学位論文

Cryptic anuran biodiversity in Bangladesh with description of a new species of genus *Hoplobatrachus* (Anura, Dicroglossidae)

（バングラデシュの無尾両生類における種多様性と *Hoplobatrachus* 属の新種記載）

MAHMUDUL HASAN

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主論文

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Hoplobatrachus 属の新種記載)

Mahmudul Hasan

CONTENTS

I. GENERAL INTRODUCTION

The study of amphibians in Bangladesh is still promising, and the number of frog species that exists in this country remains an open question. Few detailed studies on herpetofauna of Bangladesh were conducted during the British and Pakistan period, and thereafter, almost no comprehensive natural history study was conducted in present-day Bangladesh since the country has been plagued by political unrest, civil revolutions and economic instability. In spite of the paucity of an up-to-date anuran checklist in this disdain developing country, a few reports on herpetofauna have been disclosed in some non-peer review outlets but without the accompanying voucher specimen numbers or even photographic evidence, and therefore, have poor scientific merit. In addition, geographic distribution of anuran species in Bangladesh is unclear and very recently there was few reports on geographic distribution of amphibian in Bangladesh (Reza, 2008; Hasan et al., 2012c; Hasan and Sumida, 2012d). First, the herpetofaunal check list, in which 22 amphibian species were recorded, was prepared by IUCN Bangladesh in 2000, and recently, Kabir et al. (2009) assembled a list of 34 amphibian species across 20 genera of 6 families in Bangladesh based on morphology and scattered information from field research. In this list, some species were misidentified due to sharing similar external morphologies. Kabir et al. listed the presence of *Fejervarya limnocharis* in Bangladesh, but in fact, no "real" *F. limnocharis* exists in Bangladesh (Islam et al., 2008b; Hasan et al., 2012a). Thus, the present study constitutes the first attempt to review the anuran biodiversity in Bangladesh on the basis of molecular data.

Bangladesh is a riverine country nestled between the Indo-Himalayan and Indo-Chinese sub-regions of the Oriental region (Nishat et al., 2002). The country consists

predominantly of low plains comprising the Ganges-Brahmaputra River delta, one of the world's largest deltas, and lacks high mountainous regions. In the last decade, more than 60 new anuran species, including the new family Nasikabatrachidae, have been described in the neighboring India (e.g., Biju and Bossuyt, 2003, 2009; Kuramoto et al., 2007). Recently, the abundance of anuran biodiversity in northeast India, which is located adjacent to north and east Bangladesh, has been revealed in several studies. For example, Pawar and Birand (2001) listed 57 anuran species, including several possibly new species, from this area, and Ao et al. (2003) reported 19 new records of frogs from Nagaland, 5 of which are new to India. Mathew and Sen (2009) described 11 new species from northeast India. Similarly, in Myanmar, the other country bordering the southeastern corner of Bangladesh, 3 new species have been described (Wogan et al., 2003; Wilkinson et al., 2003, 2005), and more than 10 new species which were described in the last decade from Yunnan, China, and Thailand are presumed to exist in Myanmar (see Frost, 2011) and Wogan et al. (2008) added 12 anuran species to the herpetofauna of Myanmar. Notably, most of these newly added species were found in mountainous regions, including the Western Ghats and Nagaland in India, and only a few species were described from the lowlands. Considering the topographic features in Bangladesh, it can be expected that the anuran biodiversity is relatively low. Recent molecular phylogenetic studies focusing on the family Dicroglossidae have suggested the existence of many cryptic species in Bangladesh. Islam et al. (2008 a, b), using mitochondrial gene sequencing and allozyme analyses, identified 3 *Fejervarya* species that differed from *F. limnocharis* and other known congeners, and designated them as *Fejervarya* sp. large, medium and small types. In addition, Hasan et al. (2008) detected a small allozymic divergence among three populations of *Hoplobatrachus*

tigerinus in Bangladesh, while Alam et al. (2008) found notable mitochondrial 16S rRNA gene divergence among *Euphlyctis cyanophlyctis* and *E. hexadactylus* from Bangladesh and neighboring countries. Together, these studies highlight the current underestimation of anuran biodiversity and necessity for more extensive review of anuran taxonomy in Bangladesh.

Mitochondrial DNA is an effective molecular marker to examine genetic divergence and phylogenetic relationships of animal taxa (e.g., Avise, 2000). In amphibians, the mitochondrial 16S rRNA gene (*16S*) is considered a reliable marker for determining taxonomic status of frog species (Vences et al., 2005).

A few distinct species were identified in the first experiment, and among them, remarkable *16S* divergence (6.0%) was detected between two types of *H. tigerinus* (Mymensingh and Cox's Bazar specimens). One of the types (Mymensingh) is widely distributed throughout Bangladesh, whereas the other (Cox's Bazar) occurs only in the southeastern corner of Bangladesh. The systematics of this distinct taxon are also clearly different from all old synonyms of *Hoplobatachus tigerinus*, consequently distinguishing it from its known congeners (Hasan et al., 2012d).

The genus *Hoplobatrachus* comprises large robust frogs with numerous ridges or warts on the back and extensive webbing between toes. They are semi-aquatic and live mostly near water in habitats such as the edge of ponds, marshes, rivers, and flooded rice paddies. Four nominal species are currently recognized (Frost, 2011): *H. crassus*; *H. occipitalis*; *H. rugulosus;* and *H. tigerinus*. The type locality of *H. tigerinus* is "Bengale" (Bengal), India (Frost, 2011), although the type specimen is now lost (Frost, 2011). Molecular comparisons clearly indicate that the wide-ranging type corresponds to the

nomen *H. tigerinus*. Therefore, the other type (Cox's Bazar specimen) is a new species. Morphological comparisons of the new species were performed between *H. tigerinus* and *H. rugulosus*, and new molecular data are presented.

The aims of this research are 1) to survey cryptic anuran biodiversity in Bangladesh based on mitochondrial *16S* data and 2) to describe a new species of genus *Hoplobatrachus* (Anura, Dicroglossidae) from the coastal belt of Bangladesh.

II. CRYPTIC ANURAN BIODIVERSITY IN BANGLADESH REVEALED BY MITOCHONDRIAL 16S rRNA GENE SEQUENCES

ABSTRACT

To survey diversity of anuran species in Bangladesh, we compared mitochondrial 16S rRNA gene sequences (approximately 1.4 kbp) from 107 Bangladesh frog specimens. The results of genetic divergence and phylogenetic analyses incorporating data from related species revealed the occurrence of at least eight cryptic species. *Hoplobatrachus tigerinus* from two districts diverged considerably, indicating the involvement of a cryptic species. Two *Fejervarya* sp. (large and medium types) and *Hylarana* cf. *taipehensis* formed lineages distinct from related species and are probably new species. *Microhyla* cf. *ornata* differed from *M. ornata* with respect to type locality area and involved two distinct species. In addition, we found that *Hylarana* sp. and *Microhyla* sp. did not fit congeners examined to date in either morphology or 16S rRNA sequence. The occurrence of *M. fissipes* was tentatively suggested. Consequently, at least, 19 species were found from Bangladesh in this study. These findings revealed a rich anuran biodiversity in Bangladesh, which is unexpected considering the rather simple topographic features of the country.

INTRODUCTION

Bangladesh is a riverine country nestled between the Indo-Himalayan and Indo-Chinese sub-regions of the Oriental region (Nishat et al., 2002). The country consists predominantly of low plains comprising the Ganges-Brahmaputra River delta, one of the world's largest deltas, and lacks high mountainous regions. In the last decade, more than 60 new anuran species, including the new family Nasikabatrachidae, have been

described in the neighboring India (e.g., Biju and Bossuyt, 2003, 2009; Kuramoto et al., 2007). Recently, the abundance of anuran biodiversity in northeast India, which is located adjacent to north and east Bangladesh, has been revealed in several studies. For example, Pawar and Birand (2001) listed 57 anuran species, including several possibly new species, from this area, and Ao et al. (2003) reported 19 new records of frogs from Nagaland, 5 of which are new to India. Mathew and Sen (2009) described 11 new species from northeast India. Similarly, in Myanmar, the other country bordering the southeastern corner of Bangladesh, 3 new species have been described (Wogan et al., 2003; Wilkinson et al., 2003, 2005), and more than 10 new species which were described in the last decade from Yunnan, China, and Thailand are presumed to exist in Myanmar (see Frost, 2011) and Wogan et al. (2008) added 12 anuran species to the herpetofauna of Myanmar. Notably, most of these newly added species were found in mountainous regions, including the Western Ghats and Nagaland in India, and only a few species were described from the lowlands. Considering the topographic features in Bangladesh, it can be expected that the anuran biodiversity is relatively low. Recently, Kabir et al. (2009) assembled a list of 34 amphibian species across 20 genera of 6 families in Bangladesh based on morphology and scattered information from field research. In this list, however, no species endemic to Bangladesh have been recognized.

Recent molecular phylogenetic studies focusing on the family Dicroglossidae have suggested the existence of many cryptic species in Bangladesh. Islam et al. (2008 a, b), using mitochondrial gene sequencing and allozyme analyses, identified 3 *Fejervarya* species that differed from *F. limnocharis* and other known congeners, and designated them as *Fejervarya* sp. large, medium and small types. In addition, Hasan et al. (2008) detected a considerable allozymic divergence among three populations of *Hoplobatrachus tigerinus* in Bangladesh, while Alam et al. (2008) found notable

mitochondrial 16S rRNA gene divergence among *Euphlyctis cyanophlyctis* and *E. hexadactylus* from Bangladesh and neighboring countries. Together, these studies highlight the current underestimation of anuran biodiversity and necessity for more extensive review of anuran taxonomy in Bangladesh.

Mitochondrial DNA is an effective molecular marker to examine genetic divergence and phylogenetic relationships of animal taxa (e.g., Avise, 2000). In South and Southeast Asia, mitochondrial gene information has been used to identify numerous cryptic anuran species (Meegaskumbura et al., 2002; Kurabayashi et al., 2005; Stuart et al., 2006; Kuramoto et al., 2007; Sumida et al., 2007; Alam et al., 2008; Islam et al., 2008b; Inger et al., 2009; Joshy et al., 2009; Kurniawan et al., 2010). In amphibians, the mitochondrial 16S rRNA gene (*16S*) is considered a reliable marker for determining taxonomic status of frog species (Vences et al., 2005).

