimen and collected it. The air temperature after the rains was approximately 28−30°C. We could not find any other individuals. Other frog species observed immediately near the habitat were *Pulchrana baramica* (Boettger, 1900), *Fejervarya limnocharis* (Gravenhorst, 1829), and *Microhyla borneensis*. Males of *Microhyla borneensis* were calling and embryos of that species were found in the pitcher of *Nepenthes ampullaria* Jack (1835).

Morphology (measurements in mm). The specimen (Fig. 1A, B; Fig. 2A, C) showed the following features: SVL 26.8; habitus moderate; head triangular, narrower (HW 8.4) than long (HL 8.9); snout conical, strongly projecting beyond lower jaw, very long (SL 3.8), much longer than eye (EL 2.5, ED 1.5), rounded in profile; canthus rostralis rounded; lore sloping, very weakly concave; nostril lateral, below canthus rostralis, much closer to tip of snout than to eye (N-EL 2.1); interorbital distance (IOD 3.5) nearly three times width of upper eyelid (UEW 1.3), the latter about half

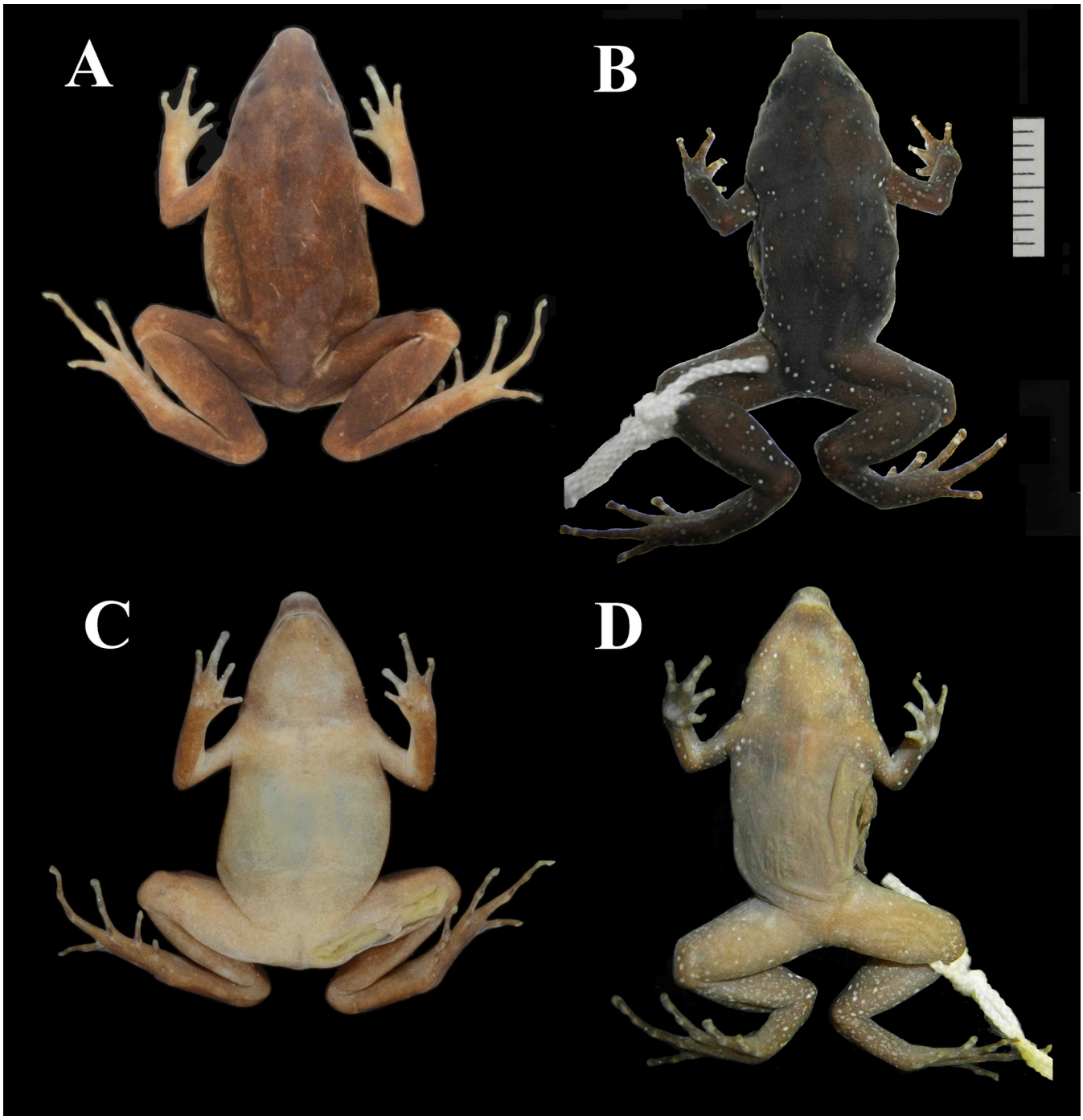

Fig. 2. Dorsal (A, B) and ventral (C, D) views of *Gastrophrynoides borneensis* from Batu Kawa, western Sarawak (UNIMAS R21555: A, C), and the same species from Tawau Hills, eastern Sabah (TBT 025: B, D) in preservative. Scale bar = 10 mm.

width of internarial distance (IND 2.5); tympanum hidden; upper jaw edentate; tongue oval, without papillae; slit-like openings to a median subgular vocal sac.

Forelimb moderately long (FLL 14.5, LAL 11.1); fingers thin, free of web; first finger much shorter than outer ones, finger formula I < II < IV < III; tips of outer fingers weakly dilated and forming weak round disks slightly wider than basal phalanges; outer palmar tubercle divided (OPTL 0.6) slightly larger than inner (IPTL 0.5); subarticular tubercles, indistinct, rounded; nuptial pad absent.

Hindlimb moderately long (HLL 42.1) about three times length of forelimb; tibia long (TL 12.7), heels overlapping when limbs are held at right angles to body; tibiotarsal articulation of adpressed limb reaching to centre of eye; foot (FL 13.8) longer than tibia; toe formula I < II < V < III <IV; tips of toes swollen, wider than those of fingers; webs between toes poorly developed on the right foot, not extending beyond basal subarticular tubercles of fourth toe, and absent on the left foot; subarticular tubercles oval, not prominent; inner metatarsal tubercle oval, much smaller (IMTL 0.5) than first toe; no outer metatarsal tubercle. Skin smooth with very weak fold from eye to axilla. Colour (in

