Zootaxa 3755 (5): 401–418
www.mapress.com/zootaxa/
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Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

http://dx.doi.org/10.11646/zootaxa.3755.5.1
http://zoobank.org/urn:lsid:zoobank.org:pub:9CC48F24-418F-4E23-9E92-5347A973DEE7

Description of two new species of *Microhyla* (Anura: Microhylidae) from Bangladesh

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Abstract

Two new frog species belonging to genus *Microhyla* from the southeast, central and northeast regions of Bangladesh are described. Based on a molecular phylogeny derived from mitochondrial DNA sequences, one of the new species forms a clade with *M. fissipes,* while the second new species is sister to this clade. The DNA sequences of the mitochondrial cytochrome *b* gene from these new species are substantially diverged from *M. fissipes* (8.9 and 10.2% [3.6 and 4.2% for 16S ribosomal RNA gene] uncorrected pairwise divergence, respectively), and the estimated phylogenetic splits from their closest relative is in the Pliocene (3.4 Mya) and middle Miocene (10.5 Mya). The first new species (*Microhyla mukhlesuri* sp. nov.) can be diagnosed from its nearest congener (*M. fissipes*) by the following characteristics: SVL: 16.5–21.0 mm, finger length 1 < 4 < 2 < 3, tips of finger and toes not swollen, subarticular tubercles distinct, an inverse U-shaped mark on the anus, and a distinct X-shaped marking on the dorsum. Although the second new species (*M. mymensinghensis* sp. nov.) shares some morphological characteristics with the first new species, it can be readily diagnosed from its close congeners by its longer hindlimbs (HLL/SVL), tibia (TIL/SVL) and forearm width (FAW/SVL), in addition to a combination of the following characteristics: SVL: 14.2–21.3 mm, snout truncate, a crescent-shaped marking on the anus, and an X-shaped marking on the dorsum. The tibio-tarsal articulation extends to the eye in *M. fissipes* but ranges from the eye to the tip of the snout in the two new species.

Key words: *Microhyla mukhlesuri* sp. nov., *Microhyla mymensinghensis* sp. nov., Microhylidae, Mitochondrial DNA, Divergence time, Morphology, Bangladesh

Introduction

Microhylidae is a large anuran family comprising 8% of all frogs (519 species), with *Microhyla* being its type genus. This genus is characterized by various morphological characteristics (e.g., smooth or warty skin, absence of vomerine teeth, a narrow and elliptical tongue, hidden tympanum, free fingers, free or webbed toes, united outer metatarsals, dilated finger and toe tips, absent omosternum, and T-shaped terminal phalanges (e.g., Chanda 2002). Members of this genus show a wide distribution across Asia from the Ryukyu Archipelago in Japan and China to the north, through India to Sri Lanka to the southwest, and through Southeast Asia to Sumatra, Borneo, Java, and Bali to the southeast (Frost 2013). Despite such a large distribution, only 31 *Microhyla* species have been documented (Frost 2013). Especially in Bangladesh, only three nominal *Microhyla* species (*Microhyla ornata*, *M. berdmorei* and *M. rubra*) are known (Kabir *et al*. 2009). Recently, we found three haplotype groups (referred to as the Chittagong, Mymensingh-Sylhet, and Dinajpur haplogroups of *M.* cf. *ornata* by Hasan *et al*. 2012) from Bangladesh genetically distinct from these three species based on mitochondrial 16S ribosomal RNA gene (*16S-rrn*) data. The *16S-rrn* divergence was >5.0% between the Chittagong and Mymensingh-Sylhet haplogroups, and ~14% between the Dinajpur and former groups. When compared with the *Microhyla* taxa from neighboring countries, it became clear that the Dinajpur haplogroup belongs to the Indian *Microhyla* group (including *M. ornata* and *M. rubra*) with the Chittagong and Mymensingh-Sylhet groups included in the Southeast Asian group (e.g., *M. fissipes, M. heymonsi*, and *M. okinavensis*). The Chittagong haplogroup therefore becomes a sister taxon to *M.*

Accepted by M. Vences: 18 Nov. 2013; published: 24 Jan. 2014

fissipes (*16S-rrn* divergence = 3.6% [compared with Chinese specimen, accession no. AB201185, Matsui *et al*. 2005]). The Mymensingh-Sylhet haplogroup also has close affinity with the *M. fissipes* + Chittagong haplogroup, but its *16S-rrn* sequence is even more strongly divergent (>5%). Yet, in our preliminary survey, the morphological characteristics of the Chittagong and Mymensingh-Sylhet haplogroups were not consistent with the description of *M. fissipes* and other nominal microhylid species from Southeast Asian countries.

In the present study, we analyze DNA sequences of the mitochondrial cytochrome *b* gene (*cytb*) and perform phylogenetic and dating analyses. This protein-encoding gene, known to exhibit rapider nucleotide substitutions than ribosomal RNA genes, is considered more phylogenetically informative when surveying relationships between conspecific populations, particularly in East and Southeast Asian microhylid frogs (Koike & Matsui 2003; Matsui *et al*. 2005). Furthermore, we compare the morphological characteristics of the Chittagong and Mymensingh-Sylhet haplogroups with their closest congener, *M. fissipes*. Molecular analysis shows clear genetic divergence of these haplogroups from the other *Microhyla* taxa, and the morphological characteristics of these groups are inconsistent with the descriptions of *M. fissipes* and other known microhylid species. Thus, here we describe two new *Microhyla* species from Bangladesh.

Materials and methods

Specimens of genus *Microhyla* were collected from five localities of Bangladesh (Fig. 1) from 2003 to 2012. Vouchers were deposited at the Institute for Amphibian Biology, Hiroshima University (IABHU), Japan.

Total DNA samples for *cytb* analysis of the *Microhyla* specimens were extracted from the clipped toe of each individual as follows: in 5 specimens of *M*. sp. from Chittagong, Bangladesh (IABHU 3879, 3956, 3958, 3959 and 3960), 19 specimens from Mymensingh (IABHU 4004–4006, IABHU 4116–4117, IABHU 4119–4120, IABHU 4129–4132, IABHU 4134, IABHU F5012 [BdMsp71, BdMsp 73–76, BdMsp 80], DFBGBAU BdMsp 306), 4 specimens from Netrokona (IABHU 22142–22145), 2 specimens from Sunamganj (IABHU 22146–22147), and 9 specimens from Sylhet (IABHU 3898– 3899, IABHU 3944–3948, IABHU 3950, IABHU 3954). The type locality of *M. fissipes* is Taiwan foo, Taiwan (Boulenger 1884) and the sequences of this species which were used to prepare phylogenetic tree in this study were retrieved from DDBJ (DNA Data Bank of Japan), including sequences obtained from Taiwan specimens (hence, topotypical sequences). The identification of that species using mtDNA data is clear and convincing as has been assessed previously (Matsui *et al*. 2005; Hasan *et al.* 2012). In addition, we incorporated 1 specimen of *M*. cf. *ornata* from Dinajpur, Bangladesh (IABHU 22136), 1 specimen of *M. berdmorei* from Gombak FSC (Field Studies Center of University of Malaya), Malaysia (IABHU 21019), 1 specimen of *M*. cf. *berdmorei* from Bandarban, Bangladesh (IABHU 3864), and 1 specimen of *Kaloula pulchra* from Sylhet, Bangladesh (IABHU 3782). Specimens and/or tissue samples were stored in the IABHU or Department of Fisheries Biology & Genetics, Bangladesh Agricultural University (BAU). Total DNA samples were used to amplify a partial portion of *cytb* corresponding to the 15,788–16,348 (ca. 560 bp) nucleotide sequence within the *Microhyla okinavensis* complete mtDNA sequence (AB303950; Igawa *et al*. 2008). PCR amplification and sequencing were performed using a primer set of Fow-1-1 (5 – ACM GGH YTM TTY YTR GCH ATR CAY TA – 3) and Rev-1 (5– TAD GCR AAW AGR AAR TAY CAY TCN GG – 3) for *cytb*. The obtained *cytb* sequences were deposited in the DDBJ/EMBL/GenBank database (accession numbers: AB819011–AB819033).

The resulting *cytb* sequences were aligned using the ClustalW program (Thompson *et al*. 1994). From this alignment, the indel and ambiguous alignment sites were removed using Gblocks Ver. 0.91b (Castresana 2000) with default parameters. The final alignment dataset was 560 bp in length and contained 170 parsimony-informative sites. The sequence divergence of *cytb* (uncorrected *p* values) was calculated using MEGA Ver. 4.0 (Tamura *et al*. 2007). Phylogenetic analysis was performed by the maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) methods. Nucleotide substitution models for ML and BI analyses were selected under the Akaike and Bayesian information criteria (AIC and BIC) respectively, by using Kakusan 3.0 (Tanabe 2007). ML analysis was performed using Treefinder (Jobb 2008) and the resulting tree was evaluated using bootstrap analysis with 1,000 replicates. BI analysis was performed using MrBayes5D (Tanabe 2011, a modified version of MrBayes Ver. 3.1.2 [Ronquist & Huelsenbeck 2003]) with the following settings: number of Markov chain Monte Carlo (MCMC) generations = 20 million, sampling frequency = 100, and the first 2 million generations discarded as burn-in. These settings were determined by checking the convergence of –log likelihood

(–ln*L*) values using Tracer ver. 1.5 (Rambaut & Drummond 2007). Statistical support of the BI tree was evaluated by Bayesian posterior probability (BPP). MP analysis was performed using PAUP 4.0b10* (Swofford 2003) with the resultant tree evaluated by BP analysis with 1,000 pseudo-replications.