In the present study, to survey anuran biodiversity in Bangladesh, we collected frog specimens from throughout Bangladesh and performed molecular phylogenetic analyses using *16S* data. Here, specimens belonging to Ranidae, Rhacophoridae, Microhylidae, and Bufonidae from Bangladesh are examined for the first time. Thus, this study constitutes the first attempt to review the anuran biodiversity in Bangladesh based on molecular data.

MATERIALS AND METHODS

Specimens

Species identification was based mainly on morphological characteristics described by Dutta and Manamendra-Arachchi (1996), Chanda (2002), and Kabir et al. (2009). We followed the species names adopted in the system of Frost (2011), with the exceptions of *Fejervarya sahyadris* (=*Minervarya sahyadris*), which is nested in the

South Asian *Fejervarya* clade (Kuramoto et al., 2007; Kotaki et al., 2010), and *F. moodiei*, which is revived from the synonymy of *F. cancrivora* (corresponding to Mangrove type) (Kurniawan et al., 2011). Most dicroglossid specimens in the present study were collected from localities that differ from those of previous studies.

A total of 107 specimens were collected from 18 localities of 14 districts of Bangladesh (Fig. 1). Based on their external morphology and relevant literature, *Euphlyctis cyanophlyctis, E. hexadactylus, Hoplobatrachus crassus, H. tigerinus, F. moodiei, Hylarana leptoglossa, Polypedates teraiensis, Kaloula pulchra, K. taprobanica*, and *Duttaphrynus melanostictus* were identified. Specimens resembling *Hylarana taipehensis* and *Microhyla ornata* are treated here as *H.* cf. *taipehensis* and *M.* cf. *ornata*, respectively. Specimens belonging to the genera *Hylarana* and *Microhyla*, but not fitting the descriptions of known congeners, are treated here as *Hylarana* sp. and *Microhyla* sp., respectively. The three unnamed *Fejervarya* taxa are referred to as *Fejervarya* sp. large, medium, and small types, following the designation of Islam et al. (2008a).

DNA extraction, PCR, and sequencing

Total genomic DNA was extracted from the clipped toe of each frog specimen using a DNeasy Tissue Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. The extracted DNA solutions were used as polymerase chain reaction (PCR) templates for amplifying a partial *16S* region corresponding to position 3093 – 4467 of the *16S* gene of *Xenopus laevis* (accession no. M10217; Roe et al., 1985).

PCR amplification and sequencing were performed using the primers F51 and R51 (Sumida et al., 2002), 12S_3'end_Fow1 (5′– AGA AGA RGY AAG TCG TAA CA –3′), 12S_3'end_Fow2 (5′–GYA AGT CGT AAC AYG GTA AG–3′), 16S_R530 (5′–

GGC GAT GTT TTT GGT AAA CAG–3′), and 16S_R723 (5′–GGA GAA DDD YDW HTT CTT RTT AC–3′). The length of the resultant *16S* fragments varied from 1332 to 1390 bp between identified haplotypes. PCR mixtures were prepared with the TaKaRa Ex Taq ™ Kit (TaKaRa Bio, Inc., Shiga, Japan), as recommended by the manufacturer's protocol. The *16S* fragments were amplified using 35 cycles, with each cycle consisting of denaturation for 10 s at 98 °C, annealing for 30 s at 47.5 °C (10 cycles), 45.0 °C (10 cycles), and 42.5 °C (15 cycles), and extension for 1 min 20 s at 72 °C. The PCR products were purified using MicroSpin™ S-300 HR columns (GE Healthcare, Buckinghamshire, UK). Both strands of the amplified *16S* fragments were directly sequenced using the BigDye Terminator Cycle Sequencing Kit (ABI) with an automated DNA sequencer (3100-Avant; ABI, Brooklyn, USA). The obtained sequences were deposited in the DNA Data Bank of Japan (DDBJ) database under the accessions numbers AB530494 to AB530547 and AB543599 to AB543609.

Alignment data and identified haplotypes

The *16S* sequences from the 107 Bangladeshi frog specimens and *X. laevis* were aligned using the ClustalW program (Thompson et al., 1994). The initial alignment consisted of 1496 nucleotide sites and showed 65 distinct haplotypes. This initial alignment was used for computing the sequence divergence (uncorrected *p* values) among the haplotypes using MEGA Ver. 4.0 (Tamura et al., 2007) with the pairwise-deletion option, in which all alignable sites were used in the calibration, but indel sites were not counted. The indel and ambiguous alignment sites were then removed using Gblocks Ver. 0.91b (Castresana, 2000) with default parameters, resulting in 1,010 well-aligned sites. After the deletion of indel and ambiguous sites, several of the haplotypes

had identical *16S* sequences, and the initial 65 haplotypes were reduced to 45 haplotypes, which were used for constructing a neighbor joining (NJ) tree (see below).

Detailed phylogenetic analyses were performed with respect to the families Dicroglossidae, Ranidae, and Microhylidae using the *16S* data of our specimens and related species in neighboring countries. The *16S* data of related species were obtained from the DDBJ/EMBL/GenBank databases. We selected the related taxa and their *16S* sequences on the basis of (1) BLAST searches, (2) most relevant congeners of Bangladeshi frogs reported by Kabir et al. (2009), and (3) results of our previous studies (Alam et al., 2008). The procedures to construct alignment datasets for each family and to calculate *16S* divergences were identical to those described above. The *16S* sequence lengths of the alignment datasets varied among the three families and were shortened from the initial alignment depending on the lengths of *16S* sequences obtained from DNA databases. The sequence lengths and total number of operational taxonomic units (OTUs) determined from the alignment data were 291 sites of 38 OTUs for dicroglossids, 308 sites of 34 OTUs for ranids, and 457 sites of 18 OTUs for microhylids.

Phylogenetic analyses

We first reconstructed a NJ tree using the alignment data of the 45 haplotypes of Bangladeshi frogs. An appropriate substitution model was estimated using Akaike information criterion (AIC) implemented in Modeltest 3.7 (Posada and Crandall, 1998), and the GTR + I + G model was selected. Support for the nodes of the resultant tree was evaluated by bootstrap probabilities (BPs) calculated from 1000 replicates for NJ analyses. *Xenopus laevis* was used as the outgroup in this analysis.

Further phylogenetic analyses of the families Dicroglossidae, Ranidae, and Microhylidae were performed by the maximum likelihood (ML), NJ, and Bayesian inference (BI) methods. The ML, NJ, and BI analyses were performed using PAUP* 4.0b10 (Swofford, 2003) and MrBayes Ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) software, respectively. Appropriate substitution models were selected using AIC (SYM + I + G, GTR + I + G, and GTR + I + G for the families Dicroglossidae, Ranidae, and Microhylidae, respectively). Node support of the resultant trees was evaluated by BPs calculated from 500 and 1000 replicates for the ML and NJ analyses, respectively. BI analysis was performed with the following settings: Markov chain Monte Carlo of 2×10^6 generations and sampling frequency of 100. The burn-in size was determined by checking the convergence of −log likelihood ($-\ln L$) values, and the first 10% generations were discarded. Statistical support of the BI tree was evaluated by Bayesian posterior probability (BPP).

RESULTS

Haplotypes and phylogeny of Bangladesh frogs

Among the *16S* sequences from 107 frog specimens, we identified 65 haplotypes (sequences with ≥ 1 nucleotide change were assigned as different haplotypes). These haplotypes and their DNA database accession numbers are shown in Table 1. The initial 65 haplotypes were reduced to 45 after indel and ambiguous sites were excluded from analysis. For the remaining haplotypes, we constructed an NJ tree (Fig. 2), which showed five well-supported major clades corresponding to the five families involved. Inter-familial relationships and generic level relationships within each family were congruent with nearly all recent molecular phylogenetic studies (e.g., Frost et al., 2006; Roelants et al., 2007). The paraphyletic nature of the genus *Fejervarya* with respect to

the genera *Hoplobatrachus* and *Euphlyctys*, which has been suggested in several studies (Frost et al., 2006; Kotaki et al., 2008, 2010), was also supported.

In Fig. 2, each species formed a clade, and in many cases, the average *16S* divergence within each species was less than 1.0%. However, slightly divergent haplotypes were detected in *F. moodiei* (2.1%), and the *16S* divergence between *H. tigerinus* from Mymensingh and Cox's Bazar was remarkably high (6.0%). Although the haplotypes of *M.* cf. *ornata* from Mymensingh and those from Sylhet were only slightly divergent (1.5%), markedly high divergence was found between *M.* cf. *ornata* from Chittagong and the above two populations (5.1% and 5.4%, respectively). Furthermore, *M.* cf. *ornata* from Dinajpur constituted a distinct clade from other *M.* cf. *ornata* specimens and exhibited 14.0% *16S* divergence with respect to the above-mentioned populations. The high *16S* divergences among the Chittagong, Dinajpur, and Mymensingh + Sylhet specimens indicated that the *M.* cf. *ornata* specimens with similar external morphology consist of three distinct species. The remaining *Microhyla* sp. from Sylhet formed a sister taxon with respect to the above three taxa in the NJ tree (Fig.2).

Genetic divergence and phylogenetic position of Bangladeshi frogs with respect to congener species

To clarify the phylogenetic relationships of the taxa in Dicroglossidae, Ranidae, and Microhylidae, we selected 20 representative haplotypes (marked with asterisks in Fig. 2) from the 45 haplotypes initially analyzed and performed further phylogenetic analyses incorporating 28, 31, and 11 *16S* sequences from the DNA database. The resultant ML trees are shown in Figs. 3 – 5. In these analyses, the majority of nodes were not strongly supported by BP or BPP values. This low statistical support may have been

due to the truncated alignment data used. However, in many cases, the sister species recovered in the resultant trees showed the lowest *16S* divergence.

For *P. teraiensis* and *D. melanostictus*, we compared our *16S* data to available sequences in DNA databases, and found that our examined *P. teraiensis* was 3.1% divergent with *P. leucomystax* from the type locality (Java, Indonesia). We could not verify our *16S* data with those of *P. teraiensis* from the type locality (East Nepal) or any other regions due to a lack of available *16S* sequences in DNA databases. In contrast, *16S* divergences of *D. melanostictus* from Bangladesh were compared with publicly available *16S* data, and it was found that our examined specimen was close (*16S* divergence = 1.1%) to one Indian population, but had diverged from the Vietnam and Yunnan (China) populations (*16S* divergence = 2.2% and 2.4%, respectively).