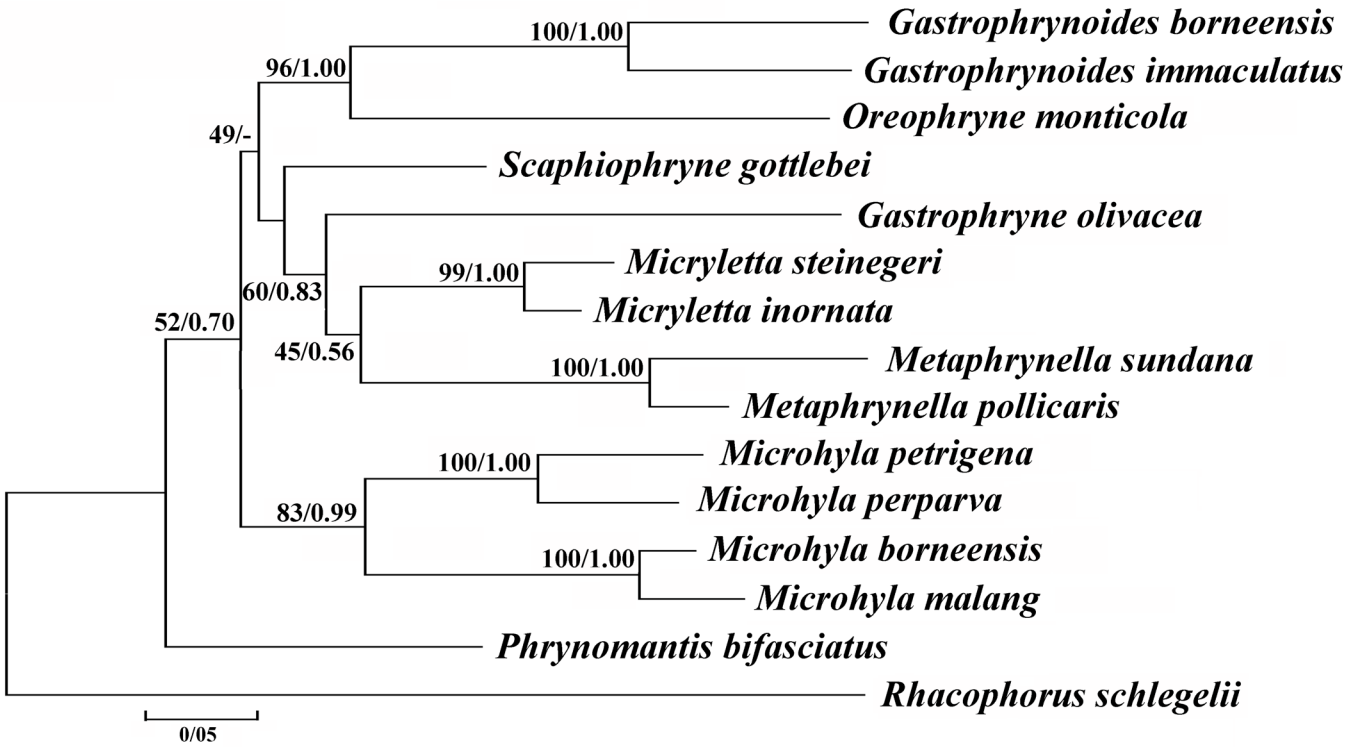

Fig. 3. ML tree from a ~2400 bp sequence of mitochondrial 12S rRNA and 16S rRNA genes for samples of *Gastrophrynoides borneensis* and representing microhylid frogs. Numbers above or below branches represent bootstrap supports for ML inference and Bayesian posterior probability (ML-BS/BPP).

life and in alcohol) of dorsum and venter brown without markings (Fig. 1, 2A, 2C).

In general, the specimen agreed well with the original description by Boulenger (1897a) and the description by Inger (1966), but slight differences were noted: lores weakly concave vs. not concave (Inger, 1966); tibiotarsal articulation reaching to centre of eye vs. articulation only reaching to the posterior border of eye (Boulenger, 1897a); fingers without notable ridges vs. second and third fingers with a ridge of skin on inner edges (Inger, 1966). Even more striking, is the uniformly brown dorsum that lacks scattered, small, light spots in the specimen from Batu Kawa in contrast to the reported descriptions of this species by Boulenger (1897a) and Inger (1966) that possess a speckled dorsum. The referred specimen from Tawau Hills, Sabah clearly shows such markings (Fig. 2B, D).

In describing *G. immaculatus*, Chan et al. (2009) noted its immaculate, greyish brown dorsum to differentiate this species from *G. borneensis*, but this does not hold for our specimen, although the ground colour observed is darker than in *G. immaculatus*. Other diagnostic characteristics shown by Chan et al. (2009) (longer snout and possession of a single, large, oval, outer metacarpal tubercle in *G. immaculatus*) were ascertained to be applicable to differentiate that species from our specimen.

Phylogeny. The best substitution models chosen for ML tree were J2 (Jobb, 2011) + G (0.246) for 12S rRNA and J2 + G (0.288) for 16S rRNA, and for Bayesian tree were General-Time-Reversible (GTR) + G (0.264) for 12S rRNA and GTR + G (0.306) for 16S rRNA. The likelihood values of the ML and Bayesian trees were lnL -13917.1008 and -13938.695, respectively.

Phylogenetic analyses employing two different optimality criteria yielded slightly different topologies, but nearly identical in terminal branches. As shown in the ML tree (Fig. 3), the monophyly of microhylid taxa (*Gastrophrynoides*, *Oreophryne*, *Scaphiophryne*, *Gastrophryne*, *Phrynomantis*, *Micryletta*, *Metaphrynella*, and *Microhyla*) with respect to *Rhacophorus* was not supported (ML BS <70%, BPP < 0.95). Although relationships among microhylid genera employed were unresolved, each congeneric species formed a clade (ML BS ≥ 83%, BPP ≥ 0.99), and the monophyly of *Gastrophrynoides* was fully supported (ML BS=100%, BPP=1.00). *Gastrophrynoides* also formed a clade with *Oreophryne monticola* (ML BS=96%, BPP=1.00).

