GENETIC VARIATION, ADVERTISEMENT CALL, AND MORPHOMETRY OF *MICROHYLA NILPHAMARIENSIS* FROM BANGLADESH

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ABSTRACT

The rich biodiversity of frogs and toads in Bangladesh is unexpected considering the rather simple topographic features of the country. Indeed, a new species, *Microhyla nilphamariensis*, has recently been described from Nilphamari District (northern region), Bangladesh. However, additional genetic, acoustic, and morphometric data are necessary to precisely delineate the newly described species as well as to measure conservation management. In this study we performed genetic, acoustic, and morphometric analyses. Our analyses showed that *M. nilphamariensis* was genetically divergent from its near congener *M. ornata* at 3.2% and 10.6% for the 16S rRNA and Cytb genes, respectively. Mean call duration of *M. nilphamariensis* was 0.42 ± 0.01 s (n = 8) and the call was composed of about 15.13 ± 0.35 rapidly repeating pulses with a pulse rate of 37.9 ± 0.4/s. Dominant frequency bands of *M. nilphamariensis* were much higher than those of *M. ornata* and *M. fissipes*. Principal component analyses showed that *M. nilphamariensis* differed from its near congeners in having shorter first and second fingers, shorter first toe, and longer inner and outer metatarsal tubercles relative to the snout-vent length.

KEYWORDS: Mitochondrial DNA, Advertisement call, Morphometry, *Microhyla*, Bangladesh

INTRODUCTION

The Microhylidae is a large family of frogs and toads in order Anura that comprises 7.7% (572 species) of all frogs (AmphibiaWeb, 2015). Members of the family are distributed across Asia from the Ryukyu

Archipelago in Japan and China to the north, through India and Sri Lanka in the southwest, and through Southeast Asia to Sumatra, Borneo, Java, and Bali (Frost, 2015; AmphibiaWeb, 2015). Despite the wide distribution, only six nominal *Microhyla* species (*Microhyla ornata*, *M. berdmorei*, *M. rubra*, *M. mukhlesuri*, *M. mymensinghensis*, and *M. nilphamariensis*) are known to occur in Bangladesh (Kabir et al., 2009; Hasan et al., 2014a; Howlader et al., 2015). The accurate description of a newly described species is an important element in both ecology and evolutionary biology research, in particular, in the development of biodiversity and conservation management strategies and plans (Bickford et al., 2007).

During herpetological fieldwork in Bangladesh conducted over a decade, we first reported *M. ornata*-like specimens (IABHU 22135–22137) from Parbatipur in Dinajpur District (Hasan et al., 2012a). Later, as a continuation of our survey we only found this species from Berakhuti, Barua (Nilphamari District) in the northern region of Bangladesh. Therefore, it seems that this species may be restricted to Dinajpur and Nilphamari districts. We reported on similar endemicity of other microhylid frogs previously (Hasan et al., 2014a).

Howlader et al. (2015) recently described *M. nilphamariensis* (the species that was identified previously as *M.* cf. *ornata* by Hasan et al., 2012a) from Nilphamari, based on 16S rRNA gene (*16S*) and morphological data. However, additional cytochrome b gene (*Cytb*), acoustic, and morphometric data are necessary to increase our understanding of *M. nilphamariensis* because a species is the fundamental unit of biology (Mayr, 1982) and it is not sufficient to define biological species on the basis of a single molecular data alone (Ferguson, 2002). Therefore, in our study keeping the *16S* , another faster evolving *Cytb* gene was used to infer the phylogenetic relationships among closely related and poorly known species. In amphibians, morphological, ecological, and/or bioacoustics characteristics have been used to define species (Hasan et al., 2014b). Acoustic data are important to understand breeding behavior of a frog as well as facilitate species tracking based upon its calling during field survey. The morphometric comparison of *M. nilpahamariensis* (newly described species) with *M. fissipes* is scientifically meaningful more than the comparison with *M. rubra* because the latter can be easily distinguished by its shovel like inner metatarsal tubercle and most importantly, it is only reported in semi-evergreen forest in Chittagong, SE Bangladesh (Asmat et al., 2003) rather than the northern region. Further, the coloration in life of any newly described species is also useful for identification among cryptic species. Therefore, the description of the new species by Howlader et al. (2015) fell short in providing these evidences.

In amphibians, *16S* is considered as a marker for outlining the taxonomic status of frog species (Vences et al., 2005), while the protein-coding *Cytb*, which is known to exhibit more rapid nucleotide substitutions

than rRNA genes, is considered to be more phylogenetically informative between conspecific populations (Koike and Matsui, 2003). Together with the molecular and morphology data, advertisement calls also play an important role in classifying cryptic species. This technique has recently been used to identify morphologically similar frog species (Hasan et al., 2012b; Matsui et al., 2014).

Important biological and ecological information that are needed to accurately describe *M. nilphamariensis* remains lacking (e.g., natural history and acoustics data are necessary to know the breeding biology which support to define future conservation strategies) in the publication of Howlader et al. (2015). Notwithstanding, biological and/or ecological data are more useful rather than the genetic data for local taxonomists in a developing country like Bangladesh where genetics/molecular research is a matter of cost. Therefore, in this study, we analyzed the mitochondrial DNA (mtDNA) sequences of both *16S* rRNA and *Cytb*, and collected detailed acoustic and morphological information of this species.

MATERIALS AND METHODS

Specimen Collection. Specimens of *M. nilphamariensis* were collected from two localities in Bangladesh: Parbatipur in Dinajpur District and Berakhuti in Barua, Nilphamari District.