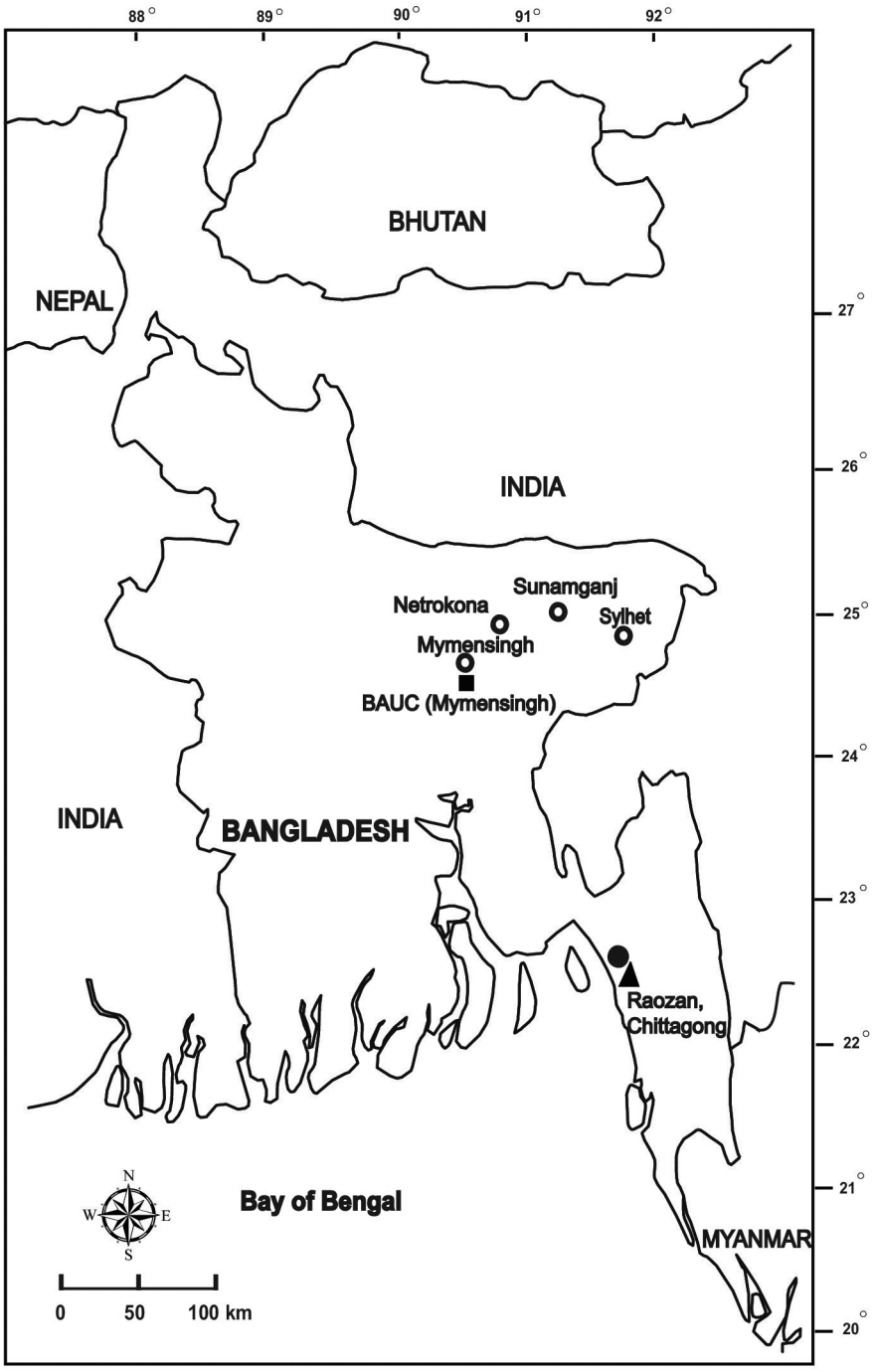

FIGURE 1. Map showing the collection sites and known occurrences of *M.* sp. C and *M.* sp. M in Bangladesh, indicated by closed and open circles, respectively. The type locality of holotype IABHU 3956 for *M.* sp. C and holotype IABHU 4116 for *M.* sp. M is indicated by closed triangles and squares, respectively.

Based on the ML and Bayesian tree topology, the divergence times of the *Microhyla* taxa, focusing especially on the new *Microhyla* taxa of interest (Chittagong and Mymensingh-Sylhet haplogroups) from Bangladesh and *M. fissipes*, were estimated by the Bayesian relaxed-clock method. Divergence time was estimated using MCMCTREE implemented in PAML software package version 4.6 (Yang 2007). General time reversal (GTR) was applied as the substitution model, and independent rate (clock = 2 in MCMCTREE) was implemented for the time

estimation model. The MCMCTREE program allows for minimum (lower) and maximum (upper) time constraints and multiple calibration points for realistic divergence time estimation. In this analysis, we applied the following two calibration constraints according to the resultant divergence ages from a previous study: (1) minimum 60.30 and maximum 85.60 Mya for the divergence of genera *Kaloula* and *Microhyla,* and (2) minimum 10.86 and maximum 22.90 Mya for the divergence of *M. heymonsi* and *M. okinavensis* (Kurabayashi *et al*. 2011; min and max 95% CI ages of these microhylid divergences are used as min and max constraints). A loose maximum value for the root was also set at 100 Mya.

For morphological comparison, the following 28 measurements were taken using digital calipers to the nearest 0.1 mm. SVL: snout-vent length; HL: head length; HW: head width; S-N: snout to nostril distance; N-N: inter-nostril distance; N-E: nostril to eye distance; ED: longitudinal eye diameter; E-E: inter-orbital distance between inner borders of upper eyelids; ELW: eyelid width; FLL: forelimb length; FHL: forearm and hand length; FAW: forearm width; HAL: hand length; F1-F4: lengths of 1st to 4th finger; HLL: hindlimb length; FEL: femur length; TIL: tibia length; TFL: tarsus and foot length; FOL: foot length; T1-T5: lengths of 1st to 5th toe; and IMT: inner metatarsal tubercle length. The examined specimens list including the samples of morphological measurement is given in Appendix 1. Statistical analysis was performed using SPSS (15.0J) software (SPSS Japan Inc., Tokyo, Japan).

Two sexually mature individuals from the Mymensingh-Sylhet haplogroup (from Mymensingh; IABHU 4005–4006) were used for karyological studies. Chromosomes were observed in the metaphase plates of femoral bone marrow cells, following the methodology of Schmid *et al*. (1979) with slight modifications.

Results and discussion

Haplogroup distribution: In our previous study (Hasan *et al*. 2012; and Fig. 1), the Chittagong haplogroup (≈*M.* sp. C) was found only in the Chittagong district in the southeastern corner of Bangladesh. Accordingly, this haplogroup was not found in our new sampling localities. Conversely, the Mymensingh-Sylhet haplogroup (≈*M.* sp. M) has been found not only in the Mymensingh and Sylhet districts (Hasan *et al*. 2012), but it is also widely distributed throughout the Netrokona and Sunamganj districts in the central and northeast regions of Bangladesh according to data herein.

Phylogenetic relationships and *cytb* gene divergence. Figure 2 shows the ML tree based on the *cytb* data. MP and BI trees showed similar topologies with only minor exceptions. For example, "*M. fissipes*" from Thailand and Laos formed a clade in both the MP and BI trees. By contrast, in the ML tree, the "*M. fissipes*" populations became paraphyletic; see below. In our trees, all individuals with the Chittagong haplotype (i.e., *M.* sp. C, IABHU 3956, IABHU 3958–3959) formed a clade with high bootstrap support (BP: 99 for ML, 99 for MP, and 100 for BI). In the ML tree, *M. fissipes* from four distinct Asian countries (China, Taiwan, Thailand and Laos) did not result monophyletic; rather *M. fissipes* was paraphyletic with respect to *M.* sp. C. Among them, Laotian "*M. fissipes*" were monophyletic with *M.* sp. C, the individual from Thailand was part of the sister taxon of the *M.* sp. C + Laotian "*M. fissipes*", and topotypic *M. fissipes* from China and Taiwan occupied the basal position within the *M. fissipes* and *M.* sp. C group. Within the *M.* sp. C group and the topotypic *M. fissipe*s group, the average *cytb* divergence was small [0.9% (range = 0.4–1.2%) and 0.5% (range = 0.4–0.6%), respectively]. By contrast, the *cytb* divergence between topotypic *M. fissipes* vs. "*M. fissipes*" from Thailand and Laos was large [8.6 % (range = 8.4–8.8%), 9.7 % (range = 9.5–9.9%), respectively]. Furthermore, topotypic *M. fissipes* vs. *M.* sp. C also showed large *cytb* divergence [8.9% (range = 8.4–9.7%)]. In comparison, the *cytb* divergence between *M.* sp. C vs. "*M. fissipes*" from Thailand and Laos was smaller [4.9% (4.7–5.4%) and 6.1% (5.8–6.6%), respectively].

The *M.* sp. M specimens formed a clade (BP: 92 for ML, 90 for MP, and 96 for BI) and became a sister taxon to the *M. fissipes* + *M.* sp. C clade (BP: 86 for ML, 78 for MP, and 100 for BI) (Fig. 2). The average *cytb* divergence was 2.7% (range = 0.2–6.0%) within the *M.* sp. M clade while this value was 10.2% (range = 8.4–11.7%) with respect to the *M. fissipes* + *M.* sp. C from Bangladesh specimens.

The *cytb* data and phylogenetic relationship reported here suggest the possible occurrence of at least two new *Microhyla* taxa in Bangladesh. Although they are closely related to *M. fissipes*, these taxa have independent phylogenetic positions from topotypic *M. fissipes* and are distinguished from *M. fissipes* by high *cytb* divergence, as well as by *16S-rrn* divergence (3.6 and 4.2% in *M. fissipes* vs *M.* sp. C and *M.* sp. M, respectively). In general,

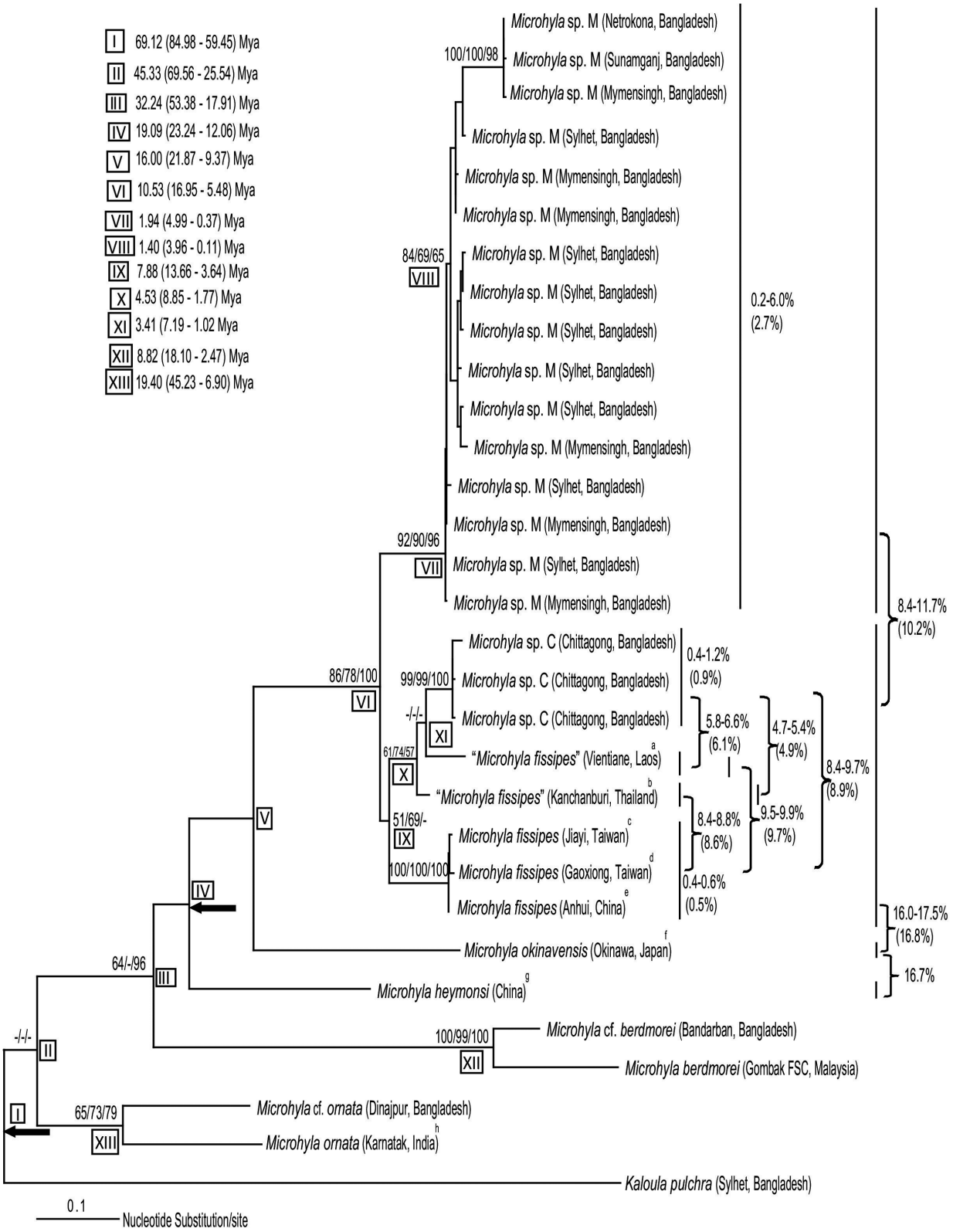

FIGURE 2. Maximum likelihood (ML) tree based on mitochondrial *cytb* gene sequences of 514 bp. *Kaloula pulchra* was used as an outgroup. Numbers on branches represent bootstrap support for ML and MP inferences, and Bayesian posterior probability. The scale bar represents 0.1 nucleotide substitutions per site. Superscript letters indicate that *cytb* data were taken from GenBank for use in constructing this tree: [a]AB201221, Matsui *et al*. 2005; [b]AB201215, Matsui *et al*. 2005; [c]AB201211, Matsui *et al*. 2005; [d]AB201212, Matsui *et al*. 2005; [e]AB201213, Matsui *et al*. 2005; [f]AB303950, Igawa *et al*. 2008; [g]AY458596, Zhang *et al*. 2005 and [h]AB201223, Matsui *et al*. 2005. Boxed Roman numerals indicate major lineage splits and estimated split ages are shown at left above. Arrows show the fixed reference points used.