The family Dicroglossidae (Fig. 3)

Euphlyctis cyanophlyctis, *E. hexadactylus*, and *H. crassus* from Bangladesh showed little genetic divergence from those of India. In *H. crassus*, the Khulna (Bangladesh) population showed only 2.9% *16S* divergence from the Assam (India) population. In *H. tigerinus*, two Bangladesh (Mymensingh and Cox's Bazar) populations showed very high *16S* diversity (6.0%). Notably, the Mymensingh and Cox's Bazar (Bangladesh) populations had diverged 3.8% and 4.8%, respectively, from the Padil (India) population.

Fejervarya sp. large type was nested in the Southeast-Asian group of *Fejervarya* and formed a clade with *F. orissaensis* (*16S* divergence = 4.0%), which is a sister group to "*F. limnocharis*" from Bangkok, Thailand (= *Fejervarya* sp. hp2, corresponds to *F. orissaensis* or an undescribed species [Kotaki et al., 2010]). The *16S* divergence between *F.* sp. large type and "*F. limnocharis*" (Thailand) was 3.5%. Three distinct

species have been recognized in "*Fejervarya cancrivora*" (designated as large, mangrove, and Sulawesi types). The large type of *F. cancrivora* was designated as the nominal *F. cancrivora* (Kotaki et al., 2010), while the mangrove and Sulawesi types were designated as *F. moodiei* and an undescribed species, respectively (Kurniawan et al. 2011). *Fejervarya moodiei* from two Bangladeshi populations (Cox's Bazar and Khulna) formed a clade with two *F. cancrivora* mangrove type from Thailand and India (BPs = 97 for ML, 100 for NJ, ≥ 95% for BI, and sequence divergence = 0.2% – 2.1%, average 1.07%). This clade became monophyly with *F. cancrivora* (large type) from Indonesia (their average sequence divergence = 9.13%), but the statistical support of this relationship is low (BP = 57 in ML). *Fejervarya* sp. small type formed a clade with *F. granosa* (Western Ghats, India), *F. pierrei* (Chitwan, Nepal), and "*F. syhadrensis*" (India and Sri Lanka) with strong support (BPs = 95 for ML, 100 for NJ, and ≥ 95% for BI). The *16S* divergence among *Fejervarya* sp. small type vs. "*F. syhadrensis*" (India), "*F. syhadrensis*" (Sri Lanka), *F. granosa* (Western Ghats, India), and *F. pierrei* (Chitwan, Nepal) were 0.2%, 2.7%, 3.3%, and 5.7%, respectively. *Fejervarya* sp. medium type formed a clade with "*F. limnocharis*" from Myanmar (BP = 64 for NJ, and *16S* divergence = 6.9%) and the clade was a sister taxon to *Fejervarya* sp. from Assam, India (= *Fejervarya* sp. hp5 in Kotaki et al., 2010). The sequence divergence between *Fejervarya* sp. medium type and *Fejervarya* sp. hp5 was 7.5%.

The family Ranidae (Fig. 4)

Among the examined Bangladesh ranid specimens, *Hylarana leptoglossa* became a sister taxon to the *H. aurantiaca* and *H. temporalis* clade (the latter two species were from Western Ghats, India). *Hylarana* cf. *taipehensis* (Sherpur) formed a clade with *H. macrodactyla* (Wenchang, Hainan, China) with 3.4% sequence divergence. *Hylarana*

cf. *taipehensis* and *H. macrodactyla* differ strikingly in many morphological traits. *Hylarana taipehensis* (Tram Lap, Vietnam) was found to be a sister species to the *H.* cf. *taipehensis* + *H. macrodactyla* clade; the *16S* divergence between *H.* cf. *taipehensis* and *H. taipehensis* (Vietnam) was 10.4%. These findings support the distinct specific status of the taxon designated here as *Hylarana* cf. *taipehensis*. *Hylarana* sp. (Bandarban) formed a clade with *H. malabarica* from the Western Ghats and high sequence divergence (15.8%) was found between these two species.

The family Microhylidae (Fig. 5)

In the constructed ML tree, *Mycrohyla* sp. formed a clade with *M. berdmorei* from Gombak, Malaysia, despite a complete difference in morphology and a relatively high *16S* divergence (5.2%). *Microhyla* cf. *ornata* from Dinajpur and *M. ornata* from Karnataka, India, formed a clade, but their sequence divergence was high (6.8%). *Microhyla* cf. *ornata* from Chittagong formed a clade with *M. fissipes* from Thailand. The *16S* sequence divergence was only 2.7% between these two species, assuming the existence of *M. fissipes* in Bangladesh. In contrast, *M.* cf. *ornata* from Mymensingh and Sylhet was found to be a sister taxon to the *M. fissipes* + *M.* cf. *ornata* (Chittagong) clade. The *16S* divergence between *M.* cf. *ornata* from Chittagong and *M.* cf. *ornata* from Mymensingh and Sylhet was 5.4%. Both *Kaloula pulchra* and *K. taprobanica* formed a clade with the respective conspecific sample from other countries and displayed low *16S* divergence (1.1% for both *K. pulchra* and *K. taprobanica*). In the ML tree, these *Kaloula* species exhibited paraphyly, a finding that is congruent with two recent molecular phylogenetic studies (Van Bocxlaer et al., 2006; Kurabayashi et al., 2011).

DISCUSSION

Recent molecular studies have demonstrated that DNA sequence information, particularly *16S* data, can help to uncover the cryptic biodiversity of anurans. Fouquet et al. (2007) reported that a divergence threshold of 3% in *16S* sequences is useful to identify species of anurans. Vences and Wake (2007) proposed the term "candidate species" for newly discovered units that likely correspond to undescribed species.

In Bangladesh, 35 frog species are currently recognized (Kabir et al., 2009; Howlader, 2011): two bufonids (*Duttaphrynus melanostictus* and *D. stomaticus*), 10 dicroglossids (*Euphlyctis cyanophlyctis*, *E. hexadactylus*, *Fejervarya limnocharis*, *F. syhadrensis*, *F. asmati*, *H. crassus*, *H. tigerinus*, *Occidozyga borealis*, *O. lima*, and *Sphaerotheca breviceps*), two megophryids (*Leptobrachium smithii* and *Xenophrys parva*), seven microhylids (*Kalophrynus interlineatus*, *K. pulchra*, *K. taprobanica*, *Microhyla berdmorei*, *M. ornata*, *M. rubra*, and *Uperodon globulosus*), eight ranids (*Amolops marmoratus*, *Clinotarsus alticola*, *Humarana humeralis*, *Hylarana erythraea*, *H. taipehensis*, *H. tytleri*, *H. leptoglossa*, and *H. nigrovittata*), and six rhacophorids (*Chiromantis simus*, *C. vittatus*, *Polypedates leucomystax*, *P. maculatus*, *Rhacophorus htunwini*, and *R. maximus*). Of these 35 species, 26 have *16S* data available in GenBank. On the basis of the *16S* data obtained in the present study and the available GenBank data, we discuss below the taxonomical status of several unresolved taxa from Bangladesh.

Taxonomic status of dicroglossid frogs from Bangladesh

Four nominal species have been described in the genus *Hoplobatrachus*. Among them, *H. tigerinus* and *H. crassus* have been identified in Bangladesh (Alam et al., 2008). In the present study, it was shown that *H. tigerinus* from Cox's Bazar and *H.*

tigerinus from Mymensingh have diverged from each, based on the detected *16S* divergence of 6.0%. As the two populations differ in size and in a few morphological traits (Hasan et al., in preparation), *H. tigerinus* from Cox's Bazar, Bangladesh represents an undescribed cryptic species. However, it remains for future studies to determine which population belongs to the nominal species with the type locality "Bengal" (Frost, 2011).

In *E. cyanophlyctis* and *E. hexadactylus*, whose type localities are Tranquebar and Pondichéry, India, respectively (Bauer, 1998; Frost, 2011), considerable *16S* divergences (4.0% – 5.9%) were detected between the India and Bangladesh populations (Alam et al., 2008). They (2008) speculated that *E. cyanophlytis* from Bangladesh might be a cryptic species compared with that from Western Ghats (India), and that *E. hexadactylus* from Bangladesh might be "real" *E. hexadactylus* if the Sri Lanka specimens correspond to the nominal species. Thereafter, Joshy et al. (2009) described two species of the genus *Euphlyctis* from Western Ghats (India) as new species: *E. mudigere* and *E. aloysii*. However, at present it is difficult to confirm that the Bangladesh specimens correspond to real *E. cyanophlyctis* and *E. hexadactylus*. Further study involving comparisons with topotypic specimens is necessary for elucidating the taxonomic status of *E. cyanophlyctis* and *E. hexadactylus* from Bangladesh.

The genus *Fejervarya* comprises 31 species that are distributed in South and Southeast Asia (Frost, 2011). Two species (*F. limnocharis* and *F. syhadrensis*) are listed as Bangladeshi *Fejervarya* species in Kabir et al. (2009) and one new species (*F. asmati*) was recently described from Bangladesh by Howlader (2011). Asmat et al. (2003) first reported the occurrence of *F. limnocharis* in Bangladesh, but Rasel et al. (2007) later suggested the presence of *F. nepalensis*, *F. pierrie*, *F. syhadrensis*, and *F.*

teraiensis, rather than *F. limnocharis*. Based on morphological, crossing, and molecular analyses, Islam et al. (2008b) claimed that four types of *Fejervarya* exist in Bangladesh: *Fejervarya* sp. large type, *Fejervarya* sp. medium type, *Fejervarya* sp. small type, and "*F. cancrivora*" mangrove type (= *F. moodiei*). In the present study, *F. moodiei* (including the previous "*F. cancrivora*" mangrove type) from Bangradesh (Cox's Bazar and Khulna), India, and Thailand formed a clade, which exhibited less than 3% (0.2% – 2.1%) *16S* divergence. *Fejervarya* sp. small type shows close relationships with "*F. syhadrensis*" from India and Sri Lanka, *F. pierreri* from Nepal, and *F. granosa* from India. Among these related species, "*F. syhadrensis*" exhibits low *16S* divergence with *Fejervarya* sp. small type (0.2% and 2.7% for India and Sri Lanka specimens, respectively). Thus, our *Fejervarya* sp. small type clearly corresponds to this taxon. However, several *F. syhadrensis*-like species have been identified in South and Southeast Asia (including the India and Sri Lanka populations), and at present, it is unclear which populations correspond to real *F. syhadrensis* (Kuramoto et al., 2007; Kotaki et al., 2010). Thus, although our results suggest that "*F. syhadrensis*" occurs in Bangladesh, final confirmation as to whether "*F. syhadrensis*" in Bangladesh actually corresponds to real *F. syhadrensis* requires *16S* sequence analysis of the topotypic *F. syhadrensis* specimens (Poona district, India). There is a possibility that "*F. syhadrensis*" from the southeastern part of Bangladesh corresponds to *F. asmati* that was recently described from Chittagong, Bangladesh (Howlader, 2011), but more investigations are needed to confirm this speculation.