DNA Extraction, Amplification, and Sequencing. Specimens and/or tissue samples were stored at the Institute for Amphibian Biology, Hiroshima University, the Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, or the Museum of Herpetology Laboratory Bangladesh, Ichamati College, Dinajpur, Bangladesh. The methods for the extraction, PCR amplification, and sequencing of mtDNA *16S* and *Cytb* fragments follow previous protocols (see Hasan et al., 2012a, b; Nasrin et al., 2014). The obtained *16S* and *Cytb* sequences are deposited in the DDBJ/EMBL/GenBank database (Accession Nos. LC090055–LC090057 for *16S* and LC090058–LC090063 for *Cytb*). The *16S* and *Cytb* sequences of other closely related species were retrieved from GenBank (Table 1).

Sequence alignment. The *16S* and *Cytb* sequences of *M. nilphamariensis* and closely related species (Table 1) were first aligned separately using the ClustalW program (Thompson et al., 1994). The divergence of the aligned sequences (uncorrected *P* values) was calculated using MEGA Ver. 6.0 (Tamura et al., 2013) with the pairwise-deletion option in which all alignable sites were used for calibration, but indel sites were not counted. Gaps and ambiguous sites were excluded using Gblocks Ver. 0.91b (Castresana, 2000) with the default parameters. Gaps in the alignments were treated as missing data. The initial two alignments (*16S* and *Cytb*) were combined into one concatenated data set, which contained a total of 1251 sites

(755 for *16S* and 496 for *Cytb*), 293 of which were parsimoniously informative.

Phylogenetic analyses. The phylogenetic analyses were performed using the maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) methods. Nucleotide substitution models for ML and BI analyses were selected based on the Akaike information criterion and Bayesian information criterion, respectively, that are implemented in the Kakusan 3.0 program (Tanabe, 2007). ML analysis was performed using Treefinder (Jobb, 2008) and the resultant tree was evaluated by bootstrap (BS) analysis with 1000 replicates. BI analysis was performed using MrBayes Ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) with the following settings: number of Markov chain Monte Carlo (MCMC) generations = 10 million; sampling frequency = 100; and the first 1 million generations were discarded as burn-in. The number of MCMC generations and burn-in size were determined by checking the convergence of −log likelihood (−ln*L*) values and tree length against generation number using Tracer Ver. 1.4 (Rambaut and Drummond, 2007). Statistical support of the BI tree was evaluated by Bayesian posterior probability (BPP). MP was performed with 1000 bootstrap (BS) replicates using PAUP* 4.0b10 (Swofford, 2003).

Recording advertisement calls. The advertisement calls of *M. nilphamariensis* were recorded at Berakhuti, Barua in Nilphamari District (25° 49.320′ N, 88° 49.732′ E) using a Sony Cybershot digital camera in video mode (model no: DSC-W730). Sound spectrograms were depicted using Avisoft-SASLab Light Software (Avisoft Bioacoustics, Germany) with Hamming window (111 Hz bandwidth). The calls of *Microhyla* species were long series of notes consisting of about 10 pulses (Kuramoto and Joshy, 2006). We measured note length, number of pulses, pulse repetition rate and dominant frequency. For comparison, calls of *M. ornata* recorded at Mudigere, Chikmagalur District, Karnataka State, India and those of *M. fissipes* recorded at Manchou, Pingtung County, Taiwan were analyzed.

Morphological measurements. For morphological comparisons, the following 29 measurements were taken to the nearest 0.1 mm using digital calipers: 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, hind limb length; FEL, femur length; TIL, tibia length; TFL, tarsus and foot length; FOL, foot length; T1-T5, lengths of 1st to 5th toe; IMT, inner metatarsal tubercle length; and OMT, outer metatarsal tubercle length. Statistical analysis was performed in SPSS (15.0J) software (SPSS Japan Inc., Tokyo, Japan).

Table 1. Samples used in this study, and GenBank accession numbers. IABHU: Institute for Amphibian Biology, Hiroshiam University; MHLB: Museum of Herpetology Laboratory' Bangladesh; BNHS: Bombay Natural History Society; RBRL: Rondano Biodiversity Research Laboratory, St. Aloysius College; ZSIK: Zoological Survey of India, Kolkata.

			Accession numbers	
Species	**Locality**	**Voucher no.**	***16S***	***Cytb***
M. nilphamariensis	Berakhuti, Barua, Nilphamari, Bangladesh	IABHU 4212	LC090055	–
M. nilphamariensis	Berakhuti, Barua, Nilphamari, Bangladesh	IABHU 4213	LC090056	–
M. nilphamariensis	Berakhuti, Barua, Nilphamari, Bangladesh	MHLB00206	LC090057	–
M. nilphamariensis	Parbatipur, Dinajpur, Bangladesh	IABHU 22135	AB530537	–
M. nilphamariensis	Parbatipur, Dinajpur, Bangladesh	IABHU 22136	AB530538	AB819032
M. nilphamariensis	Parbatipur, Dinajpur, Bangladesh	IABHU 22137	AB530539	LC090058
M. ornata	Bajipe, India	Released	AB530627	LC090059
M. ornata	Karnoor, India	BNHS 5028	AB530628	LC090060
M. ornata	Talagini, Shimoga, India	RBRL 040723-04	AB530630	LC090061
M. ornata	Dharwad, Karnataka, India	ZSIK-A9119	AB201188	AB201223
M. rubra	Dharwad, Karnataka, India	Released	AB201192	AB201224
M. berdmorei	Gombak, Malaysia	IABHU21019	AB530638	AB819031
M. cf. *berdmorei*	Sylhet & Bandarban, Bangladesh	IABHU 3864	AB530541	AB819030
M. mymensinghensis	Mymensingh, Bangladesh	IABHU3899	AB543607	AB819019
M. mukhlesuri	Chittagong, Bangladesh	IABHU 3880	AB543609	LC090062
M. fissipes	Huangshan, Anhui, China	KUHE32943	AB201185	AB201213
M. fissipes	Thong Pha Phum, Kanchanaburi, Thailand	KUHE35165	AB201186	AB201215
M. okinavensis	Ishigaki Island, Okinawa, Japan	IABHU5263	AB303950	AB303950
M. heymonsii	China	Living Animals	AY458596	AY458596
M. heymonsii	University Malaya Campus, Malaysia	IABHU 21026	AB530637	LC090063
K. pulchra	Bandarban, Bangladesh	IABHU3781	AB530543	AB819033