16S-rrn is considered a reliable marker for determining the taxonomic status of frog species (Vences *et al*. 2005) in amphibians and it has been shown that a 3% *16S-rrn* divergence between taxa to target lineages that could correspond to first preliminary evidence of differentiation at the species level of anurans (Fouquet *et al*. 2007). By contrast, some researchers recently also found populations meriting recognition as two distinct species at lower *16S-rrn* divergences (<3%) (for e.g. Kuramoto *et al*. 2011). Our results also suggest that the currently recognized *M. fissipes* (sensu lato) from Taiwan and a large portion of China and Southeast Asia (Matsui *et al*. 2005) do not constitute a single species, and that at least "*M. fissipes*" from Laos and Thailand has a close relationship with respect to *M*. sp. C rather than to topotypic *M. fissipes* specimens.

The *cytb* data of *M. fissipes* specimens from Laos and Thailand were originally submitted by other authors (Matsui *et al*. 2005), and these data were retrieved from the DDBJ; thus, we could not include these taxa in our morphological comparisons, and their detailed taxonomic statuses have not been clarified. However, based on *cytb* data and our phylogenetic analysis results, two distinct scenarios can be hypothesized: "*M. fissipes*" specimens from Thailand and Laos might be the same species as *M*. sp. C (newly described in this paper), or they correspond to an undescribed and/or cryptic species. To clarify the taxonomic status of these "*M. fissipes*" specimens, additional sampling is necessary in Laos and Thailand.

Estimation of evolutionary time. We estimated divergence times among *Microhyla* taxa (Fig. 2). Among the *Microhyla* species examined here, the time of divergence of *M*. sp. M from its close relatives (Fig. 2, node VI) was estimated as 10.5 Mya (17.0–5.5 Mya, 95% CI). Throughout the *M*. sp. M clade, the branching estimation date was <2 Mya (nodes VII and VIII). Within the *M. fissipes* (sensu lato) and *M*. sp. C clades, topotypic *M. fissipes* (from China and Taiwan) first split from *M. fissipes* (from Thailand and Laos) and *M*. sp. C at 7.9 Mya (node IX). *Microhyla* sp. C split from Thai and Laotian *M. fissipes* 4.5 and 3.4 Mya, respectively (nodes X and XI).

It has been proposed that during the uplifting of the Himalayas through the North and Indo-Burma ranges, the Bengal basin (present Bangladesh) was formed through sedimentation of the Ganges-Brahmaputra and other associated or ancestral rivers between 20 and 14 Mya (Alam *et al*. 2003; Uddin & Lundberg 2004). Following this, the formation of Asian dry and wet zones occurred between 10 and 1.6 Mya (Karanth 2003; Alam *et al*. 2008). The divergence of *M*. sp. M and its nearest relatives *M. fissipes* (sensu lato) and *M*. sp. C was 10.5 Mya (17.0–5.5 Mya) and the estimated divergence of Laotian "*M. fissipes*" vs. *M*. sp. C from Bangladesh was 3.4 Mya (7.2–1.0 Mya). Both of these events thus appear to have occurred after the land formation of Bangladesh during the middle Miocene to Pliocene. The sister taxa of the clade formed by *M. fissipes* and our newly discovered Bangladesh species group are *M. okinavensis* and *M. heymonsi* in the East Asian group; and topotypic *M. fissipes* and its close relatives are distributed in East and Southeast Asia. Thus, the common ancestor of these taxa seems to have evolved somewhere in East or Southeast Asia before the formation of the Bangladesh landmass; and *M*. sp. M and *M*. sp. C may have independently colonized Bangladesh from these areas at different times (10.5 Mya and 3.4 Mya, respectively) following land formation. Further extensive sampling in this area is needed to fully understand the evolutionary process of these microhylid taxa.

Morphological comparisons. Among the five *Microhyla* species in Bangladesh (*M*. sp. C, *M*. sp. M, *M. ornata*, *M. berdmorei* and *M. rubra*), *M. rubra* is easily distinguishable from the others by its tibiotarsal articulation reaching the orbit, one-third webbed toes, and shovel-like metatarsal tubercles (outer tubercle is semicircular and the inner is crescent-shaped). In contrast, *M. berdmorei* is a comparatively large species having fully webbed toes, tibiotarsal articulation reaching beyond the tip of the snout, and a chevron-shaped black marking near the anus. *Microhyla ornata* can be distinguished by its tibiotarsal articulation reaching slightly in front of the shoulders, toes with rudimentary webbing, an oval inner metatarsal tubercle, and the absence of an outer metatarsal tubercle (Duméril & Bibron 1841). *Microhyla* sp. C and M can be separated from the aforementioned species through a combination of the following characteristics: slender fingers, with the first finger notably shorter than the second, tips of fingers and toes not swollen, a distinct subarticular tubercle, and tibiotarsal articulation extending from between the eyes to the snout. From *M. berdmorei* with its long hindlimb, *M.* sp. C and M are readily separated by the absence of toe webbing. The chief difference among *M. fissipes*, *M*. sp. C and *M*. sp. M specimens is the decreasing values of SVL, while the TIL/SVL and HLL/SVL ratios increase in ascending order; particularly tibiotarsal articulation, which extends to the eye in *M. fissipes*, while ranging from the eye to the tip of the snout in the new species. Furthermore, significant differentiation of FAW/SVL ratio among these three species makes them completely separated from each other (Fig. 3A).

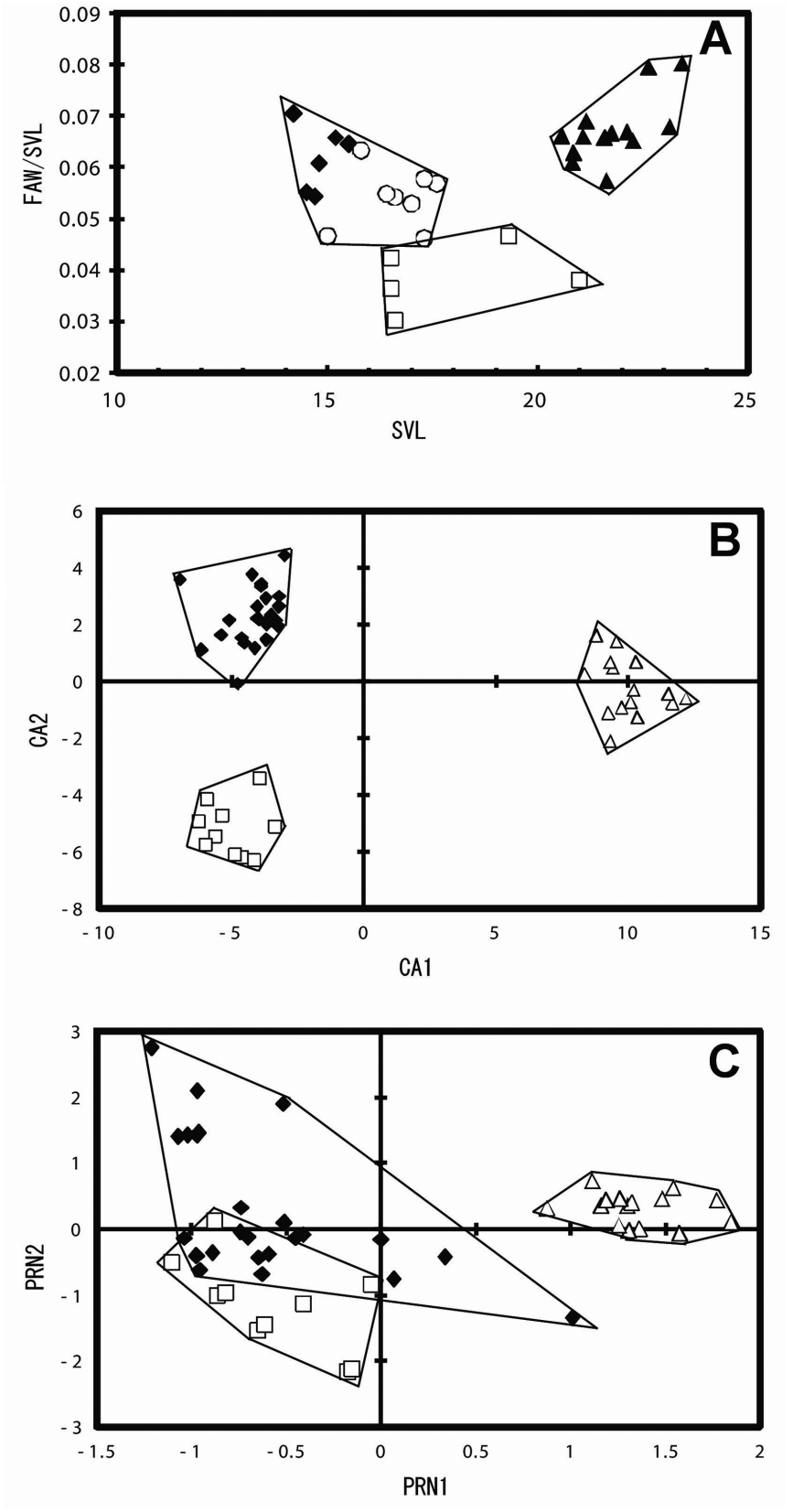

FIGURE 3. (A) Univariate scatterplot of male individuals of *M.* sp. C from Chittagong (open square), *M.* sp. M from Mymensingh (solid rhombus), Sylhet (open circle) and *M. fissipes* (solid triangle) from Taiwan. (B) Scatter plot of individual discriminant scores on the first (CA1) and second canonical axes (CA2) for *M.* sp. C (open square), *M.* sp. M (solid rhombus) and *M. fissipes* (open triangle). (C) Scatterplot of principal component1 (PRN1) versus principal component 2 (PRN2) from the principal component analysis of *M.* sp. C (open square), *M.* sp. M (solid rhombus) and *M. fissipes* (open trangle).

Morphometric comparisons. From both DNA analysis and morphological comparison, it became clear that the new taxa *M.* sp. C and *M.* sp. M are closely related to *M. fissipes* and distantly related to *M. ornata,* which encouraged us to make detailed morphological comparisons between *M.* sp. C and M with *M. fissipes*. The measurements of 28 body parts of *M.* sp.C, *M.* sp. M and *M. fissipes* are summarized in Table 1.

Microhyla sp. C, as well as *M.* sp. M, is notably smaller in size than topotypic *M. fissipes*, and there are highly significant differences in almost all comparisons between the former two and the latter taxa. *Microhyla* sp. C differed significantly from *M.* sp. M, especially with regard to HL, HW, E-E, FAW, and T3 ($P < 0.01$) (Table 1). All three taxa are clearly separated in discriminant analysis (Fig. 3B), with an Eigen value of 47.058 (for function 1) and a Wilks' lambda value of 0.002 (for functions 1 to 2). In principal component analysis (Fig. 3C), *M.* sp. C and M completely separated from *M. fissipes,* with some separation despite considerable overlap of the scores of *M.* sp. C and M.