Call characteristics. Calls of the male consisted of two different types, one composed of multi-pulsed notes (Type 1 call: Fig. 4A–C), and another of whistle like vaguely defined two-pulsed notes (Type 2 call: Fig. 4 D–F). However, we could not ascertain the sequence directly because the male called secretively. We observed calling starting with the Type 1 calls that lasted ca. 5.9 s, followed by a long pause of ca. 10.6 s, before it continued with a long series of Type 2 calls for ca. 58.6 s. After a short pause of ca.1.4 s, a series of Type 1 calls resumed for 11.0 s.

The Type 1 call consisted of a series of notes each emitted at a long interval (between the beginnings of two successive notes) of 1.03−2.34 (mean ± SD = 1.27 ± 0.38, n=11) s (Fig. 4A). The note repetition rate was 0.70–0.87 (mean ± SD = 0.80 ± 0.07, n=4) notes per s. Each note was composed

Table 1. Sample of Microhylidae and outgroup species used for mtDNA analysis in this study together with the information on voucher, collection locality and GenBank accession numbers. Voucher abbreviations: BORN = BORNEENSIS Collection, University Malaysia Sabah, KUHE = Graduate School of Human and Environmental Studies, Kyoto University; KUZ = Department of Zoology, Graduate School of Science, Kyoto University; MZB = Museum Zoologicum Bogoriense; UKMHC = Herpetological Collection, University Kebangsaan Malaysia; UNIMAS = Universiti Malaysia Sarawak.

S/No.	Species	Voucher	Locality	GenBank 12S, 16S
1	*Gastrophrynoides borneensis*	UNIMAS R21555	Malaysia, Sarawak	LC208814
2	*Gastrophrynoides immaculatus*	UKM HC 279	Malaysia, Negeri Sembilan	AB634647, AB634705
3	*Oreophryne monticola*	MZB Amp 16265	Indonesia, Bali	AB634651, AB634709
4	*Micryletta steinegeri*	KUHE 35937	Taiwan, Yunlin	AB634638, AB634696
5	*Micryletta inornata*	KUHE 23858	Thailand, Ranong	AB634637, AB634695
6	*Metaphrynella sundana*	BORN 8191	Malaysia, Sabah	AB634635, AB634693
7	*Metaphrynella pollicaris*	KUZ 21655	Malaysia, Pahang	AB634634, AB634692
8	*Microhyla borneensis*	KUHE 53165	Malaysia, Sarawak	AB59830,5 AB598329
9	*Microhyla malang*	KUHE 53018	Malaysia, Sarawak	AB598295, AB598319
10	*Microhyla petrigena*	KUHE 53743	Malaysia, Sarawak	AB634617, AB634675
11	*Microhyla perparva*	KUHE 53675	Malaysia, Sarawak	AB634615, AB634673
12	*Scaphiophryne gottlebei*	KUHE 34977	Pet trade	AB634653, AB634711
13	*Phrynomantis bifasciatus*	KUHE 33277	Pet trade	AB634652, AB634710
14	*Gastrophryne olivacea*	KUHE 33224	USA, Texas	AB634650, AB634708
15	*Rhacophorus schlegelii*	–	Japan, Hiroshima	AB202078, AB202078

of 6.21 ± 0.58 (5−7, n=14) short pulses and lasted for 0.11 ± 0.01 (0.11−0.13, n=14) s (Fig. 4B, C). Within a note, frequencies did not differ between the pulses, and the dominant frequency was 1.89 ± 0.05 (1.79−2.03, n=87) kHz. The note began at 2.23 ± 0.11 (1.93−2.45, n=37) kHz and there was a sight frequency modulation (Fig. 4C). No harmonic bands were detected.

In the whistle-like Type 2 call, the note repetition rate was 0.54–1.12 (mean ± SD = 0.74 ± 0.16, n=13) notes per s. Each note lasted 0.06–0.12 (mean ± SD = 0.09 ± 0.02, n=12) s, and time interval between two notes varies from 0.56–1.50 (mean ± SD = 1.49 ± 0.53, n=40) s (Fig. 4D). The dominant frequency lies at 1.89–2.07 (mean ± SD = 1.99 ± 0.04, n=27) kHz, and no harmonics were recognised. The call has marked frequency modulation, and the frequency decreased towards the end of a note to 1.77–1.97 (mean ± SD = 1.84 ± 0.06, n=17) kHz (Fig. 3E, F).