RESULTS AND DISCUSSION

Molecular phylogeny. In the phylogenetic trees that we constructed (Fig. 1), *M. nilphamarienisis* from the Dinajpur District formed a clade with high bootstrap support (BS: 86 for ML; 100 for MP, and 1.0 for BI), and the topotypic *M. ornata* from Bajipe, Karnoor, and Talagini in India formed another clade also with high bootstrap support (BS: 92 for ML; 100 for MP, and 1.0 for BI). The *M. nilphamariensis* from Dinajpur District formed a sister clade with the topotypic *M. ornata* clade with high bootstrap support (BS: 90 for ML; 97 for MP, and 1.0 for BI). The sequence divergence between these two clades was 3.2% for *16S* and 10.6% for *Cytb*. We found that all the specimens from India and Bangladesh formed a clade, while the *Microhyla* frogs from Southeast Asia (China, Japan, Malaysia, and Thailand) and two newly described *Microhyla* species from Bangladesh formed another clade. This result may reflect a sister relationship between Indian subcontinental species and other Southeast Asian frogs.

The herpetofauna of Bangladesh is poorly known and identification of the morphologically conserved frogs of the genus *Microhyla* is a difficult task (Mahony et al., 2009; Hasan et al., 2012a). The accurate delineation of *Microhyla* species has been challenging because of their minute body size and high level of homoplasy as a result of the loss of pectoral girdle elements (Zweifel, 1986). Recently, Howlader et al. (2015) described a new species, *M. nilphamariensis*, from the northern part of Bangladesh, but *Cytb* data and acoustic information was not provided. *Microhyla nilphamariensis* was found to be close to *M. ornata* in both the phylogenetic and morphometric analyses. Because of their morphological homoplasy, a clear-cut separation among microhylid frogs is very difficult (Kuramoto and Joshy, 2006). In the present study, *M. nilphamariensis* formed a sister clade with the topotypic *M. ornata* from Bajipe, Karnoor, and Talagini in India, and exhibited a high degree of genetic divergence (3.2% for *16S* and 10.6% for *Cytb*). It has been suggested that there may be more than two lineages in the currently known *M. ornata* species from India (Hasan et al., 2014b). Further, the *M. nilphamariensis* embedded within the Indian microhylid clade, which was remotely related with Southeast Asian microhylid frogs. Similar evolutionary relationships have been observed among frogs in the genus *Fejervarya* (Hasan et al., 2014b).

Generally, it is hypothesized that during the uplift of the Himalayas, the Bengal basin (including present-day Bangladesh) was formed between 20 and 14 million years ago (Mya) (Alam et al., 2003; Uddin and Lundberg, 2004). Recently, Hasan et al. (2014a) proposed that *M. nilphamariensis* and *M. ornata* diverged from each other approximately 19.40 (45.23–6.90) Mya. This event seems to have occurred before the land formation of Bangladesh and was continued until the late Miocene period. The common ancestor of *M. nilphamariensis* may have evolved somewhere in South Asia, and this species split from its near congener before 19.40 Mya (Hasan et al., 2014a).