Fejervarya sp. large and medium types have been examined in previous studies, which have suggested that these taxa are possibly undescribed species (Islam et al., 2008b). The present results are consistent with the findings of Islam et al. (2008b). *Fejervarya* sp. large type shows a close relationship with *F. orissanensis*, but the *16S*

divergence (4%) is larger than the species threshold value. *Fejervarya* sp. medium type constitutes a clade with "*F. limnocharis*" from Myanmar, but their *16S* divergence is high (6.9%). It was suggested that "*F. limnocharis*" from Myanmar is not real *F. limnocharis* (Islam et al., 2008b), a view that is also supported by our results. Consequently, our study confirmed the occurrence of two possibly undescribed species, namely *Fejervarya* sp. large and medium types, from Bangladesh. Although our sampling areas covered a wide range in Bangladesh, *F. limnocharis* specimens corresponding to the haplotype from the type locality area (Indonesia) were not found. As previous molecular studies also failed to detect *F. limnocharis* in Bangladesh, we propose that the name *F. limnocharis* should be removed from the list of Bangladesh anurans.

The species in the genus *Fejervarya* constitute two distinct groups, the Southeast-Asian and South-Asian groups (Fig. 3), with *F. moodiei* and *Fejervarya* sp. large type belonging to the former, and *Fejervarya* sp. medium and small types belonging to the latter. Thus, the intermingling nature of anuran fauna of Bangladesh is evident. Two species of "*F. limnocharis*" (large and small, which also differ in their habitat) were recognized in Myanmar (Zug et al., 1998), but the relationship between *Fejervarya* taxa of Bangladesh and Myanmar remain to be determined in future studies.

Taxonomic status of ranid frogs from Bangladesh

The genus *Hylarana* consists of 86 nominal species, and 75 *Hylarana* species are distributed in Asia and northern Australia (Frost, 2011). It has been reported that five species of this genus (*H. erythraea*, *H. taipehensis*, *H. leptoglossa*, *H. tytleri*, and *H. nigrovittata*) are distributed in Bangladesh (Kabir et al., 2009). Our present specimens contained *H. leptoglossa* and two unidentified species (*H.* cf. *taipehensis* and *Hylarana*

sp.). Among these species, *H.* cf. *taipehensis* has a close affinity with *H. macrodactyla* (Wenchang, Hainan, China), with 3.4% *16S* divergence, but the external morphologies of the two differ completely (Hasan et al., in preparation). In contrast, the *16S* divergence between *H.* cf. *taipehensis* and *H. taipehensis* (Vietnam) is very high (10.4%). Thus, our results show that *H.* cf. *taipehensis* does not correspond to either *H. macrodactyla* or *H. taipehensis*, and likely represents a new cryptic species. Specimens of *H.* cf. *taipehensis* were collected from many regions of Bangladesh and it is probable that this taxon has long been confused with *H. taipehensis*. Thus, the name *H. taipehensis* should be removed from the anuran list of Bangladesh.

Hylarana sp. (Bandarban, Bangladesh) and *H. malabarica* (India) formed a clade and exhibited 15.8% *16S* divergence. Due to the limited number of available *16S* sequences of nominal *Hylarana* species (15 of 86) and lack of *16S* data for *H. tytleri* specimens, our analyses could not verify the taxonomic status of this unidentified *Hylarana* taxon. However, the present phylogenetic analyses, together with morphological comparisons (Hasan et al., in preparation), suggests that *Hylarana* sp. does not correspond to four *Hylarana* species (*H. leptoglossa*, *H. erythraea*, *H. taipehensis*, and *H. nigrovittata*) currently recognized in Bangladesh. Although usable *16S* data is lacking for *H. tytleri*, the morphologies of our *Hylarana* sp. differ from those of the remaining Bangladeshi *Hylarana* species (*H. tytleri*). Detailed morphological comparisons are now in progress.

Taxonomic status of microhylid frogs from Bangladesh

The genus *Microhyla* consists of 31 species that are widely distributed throughout South and Southeast Asia (Frost, 2011). In Bangladesh, only three nominal species (*M. ornata*, *M. berdmorei*, and *M. rubra*) are reported to exist (Kabir et al., 2009). In the

present study, we identified four distinct taxa in the genus *Microhyla*. *Microhyla* cf. *ornata* from Chittagong formed a clade with *M. fissipes* (Thailand) and displayed a *16S* divergence of only 2.7%. Thus, we speculated this taxon to *M. fissipes*, which needs further taxonomic study to confirm this idea. *Microhyla fissipes* has long been confused with *M. ornata* (Matsui et al., 2005) and is presumed to occur in Myanmar (Frost, 2011). *Microhyla* cf. *ornata* from Mymensingh and Sylhet showed a considerable genetic divergence (> 5.0%) from these above taxa, although they share similar external morphologies. Thus, it is highly probable that *M.* cf. *ornata* from Mymensingh and Sylhet is a cryptic species. *Microhyla* cf. *ornata* from Dinajpur is morphologically similar to *M. ornata* (Karnataka, India; type locality area), but a relatively high *16S* divergence (6.8%) exists between them. Therefore, this taxon is apparently a new cryptic species, as suggested by Matsui et al. (2005). *Microhyla* sp. from Sylhet has 5.2% *16S* divergence from *M. berdmorei* (Gombak, Malaysia). As these two taxa differ morphologically, *Microhyla* sp. from Sylhet is likely a cryptic species.

In conclusion, the present study revealed the presence of at least eight undescribed frog taxa in Bangladesh. This finding is remarkable in view of the relatively simple topographic features of Bangladesh, which mainly consists of lowlands and lacks high mountainous regions. In addition, our results clearly indicate that anuran biodiversity has been underestimated in Bangladesh and emphasize the necessity for further taxonomic studies of anurans in this country.

III. A NEW SPECIES OF GENUS *HOPLOBATRACHUS* (ANURA, DICROGLOSSIDAE) FROM THE COASTAL BELT OF BANGLADESH

ABSTRACT

A new cryptic species of the genus *Hoplobatrachus* from Cox's Bazar district of Bangladesh is described and compared with its relevant congeners both in morphology and mitochondrial gene sequences. The new species differs from its close relative *H. tigerinus* in having a distinct broad black band from the eye, through the nostrils, to the anterior edge of the upper jaw, another black band along the lateral margin of the upper jaw, and a narrow inter-orbital distance relative to eyelid width and inter-nostril distance. Advertisement calls of the new species are similar to those of *H. tigerinus* but differ in dominant frequency and number of pulses. Based on mitochondrial DNA sequence data, this species was proved to genetically diverge from *H. tigerinus* at 3.2% for the 16S rRNA gene and 14.2% for the *Cytb* gene, which are higher values than suggested for species threshold in anura. The distribution range of the new species is restricted to the southeastern corner of Bangladesh and is endemic in this coastal belt.

INTRODUCTION

The genus *Hoplobatrachus* comprises large robust frogs with numerous ridges or warts on the back and extensive webbing between toes. Individuals are semi-aquatic and live mostly near water edge of ponds, marshes, rivers, and flooded rice paddies. The following four species are currently recognized (Frost, 2011): *H. crassus* in south to east India, Sri Lanka, Nepal, and Bangladesh; *H. occipitalis* in western and central Africa; *H. rugulosus* (= *H. chinensis*, used by some authors [eg., Kosuch et al., 2001] in Myanmar, southern China, Taiwan, Thailand, and peninsular Malaysia; and *H. tigerinus* in east Afghanistan, north Pakistan, India, Sri Lanka, Nepal, Bangladesh, and

Myanmar. All of these species were described during the early to middle 19th century, and no new species were reported far more than a century thereafter.

In our previous study (Hasan et al., 2012a), we revealed the existence of two genetically different types of *H. tigerinus* in Bangladesh. Divergence in mitochondrial 16S rRNA gene sequences was 6.0% between specimens from Mymensingh and Cox's Bazar districts. One of the types is widely distributed throughout Bangladesh, whereas the other occurs only in the southeastern corner of Bangladesh. The type locality of *H. tigerinus* is "Bengale" (Bengal), India (Frost, 2011). The distribution and molecular comparison clearly indicate that the wide-ranging type corresponds to the nomen *H. tigerinus*. Therefore, the other type is described as a new species. Morphological comparisons of the new species were performed between *H. tigerinus* and *H. rugulosus*, and new molecular data are presented.

MATERIALS AND METHODS

Specimens of the new species were found in Cox's Bazar district, whereas *H. tigerinus* were collected from throughout Bangladesh from 2000 to 2011. Finger tips were cut for DNA analysis, and voucher specimens were deposited in the Institute for Amphibian Biology, Hiroshima University (IABHU).