We compared 37 body ratios and found that *M.* sp. C differed significantly from its close relative *M.* sp. M, having a larger HW and smaller ELW, FAW, HAL, F1, F2, and IMT relative to SVL ($P < 0.01$) (Table 2). *Microhyla* sp. C and *M.* sp. M differ from topotypic *M. fissipes* in having a longer head length (HL/SVL), shorter forelimb length ((FLL/SVL), thinner forearms (FAW/SVL), and longer tibias (TIL/SVL). Lastly, the hindlimbs (HLL/SVL) in *M.* sp. M are significantly longer than in *M. fissipes* ($P < 0.01$) (Table 2), which apparently correlates with *M.* sp. M's excellent jumping ability.

Microhylids show a high level of homoplasy due to the loss of pectoral girdle elements (Zweifel 1986) which complicates the use of morphological characteristics for microhylid taxonomy. Furthermore, as some other factors such as seasonal breeding behavior and minute body size (Vences *et al.* 2010) are difficult to study, it is particularly difficult to make clear-cut separations between the three morphologically poorly defined species—*M. fissipes*, *M. ornata,* and *M. okinavensis* (Kuramoto & Joshy 2006). Despite *M.* sp. C and *M.* sp. M sharing some morphological characteristics with previously examined *Micohyla* species; our canonical discriminant analysis clearly separated them from their near congener *M. fissipes*. In addition, molecular phylogenetic analysis revealed that *M.* sp. C and *M.* sp. M had their own evolutionary lineage independent of topotypic *M. fissipes*. Finally, some characteristics (e.g., tibiotarsal articulation) and body ratios (e.g., FAW/SVL) can further be used to distinguish these species from their closest relatives. Consequently, based on the result of multiple data sets, it became clear that *M.* sp. C and *M.* sp. M are completely separated from their closest relative, *M. fissipes*. Therefore, in the next section we describe them as two distinct, new frog species of genus *Microhyla* from Bangladesh.

Systematics

Microhyla mukhlesuri sp. nov.

Microhyla ornata (Bangladesh): Kabir *et al*. (2009), p. 25 (part). *Microhyla* cf. *ornata* (Chittagong, Bangladesh): Hasan *et al.* (2012), p. 168.
Microhyla sp. C: above discussion

Holotype. IABHU 3956, adult female (SVL: 17.9 mm; if not otherwise specified, the following body parts are measured in mm) collected from Raozan, Chittagong (22° 35′ N, 91° 55′ E, > 9 m asl.), Bangladesh on 14 November 2009 by M. M. Islam (Figs. 4A, 4B).

Paratypes. IABHU 3878, adult female (SVL: 17.3); IABHU 3879, adult male (SVL: 21.0); IABHU 3880, adult male (SVL: 19.3); IABHU 3881, adult female (SVL: 17.5); IABHU 3882, adult male (SVL: 16.5); IABHU 3957, adult female (SVL: 18.4); IABHU 3958, adult female (SVL: 17.3); IABHU 3959, adult male (SVL: 16.5); and IABHU 3960, adult male (SVL: 16.6) collected from Raozan, Chittagong, Bangladesh on 14 November 2009 by M. M. Islam.

Etymology. We dedicate the species name "*mukhlesuri*" to the late Dr. Md. Mukhlesur Rahman Khan, Professor of the Department of Fisheries Biology & Genetics, Bangladesh Agricultural University (BAU), who significantly contributed to amphibian research in both Bangladesh and the international community by establishing collaborations between BAU, Bangladesh, and the Institute for Amphibian Biology, Hiroshima University, Japan.

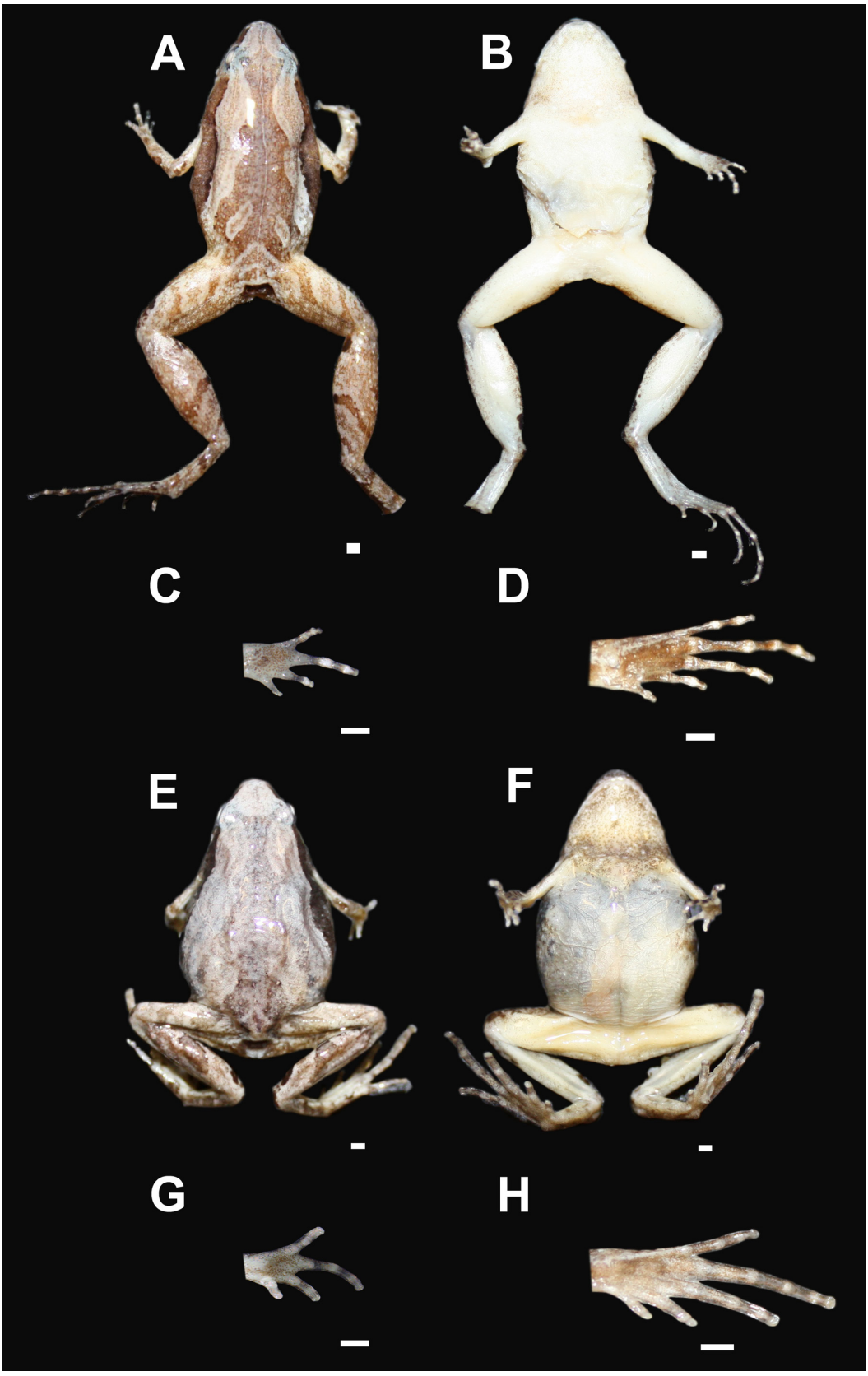

FIGURE 4. (A) Dorsal view and (B) Ventral view of the holotype of *Microhyla mukhlesuri* sp. nov (IABHU 3956). Ventral view of right (C) hand and (D) foot of paratype (3960) of *M. mukhlesuri* sp. nov.. (E) Dorsal view and (F) Ventral view of the holotype of *M. mymensinghensis* sp. nov (IABHU 4116). Ventral view of right (G) hand and (H)foot of holotype (4116) of *M. mymensinghensis* sp. nov.. All pictures of specimens were taken after preservation in alcohol. Scale bar = 1 mm.

TABLE 1. Measurements of 28 body parts in three *Microhyla* taxa examined (mean±S. D. in mm) and probabilities obtained by Mann-Whitney *U* tests. Symbols * and ** indicate the 5% and 1% significance levels, respectively.

	Microhyla sp. C		*Microhyla* sp. M		*Microhyla fissipes*		*M.* sp. C vs. *M.* sp. M			*M.* sp. C vs. *M. fissipes*			*M.* sp. M vs. *M. fissipes*		
	(n=10)		(n=24)		(n=15)		U	P		U	P		U	P	
SVL	17.83	1.424	16.50	1.872	21.82	0.850	53.5	0.0118	*	3	0.0001	**	5	0.0000	**
HL	7.07	1.222	5.70	1.050	5.20	0.458	50.5	0.0085	**	5	0.0001	**	103	0.0261	*
HW	6.54	1.004	5.38	0.720	6.63	0.497	42	0.0031	**	69	0.7392		27	0.0000	**
S-N	0.75	0.135	1.23	1.881	1.43	0.193	85.5	0.1866		0	0.0000	**	27	0.0000	**
N-N	1.56	0.201	1.56	0.279	2.02	0.109	117	0.9088		0	0.0000	**	24	0.0000	**
N-E	1.52	0.266	1.49	0.360	1.82	0.169	102	0.4930		28	0.0091	**	72	0.0018	**
ED	1.62	0.204	1.60	0.336	1.92	0.212	113	0.7894		23.5	0.0041	**	75	0.0024	**
E-E	2.47	0.275	2.05	0.440	2.42	0.137	50	0.0078	**	65	0.5786		71.5	0.0017	**
ELW	1.11	0.152	1.28	0.251	1.51	0.127	63	0.0287	*	3.5	0.0001	**	56	0.0003	**
FLL	8.59	0.695	8.57	0.942	12.46	0.634	116.5	0.8946		0	0.0000	**	0	0.0000	**
FHL	6.91	0.507	6.57	0.816	8.77	0.752	83	0.1612		8	0.0002	**	15	0.0000	**
FAW	0.71	0.120	0.90	0.143	1.46	0.176	33	0.0008	**	0	0.0000	**	0	0.0000	**
HAL	3.86	0.341	4.00	0.374	5.22	0.231	100.5	0.4590		0	0.0000	**	1.5	0.0000	**
F1	0.69	0.173	0.84	0.193	1.20	0.156	65	0.0354	*	1.5	0.0000	**	18	0.0000	**
F2	1.44	0.250	1.60	0.120	2.40	0.227	69	0.0456	*	0	0.0000	**	0	0.0000	**
F3	2.52	0.421	2.48	0.236	3.72	0.270	109.5	0.6881		0	0.0000	**	0	0.0000	**
F4	1.15	0.242	1.29	0.224	2.22	0.218	76	0.0929		0	0.0000	**	0	0.0000	**
HLL	28.26	1.855	27.77	3.135	33.90	1.838	97	0.3846		6	0.0001	**	25	0.0000	**
FEL	7.46	0.738	7.52	1.063	9.41	0.549	113.5	0.8056		3	0.0001	**	32	0.0000	**
TIL	9.62	0.483	9.41	0.925	10.93	0.345	98.5	0.4156		0	0.0000	**	24	0.0000	**
TFL	12.42	1.357	12.88	1.856	15.71	0.621	104.5	0.5577		0	0.0000	**	17	0.0000	**
FOL	9.00	0.775	8.47	1.243	11.48	0.495	98.5	0.4156		0	0.0000	**	2	0.0000	**
T1	0.94	0.227	1.07	0.254	1.54	0.241	86.5	0.2003		3	0.0001	**	24.5	0.0000	**
T2	2.52	0.368	2.45	0.439	3.56	0.302	106	0.5945		0	0.0000	**	3	0.0000	**
T3	4.22	0.410	3.73	0.578	5.98	0.296	50.5	0.0084	**	0	0.0000	**	0	0.0000	**
T4	5.17	0.564	5.07	0.524	8.09	0.356	110.5	0.7186		0	0.0000	**	0	0.0000	**
T5	2.70	0.624	2.33	0.459	4.19	0.290	75.5	0.0907		0	0.0000	**	0	0.0000	**
IMT	0.78	0.225	0.95	0.189	0.95	0.129	63.5	0.0307	*	38.5	0.0426	*	178.5	0.9653	