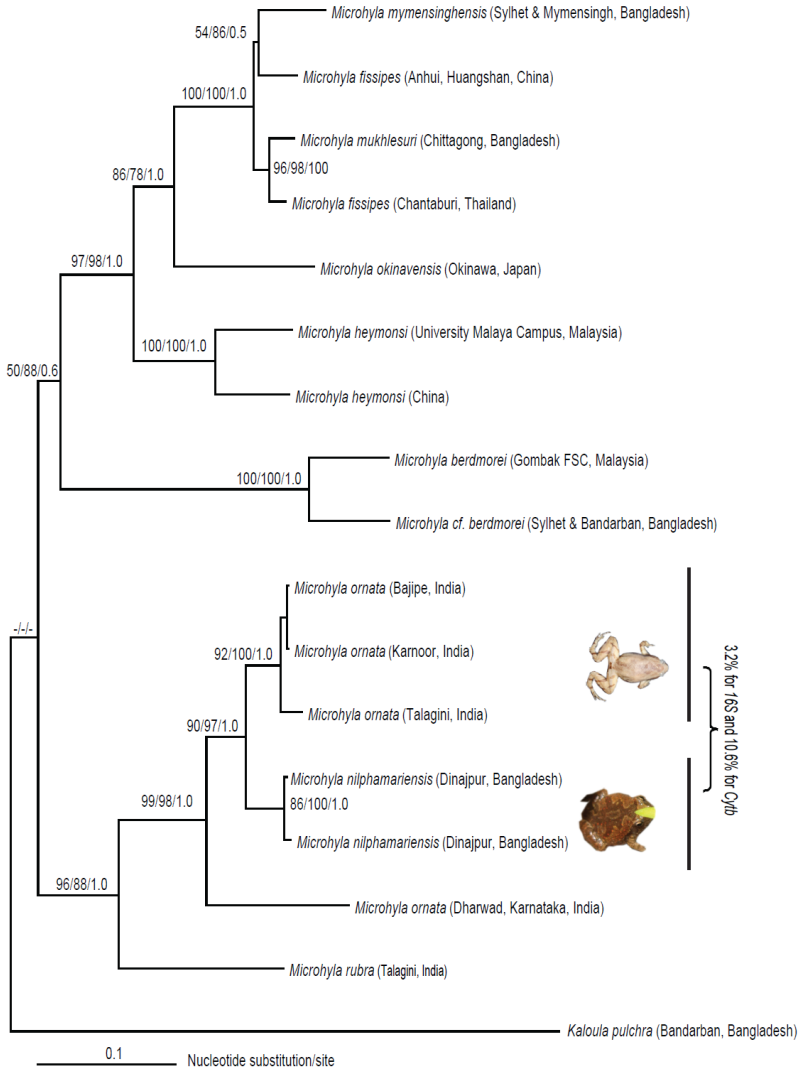

Figure 1: Maximum Likelihood (ML) tree based on the 1251 bp of mitochondrial (*16S* + *Cytb*) genes. The numbers near branches represent bootstrap supports for ML and MP inferences, and Bayesian posterior probability (ML-BPs/MP-BPs/BPP). The scale bar represents 0.1 nucleotide substitution/site.

Advertisement call. Sound spectrograms and acoustic parameters of advertisement calls are given in Fig. 2 and Table 2. Notes of *M. nilphamariensis* were slightly longer and involved more pulses than those of *M. ornata* and *M. fissipes.* Pulse repetition rates were nearly the same in *M.*

Table 2: Acoustic parameters of advertisement calls of three *Microhyla* species (mean ± S. D.).

Species	n	Note length (s)	No. pulses	Pulse rate (pulses/s)	Lower dom. freq. (kHz)	Higher dom. freq. (kHz)
M. nilphamariensis	9	0.41 ± 0.01	15.11 ± 0.33	36.13 ± 0.39	1.65 ± 0.04	3.62 ± 0.06
M. ornata	14	0.34 ± 0.05	11.79 ± 1.67	32.67 ± 0.90	1.24 ± 0.07	2.62 ± 0.07
M. fissipes	11	0.36 ± 0.05	13.09 ± 2.07	35.27 ± 1.50	1.36 ± 0.04	2.87 ± 0.01

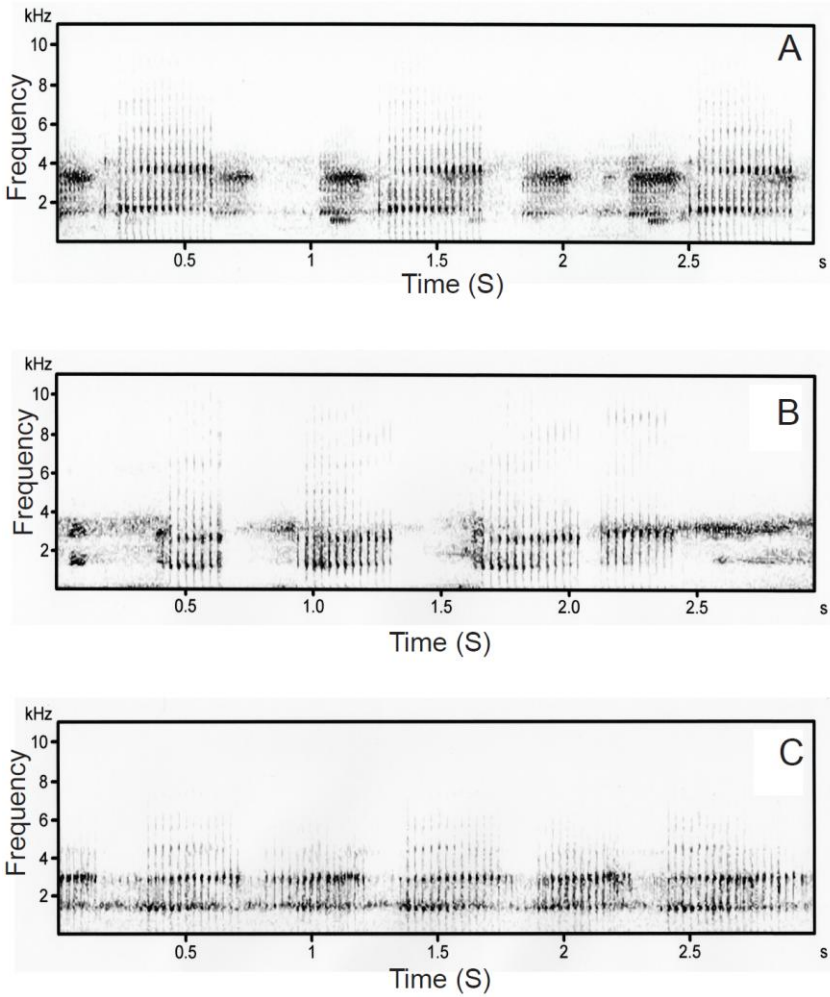

Figure 2: Sound spectrograms showing advertisement call structures of *M. nilphamariensis* (A), *M. ornata* (B) and *M. fissipes* (C).

nilphamariensis and *M. fissipes*, but the range in *M. nilphamariensis* (35.4-36.5 pulses/s) did not overlap with that of *M. ornata* (31.6-34.4 pulses/s). Pulse rate of *M. ornata* from Assam and Meghalaya, India (Roy, 1996; 4.6 pulses/s) and that of *M. ornata* from Thailand (Heyer, 1971; 53-63 pulses/s) differed completely from our values, and the taxonomic status of their materials should be examined in future studies.