The following 29 body parts were measured for both sexually matured individuals of male and female using digital calipers to the nearest 0.1 mm. Mature male frogs were identified by their secondary sexual character, i.e., presence of vocal sac, and big sized females were considered as mature, although few smaller female specimens were confirmed for maturity by checking their gonad. 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: horizontal eye diameter; E-E: inter-orbital distance between inner borders of upper eyelids; ELW: eyelid width; TD: horizontal tympanum

diameter; FLL: forelimb length; FHL: forearm and hand length; FAW: forearm width; HAL: hand length; F1-F4: length of 1st to 4th finger; HLL: hindlimb length; FEL: femur length; TIL: tibia length; TFL: tarsus and foot length; FOL: foot length; T1-T5: length of 1st to 5th toe; and IMT: inner metatarsal tubercle length. For comparison of morphometric data, we considered 27 mature *H.* sp from Ukhia and Teknaf of Cox's Bazar district; 15 mature *H. tigerinus* as representative samples from a single locality of Mymensingh district, as there was a negligible genetic variation among the *H. tigerinus* distributed all over Bangladesh (Alam et al., 2008; Hasan et al., 2012a and Islam et al., unpublished); 7 mature individuals of *H. rugulosus* from Nong Khai and Chachoengsao of Thailand. Statistic analysis was performed in SPSS (15.0J) software (SPSS Japan Inc., Tokyo, Japan).

Advertisement calls of multiple males of new species were recorded at Ukhia, Cox's Bazar district, with an ICD-UX300F IC recorder (Sony Corp., Tokyo, Japan) on 19 June 2011. These specimens of male were also recorded in our note book and underwent for further molecular and morphological analyses in this study. Sound spectrograms were depicted using Avisoft-SASLab Light software (Avisoft Bioacoustics).

Total DNA was extracted from a clipped toe of each individual in 4 specimens of *H. tigerinus* (= 2 haplotypes) from Mymensingh and in 26 specimens of *H.* sp. (= 9 haplotypes) from Cox's Bazar using the DNeasy Tissue Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. The extracted DNA solutions were used to amplify partial portions of the 16S rRNA gene (*16S*) and Cytb gene (*Cytb*) corresponding to nucleotide position 6,205–6,753 and 16,761–17,372, respectively, in the *Fejervarya limnocharis* complete mtDNA sequence (accession no.: AY158705; Liu et al., 2005). PCR amplification and sequencing were performed using the primers F51 (5́-CCC GCC TGT TTA CCA AAA ACA T-3́) and R51 (5́-GGT CTG AAC TCA

GAT CAC GTA-3́) (Sumida et al., 2002) for *16S*, and Fow-1-1 (5́-ACM GGH YTM TTY YTR GCH ATR CAY TA-3́) and Rev-1 (5́-TAD GCR AAW AGR AAR TAY CAY TCN GG-3́) for *Cytb*. Partial *16S* (556 bp) and *Cytb* (616 bp) portions were sequenced, and the obtained sequences were deposited in the DDBJ/EMBL/GenBank database (accessions numbers: AB671173–AB671184 for *16S* and AB671185–AB671196 for *Cytb*).

The resultant nucleotide sequences of the *16S* and *Cytb* genes were separately aligned using the ClustalW program (Thompson et al., 1994) with their counterparts from *H. tigerinus* (n = 2) of India, three congener species, *H. crassus* (n = 1), *H. rugulosus* (n = 3), and *H. occipitalis* (n = 2), and two *Euphlyctis* species, *E. cyanophlyctis* (n = 1) and *E. hexadatylus* (n = 1) (Alam et al., 2008). From these two alignment data sets, sequence divergences (uncorrected *p* values) were calculated using MEGA Ver. 4.0 (Tamura et al., 2007) 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 default parameters. Gap sites in alignments were treated as missing data. Initial two alignments (*16S* and *Cytb*) were combined into one alignment data set and this concatenated alignment data set contained a total of 1036 sites (478 for *16S* and 558 for *Cytb*), 294 of which were parsimoniously informative, and this data set underwent further phylogenetic analyses. Phylogenetic analyses were performed by 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 (AIC) and Bayesian information criterion (BIC) respectively, which 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 analysis with 1,000 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 was determined by checking the convergence of –log likelihood (–ln*L*) values and tree length against generation number by using Tracer ver. 1.4 (Rambaut and Drunnond, 2007). Statistical support of the BI tree was evaluated by Bayesian posterior probability (BPP). MP was performed with 1,000 bootstrap replicates using PAUP* 4.0b10 (Swofford, 2003).

SYSTEMATICS

***Hoplobatrachus litoralis* sp. nov.**

Holotype

IABHU 3993, adult female (SVL: 100.6 mm) collected from Teknaf, Cox's Bazar district (20° 52′ N, 92° 18′ E, > 5 m asl.), Bangladesh on 20 June 2011 by M. Hasan (Figs. 6A, 6B).

Paratypes

IABHU 3974, adult female (SVL: 121.3 mm), IABHU 3979, adult female (SVL: 109.1 mm), and IABHU 3980, adult female (SVL: 119.3 mm) collected from Ukhia, Cox's Bazar district, Bangladesh on 19 June 2011 by M. Hasan. IABHU 3989, adult male (SVL: 89.5 mm), IABHU 3992, adult male (SVL: 83.7 mm), IABHU 3994, adult male (SVL: 84.7 mm) and IABHU 3997, adult female (SVL: 96.1 mm) collected from Teknaf, Cox's Bazar district, Bangladesh on 20 June 2011 by M. Hasan.

Diagnosis

Large frog with SVL of 81.3–102.1 mm in males and 83.2–121.3 mm in females.

A broad black band from anterior corner of eye through the nostrils to anterior edge of upper jaw, and another band along the lateral margin of upper jaw (Fig. 6C) are much distinct than in its close relatives *H. tigerinus* and *H. rugulosus* where the band widths are uneven and often discontinuous (Fig. 6D). There is distinct black margin in the inner side of the upper arm in the new species (Fig. 6E), but such characteristics is absent in *H. tigernius* (Fig. 6F). Inner metatarsal tubercle of the new species is black (Fig. 6G), whereas it is pigment-less in *H. tigerinus* (Fig. 6H). Inter-orbital distance is much narrower than eyelid width and inter-nostril distance in the new species (ELW/E-E = 1.875, N-N/E-E = 1.575, on average), whereas these values are nearly the same in *H. tigerinus* (ELW/E-E = 1.060, N-N/E-E = 1.002) and *H. rugulosus* (ELW/E-E = 1.021, N-N/E-E = 1.004).

Description of holotype (measurements in mm)

Vomerine teeth, long oblique lines between choanae. Tongue tip bifurcated. Distinct symphysial knob on anterior edge of lower jaw.

Head longer than wide (HL: 43.9; HW: 40.2), obtusely pointed. Canthus rostralis blunt. Loreal region concave. Nostril nearer to tip of snout than to eye (S-N: 6.6; N-E: 10.7). Tympanum large, slightly smaller than eye (TD: 8.3; ED: 8.9). Inter-orbital space much narrower than eyelid width and inter-nostril space (E-E: 3.9; ELW: 7.2; N-N: 6.5).

Fingers free, finger tips blunt without disk. Finger length F3 > F1 > F2 > F4 (F1: 12.9; F2: 10.3; F3: 13.2; F4: 7.7). Subarticular tubercles moderate. Thenar and palmar tubercles distinct.

Hindlimb about 1.6 times SVL (HLL: 165.3; SVL: 100.6). Femur length subequal to tibia length (FEL: 51.0; TIL: 51.8). Toe tips blunt, slightly rounded. Toe length T4 > T5 > T3 > T2 > T1 (T1: 11.8; T2: 17.8; T3: 26.2; T4: 34.6; T5: 27.9). Wide web,

reaching the base of toe-tip disk (Fig. 6G). Subarticular tubercles were weak. Inner metatarsal tubercle moderate (IMT: 4.6). No outer metatarsal tubercle.

Many thin longitudinal ridges on the dorsum (Fig. 6A). Small round warts over dorsal and lateral side. Supra-tympanic fold from behind eye to posterior margin of tympanum. Weak tarsal ridge extending from proximal end of inner metatarsal tubercle to heel. Ventral side including thigh and tibia smooth.

Color in alcohol

Dorsum dark gray with many large black spots (Fig. 6A). Thin whitish mid-dorsal stripe from tip of snout to vent. Lateral side with many small black dots. Wide black band from anterior corner of eye through the nostrils to anterior margin of upper jaw (Fig. 6C). Another band on the lateral margin of upper jaw (Fig. 6C). Ventral side immaculate, except for large black blotches along the edge of lower jaw to the base of forelimb (Fig. 6B).

Large transverse black bands on the upper surface of thigh, tibia, and tarsus to the outer edge of foot. Rear side of thigh heavily mottled. Outer side of tarsal ridge dark, whereas inner side yellowish with several dark irregular blotches. Web dark gray, except for whitish upper side of inner web (between toes 1–4).

Color in life

Dorsal ground color varies from yellowish to dark brown with many dark brown to black spots. Large transverse black bands are present on the dorsal surface of the thigh, tibia, and tarsus region (Fig. 7A). Mid-dorsal stripe is yellowish white. Bands running from the anterior part of eye to upper jaw margin and on the lateral margin of upper jaw are black. Many fused spots on the posterior surface of thigh are black with thin yellow reticulations between them. Tympanum is dark gray with pale central

circle. A short discontinuous black line is present below the eye. Venter is creamy white with a few black blotches from the margin of lower jaw to base of forelimb (Fig. 7B).

Variation

Males have a pair of gray (in alcohol) subgular vocal sac on underside of jaw angle, and a well-developed nuptial pad on the base of 1st finger.

Of the 26 specimens examined, three (12%) lacked a mid-dorsal stripe. A broad mid-dorsal stripe, often found in *Fejervarya* species, was absent. Number and size of dark spots along the margin of the lower jaw varied, and only one specimen lacked these spots. Usually, the lateral upper jaw stripe is continuous, but in 6 specimens the stripe is fragmented into irregular markings.

Morphological comparisons

Among the three *Hoplobatrachus* species in Bangladesh (*H. litoralis*, *H. tigerinus*, and *H. crassus*), *H. crassus* is easily distinguishable from the other two by large shovel-like inner metatarsal tubercle and usual occurrence of large dark dots in the gular and pectoral region. In the following, we compare the morphological characters of *H. litoralis* with those of *H. tigerinus* and *H. rugulosus*. *Hoplobatrachus rugulosus* is so similar to *H. tigerinus* in that it was previously regarded as a subspecies of *H. tigerinus*. Because *H. rugulosus* occurs in Myanmar, just adjacent to the distribution range of *H. litoralis*, comparisons of the two are relevant.