Diagnosis. The new species *M. mukhlesuri* is assigned to the genus *Microhyla* based on smooth or warty skin, absence of vomerine teeth, a narrow and elliptical tongue, hidden tympanum, and molecular phylogenetic relationships (Hasan *et al*. 2012; Hasan *et al*. unpublished data). The genus *Microhyla* comprises 31 species and among them, only three (*Microhyla ornata*, *M. berdmorei* and *M. rubra*), five (*M. ornata*, *M. rubra*, *M. berdmorei*, *M. heymonsi* and *M. butleri*) and seven (*M. ornata*, *M. rubra*, *M. berdmorei*, *M. heymonsi*, *M. butleri*, *M. chakrapanii* and *M. sholigari*) nominal species are known to occur in Bangladesh, Myanmar and India, respectively (Kabir *et al*. 2009; AmphibiaWeb 2013). After documentation of all available *16S-rrn* and/or *cytb* sequences of *Microhyla* species from DDBJ/EMBL/GenBank databases, it became clear that this species (*M. mukhlesuri*) do not fit with any other previous sequenced species of *Microhyla*. Further, tibiotarsal articulation and an inverse U-shaped marking on the anus, and a distinct X-shaped marking on the dorsum made it stand out from other *Microhyla* species. There is no available *16S-rrn*/*cytb* data of *M. chakrapanii* and *M. sholigari* in GenBank, but the new species differentiates from them by the absence of minute tubercle on the dorsal part of tibia and thin forelimbs (Pillai 1977; Chanda 2002) and longitudinal groove dorsally on dilated toe tips, respectively (Dutta & Ray 2000). The new species differs from *M. rubra* by its tibiotarsal articulation reaching eye to the tip of the snout (vs. tibiotarsal articulation reaching until the orbit in *M. rubra*), from *M. berdmorei* with no or rudimentary webbing (vs. fully webbed toes in *M. berdmorei*) and from *M. ornata* by the presence of an outer metatarsal tubercle (vs. outer metatarsal tubercle is absence in *M. ornata*). In addition, the new species differs from *M. butleri* by the projection of first finger from the palm is narrow (vs. this kind of projection is far in *M. butleri*) and absence of disc on finger (vs. with disc in *M. butleri*) (Inger 1966). The new species can be separated from *M. heymonsi* by

the presence of X-shaped marking on the dorsum and smaller size (SVL = 16.5–21 mm) (vs. 22–26 mm in *M. heymonsi*) (Chanda 2002). Lastly, the new species can be distinguished from its most near congener *M. fissipes* by the extension of tibiotarsal articulation until eye to the tip of snout (vs. reached only until eye in *M. fissipes*). *Microhyla inornata* (Boulenger 1890) in India currently treated as *Micryletta inornata* based on a specimen collected by Pillai (1977) from Andaman Islands. Jerdon (1853) described two new spcies i.e. *Engystoma malabaricum* and *E. carnaticum* from "Malabar" (present Kerala) and "Caranatic" (present Karnataka), respectively. Later, Parker (1934) synonymised the former two species as a singles species *M. ornata*. Despite the unavailability of type specimen, it seems quite untenable to resurrect of the former two *Engystoma* species from the synonymy of *M. ornata* due to following reasons: 1) insufficient description of *Engystoma* species by Jerdon ("1853" 1854), 2) genetically, our new species has closer affinity with Myanmar "*M. ornata*" (Hasan *et al.* unpublished data) and evolutionary, its immediate ancestors likely occurred and diverged in East or Southeast Asia rather than India, 3) known occurrence of the new species reveals that the radiation of this species restricted into a particular biogeographical region, i.e. Chittagong area, and ecologically this area has some similarity with Myanmar rather than Kerala and Karnataka (Western Ghats), India. Hence, from a biogeographical point of view, the dispersal of the new species might be extending to the south-western part of Myanmar instead of Kerala and Karnataka, India. *Pyxicephalus frithi* Theobald (1868) described from Jessore, SW Bangladesh reported to have vinous coloration and recently it became invalid (Frost 2013). Also this nomen does not fit with our new species.

TABLE 2. Body ratios in three *Microhyla* taxa examined (mean ±S. D.) and results of comparisons using Mann-Whitney *U* tests. *U* and *P* values are given. Symbols * and ** indicate the 5% and 1% significance levels, respectively.

	Microhyla sp. C (n=10)	*Microhyla* sp. M (n=24)	*M. fissipes* (n=15)	Sp. C vs. Sp. M		Sp. C vs. *fissipes*		Sp. M vs. *fissipes*	
				U	*P*	*U*	*P*	*U*	*P*
HL/SVL	0.39 ± 0.046	0.35 ± 0.066	0.24 ± 0.024	74	0.0821	0	0.0000 **	23	0.0000 **
HW/SVL	0.37 ± 0.033	0.33 ± 0.032	0.30 ± 0.024	47.5	0.0061 **	13	0.0006 **	106	0.0327 *
S-N/SVL	0.04 ± 0.008	0.08 ± 0.120	0.07 ± 0.010	71	0.0639	5	0.0001 **	85	0.0061 **
N-N/SVL	0.09 ± 0.011	0.09 ± 0.014	0.09 ± 0.006	85	0.1857	56	0.2918	146	0.3263
N-E/SVL	0.09 ± 0.015	0.09 ± 0.020	0.08 ± 0.006	106	0.5967	71	0.8244	165	0.6650
ED/SVL	0.09 ± 0.010	0.10 ± 0.014	0.09 ± 0.011	80.5	0.1354	67	0.6572	121.5	0.0912
E-E/SVL	0.14 ± 0.016	0.12 ± 0.020	0.11 ± 0.006	70	0.0588	2	0.0001 **	94	0.0130 *
ELW/SVL	0.06 ± 0.007	0.08 ± 0.011	0.07 ± 0.006	26.5	0.0004 **	34	0.0229 *	85	0.0061 **
FLL/SVL	0.48 ± 0.019	0.52 ± 0.049	0.57 ± 0.021	59	0.0211 *	0	0.0000 **	70	0.0015 **
FHL/SVL	0.39 ± 0.023	0.40 ± 0.035	0.40 ± 0.029	88	0.2264	44	0.0855	163	0.6236
FAW/SVL	0.04 ± 0.005	0.06 ± 0.009	0.07 ± 0.006	13.5	0.0001 **	0	0.0000 **	46	0.0001 **
HAL/SVL	0.22 ± 0.012	0.24 ± 0.018	0.24 ± 0.008	20	0.0002 **	8	0.0002 **	157	0.5067
F1/SVL	0.04 ± 0.009	0.05 ± 0.013	0.05 ± 0.007	50.5	0.0086 **	10	0.0003 **	153	0.4357
F2/SVL	0.08 ± 0.015	0.10 ± 0.010	0.11 ± 0.011	36	0.0015 **	7	0.0002 **	66	0.0010 **
F3/SVL	0.14 ± 0.024	0.15 ± 0.021	0.17 ± 0.014	99.5	0.4384	19	0.0019 **	73	0.0020 **
F4/SVL	0.06 ± 0.014	0.08 ± 0.017	0.10 ± 0.010	57	0.0172 *	0	0.0000 **	56	0.0003 **
HLL/SVL	1.59 ± 0.123	1.69 ± 0.181	1.55 ± 0.076	72	0.0696	59	0.3748	81	0.0043 **
FEL/SVL	0.42 ± 0.032	0.46 ± 0.044	0.43 ± 0.028	61	0.0257 *	58	0.3457	122	0.0941
TIL/SVL	0.54 ± 0.026	0.57 ± 0.040	0.50 ± 0.015	66	0.0413 *	15	0.0009 **	17	0.0000 **
TFL/SVL	0.70 ± 0.096	0.78 ± 0.057	0.72 ± 0.022	70	0.0587	70	0.7815	57	0.0004 **
FOL/SVL	0.51 ± 0.044	0.51 ± 0.050	0.53 ± 0.030	105	0.5707	57	0.3181	165	0.6650
T1/SVL	0.05 ± 0.012	0.07 ± 0.017	0.07 ± 0.010	62	0.0284 *	18	0.0016 **	123	0.0999
T2/SVL	0.14 ± 0.019	0.15 ± 0.029	0.16 ± 0.011	107	0.6098	26	0.0066 **	101	0.0226 *
T3/SVL	0.24 ± 0.016	0.23 ± 0.035	0.27 ± 0.014	105	0.5708	4	0.0001 **	35	0.0000 **
T4/SVL	0.29 ± 0.037	0.31 ± 0.037	0.37 ± 0.020	98	0.4057	0	0.0000 **	22	0.0000 **
T5/SVL	0.15 ± 0.031	0.14 ± 0.026	0.19 ± 0.014	98	0.4057	17	0.0013 **	9	0.0000 **
IMT/SVL	0.04 ± 0.011	0.06 ± 0.014	0.04 ± 0.006	48.5	0.0069 **	71	0.8244	59	0.0005 **
HL/HW	1.08 ± 0.084	1.06 ± 0.165	0.79 ± 0.077	101	0.4725	0	0.0000 **	37	0.0000 **
S-N/N-E	0.50 ± 0.117	0.90 ± 1.590	0.79 ± 0.134	79.5	0.1256	9	0.0003 **	71	0.0017 **
N-E/E-E	0.62 ± 0.116	0.76 ± 0.231	0.75 ± 0.065	84	0.1734	26	0.0066 **	144	0.2986
ED/E-E	0.66 ± 0.065	0.80 ± 0.187	0.80 ± 0.094	61	0.0256 *	19	0.0019 **	178	0.9540
N-N/E-E	0.64 ± 0.094	0.78 ± 0.140	0.84 ± 0.062	46	0.0051 **	2	0.0001 **	119	0.0782
ELW/E-E	0.46 ± 0.095	0.65 ± 0.162	0.63 ± 0.054	31.5	0.0008 **	11	0.0004 **	177	0.9310
F1/F2	0.48 ± 0.098	0.53 ± 0.140	0.50 ± 0.067	86.5	0.2043	66	0.6175	140	0.2478
TIL/FEL	1.30 ± 0.106	1.26 ± 0.139	1.16 ± 0.058	100	0.4496	18	0.0016 **	108	0.0377 *
FOL/FEL	1.21 ± 0.092	1.14 ± 0.179	1.22 ± 0.102	91.5	0.2813	72	0.8678	128	0.1333
TIL/FOL	1.07 ± 0.075	1.12 ± 0.109	0.95 ± 0.057	89	0.2413	17	0.0013 **	23	0.0000 **

Summarizing, the new species is small frog with SVL of 16.5–21.0 mm in males and 17.3–18.4 mm in females. Head length subequal head width, finger formula 1 < 4 < 2 < 3, toe formula 1 < 2 < 5 < 3 <4, fingers free and slender, tips of fingers and toes not widened, rudimentary web between toes and subarticular tubercles relatively prominent (Figs. 4A, 4B). TIL/SVL ratio was 0.54 ± 0.03, whereas this value was 0.57 ± 0.04 in *M. mymensinghensis*, and 0.50 ± 0.02 in *M. fissipes* from Taiwan. Tibiotarsal articulation reaches between the eyes to tip of snout, whereas it reaches near the eye in *M. fissipes*. Phylogenetically, it appears to closer to "*M. ornata*" from Myanmar plus "*M. fissipes*" from Laos and Thailand and to be sister respectively with Taiwanese topotypic *M. fiissipes* with significant genetic divergences (Hasan *et al*. 2012; Hasan *et al*. unpublished data; this study).