The call of *M. nilphamariensis* had two distinct dominant frequency bands at 1.65 and 3.62 kHz (Fig. 2A). Similarly, the calls of *M. ornata* and *M. fissipes* exhibited two dominant frequency bands (Figs. 2B, 2C), but they were much lower than those of *M. nilphamariensis*. The ranges in *M. nilphamariensis* (1.59-1.71 and 3.52-3.70 kHz) did not overlap those of *M. ornata* (1.14-1.36 and 2.54-2.81 kHz) and *M. fissipes* (1.31-1.42 and 2.85-2.88 kHz). The fundamental acoustic parameters reported for *M. rubra* (note duration 0.17 s and pulse rate 108 pulse/s) (Kanamadi et al., 1994) were clearly very different from those of the other *Microhyla* species mentioned above.

***Morphometric comparisons*.** The measurements of 29 characters of *M. nilphamariensis*, *M. fissipes*, and *M. ornata* are listed in Table 3. *Microhyla nilphamariensis* was smaller in size (SVL) = 14.74 ± 2.57 mm) than topotypic *M. ornata* (SVL = 24.10 ± 1.17 mm) and *M. fissipes* (SVL = 21.82 ± 0.85 mm). Further, *M. nilphamariensis* differed significantly from its close relatives in relation to the lengths of the first, second, and fourth fingers (F1, F2, and F4), the first toe (T1), the inner metatarsal tubercle (IMT), and the outer metatarsal tubercle (OMT) ($P < 0.04$) (Table 3).

The three species were clearly separated by principal component analysis (PCA) (Figs. 3A, 3B). Eigen values were 18.782 for function 1, and 2.982 for function 2. Coefficients were large in SVL, F2, HLL, TIL, FOL, and T5 for function 1 and in FLL and TIL for function 2. In the PCA (Fig. 3B), *M. nilphamariensis* formed a cluster that was separate from the other two species, while the scores of *M. ornata* and *M. fissipes* overlapped considerably (Fig. 3A).

Body ratios relative to SVL (e.g., head length (HL)/SVL and head width (HW)/SVL) and 10 other ratios of *M. nilphamariensis* vs. *M. ornata* and *M. fissipes* as well as the results of the Mann–Whitney *U* test between ratios are shown in Table 4. The results showed that *M. nilphamariensis* differed significantly from both *M. ornata* and *M. fissipes*, having smaller F1, F2, and T1 and larger IMT and OMT ratios relative to SVL ($P < 0.04$) (Table 4). Further, *M. nilphamariensis* differed from *M. ornata* in having smaller forearm width (FAW) and shorter 2nd and 4th toes (T2 and T4) relative to SVL, and from *M. fissipes*, in having smaller nostril to eye distance (N-E), FAW, F4, hind limb length (HLL), and tibia length (TFL) relative to SVL ($P < 0.04$) (Table 4).

Morphologically, *M. nilphamariensis* was easily distinguished from its near congeners, having SVL of 11.7–18.3 mm, finger lengths with 1 < 2 < 4 < 3, and rudimentary toe webbings.

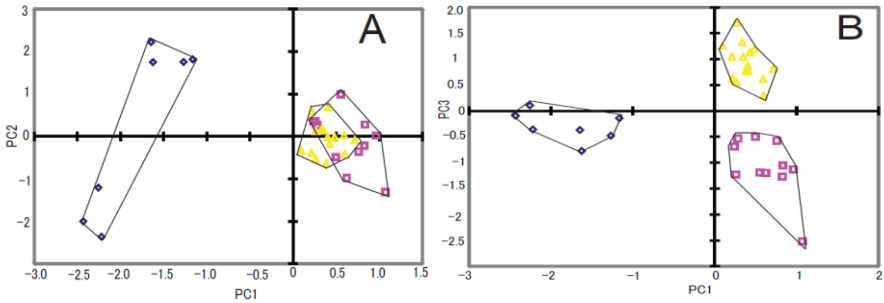

Figure 3: Results of the multivariate analyses of morphometric variability in three *Microhyla* species. Scatterplot of (A) principal component 1 (PCA1) versus principal component 2 (PCA2) and (B) principal component 1 (PCA1) versus principal component 3 (PCA3) from the principal component analysis of *M. nilphamariensis* (rhombus [blue]), *M. ornata* (rectangle [purple]) and *M. fissipes* (triangle [yellow]).

Color in life. In life, the dorsum varied from brown or slightly reddish with a black band-type mark running from the head to the vent. A few black spots were observed on the dorsal surface of the thigh and femur regions. The ventral surface was slightly whitish with a black mark on the throat and a few big black dots between the bases of the forelimbs in males of *M. nilphamariensis* (Figs. 4A, 4B). Photographs in life of *M. ornata* and *M. fissipes* were shown in Figs. 4C and 4D, respectively. The X-shaped mark on the dorsum of these three species facilitated to separate them from each other.

Distribution. *Microhyla ornata* is widely distributed in poorly documented areas including Kashmir (Pakistan and India); Nepal; throughout India, including the Andaman and Nicobar Islands, Sri Lanka and Bangladesh. *Microhyla fissipes* is distributed in southern and central China (including Taiwan and Hainan) from southern Yunnan northward and east to Shanxi and Shaanxi, Thailand and Indochina and through the Malay Peninsula to Singapore and possibly to Bangladesh (Frost, 2015) (Fig. 5A).