All specimens (n = 15) of *H. tigerinus* from Mymensingh, Bangladesh had a thin mid-dorsal stripe. Five specimens (33%) had a thin yellowish stripe along the inner side of the tibia, usually extending over the upper side of the thigh to the base of the thigh. This line was not observed in *H. litoralis*. The underside of the mandible is

finely dotted in most specimens. A marginal stripe along the jaw margin is usually fragmented into narrow irregular markings. The stripe from the eye through the nostrils to the anterior edge of the upper jaw is not as broad as in *H. litoralis* and is usually fragmented. In *H. tigerinus*, the broad light line from behind the eye to the groin is more distinct than in *H. litoralis*. Webs are mottled with an irregular dark pattern, in contrast to the uniform gray in *H. litoralis*.

Specimens of *H. rugulosus* from Thailand (n = 7) lacked a mid-dorsal stripe. The snout was rounded rather than pointed. The dorsal dermal ridges were wider and shorter than that of *H. litoralis* and *H. tigerinus*.

There are several old nomina which have been treated as junior synonyms of currently recognized *Hoplobatrachus* species. *Rana brama* Lesson, 1834, a large frog described from Bengale, was reported as having very smooth surface, depressed dorsum, and short depressed head. Femur is much shorter than tibia. Obviously, *H. litoralis* differs from *R. brama* in these characteristics. *Pyxicephalus frithi* Theobald, 1868 described from Jessore, SW Bangladesh was reported to have quite smooth skin and uniform vinous coloration. These do not fit *H. litoralis*. *Rana burkilli* Annandale, 1910 was described from Tavoy, Myanmar. This species differs from *H. litoralis* in having ventral surface "marked with black, the markings sometimes taking on a reticulate character all over the belly". The inner metatarsal tubercle was described as feebly developed, but *H. litoralis* has well-developed inner metatarsal tubercle. *Rana gracilis* (not of Gravenhorst, 1829) Boulenger, 1920, a small frog (SVL 50 mm for male, 41– 64 mm for female) described from Sri Lanka, was disclosed as having a oval inner metatarsal tubercle and smooth skin or feebly granulate above. *Rana gracilis var. pulla* (Stocliczka 1870) described from Penang hill, Malaysia was reported to be a very small frog (SVL is 7/8 inch [< 23 mm] and HLL is 1 1/2 inch [nearly 38.1 mm]). *Hoplobatrachus littoralis* is distinguished from both *R. gracilis* and *R. gracilis var.*

pulla considering its very large size (SVL = 89.96 mm and 101.42 mm; and HLL = 143.85 mm and 159.74 mm for male and female, respectively). The skin of *H. litoralis* is rough and its inner metatarsal tubercle is comparatively elongated rather than oval. *Rana picta* Gravenhorst, 1829 whose type locality is unknown (Frost 2011) was reported to have three spots below one eye and there is no trace of tubercle on dorsal skin. These characteristics do not fit with the new species of *H. litoralis*.

Morphometric comparisons

Measurements of 29 body parts of *H. litoralis*, *H. tigerinus*, and *H. rugulosus* are summarized in Table 2. Mean SVL of males differs significantly (U test, $P < 0.05$) between the three species, but that of females does not. Size of *H. litoralis* is slightly smaller than that of the other two species. SVL of females is larger than that of males ($P < 0.05$) in *H. litoralis* and *H. rugulosus*.

The three species are clearly separated by canonical discriminant analysis (Fig. 8 A). Eigenvalues are 11.606 for function 1, and 5.684 for function 2. Coefficients for function 1 are large especially in E-E, N-N, N-E, HAL, and ED for function 1 and in E-E, HAL, TD, and TIL for function 2. In principal component analysis (Fig. 8 B), *H. litoralis* made completely separate cluster from *H. rugulosus,* but the scores of *H. litoralis* and *H. tigerinus* overlapped considerably.

Body ratios relative to SVL (e.g., HL/SVL and HW/SVL) and 10 other ratios (e.g., HL/HW and S-N/N-E) are shown in Table 3, and the results of the Mann-Whitney U test between ratios of *H. litoralis* vs. *H. tigerinus* and *H. rugulosus* are shown in Table 4. Mean values of ED/SVL, ELW/SVL, ED/E-E, N-N/E-E, and ELW/E-E are significantly large ($P < 0.01$) in *H. litoralis* compared with those in *H. tigerinus* and *H. rugulosus*. These are apparently derived from relatively large ED, ELW, and N-N and relatively small SVL and E-E of *H. litoralis*.

Advertisement calls

Advertisement calls of *H. litoralis* are low-pitched groans emitted at about 4.4 s interval. Mean call duration is 0.28 s (n = 34). The call is composed of about 20 rapidly repeating pulses (Fig. 9A). Fundamental frequency is 0.30 kHz and the 3rd and 4th harmonic bands (at about 1.2 kHz) are dominant (Fig. 9B).

Roy and Elepfandt (1993) and Kanamadi et al. (1994) reported the acoustic features of *H. tigerinus* from northeast India (Assam and Meghalaya) and southwest India (Karnataka), respectively. Call durations are reported as 0.30 s (NE India) and 0.22 s (SW India); these values do not differ significantly from those of *H. litoralis.* Dominant frequency bands in *H. tigerinus* are 1.65 kHz and 0.52 kHz in NE India populations and 1.5–2.2 kHz and 0.2–1.2 kHz in SW India populations. These dual-dominant bands are absent in *H. litoralis*. The number of pulses in a call is larger in *H. litoralis* than in *H. tigerinus* (16 in NE India and 12.6 in SE India).

Advertisement calls of *H. crassus* (Kanamadi et al., 1992) are different from those of *H. litoralis* and *H. tigerinus* with regard to distinctly few number of pulse groups (2-4 per call) and show many intense harmonic bands.

Divergence in mitochondrial 16S rRNA and Cytb gene sequences

The average sequence divergence for *16S* and *Cytb* was 3.2% and 14.2%, respectively, between *H. litoralis* from Cox's Bazar and *H. tigerinus* from Mymensingh, while these values were only 2.1% and 9.1%, respectively, between the Mymensingh and Western Ghats populations of *H. tigerinus* (Fig. 10). *Hoplobatrachus litoralis* is greatly diverged from *H. tigerinus* of the Western Ghats by 3.3% for *16S* and 12.0% for *Cytb*. *Hoplobatrachus litoralis* clearly differ from *H. tigerinus* from Mymensingh and the Western Ghats (BP: 92 for ML, 93 for MP, and 100 for BI). All

H. litoralis specimens formed one clade supported by high bootstrap values (BP: 100 for ML, MP, and BI), while the *H. tigerinus* clade from Mymensingh and the Western Ghats was supported by medium bootstrap values (BP: 54 for ML, 81 for MP, and 67 for BI).

Distribution

Hoplobatrachus litoralis occurs in the southeastern coastal belt in Ukhia, Teknaf Upazilla (sub-district) and Cox's Bazar town of Cox's Bazar district (21° 45′ N, 91° 97′ E, > 3 m asl.) in Bangladesh. The preferable habitat of this species is a vegetated, marshy ditch/ pond, beside the wetland created by hill stream (locally called "Jiri") and/or sometimes the base of mountains having different soil texture from the mainland Bangladesh. This new species is sympatrically occurred with other anuran species such as *H. crassus*, as well as some *Euphlyctis, Fejervarya*, and *Polypedates* species. *Hoplobatracus tigerinus* is widely distributed in mainland Bangladesh (Alam et al., 2008) as well as also in coastal region ranging from southwestern Shatkhira to southeastern Bandarban including Shatkhira, Barguna, Patuakhali, Bhola, Sandwip and Raojan of Chittagong and Bandarban districts (Islam et al., unpublished); but no *H. tigerinus* specimens have been obtained from Cox's Bazar district area. Thus we have no confirmation about the overlapping region between this two species, but we speculate that this species is endemic in Cox's Bazar district. The adjacent area of Myanmar (Teknaf) is only separated by the Naaf river, which probably does not constitute a strong barrier of *H. litoralis* to migration. Thus it is possible that our new species may also occur in the adjacent coastal geographic region of Myanmar. But as we have no access for sampling in that region, we cannot confirm whether our new species is distributed in that region of Myanmar or not. Approximate distribution of all Asian *Hoplobatrachus* species is shown in Fig. 11A, whereas the detail distribution of

three genetically different *Hoplobatrachus* species in Bangladesh is shown in Fig. 11B.

The Teknaf-Ukhia peninsula is a long, narrow and forested area rising up to 300 meters above the sea level and, encompassed by the Bay of Bengal to the west and the Naaf river to the east. This is a diverse habitat for many flora and fauna due to its special characteristics i.e. estuarine habitat and wetland at the base of the mountain. In April 1999, Department of Environment (DOE) of Bangladesh declared that Teknaf peninisula is a Ecologically Critical Area (ECA) due to adverse change of its ecosystems by the human activities. It is an important nesting site for at least four species (*Chelonia mydas*, *Eretmochelys imbricata*, *Lepidochelys olivacea* and *Dermochelys coraicea*) of marine turtle listed as globally threatened by IUCN. A few of globally threatened shorebirds (*Eurynorhynchus pygmeus*, *Limnodromus semiplamatus*, *Tringa guttifer*) are also prefer this region for their habitat (GoB/GEF/UNDP, 1999).

Etymology

The specific name is derived from the Latin *litoralis* meaning coastal, in reference to the distribution range of this species; the Coastal belt of Bangladesh.

Examined specimens list

Hoplobatrachus litoralis (27 specimens): Institute for Amphibian Biology, Hiroshima University: IABHU 3939, 3974–3999.

Collection localities: Ukhia, Teknaf (Cox's Bazar district, Bangladesh).

Hoplobatrachus tigerinus (15 specimens): Institute for Amphibian Biology, Hiroshima University: IABHU 3066–3068, 3087, 3589, 3776, 4000–4003, 20008–20010, 20022, 20027.

Collection locality: Bangladesh Agricultural University Campus (BAUC),

Mymensingh district, Bangladesh.

Hoplobatrachus rugulosus (7 specimens): Institute for Amphibian Biology, Hiroshima University: IABHU 3576–3578, 3583–3584, 3663–3664.

Collection localities: Nong Khai, Chachoengsao (Thailand).