Description of holotype. Body small (SVL: 17.9) and slightly elongated. Vomerine teeth absent, tongue elliptical. Head length greater than width (HL: 8.1; HW: 6.9), snout rounded. Canthus rostralis steep, lore sloping and weakly concave. Nostril nearer to tip of snout than to eye (S-N: 0.7; N-E: 1.7). Tympanum hidden. Inter-orbital space wider than inter-nostril space and eyelid width (E-E: 2.2; N-N: 1.5 and ELW: 1.1). Fingers slender, free and tips not swollen. Finger length F1 < F4 < F2 < F3 (F1: 0.8; F2: 1.4; F3: 3.0; F4: 1.2) (Fig. 4C). Hindlimb about 1.6 times SVL (HLL: 28.8; SVL: 17.9). Femur length significantly less than tibia length (FEL: 7.1; TIL: 10). Toe tips rounded, not swollen. Toe length T1 < T2 < T5 < T3 < T4 (T1:1.1; T2: 2.5; T3: 4.0; T4: 5.8; T5: 3.0) (Fig. 4D). Hindlimb long and stout. Subarticular tubercles relatively prominent.

Skin smooth with dense, dark X-shaped mark on the dorsum, arising from the eyes, narrowing on the front of forelimbs, with little expanse between the shoulders, then delivering two broad longitudinal lines between the post-belly and the groin; finally two additional repeated longitudinal lines that reach to the thigh, passing the corner of the groin. Black band starts from the tip of snout, passing through the eyes, but interrupted at the post corner of eyes, eventually fusing before reaching the groin. Many oblique bars present on thigh, tibia, and tarsal region. Inverse U-shaped black mark above the anus. Many irregular, speckled dots below both sides of the anus (Fig. 4A). Ventral side is a slightly whitish with few very small fine speckles along the throat (Fig. 4B).

Color in alcohol. Dorsum dark gray to brownish. Ventral side of throat is slightly whitish with a few small speckles along the edge of throat. Lateral side is gray (Figs. 4A, 4B).

Distribution. The known occurrence of *M. mukhlesuri* is Raozan, Chittagong District, southeastern corner of Bangladesh (Fig. 1).

Variation. Of the examined 10 specimens from Chittagong, the ratio of males to females was 50%. Among these 5 males, one individual (IABHU 3879, 10%) had a distinct black vocal sac, but this characteristic is not prominent in other males. All 5 female (50%) specimens from Chittagong had a whitish ventral throat along with a few fine speckles along the border of the chin and base of the forelimbs. In addition, all specimens had distinct oblique bars on the hindlimbs, except for two specimens (IABHU 3957–8, [n = 2, 20%]) with dim bars, even the bars were sometimes fragmented. All specimens (n = 10, 100%), except one (IABHU 3958), had a conspicuous X mark on their back with branching at the end forming an inverse 'Y' shape. However, this stripe was present on only the left side of specimen IABHU 3959, and this stripe became two spots in IABHU 3880.

Natural history. *Microhyla mukhlesuri* was found in the grass near the bank of a pond in a calm, cold environment where soil was wet and slightly loose. Although no other *Microhyla* sp. were found, many *Fejervarya* sp. were caught in the same locality (Raozan, Chittagong) at the time of observation.

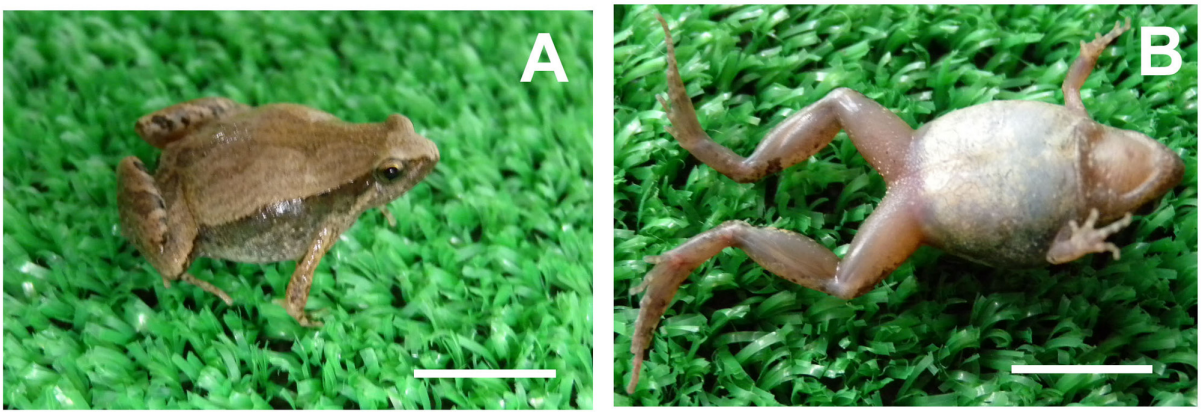

FIGURE 5. Holotype (IABHU 4116) of *M. mymensinghensis* sp. nov. in life. (A) Dorsal view. (B) Ventral view. Scale bar = 10 mm.

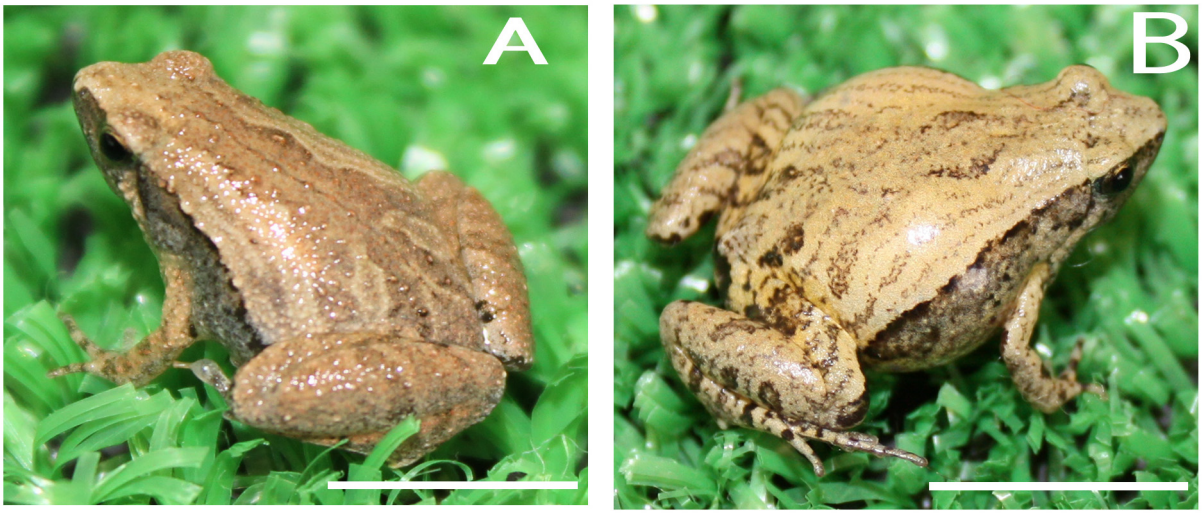

FIGURE 6. *Microhyla mymensinghensis* sp. nov. from Bangladesh Agricultural University Campus (BAUC), Mymensingh, Bangladesh. A and B showing color variations between individuals from BAUC. Scale bar = 10 mm.

***Microhyla mymensinghensis* sp. nov.**

Microhyla ornata (Bangladesh): Kabir *et al*. (2009), p. 25 (part). *Microhyla* cf. *ornata* (Mymensingh, Bangladesh): Hasan *et al.* (2012), p. 168. *Microhyla* cf. *ornata* (Sylhet, Bangladesh): Hasan *et al.* (2012), p. 168.
Microhyla sp. M: above discussion

Holotype. IABHU 4116, adult female (SVL: 21.3 mm, if not otherwise specified, the following body parts are measured in mm) collected from Bangladesh Agricultural University Campus (24° 44′ 50″ N, 90° 24′ 24″ E, > 18 m asl.), Mymensingh, Bangladesh on June 25, 2012 by M. Hasan (Figs. 4E, 4F).

Paratypes. IABHU 4004, adult male (SVL: 14.8); IABHU 4117, adult female (SVL: 20.2); and IABHU 4120, adult female (SVL: 20.5) collected from Bangladesh Agricultural University Campus, Mymensingh, Bangladesh on 9 June 2011 and 25 June 2012 by M. Hasan. IABHU 3947, adult male (SVL: 17.6); IABHU 3948, adult male (SVL: 17.3); and IABHU 3899, adult female (SVL: 16.7) collected from Golapganj, Sylhet on 6 June 2011 by M.M.R. Khan.

Etymology. The specific name refers to Mymensingh, the type locality of this species.

Diagnosis. The new species *M. mymensinghensis* is allocated to genus *Microhyla* due to its smooth or warty skin, absence of vomerine teeth, hidden tympanum, and sister relationships with other microhylid frogs (Hasan *et al*. 2012; Hasan *et al*. unpublished data). Comparison of the *16S-rrn* and/or *cytb* sequences of the new species with its congeners in DDBJ/EMBL/GenBank databases from South and Southeast Asia including Bangladesh, India and Myanmar, denotes that any known microhylid frogs do not correspond to this new species and merits its distinctness.

Considering the molecular analyses and morphological characters i.e. a crescent-shaped marking on the anus, an X-shaped marking on the dorsum and the tibio-tarsal articulation of the new species deduct it from all of the known *Microhyla* species from South and Southeast Asia, particularly from Bangladesh and its neighbor countries; but make confusion with only *M. mukhlesuri*, *M. fissipes*, *M. heymonsi* and *M. ornata*. However, the new species differs from *M. ornata* by the presence of an outer metatarsal tubercle (vs. outer metatarsal tubercle is absence in *M. ornata*) and from *M. heymonsi* by smaller size (SVL = 14.2–21.3 mm) (vs. 22–26 mm in *M. heymonsi*) (Chanda 2002) and presence of X-shaped marking on the dorsal (this kind of marking absence in *M. heymonsi*). The new species can be distinguished from its most near congener *M. fissipes* by the extension of tibiotarsal articulation until eye to the tip of snout (vs. reached only until eye in *M. fissipes*). Most importantly, the new species differs from *M. mukhlesuri* by smaller body size, SVL = 16.5 ± 1.8 mm (vs. SVL = 17.8 ± 1.4 mm in *M. mukhlesuri*), higher forearm width, FAW = 0.9 ± 0.1 mm (vs. FAW = 0.7 ± 0.1 mm in *M. mukhlesuri*) (see Table 1), and presence of crescent shape marking on the anus (vs. this marking looks "U" shape in the *M. mukhlesuri*). Further, like with *M. mukhlesuri*, none of the old nomina of *Microhyla* corresponds to this new species.

In conclusion, this new species is small frog with SVL of 14.2–17.6 mm in males and 15.2–21.3 mm in females. Head length subequal head width, finger formula 1 < 4 < 2 < 3, toe formula 1 < 5 < 2 < 3 < 4, fingers free and slender, tips of fingers and toes not widened, rudimentary web between toes and subarticular tubercles distinct (Figs. 4G, 4H). TIL/SVL ratio was 0.57 ± 0.04, whereas this value was 0.54 ± 0.03 in *M. mukhlesuri*, and 0.50 ± 0.02 in *M. fissipes* from Taiwan. Tibiotarsal articulation extends from between the eyes to the tip of the snout, while it reaches near the eye in *M. fissipes* and in front of the shoulders in *M. ornata*. Phylogenetically, it shows sister relationship with *M. mukhlesuri* plus *M. fissipes* with high genetic divergences (Hasan *et al*. 2012; Hasan *et al*. unpublished data; this study).