In this study, we collected individuals of *M. nilphamariensis* from Berakhuti, Barua in Nilphamari District. Berkahuti is around 7 km from Koya Golahut, Saidpur (also in Nilphamari District), which was considered as the type locality of *M. nilphamariensis* by Howlader et al. (2015). *M. nilphamariensis* has been found in the northern part of Bangladesh (Dinajpur and Nilphamari districts); however, its range may extend around the currently known distribution area (Fig. 5B).

Table 3: Measurements (mean ± S. D., in mm) for 29 body parts of three species of the genus *Microhyla* and obtained by Mann-Whitney *U* tests. Symbols * and ** indicate the 4% and 5% significant levels, respectively.

	M. nilphamariensis (n = 7)	*M. ornata* (n = 11)	*M. fissipes* (n = 15)	*M. nilphamariensis* vs. *M. ornata*		*M. nilphamariensis* vs. *M. fissippes*		*M. ornata* vs. *M. fissipes*	
				U	P	U	P	U	P
SVL	14.74 ± 2.57	24.10 ± 1.17	21.82 ± 0.85	0	0.0005 **	0	0.0002 **	10	0.0002 **
HL	4.29 ± 0.32	5.56 ± 0.69	5.20 ± 0.46	0	0.0005 **	5	0.0008 **	56.5	0.1771
HW	5.00 ± 0.42	7.91 ± 0.61	6.63 ± 0.50	0	0.0005 **	0	0.0002 **	4	0.0000 **
S-N	0.77 ± 0.26	1.36 ± 0.11	1.43 ± 0.19	0	0.0005 **	0	0.0002 **	57	0.1852
N-N	1.34 ± 0.25	2.10 ± 0.22	2.02 ± 0.11	0	0.0005 **	0	0.0002 **	69	0.4835
N-E	0.96 ± 0.46	1.89 ± 0.18	1.82 ± 0.17	0	0.0005 **	0	0.0002 **	66	0.3908
ED	1.63 ± 0.68	1.89 ± 0.16	1.92 ± 0.21	36.5	0.8562	50.5	0.8878	78	0.8152
E-E	1.63 ± 0.66	2.56 ± 0.32	2.42 ± 0.14	1	0.0007 **	2.5	0.0004 **	63.5	0.3236
ELW	1.09 ± 0.25	1.71 ± 0.17	1.51 ± 0.13	0	0.0005 **	4	0.0006 **	29.5	0.0059 **
FLL	7.43 ± 1.98	12.77 ± 0.88	12.46 ± 0.63	0	0.0005 **	0	0.0002 **	67.5	0.4362
FHL	5.64 ± 1.10	9.48 ± 0.53	8.77 ± 0.75	0	0.0005 **	2	0.0004 **	29.5	0.0059 **
FAW	0.80 ± 0.22	1.65 ± 0.27	1.46 ± 0.18	0	0.0005 **	0	0.0002 **	40.5	0.0291 *
HAL	3.24 ± 0.61	5.80 ± 0.47	5.22 ± 0.23	0	0.0005 **	0	0.0002 **	18.5	0.0009 **
F1	0.78 ± 0.23*	1.27 ± 0.22	1.20 ± 0.16	3	0.0130 *	4.5	0.0107 *	70	0.5164
F2	0.95 ± 0.26*	2.28 ± 0.33	2.40 ± 0.23	0	0.0041 **	0	0.0027 **	66.5	0.4059
F3	2.49 ± 0.38	3.70 ± 0.43	3.72 ± 0.27	1	0.0007 **	0	0.0002 **	77.5	0.7952
F4	1.50 ± 0.18*	2.04 ± 0.29	2.22 ± 0.22	1	0.0061 **	0	0.0027 **	53	0.1253
HLL	20.96 ± 3.20	35.61 ± 2.65	33.90 ± 1.84	0	0.0005 **	0	0.0002 **	54	0.1390
FEL	5.76 ± 0.29	10.65 ± 0.77	9.41 ± 0.55	0	0.0005 **	0	0.0002 **	14	0.0004 **
TIL	6.47 ± 0.50	11.49 ± 0.95	10.93 ± 0.34	0	0.0005 **	0	0.0002 **	53.5	0.1322
TFL	10.00 ± 1.58	16.47 ± 1.24	15.71 ± 0.62	0	0.0005 **	0	0.0002 **	53	0.1256
FOL	5.97 ± 1.75	11.88 ± 0.52	11.48 ± 0.49	0	0.0005 **	0	0.0002 **	47.5	0.0692
T1	1.08 ± 0.26*	1.55 ± 0.38	1.54 ± 0.24	2	0.0090 **	3	0.0069 **	67	0.4207
T2	1.60 ± 0.26**	3.16 ± 0.44	3.56 ± 0.30	0	0.0018 **	0	0.0011 **	34.5	0.0127 *
T3	2.76 ± 0.28	5.29 ± 0.30	5.98 ± 0.30	0	0.0005 **	0	0.0002 **	6	0.0001 **
T4	3.84 ± 0.61	7.30 ± 0.34	8.09 ± 0.36	0	0.0005 **	0	0.0002 **	9	0.0001 **
T5	2.20 ± 0.24	3.46 ± 0.37	4.19 ± 0.29	0	0.0005 **	0	0.0002 **	7	0.0001 **
IMT	0.75 ± 0.05*	1.02 ± 0.15	0.95 ± 0.13	1	0.0058 **	6	0.0162 *	56	0.1687
OMT	0.98 ± 0.36*	1.04 ± 0.30	0.94 ± 0.12	17	0.5109	22	0.4227	60	0.2419

Table 4: 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 4% and 5% significant levels, respectively.