DISCUSSION

The type locality of *Engystoma borneense* Boulenger, 1897a (now *Gastrophrynoides*), is Baram district, Sarawak (Inger, 1966), but probably due to its fossorial habits, records of the species are still limited. Apart from Baram district, (Miri Division), other reported localities for the species listed by Inger (1966) were Kuching, (Kuching Division) and Mengiong river, (Kapit Division). All these localities are in Sarawak, Malaysian Borneo, and Inger (1966) doubted the record from Sumatra, Indonesia. Recently, the range of this species has been expanded to include Tawau Hills, Sabah (Kueh & Sudin, 2008), and Pesu river, Bintulu, Sarawak (Chan et al., 2009). We believe the report from Tekalit river in Chan et al. (2009) should be identical with Mengiong river in Inger (1966).

In addition to these five localities in Malaysian Borneo (Sabah and Sarawak), a record from Kalimantan, Indonesian Borneo is reported on the Frogs of Borneo website (Haas et al., 2016). Furthermore, the species has also been photographed at two localities in Sumatra (Fig. 5: Amir Hamidy, unpublished data). Specimens from Sarawak have been found below 500 m in elevation (Inger & Stuebing, 2005), but Kueh & Sudin (2008) recorded their specimen at 878 m asl in Sabah. The species is not restricted to undisturbed, pristine habitats, but can inhabit disturbed areas as shown in this report. Thus, the species might actually be distributed widely from lowland to montane regions, but is difficult to detect due to its highly secretive habits.

Chan et al. (2009) noted that the peninsular species, *G. immaculatus*, is distinguished from *G. borneensis* in having an immaculate, greyish brown dorsum as opposed to a spotted dorsum. Such spotted markings could not be observed in our specimen from Batu Kawa, although they are present in the Tawau specimen. Unfortunately, we could not examine museum specimens except for a very old one stored in Sarawak Museum, probably examined by Inger (1966), but the body colour in that specimen has now totally faded. Because all the previous authors (Boulenger, 1897; Inger, 1966; Chan et al., 2009) noted the presence of scattered, small, light spots on brown dorsum in *G. borneensis*, the specimen from Batu Kawa is unique. There may be either geographic variation in body colour in *G. borneensis* or the forms with and without dorsal marking may represent different taxa. Through unpublished evidence of the two specimens photographed in Sumatra (see above), one was immaculate and very similar to the Batu Kawa specimen, while another had light spots, as in *G. borneensis* (Fig. 5).