IV. GENERAL DISCUSSION

The application of molecular genetics and bioacoustic tools in systematic studies have been particularly effective for revealing morphologically "cryptic" species within the same group of taxa that were previously considered a single species (Hillis et al., 1986; Gower et al., 2005). Mitochondrial (mt) DNA is generally considered to be an effective molecular marker for revealing genetic and phylogenetic relationships between animal taxa (Avise, 2000). Furthermore, mtDNA analyses have occasionally triggered the discovery of undescribed and/or cryptic frog species (Staurt et al., 2006). The mt Cox1 gene sequence (DNA barcoding) is often used as a marker for species identification in many animal taxa (Herbert et al., 2003; Herbert et al., 2004). In amphibians, however, the mt 16S rRNA gene (*16S*) is considered a more usable marker for determining taxonomic affiliation of frog species (Vences et al., 2005). According to recent studies, a 3% difference of *16S* sequence corresponds to species threshold in many frog taxa (Fouquet et al. 2007; Vieties et al., 2009; Kotaki et al., 2010).

In Bangladesh, 35 frog species are currently recognized (Kabir et al., 2009; Howlader, 2011). Of these, 26 have *16S* data available in GenBank (until July, 2011). On the basis of the *16S* data obtained in the present study and the available GenBank data, we discuss the taxonomical status of several unresolved taxa from Bangladesh.

Taxonomic status of dicroglossid frogs from Bangladesh

There are four nominal species have been described in the genus *Hoplobatrachus*. Among them, *H. tigerinus* and *H. crassus* have been identified in Bangladesh (Alam et al., 2008). In the present study, it was shown that *H. tigerinus* from Cox's Bazar and *H.*

tigerinus from Mymensingh have diverged from each, based on the detected *16S* divergence of 6.0%. As the two populations differ in size and in a few morphological traits (Hasan et al., 2012b), *H. tigerinus* from Cox's Bazar, Bangladesh represents an undescribed cryptic species.

In *E. cyanophlyctis* and *E. hexadactylus*, whose type localities are Tranquebar and Pondichéry, India, respectively (Bauer, 1998; Frost, 2011), considerable *16S* divergences (4.0% – 5.9%) were detected between the India and Bangladesh populations (Alam et al., 2008). They (2008) speculated that *E. cyanophlytis* from Bangladesh might be a cryptic species compared with that from Western Ghats (India), and that *E. hexadactylus* from Bangladesh might be "real" *E. hexadactylus* if the Sri Lanka specimens correspond to the nominal species. Thereafter, Joshy et al. (2009) described two species of the genus *Euphlyctis* from Western Ghats (India) as new species: *E. mudigere* and *E. aloysii*. However, at present it is difficult to confirm that the Bangladesh specimens correspond to real *E. cyanophlyctis* and *E. hexadactylus*. Further study involving comparisons with topotypic specimens is necessary for elucidating the taxonomic status of *E. cyanophlyctis* and *E. hexadactylus* from Bangladesh.

The genus *Fejervarya* comprises 31 species that are distributed in South and Southeast Asia (Frost, 2011). Two species (*F. limnocharis* and *F. syhadrensis*) are listed as Bangladeshi *Fejervarya* species in Kabir et al. (2009) and one new species (*F. asmati*) was recently described from Bangladesh by Howlader (2011). Asmat et al. (2003) first reported the occurrence of *F. limnocharis* in Bangladesh, but Rasel et al. (2007) later suggested the presence of *F. nepalensis*, *F. pierrie*, *F. syhadrensis*, and *F. teraiensis*, rather than *F. limnocharis*. Based on morphological, crossing, and molecular analyses, Islam et al.

(2008b) claimed that four types of *Fejervarya* exist in Bangladesh: *Fejervarya* sp. large type, *Fejervarya* sp. medium type, *Fejervarya* sp. small type, and "*F. cancrivora*" mangrove type (= *F. moodiei*). In the present study, *F. moodiei* (including the previous "*F. cancrivora*" mangrove type) from Bangradesh (Cox's Bazar and Khulna), India, and Thailand formed a clade, which exhibited less than 3% (0.2% – 2.1%) *16S* divergence. *Fejervarya* sp. small type shows close relationships with "*F. syhadrensis*" from India and Sri Lanka, *F. pierreri* from Nepal, and *F. granosa* from India. Among these related species, "*F. syhadrensis*" exhibits low *16S* divergence with *Fejervarya* sp. small type (0.2% and 2.7% for India and Sri Lanka specimens, respectively). Thus, our *Fejervarya* sp. small type clearly corresponds to this taxon. However, several *F. syhadrensis*-like species have been identified in South and Southeast Asia (including the India and Sri Lanka populations), and at present, it is unclear which populations correspond to real *F. syhadrensis* (Kuramoto et al., 2007; Kotaki et al., 2010). Thus, although our results suggest that "*F. syhadrensis*" occurs in Bangladesh, final confirmation as to whether "*F. syhadrensis*" in Bangladesh actually corresponds to real *F. syhadrensis* requires *16S* sequence analysis of the topotypic *F. syhadrensis* specimens (Poona district, India).

Fejervarya sp. large and medium types have been examined in previous studies, which have suggested that these taxa are possibly undescribed species (Islam et al., 2008b). The present results are consistent with the findings of Islam et al. (2008b). *Fejervarya* sp. large type shows a close relationship with *F. orissanensis*, but the *16S* divergence (4%) is larger than the species threshold value. *Fejervarya* sp. medium type constitutes a clade with "*F. limnocharis*" from Myanmar, but their *16S* divergence is high (6.9%). It was suggested that "*F. limnocharis*" from Myanmar is not real *F. limnocharis* (Islam et al.,

2008b), a view that is also supported by our results. Consequently, our study confirmed the occurrence of two possibly undescribed species, namely *Fejervarya* sp. large and medium types, from Bangladesh.

Taxonomic status of ranid frogs from Bangladesh

The genus *Hylarana* consists of 86 nominal species and 75 *Hylarana* species are distributed in Asia and northern Australia (Frost, 2011). It has been reported that five species of this genus (*H. erythraea*, *H. taipehensis*, *H. leptoglossa*, *H. tytleri*, and *H. nigrovittata*) are distributed in Bangladesh (Kabir et al., 2009). Our present specimens contained *H. leptoglossa* and two unidentified species (*H.* cf. *taipehensis* and *Hylarana* sp.). Among these species, *H.* cf. *taipehensis* has a close affinity with *H. macrodactyla* (Wenchang, Hainan, China), with 3.4% *16S* divergence, but the external morphologies of the two differ completely (Hasan et al., in preparation). In contrast, the *16S* divergence between *H.* cf. *taipehensis* and *H. taipehensis* (Vietnam) is very high (10.4%). Thus, our results show that *H.* cf. *taipehensis* does not correspond to either *H. macrodactyla* or *H. taipehensis,* and likely represents a new cryptic species. Specimens of *H.* cf. *taipehensis* were collected from many regions of Bangladesh and it is probable that this taxon has long been confused with *H. taipehensis*.

Hylarana sp. (Bandarban, Bangladesh) and *H. malabarica* (India) formed a clade and exhibited 15.8% *16S* divergence. Due to the limited number of available *16S* sequences of nominal *Hylarana* species (15 of 86) and lack of *16S* data for *H. tytleri* specimens, our analyses could not verify the taxonomic status of this unidentified *Hylarana* taxon. However, the present phylogenetic analyses, together with morphological comparisons

(Hasan et al., in preparation), suggests that *Hylarana* sp. does not correspond to four *Hylarana* species (*H. leptoglossa*, *H. erythraea*, *H. taipehensis*, and *H. nigrovittata*) currently recognized in Bangladesh. Although usable *16S* data is lacking for *H. tytleri*, the morphologies of our *Hylarana* sp. differ from those of the remaining Bangladeshi *Hylarana* species (*H. tytleri*). Detailed morphological comparisons are now in progress.

Taxonomic status of microhylid frogs from Bangladesh

The genus *Microhyla* consists of 31 species that are widely distributed throughout South and Southeast Asia (Frost, 2011). In Bangladesh, only three nominal species (*M. ornata*, *M. berdmorei*, and *M. rubra*) are reported to exist (Kabir et al., 2009). In the present study, we identified four distinct taxa in the genus *Microhyla*. *Microhyla* cf. *ornata* from Chittagong formed a clade with *M. fissipes* (Thailand) and displayed a *16S* divergence of only 2.7%. *M. fissipes* has long been confused with *M. ornata* (Matsui et al., 2005) and is presumed to occur in Myanmar (Frost, 2011). *Microhyla* cf. *ornata* from Mymensingh and Sylhet showed a considerable genetic divergence (> 5.0%) from these above taxa, although they share similar external morphologies. Thus, it is highly probable that *M.* cf. *ornata* from Mymensingh and Sylhet is a cryptic species. *M.* cf. *ornata* from Dinajpur is morphologically similar to *M. ornata* (Karnataka, India; type locality area), but a relatively high *16S* divergence (6.8%) exists between them. Therefore, this taxon is apparently a new cryptic species, as suggested by Matsui et al. (2005). *Microhyla* sp. from Sylhet has 5.2% *16S* divergence from *M. berdmorei* (Gombak, Malaysia). As these two taxa differ morphologically, *Microhyla* sp. from Sylhet is likely a cryptic species.

Description of a new species of genus *Hoplobatrachus* (Anura, Dicroglossidae)

In our previous study (Hasan et al., 2012a), we revealed the existence of two genetically different types of *H. tigerinus* corresponding to Mymensingh and Cox's Bazar Districts in Bangladesh. Of these, one is widely distributed in Bangladesh (Alam et al., 2008), while the other is confined to the south-eastern corner of Bangladesh. The new species *H. litoralis* (also *H.* cf. *tigerinus*) differs from its close relative *H. tigerinus* in having a broad black band from the anterior corner of the eye through the nostrils to the anterior edge of the upper jaw. It also has another distinct band along the lateral margin of the upper jaw where the band widths are uneven and often discontinuous. The inner metatarsal tubercle of the new species is easily distinguishable from the other part of the foot compared with that of *H. tigerinus*. There is a distinct black margin on the inner side of the upper arm in the new species, but such characteristics are absent in *H. tigerinus,* or sometimes pale coloration is evident. Inter-orbital distance is much narrower than eyelid width and inter-nostril distance in the new species (ELW/E-E = 1.875, N-N/E-E = 1.575, on average), whereas these values are nearly the same in *H. tigerinus* (ELW/E-E = 1.060, N-N/E-E = 1.002) and *H. rugulosus* (ELW/E-E = 1.021, N-N/E-E = 1.004).