	M. nilphamariensis (n = 7)	*M. ornata* (n = 11)	*M. fissipes* (n = 15)	*M. nilphamariensis* vs. *M. ornata* U	P	*M. nilphamariensis* vs. *M. fissippes* U	P	*M. ornata* vs. *M. fissipes* U	P
HL/SVL	0.300 ± 0.068	0.231 ± 0.026	0.239 ± 0.024	8	0.0057 **	25	0.0526	63	0.3115
HW/SVL	0.346 ± 0.052	0.329 ± 0.025	0.304 ± 0.024	32	0.5561	34	0.1922	35	0.0137 *
S-N/SVL	0.052 ± 0.016	0.056 ± 0.005	0.066 ± 0.010	35	0.7513	26	0.0618	31	0.0075 **
N-N/SVL	0.095 ± 0.030	0.087 ± 0.009	0.093 ± 0.006	35	0.7513	50	0.8601	50	0.0917
N-E/SVL	0.062 ± 0.023	0.079 ± 0.007	0.083 ± 0.006	21	0.1130	19	0.0182 *	53	0.1258
ED/SVL	0.107 ± 0.033	0.079 ± 0.008	0.088 ± 0.011	20	0.0938	32	0.1484	44	0.0457 *
E-E/SVL	0.107 ± 0.029	0.106 ± 0.012	0.111 ± 0.006	37	0.8919	45	0.5970	49	0.0821
ELW/SVl	0.073 ± 0.006	0.071 ± 0.008	0.069 ± 0.006	28	0.3416	30	0.1127	76	0.7359
FLL/SVL	0.499 ± 0.062	0.531 ± 0.036	0.571 ± 0.021	29	0.3896	15	0.0082 **	25	0.0028 **
FHL/SVL	0.383 ± 0.034	0.394 ± 0.019	0.402 ± 0.029	29	0.3896	30	0.1127	56	0.1690
FAW/SVl	0.054 ± 0.012	0.068 ± 0.010	0.067 ± 0.006	13	0.0209 *	16	0.0101 *	87	0.8153
HAL/SVl	0.222 ± 0.041	0.241 ± 0.017	0.239 ± 0.008	24	0.1891	30	0.1127	66	0.3918
F1/SVL	0.047 ± 0.015*	0.053 ± 0.009	0.055 ± 0.007	13	0.2400	16	0.1615	64	0.3370
F2/SVL	0.058 ± 0.018*	0.094 ± 0.012	0.110 ± 0.011	1	0.0061 **	0	0.0027 **	24	0.0024 **
F3/SVL	0.173 ± 0.043	0.154 ± 0.017	0.171 ± 0.014	30	0.4414	47	0.6982	36	0.0158 *
F4/SVL	0.090 ± 0.008*	0.085 ± 0.011	0.102 ± 0.010	15	0.3608	10	0.0455 *	19	0.0010 **
HLL/SVl	1.430 ± 0.105	1.477 ± 0.068	1.554 ± 0.076	27	0.2976	17	0.0123 *	34	0.0118 *
FEL/SVL	0.402 ± 0.080	0.442 ± 0.020	0.432 ± 0.028	31	0.4970	45	0.5970	70	0.5165
TIL/SVL	0.448 ± 0.071	0.476 ± 0.021	0.501 ± 0.015	28	0.3416	30	0.1127	23	0.0020 **
TFL/SVL	0.681 ± 0.037	0.683 ± 0.038	0.720 ± 0.022	38	0.9639	21	0.0264 *	36	0.0158 *
FOL/SVL	0.398 ± 0.060	0.493 ± 0.014	0.527 ± 0.030	8	0.0057 **	3	0.0005 **	27	0.0040 **
T1/SVL	0.064 ± 0.016*	0.064 ± 0.014	0.071 ± 0.010	17	0.5139	25	0.6171	41	0.0313 *
T2/SVL	0.102 ± 0.020**	0.131 ± 0.015	0.163 ± 0.011	5	0.0108 *	0	0.0011 **	3	0.0000 **
T3/SVL	0.191 ± 0.033	0.220 ± 0.008	0.274 ± 0.014	19	0.0774	0	0.0002 **	0	0.0000 **
T4/SVL	0.264 ± 0.039	0.303 ± 0.009	0.371 ± 0.020	16	0.0416 *	0	0.0002 **	0	0.0000 **
T5/SVL	0.152 ± 0.026	0.143 ± 0.014	0.192 ± 0.014	31	0.4970	11	0.0034 **	1	0.0000 **
IMT/SVL	0.045 ± 0.003*	0.043 ± 0.007	0.044 ± 0.006	19	0.6953	24	0.5485	78	0.8153
OMT/SVl	0.058 ± 0.019*	0.043 ± 0.012	0.043 ± 0.006	13	0.2400	15	0.1336	68	0.4517
HL/HW	0.863 ± 0.105	0.702 ± 0.046	0.787 ± 0.077	7	0.0043 **	28	0.0842	34	0.0118 *
S-N/N-E	0.943 ± 0.493	0.721 ± 0.078	0.793 ± 0.134	38	0.9639	44	0.5489	52	0.1134
N-E/E-E	0.571 ± 0.098	0.744 ± 0.064	0.753 ± 0.065	5	0.0024 **	4	0.0006 **	70	0.5165
ED/E-E	0.999 ± 0.171	0.748 ± 0.101	0.796 ± 0.094	5	0.0024 **	14	0.0066 **	66	0.3918
N-N/E-E	1.019 ± 0.584	0.831 ± 0.122	0.839 ± 0.062	35	0.7513	45	0.5970	73	0.6220
ELW/E-E	0.728 ± 0.210	0.671 ± 0.071	0.627 ± 0.054	35	0.7513	46	0.6468	52	0.1134
F1/F2	0.875 ± 0.370*	5.834 ± 0.961	0.501 ± 0.067	10	0.1172	7	0.0214 *	54	0.1391
TIL/FEL	1.124 ± 0.065	1.080 ± 0.052	1.164 ± 0.058	20	0.0938	36	0.2448	20	0.0012 **
FOL/FEL	1.042 ± 0.313	1.118 ± 0.046	1.225 ± 0.102	33	0.6184	46	0.6468	21	0.0014 **
TIL/FOL	1.164 ± 0.335	0.967 ± 0.055	0.953 ± 0.057	37	0.8919	46	0.6468	72	0.5858