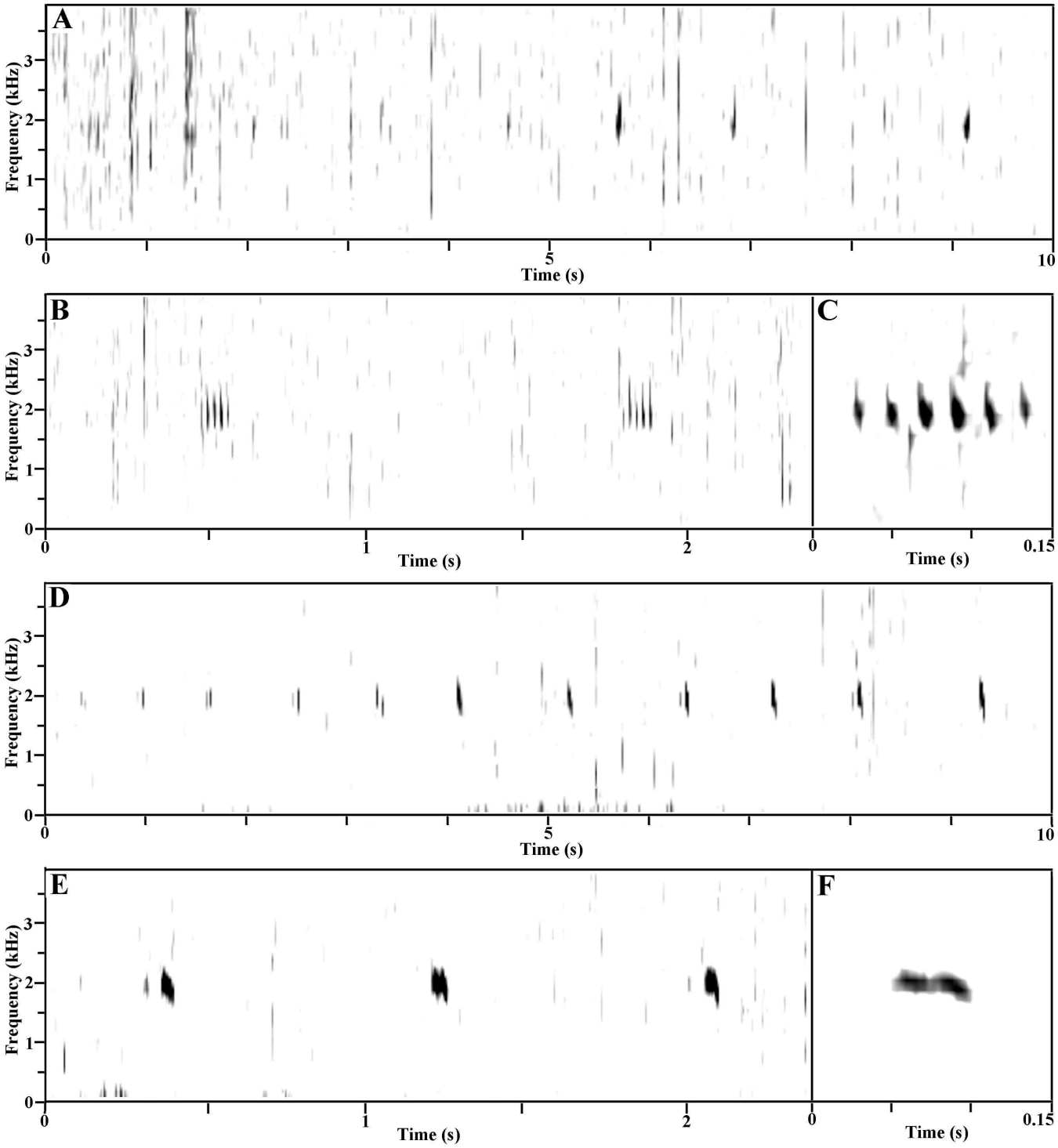

Fig. 4. Sonogram showing Type 1 (A–C) and Type 2 (D–F) calls of *Gastrophrynoides borneensis* (UNIMAS R21555) from Batu Kawa, western Sarawak, recorded at an air temperature around 28–30C.

Gastrophrynoides borneensis is monophyletic with *G. immaculatus* and together formed a clade with an asterophryine species *Oreophryne monticola*. This result concurs with Matsui et al. (2011) and Kurabayashi et al. (2011). As shown in Table 2, genetic distances (short and long fragments of *16S* rRNA) between each pair of sister species, *Micryletta inornata* and *Micryletta steinegeri* (4.9% and 5.2%), *Metaphrynella pollicaris* and *Metaphrynella sundana* (6.7% and 7.6%), *Microhyla malang* and *Microhyla borneensis* (1.9% and 5.0%), and *Microhyla petrigena* and *Microhyla perparva* (5.1% and 7.1%), were all smaller than the distance between the two species of *Gastrophrynoides* (9.5% and 10.7%). Thus the two species are genetically confirmed to be heterospecific.

In two species of *Metaphrynella,* the peninsular species *M. pollicaris* has also been recorded from Sumatra, while *M. sundana* is endemic to Borneo; both have less genetic divergence to each other than the two species of *Gastrophrynoides*. Also, *Micryletta inornata* occurs on Sumatra but not on Borneo. These patterns of geographic distribution in other microhylids suggest the possibility

Table 2. Uncorrected p-distances (%) among samples of *Gastrophrynoides* and other species for long fragments (794 bp: above diagonal) and short fragments (430 bp: below diagonal) of 16S rRNA.