Description of holotype. Body small (SVL: 21.3) and stocky. Vomerine teeth absent, tongue elliptical. Head width greater than length (HW: 7.2; HL: 5.9), snout truncate, projecting slightly beyond the lower jaw. Canthus rostralis rounded, lore sloping and weakly concave. Nostril nearer to tip of snout than to eye (S-N: 1.3; N-E: 2.3). Tympanum hidden. Inter-orbital space wider than eyelid and inter-nostril space (E-E: 2.9; ELW: 2.1 and N-N: 2.1). Fingers slender, free, and tips not swollen. Finger length F1 < F4 < F2 < F3 (F1: 1.1; F2: 1.7; F3: 2.8; F4: 1.3) (Fig. 4G). Hindlimb about 1.6 times SVL (HLL: 34.0; SVL: 21.3). Femur length almost equal to tibia length (FEL: 10.1; TIL: 10.6). Toe tips rounded, not swollen. Toe length T1 < T2 < T5 < T3 < T4 (T1:1.2; T2: 2.8; T3: 4.9; T4: 6.0; T5: 3.3). Hindlimb long and stout. Subarticular tubercles distinct.

Skin smooth with distinctive brown pattern on the back similar to an X shape which commences from between the eyes, narrows on the nape, again widens below the shoulders, and finally broadens out, and sending many longitudinal lines to the groin. Crescent-shaped black mark above the anus. Black streak starts from the snout, passing through the eyes, becoming wide on the side of the belly and ultimately narrowing before reaching the groin (Fig. 5A). Ventral side is immaculate with some speckles along the throat and between the bases of forelimbs.

Color in life. Dorsal ground color varies from brown to slightly yellowish (Fig. 5A). A distinctive pattern on the back shaded in dark brown initiates between the eyes, narrowing on the nape, again enlarging at the lower part of dorsal surface, and finally rejoins at the groin to anal region. Another black band starting from the snout, passing through the nostril and eye also terminates in the groin. Between these two bands, many brownish to off-white comb-like longitudinal lines pass from the eyes to the groin on both sides of the body. Ventral surface is cream colored, but chest is stippled with brown speckles (Fig. 5B)**.**

Color in alcohol. Dorsum gray to ash. Color arrangement of total body becomes faint. Ventral side of throat slightly black with a few small speckles along the edge of the lower jaw to the base of forelimbs. Lateral side pale to ash whereas dorsolateral side is black, originating from tip of snout through the nostrils and eyes, and eventually ending in the lower groin. Many black oblique lines present above the hindlimbs (Figs. 4E, 4F).

Distribution. The known occurrence of *M. mymensinghensis* includes the Mymenisngh, Netrokona, Sylhet and Sunamganj districts in the in the central and northeastern regions of Bangladesh (Fig. 1).

Variation. The Sylhet population has a longer head than the Mymensingh population (HL/SVL: 0.39 vs. 0.29, HL/HW: 1.17 vs. 0.91), the differences being highly significant.

Dorsal pattern is variable among individuals from the Mymensingh population (Figs. 6A, 6B), but Netrokona, Sylhet and Sunamganj specimens follow the dorsal pattern noted in Fig. 6A. Usually, the dorsal color of Mymensingh population is reddish to yellowish, while a few individuals have a browner dorsal color (Fig. 6B). Of the 15 specimens examined from Mymensingh, 9 individuals (60%) have a whitish ventral throat while 6 individual (40%) have a slightly ash ventral throat, along with a few speckles from their chin to base of forelimbs and rarely between the bases of forelimbs, not correlated with the male vocal sac. Of the examined 14 specimens from Sylhet, 12 individuals (86%) have two parallel small dark lines at the terminus of their X-shaped distinct pattern, located near the crescent-shaped markings on the anus, but the remaining two individuals (IABHU 3945 and 3950) lacked these lines. These lines are irregular in the Mymensingh population.

Chromosomes. *Microhyla mymensinghensis* has 2n = 24 chromosomes (Fig. 7). The frogs of genus *Microhyla* from Southeast Asia have been reported to possess 2n = 24 or 2n = 22 chromosomes (King 1990; Kuramoto 1990; Kuramoto & Yong 1992). *M. okinavensis* (Ryukyu Archipelago, Japan), *M. fissipes* (China) and *M. berdmorei* (Thailand) have 2n = 24 chromosomes. Joshy & Kuramoto (2011) suggested that the 2n = 24 and 2n = 22 karyotypes derive from the 2n = 26 karyotype of the Indian *M. ornata* and *M. rubra.* The karyotype of *M. mymensinghensis* supports a close relationship with *M. fissipes.*

Natural history. *Microhyla mymensinghensis* is sympatric with *M. berdmorei*, in the northeastern part of Bangladesh. We observed *M. mymensinghensis* either in grass under large trees (locally called “Lendi Korui”) or in

open fields with some vegetation and slightly wet and loose soil. After dissection, we found many small insect parts, as well as some sand in the gut. Their breeding season is likely June-July, as we caught several females in June from Bangladesh Agricultural University Campus (BAUC) just after light rains, which contained about 40–50 mature ova in the ovaries. Each ovum is very small, approximately 650 µm in diameter.

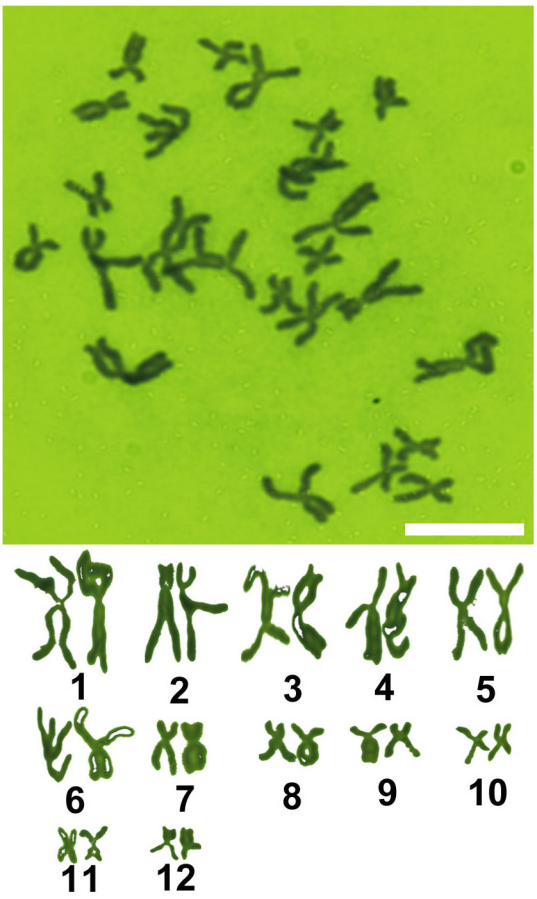

FIGURE 7. Metaphase spread and karyotype from bone marrow cells of *M. mymensinghensis*. Scale bar = 10 µm.

Acknowledgments

We thank the Wildlife Management and Nature Conservation Division, Bangladesh for their permission to collect and transport specimens from Bangladesh to Japan. This work was supported by Grants-in-Aid for Scientific Research (C) (Nos. 17570082 and 20510216) and (B) (No. 24310173) to M. Sumida from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Appendix 1. Examined specimens list

***Microhyla mukhlesuri*:** Institute for Amphibian Biology, Hiroshima University: IABHU 3956–3960, 3978, 3879–3882.
Collection localities: Raozan, Chittagong, Bangladesh.
***Microhyla mymensinghensis*:** Institute for Amphibian Biology, Hiroshima University: IABHU 4004–4006, IABHU 4117–4120, IABHU 4129–4134, F5012 and IABHU 3898–3899, IABHU 3944–3955.
Collection localities: Bangladesh Agricultural University Campus (BAUC), Mymensingh and Golapganj, Sylhet, Bangladesh.
***Microhyla fissipes*:** Osaka Museum of Natural History: OMNH Am 20028–20042.
Collection localities: Manchou of Pingtung district, Taiwan.

Supplementary Table: Original measurement data of *Microhyla* sp. M, *M*. sp. C and *M. fissipes* from Bangladesh and Taiwan.

Microhyla sp. M (Mymensingh, Bangladesh)

Voucher No.	SVL	HL	HW	S-N	N-N	N-E	ED	E-E	ELW	FLL	FHL	FAW	HAL	F1	F2	F3	F4	HLL	FEL	TIL	TFL	FOL	T1	T2	T3	T4	T5	IMT	Sex
IABHU4004	14.8	4.1	5.4	0.9	1.3	2	1.2	1.8	1.2	6.9	6	0.9	3.7	1	1.4	2.6	1.3	25.5	7.7	7.9	11.1	6.6	1	2.4	3.8	4.2	2.2	0.9	♂
IABHU4116	21.3	5.9	7.2	1.3	2.1	2.3	2.3	2.9	2.1	10.2	8.3	1.2	4.9	1.1	1.7	2.8	1.3	34	10.1	10.6	20	10.9	1.2	2.8	4.9	6	3.3	0.9	♀
IABHU4120	20.5	5.6	6.5	1	2	1.4	2.1	2.6	1.7	9.2	7.2	1	4.6	0.9	1.6	2.6	1.3	25.7	8.1	10.4	14	9.9	1.3	2.7	3.5	5.2	3	0.9	♀
IABHU4117	20.2	4	5.6	0.8	2	2.1	2.3	2.6	1.5	10.1	7.7	0.9	4.2	1	1.6	2.5	1.3	29.6	9.8	11.2	14.2	10	1.3	2.5	4.7	5.5	2.9	0.8	♀
IABHU4130	15.2	4.2	5.2	0.9	1.7	1.5	1.1	1.6	1.4	9	6.3	1	3.6	1	1.4	2.2	1.3	24.8	7.3	8.4	11.5	6.2	1.1	2	3.4	5.6	2	0.8	♂
IABHU4132	14.5	3.5	3.7	0.5	1.1	1.2	1.3	1.1	1.1	6.2	4.1	0.8	3.3	0.9	1.5	2.4	1.3	24.9	6.5	8.9	12	6.9	1.4	3.2	3.9	5.6	2.5	1.1	♂
IABHU4134	14.7	4.5	4.5	1	1.2	1.2	1.8	1.4	1.1	7.7	6.1	0.8	4.1	1.1	1.5	2.6	1.5	25.9	7.6	8.6	11.7	6.5	0.8	2	2.9	4.6	2.5	0.7	♂
IABHU4129	14.2	4.5	4.8	1.2	1.4	1.5	1.2	1.4	1.5	8.3	6.4	1	3.7	1	1.7	2.7	1.5	22	6.5	8.2	11	7	1.1	2.1	3.9	4.8	2.6	1.1	♂
IABHU4131	15.5	5.5	4.7	1.1	1.2	2.2	1.5	1.7	1	8.6	6.2	1	3.9	1	1.5	2.5	1.6	24.5	6.6	8	11.2	6.5	1	2.5	3.5	4.6	2.2	1	♂
IABHU4119	15.2	5.1	4.8	1.4	1.6	1.8	1.7	1.6	1.2	8.5	5.5	1	3.8	1	1.5	3.1	1.7	26.8	6.8	8.6	11.5	8.8	1.8	2.8	4	5.3	2.8	1.3	♂

Microhyla sp. M (Sylhet, Bangladesh)