Figure 4: Comparisons of three *Microhyla* species in life. (A) *Microhyla nilphamariensis* from Parbatipur, Dinajpur [IABHU 22135], (B) Berakhuti, Barua, Nilphamari [IABHU 4212], Bangladesh; (C) *M. ornata* from Bajipe, Mangalore, Dakshin Kannad District, Karnataka State, India and (D) *M. fissipes* from Manchou, Pingtung County, Taiwan. Scale bar = 10 mm.

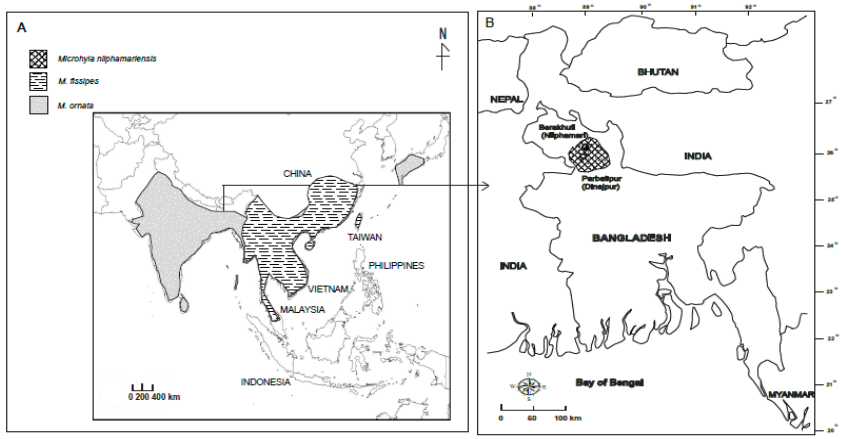

Figure 5: (A) Map showing the approximate distribution areas of *M. nilphamariensis*, *M. ornata* and *M. fissipes*. (B) Bangladesh map showing the two collecting localities (open circle) and reported type locality by Howlader et al., 2015 (solid triangle) of *M. nilphamariensis*.

***Natural history*.** We observed *M. nilphamariensis* either on grass in an open field or near shallow water of croplands. This species is abundant in agricultural croplands and home state forest areas. Although no other *Microhyla* species were found, many *Fejervarya* sp. large type were observed in the same locality (Berakhuti, Barua, Nilphamari in Bangladesh).

CONCLUSIONS

In conclusion, *M. nilphamariensis* can be separated from other microhylid frogs based on the sequences of its mtDNA (*16S* and *Cytb*), and acoustic and morphological data. In the future, it will be important to do more sampling in the whole of Bangladesh to clearly understand the distribution status of this frog species as well as to study the fine-scale population structure and genetic variation within the population. Habitat loss and fragmentation have reduced population sizes and have led to extinction of many amphibian species in human altered landscapes (Becker et al., 2007); similar anthropological and environmental changes are frequently occurring in Bangladesh (Islam et al., 2012). Therefore, the existing natural population might be under threat and necessary measures will need to be taken for proper conservation of this species. Likewise other microhylid frogs, for example, *Kaloula pulchra* and *Uperodon globulosus* have been marked as Vulnerable and Endangered, respectively, in Bangladesh (IUCN-Bangladesh, 2000). Therefore, the Government of Bangladesh formulated the Wildlife (Conservation and Security) Act, 2012 to protect threatened amphibian and reptiles and has declared 17 National Parks, 20 Wildlife Sanctuaries, 12 Ecologically Critical Areas, 6 Eco-Parks, 2 Safari Parks and 2 Botanical Gardens that are the home of many amphibians and reptiles (Hasan et al., 2014c).

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Appendix: Examined specimens list

***Microhyla ornata*:** (13 specimens): Rondano Biodiversity Research Laboratory, St. Aloysius College: RBRL 040723-04, 00062617─18, 04071113─122.
Collection locality: Talagini and Karnoor, Karnataka, India.

***Microhyla fissipes*:** (15 specimens): Osaka Museum of Natural History: OMNH am 20028─20042.
Collection locality: Pingtung, Taiwan.

Microhyla nilphamariensis (9 specimens): Institute for Amphibian Biology, Hiroshima University: IABHU 22135─22137, 4212-4213 and MHLB 201-202, 205-206.
Collection locality: Parbatipur, Dinajpur and Berakhuti, Barua, Nilphamari Sadar Upazila, Nilphamari, Bangladesh.