S/No.	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*Gastrophrynoides borneensis*	–	10.7	15.9	16.5	15.7	19.8	18.0	18.9	19.6	17.4	17.5	16.5	17.1	19.9	24.4
2	*Gastrophrynoides immaculatus*	9.5	–	17.6	16.0	16.8	20.3	17.8	19.1	19.8	18.8	18.1	16.0	18.5	20.9	24.8
3	*Oreophryne monticola*	13.0	14.0	–	16.1	17.0	18.9	18.8	19.3	19.1	17.6	17.6	16.1	16.8	20.2	24.7
4	*Micryletta steinegeri*	14.7	14.9	13.3	–	5.2	14.6	13.9	15.7	16.2	14.6	13.4	11.3	13.1	14.5	21.2
5	*Micryletta inornata*	13.7	14.9	14.7	4.9	–	15.1	13.9	16.6	16.8	13.4	12.2	11.3	13.1	15.4	20.5
6	*Metaphrynella sundana*	18.1	18.1	14.7	11.4	13.3	–	7.6	17.9	18.8	16.8	16.2	16.2	18.1	18.6	21.9
7	*Metaphrynella pollicaris*	15.6	16.3	14.9	11.4	12.1	6.7	–	17.0	17.4	15.5	15.5	14.5	17.1	16.9	21.3
8	*Microhyla borneensis*	17.7	15.8	15.6	12.1	13.0	14.2	14.0	–	5.0	14.6	14.6	16.0	17.1	19.8	21.8
9	*Microhyla malang*	17.2	15.4	15.3	11.4	12.6	14.9	13.7	1.9	–	16.0	15.5	16.2	17.8	20.2	21.4
10	*Microhyla petrigena*	15.4	15.8	13.7	10.2	10.0	13.3	11.9	12.1	12.1	–	7.1	12.7	15.5	18.8	20.3
11	*Microhyla perparva*	14.2	14.9	13.5	9.8	9.1	13.5	11.9	11.6	11.9	5.1	–	13.0	14.5	18.5	20.8
12	*Scaphiophryne gottlebei*	14.7	14.9	12.6	10.7	11.4	14.0	11.9	13.5	12.6	10.2	11.6	–	13.2	15.7	22.0
13	*Phrynomantis bifasciatus*	13.5	14.9	13.3	10.5	10.2	15.4	13.5	12.8	12.6	11.2	11.2	10.5	–	15.9	22.2
14	*Gastrophryne olivacea*	16.7	17.4	15.6	11.4	12.6	15.6	14.2	17.2	16.5	14.7	15.4	13.0	12.3	–	23.4
15	*Rhacophorus schlegelii*	23.7	22.8	23.3	18.8	18.8	21.2	20.5	19.8	19.3	18.1	18.8	22.3	20.7	23.0	–

Fig. 5. Sumatran *Gastrophrynoides* without (A) and with (B) light spots. Photographs courtesy of Muhammad Arifin (A) and Ade Prasetyo Agung and Hon Tjong Djong (B).

that the unpublished record of *G. borneensis* from Sumatra (see above) may be conspecific with the peninsular species, *G. immaculatus* and not with Bornean *G. borneensis*. However, as noted above, dorsal ground colour of the immaculate specimen found in Sumatra is deep brown (Fig. 5A) and not identical with *G. immaculatus* (dorsal ground colour greyish brown). Thus, it would be plausible to postulate that the continental and Sumatran populations are heterospecific. If this is the case, the pattern of differentiation in *Gastrophrynoides* would be different from *Metaphrynella* or *Micryletta inornata*.

The call of *G. borneensis* was found to consist of two distinct types. Calls of other asterophryine species have been extensively studied (e.g., Zweifel & Allison, 1982; Zweifel, 1985; Richards et al., 2007; Kraus, 2013), but none are similar to *G. borneensis* in call characteristics. Furthermore, in studying New Guinean species of *Cophixalus* Boettger, 1892, Zweifel (1985) found call parameters to show essentially no correlation with relationships as deduced from morphology, and doubted the validity of using call structure as a character when inferring relationships.

Finally, because of its secretive habits with most of its time probably spent underground, the reproductive mode of *Gastrophrynoides* is totally unknown and requires future study. Direct development is suggested by the possession of large, non-pigmented ova (Inger, 1966).

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