Voucher No.	SVL	HL	HW	S-N	N-N	N-E	ED	E-E	ELW	FLL	FHL	FAW	HAL	F1	F2	F3	F4	HLL	FEL	TIL	TFL	FOL	T1	T2	T3	T4	T5	IMT	Sex
IABHU3898	17	6.3	5.3	0.6	1.4	1.5	1.4	2.5	1.3	8.2	6.5	0.9	4	0.7	1.6	2	1	25.1	7.4	9.1	12.3	8.5	0.6	1.8	3	5	1.7	1.1	♂
IABHU3944	16.6	6.3	5.3	0.5	1.2	1.6	1.5	2.2	1.2	8.3	6.5	0.9	4.1	0.7	1.8	2.4	1.1	29.3	7.7	10.5	13	9.2	0.8	2.7	3.8	5.4	2.5	0.7	♂
IABHU3945	15.8	6.3	5.3	10	1.5	1.2	1.3	2.1	0.9	8.1	7	1	4.3	0.8	1.6	2.4	1.2	27.6	7.1	9.4	12.6	9.2	0.9	2.5	3.7	5	2.3	0.9	♂
IABHU3947	17.6	7.2	6.1	0.9	1.3	1.3	1.7	2	1.4	8.2	6.9	1	4.2	0.9	1.5	2.4	1.2	28.2	7.2	10.2	14	8.8	0.8	2.3	2.7	4.9	1.9	1.1	♂
IABHU3948	17.3	6	5	0.7	1.6	1.8	1.6	2.4	1.2	10	7.6	1	4	0.8	1.6	2.8	1.2	32	8.8	11	15.1	9.2	1.2	3.2	4.6	5.9	3	1	♂
IABHU950	17.3	6.4	5.8	0.7	1.7	1.4	1.9	2.2	1.4	8.9	6.9	0.8	4.1	0.9	1.8	2.4	1.7	26.8	8.3	9.4	12.4	8.9	1.1	2.5	3.5	5	2.3	1.1	♂
IABHU3951	16.4	6.7	5.6	0.8	1.6	1.4	1.9	2.2	1.1	8.8	6.6	0.9	3.7	0.9	1.6	2.6	1.5	34.8	7.1	9.8	12.9	9.1	1.1	2.2	3.8	5.6	2.2	1.3	♂
IABHU3952	15	6.9	6	0.8	1.8	1.3	1.4	2.6	1.2	7.6	6	0.7	3.6	0.9	1.5	2.5	1.1	25.2	6.4	9	11.3	8.4	0.8	2.2	3.7	4.8	2.2	1	♂
IABHU3899	16.7	6	5.1	0.6	1.6	1.4	1.6	2	1.4	7.8	6.2	0.8	4.1	0.4	1.8	2.5	0.9	26.6	8.7	9.2	12.6	8.8	1	2.4	4	4.8	1.9	1	♀
IABHU3946	16.2	6	5	0.8	1.3	1.2	1.1	2.1	1	9.2	6.7	0.8	4.6	0.5	1.6	2.5	1.1	30	6.8	10.2	13	9	0.8	1.4	2.7	3.8	1.2	1.1	♀
IABHU3949	15.7	6	4.8	0.7	1.7	1.1	1.7	2	1.2	9.2	6.8	0.8	3.9	0.7	1.6	2.2	1.2	31.6	8.5	9.7	13.4	7.9	1.3	3.2	4.4	5.4	2.1	0.8	♀
IABHU3953	16.7	6.1	5.8	0.7	1.6	1.2	1.6	1.8	1.2	9.1	7.3	0.9	4.3	0.5	1.8	2.2	1	29.4	6.3	9.4	12.6	9.3	1	2.6	3.5	4.5	2.3	1	♀
IABHU3954	15.2	6.7	5.5	0.9	1.7	1.1	1.5	2.2	1.2	9	6.3	0.5	3.7	0.8	1.6	2.4	1.1	27.6	6.7	9.1	12.5	8.6	1.2	2.1	3.5	4.9	2.2	0.8	♀
IABHU3955	16.3	7	6.1	0.8	1.9	1.1	1.6	2.1	1.3	8.6	6.6	1.1	3.6	0.6	1.5	2.2	1.6	28.6	6.4	9	13.2	9.1	1.1	2.6	4.2	5.2	2.2	0.5	♀

Microhyla sp. C (Chittagong, Bangladesh)

Voucher No.	SVL	HL	HW	S-N	N-N	N-E	ED	E-E	ELW	FLL	FHL	FAW	HAL	F1	F2	F3	F4	HLL	FEL	TIL	TFL	FOL	T1	T2	T3	T4	T5	IMT	Sex
IABHU3880	19.3	9	7.8	1	1.6	1.7	1.8	2.6	1.1	9.3	7	0.9	4.2	0.8	1.7	2.8	1.5	28.5	9.2	10	11.3	10.1	0.8	2.2	4.1	5.6	3.4	0.6	♂
IABHU3882	16.5	6.8	5.8	0.6	1.6	1.4	1.5	2.5	1	8.3	6.5	0.6	3.3	0.6	1.6	2.2	1.4	25.6	7	9	12.5	8.8	0.7	2.5	4.1	4.6	3.3	0.6	♂
IABHU3879	21	8.9	8.1	0.6	1.8	1.5	1.9	2.7	1.3	10	7.6	0.8	4.3	0.8	1.5	2.1	1	28.7	7.7	10.2	11.4	8.5	1.1	3	5	4.3	3.3	1.2	♂
IABHU3959	16.5	5.4	5	0.8	1.5	1.3	1.5	2	1.1	8.4	6.7	0.7	3.9	0.5	1.6	2.6	1	28.4	6.3	9.2	12.8	8	0.9	2.6	4	5.4	2	0.8	♂
IABHU3960	16.6	6	5.3	0.9	1.4	1.3	1.5	2.4	1.2	7.7	6.6	0.5	3.4	0.7	1.5	2.4	0.8	26.1	7.5	8.8	11.9	8	0.6	1.8	3.6	4.8	2.2	0.9	♂
IABHU3878	17.3	7	6.7	0.7	1.7	1.2	2	2.9	1	7.7	6.4	0.6	3.8	0.6	1.3	2.5	1	29	7.4	9.9	10.7	8.7	1.3	2.5	4.1	5.1	2.4	1	♀
IABHU3881	17.5	6.9	6.5	0.8	1.1	1.4	1.6	2.5	1	8.7	6.3	0.8	3.6	1	1.5	2.6	1.5	26	7.2	9.4	11.2	9	0.8	2.3	4.1	4.8	1.6	0.4	♀
IABHU3956	17.9	8.1	6.9	0.7	1.5	1.7	1.5	2.2	1.1	8.5	7.2	0.7	4	0.8	1.4	3	1.2	28.8	7.1	10	14.4	9.1	1.1	2.5	4	5.8	3	0.8	♀
IABHU3957	18.4	6.5	7.1	0.6	1.7	1.6	1.5	2.2	1.4	8.9	7.8	0.7	4.2	0.7	1.5	3.2	1.1	31.3	7.7	9.9	14.5	10.2	1.2	3	4.8	6.1	3.1	0.8	♀
IABHU3958	17.3	6.1	6.2	0.8	1.7	2.1	1.4	2.7	0.9	8.4	7	0.8	3.9	0.4	0.8	1.8	1	30.2	7.5	9.8	13.5	9.6	0.9	2.8	4.4	5.2	2.7	0.7	♀

Microhyla fissipes (Pingtung, Taiwan)

Voucher No.	SVL	HL	HW	S-N	N-N	N-E	ED	E-E	ELW	FLL	FHL	FAW	HAL	F1	F2	F3	F4	HLL	FEL	TIL	TFL	FOL	T1	T2	T3	T4	T5	IMT	Sex
OMNH Am 20028	20.8	5.4	6.5	1.6	2.1	1.7	1.9	2.2	1.3	11.2	8	1.3	5	1	2.1	3.5	2.1	28.4	8.3	10.4	15.2	11.5	1.5	3.1	5.9	8.2	3.8	0.9	♂
OMNH Am 20029	20.6	5.1	7.4	1.5	1.9	1.6	2.3	2.3	1.7	12.2	6.5	1.4	4.9	1.2	2.5	3.5	2.2	33.1	9.5	11	15.2	11.1	1.7	3.3	5.5	7.9	4.1	1.1	♂
OMNH Am 20030	23.4	4.9	7.5	1.2	2.2	2	1.9	2.5	1.6	13.3	8.9	1.9	5.3	1.1	2.5	3.8	2.6	34.9	9.9	11.7	17	11.8	1.5	4.1	6.7	8.6	4.7	0.9	♂
OMNH Am 20031	23.1	4.4	6	1.4	2	2	1.9	2.5	1.6	13.7	9.6	1.6	5.7	1.2	2.2	3.3	2.3	35.6	9.6	11.1	15.7	10.9	1.9	3.9	5.9	8.1	3.7	1	♂
OMNH Am 20032	22.1	4.6	5.9	1.5	2.1	1.7	1.9	2.3	1.6	13.1	9.2	1.4	5	1.1	2.3	3.9	1.9	32.5	9.3	10.7	16.1	12.7	1.3	3.5	6.1	7.9	3.8	1	♀
OMNH Am 20033	22.2	5.6	6.9	1.4	2	1.9	1.8	2.4	1.2	12.1	8.9	1.5	5.4	1.2	1.9	3.6	2.1	34.9	9.9	11.2	16.4	10.7	1.4	3.6	5.9	7.6	4.4	1.1	♀
OMNH Am 20034	22.1	5.9	6.5	1.2	2	1.8	2.2	2.5	1.4	12.1	8.7	1.5	5.4	1	2.3	4.1	2.5	34.4	8.4	10.7	15.3	12.1	1.6	3.6	5.7	8.2	4.5	1	♂
OMNH Am 20035	22.6	5.6	7.1	1.1	1.9	1.9	1.7	2.7	1.6	13.1	9.6	1.8	5.3	1.5	2.8	4	2.3	36.2	9.4	11.2	16.5	11.5	2.1	3.2	5.9	8.8	4.5	0.8	♂
OMNH Am 20036	21.6	4.8	6.7	1.3	2	1.6	1.5	2.5	1.6	12.1	8.9	1.4	5.1	1.2	2.5	3.6	2.1	34.7	10	10.8	15.3	11.6	1.7	3.8	5.9	7.7	4.1	0.7	♂
OMNH Am 20037	21.6	5	6	1.4	1.9	1.9	1.6	2.3	1.6	12	8.5	1.2	5.4	1	2.5	3.9	2	33.4	9.2	10.6	14.9	11.1	1.4	3.6	5.9	7.7	4.2	1.2	♂
OMNH Am 20038	21.2	5.8	6.4	1.9	2.2	1.9	2.1	2.5	1.5	12	8.7	1.5	5	1.2	2.5	3.9	2.3	33.2	9.4	10.7	15.2	11.6	1.4	3.4	6	8	4.1	0.8	♂
OMNH Am 20039	21.1	4.9	6.8	1.4	1.9	1.9	1.8	2.3	1.5	12.4	8.9	1.4	5.4	1.2	2.7	4.1	2.7	35.6	9.9	10.6	15.9	11.7	1.1	3.9	6.3	8.7	4.1	0.8	♂
OMNH Am 20040	20.9	5.1	6.1	1.5	1.9	1.6	2.1	2.3	1.4	12.5	9.1	1.3	4.9	1.3	2.3	3.6	2.2	33.9	9.5	10.8	15.1	11.5	1.4	3.2	5.8	8.1	4.6	1	♂
OMNH Am 20041	22.3	4.9	6.8	1.6	2.1	1.9	1.9	2.3	1.5	12.5	9.1	1.5	5.2	1.3	2.5	3.3	2.1	33.6	8.9	11.1	15.7	11.1	1.5	3.8	5.7	7.8	4.3	0.9	♂
OMNH Am 20042	21.8	5.8	6.7	1.5	2.1	2	2.1	2.6	1.6	12.9	9	1.5	5.3	1.4	2.4	3.9	2.1	33.9	10.1	11.3	16.2	11.4	1.6	3.4	6.4	8	4.1	0.9	♂

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OMNH Osaka Museum of Natural History