Advertisement calls of the new species are similar to those of *H. tigerinus* but differ in dominant frequency and number of pulses. Based on mitochondrial DNA sequence data, this species was proved to diverge genetically from *H. tigerinus* by 3.2% in the 16S rRNA gene and 14.2% in the Cytb gene, which are higher than suggested values for species threshold in anura. Recently, another new species *F. limnocharis* was described from the Chittagong University Campus, Chittagong, Bangladesh (Howlader, 2011). Although, Bangladesh has simple topographic features, the south-eastern part of Bangladesh has

relatively high species richness. Reza (2010) reported that the Kaptai National Park has the highest species richness of the 8 selected study sites and lies in the south-eastern region of Bangladesh, which is an agreement with our speculation. Still, many distinct species which were screened in our first study await further description in these regions. *Microhyla* cf. *ornata* from the Sylhet and Chittagong regions is quite different from the available so-called *M. ornata* found in the rest of the country. A detailed description of this species is now under progress by the present authors.

In conclusion, at least 19 species were recognized with eight undescribed taxa in this present study. The new species *H. litoralis* is one such species which indicates that anuran biodiversity has been underestimated in Bangladesh and emphasizes the necessity for further taxonomic studies of anurans in this country.

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Tables and Figures

Table 1. Specimens used and identified *16S* haplotypes found in this study. District names are used as population names in the text.

Family	Species	Collection station		No. of	Specimen	16S rRNA gene haplotype		
		Locality	(District)	frogs used	Voucher No. [b]	No.	Kind	Accession Number
Dicroglossidae	*Euphlyctis cyanophlyctis*	Laboni point	(Cox's Bazar)	8	DFBGBAU Ecya 3-10	4	Ecya-Bd1, 3-5	AB530494, AB530496-AB530498
		Char Nilokhia	(Mymensingh)	1	IABHU 3758	1	Ecya-Bd2*	AB530495
	Euphlyctis hexadactylus	Dacope	(Khulna)	3	IABHU F2242 1-3	1	Ehex-Bd1*	AB530499
		Satkhira	(Satkhira)	1	DFBGBAU Ehex 510	1	Ehex-Bd2	AB543599
	Hoplobatrachus tigerinus	BAUC [a]	(Mymensingh)	1	IABHU 3902	1	Htig-Bd1*	AB530500
		Ukhia	(Cox's Bazar)	2	DFBGBAU Htig 405- 406	2	Htig-Bd2*-3	AB530501, AB530502
		Teknaf	(Cox's Bazar)	1	IABHU 3857	1	Htig-Bd4	AB543600
	Hoplobatrachus crassus	Dacope	(Khulna)	1	DFBGBAU Hrca 1	1	Hcra-Bd1*	AB530503
		Sandwip	(Chittagong)	1	IABHU 3859	1	Hcra-Bd2	AB543601
	Fejervarya sp. large type	Golapganj	(Sylhet)	4	IABHU F2246 1-4	1	Fsp. L-Bd1	AB530504
		BAUC [a]	(Mymensingh)	2	DFBGBAU FspL 313-314	2	Fsp. L-Bd2*-3	AB530505, AB530506
		Dacope	(Khulna)	1	DFBGBAU FspL 156	1	Fsp. L-Bd4	AB530507
	Fejervarya moodiei	Dacope	(Khulna)	1	DFBGBAU Fmod 315	1	Fmod-Bd1*	AB530508
		Teknaf	(Cox's Bazar)	1	IABHU 3860	1	Fmod-Bd2*	AB543602
	Fejervarya sp. small type	Char Nilokhia	(Mymensingh)	1	DFBGBAU FspS 31	1	Fsp. S-Bd1*	AB530509
		Laboni point	(Cox's Bazar)	1	DFBGBAU FspS 11	1	Fsp.S-Bd2	AB530510
	Fejervarya sp. medium type	BAUC [a]	(Mymensingh)	1	DFBGBAU FspM 312	1	Fsp. M-Bd*	AB530511
Rhacophoridae	*Polypedates teraiensis*	Char Nilokhia	(Mymensingh)	13	DFBGBAU Pter 50-52, 202-211	2	Pter-Bd1-2	AB530512, AB530513
		Bisampur	(Sunamganj)	4	DFBGBAU Pter 179, 181, 178, 180	3	Pter-Bd3, 7-8	A B530514, AB530518, AB530519
		Vowal	(Gazipur)	3	IABHU F4040 1-3	2	Pter-Bd4, 6	AB530515, AB530517
		Modhupur	(Tangail)	1	IABHU F4040	1	Pter-Bd5	AB530516
		Sadar Thana	(Bandarban)	2	DFBGBAU Pter 401-402	2	Pter-Bd9-10	AB530520, AB530521
Ranidae	*Hylarana* cf. *taipehensis*	Ghazni	(Sherpur)	5	DFBGBAU Htai 216, 225, 229-231	1	Htai-Bd1*	AB530522
		BAUC [a]	(Mymensingh)	1	DFBGBAU Htai 228	1	Htai-Bd2	AB530523
		Ghorasal	(Narsingdi)	2	IABHU 3893-3894	2	Htai-Bd3-4	AB530524, AB530525
		Barguna	(Barguna)	1	IABHU 3892	1	Htai-Bd5	AB543603
	Hylarana leptoglossa	Kewatkhali, BAUC [a]	(Mymensingh)	3	IABHU 3897, IABHU F2243 1-2	2	Hlep-Bd1*-2	AB530526, AB530527
		Golapganj	(Sylhet)	1	IABHU 3784	1	Hlep-Bd3	AB530528
	Hylarana sp.	Bandarban	(Bandarban)	2	IABHU 3865-3866	2	Hsp. -Bd1*-2	AB543604, AB543605
Microhylidae	*Microhyla* cf. *ornata*	Char Nilokhia	(Mymensingh)	14	IABHU F5012 1-6, BdMsp 75-76, 81, 70, 72-73, 77-78	7	Morn -Bd1*-7	AB530529-AB530535
		BAUC [a]	(Mymensingh)	1	DFBGBAU Msp 306	1	Morn -Bd8	AB530536
		Golapganj	(Sylhet)	2	IABHU 3898-3899	2	Morn -Bd9*-10	AB543606, AB543607
		Raozan	(Chittagong)	2	IABHU 3879-3880	2	Morn -Bd11*-12	AB543608, AB543609
		Parbatipur	(Dinajpur)	3	IABHU 22135-22137	3	Morn-Bd1*-3	AB530537-AB530539
	Microhyla sp.	Golapganj	(Sylhet)	8	DFBGBAU Msp 411-413, 415-416, 418-419, IABHU 3786	2	Msp.-Bd1*, Msp.-Bd3	AB530540, AB530542
		Golapganj + Bandarban	(Sylhet + Bandarban)	2	DFBGBAU Msp 414, IABHU3864	1	Msp.-Bd2	AB530541
	Kaloula pulchra	Golapganj + Sadar Thana	(Sylhet + Bandarban)	3	IABHU 3781-3783	2	Kpul-Bd1*-2	AB530543, AB530544
	Kaloula taprobanica	BAUC [a]	(Mymensingh)	1	IABHU F5013	1	Ktap-Bd*	AB530545
Bufonidae	*Duttaphrynus melanostictus*	BAUC [a]	(Mymensingh)	1	DFBGBAU Dmel 226	1	Dmel-Bd1	AB530546
		Ukhia	(Cox's Bazar)	1	DFBGBAU Dmel 407	1	Dmel-Bd2	AB530547
	Total			107		65		

[a] BAUC, Bangladesh Agricultural University Campus.

[b] DFBGBAU, Department of Fisheries Biology and Genetics, Bangladesh Agricultural University; IABHU, Institute for Amphibian Biology, Hiroshima University.

Table 2. Measurements (mean ± S. D., in mm) for 29 body parts of three species of the genus *Hoplobatrachus* .

	Hoplobatrachus litoralis		*Hoplobatrachus tigerinus*		*Hoplobatrachus rugulosus*	
	Male (n = 14)	Female (n = 13)	Male (n = 9)	Female (n = 6)	Male (n = 3)	Female (n = 4)
SVL	89.96 ± 5.92	101.42 ± 12.01	114.04 ± 6.52	113.67 ± 15.43	99.43 ± 0.71	117.33 ± 10.09
HL	34.09 ± 2.77	38.21 ± 5.00	44.13 ± 2.47	41.53 ± 4.68	35.10 ± 1.05	44.13 ± 2.23
HW	32.24 ± 2.93	36.46 ± 4.89	42.46 ± 3.07	39.95 ± 8.37	37.17 ± 1.56	46.20 ± 2.02
S-N	5.76 ± 0.77	6.05 ± 0.55	7.21 ± 0.65	7.38 ± 1.09	7.33 ± 1.36	8.68 ± 0.91
N-N	5.94 ± 0.57	6.06 ± 0.63	7.03 ± 0.95	6.32 ± 0.79	4.53 ± 1.19	6.58 ± 0.90
N-E	8.41 ± 0.90	8.81 ± 1.19	10.07 ± 0.99	10.18 ± 1.28	8.60 ± 0.79	8.58 ± 0.81
ED	7.76 ± 0.90	7.82 ± 0.63	8.71 ± 1.26	7.70 ± 1.19	5.97 ± 0.93	7.38 ± 1.48
E-E	3.89 ± 0.56	3.88 ± 0.75	7.42 ± 1.52	6.05 ± 0.40	4.57 ± 1.19	6.55 ± 0.64
ELW	6.96 ± 0.93	7.32 ± 1.00	7.23 ± 0.90	6.85 ± 0.55	5.50 ± 0.20	5.53 ± 1.13
TD	6.24 ± 0.51	6.89 ± 1.19	7.84 ± 0.79	7.80 ± 1.48	5.20 ± 0.35	6.30 ± 1.06
FLL	49.72 ± 5.79	53.62 ± 6.47	61.57 ± 4.34	58.17 ± 5.65	50.43 ± 6.56	62.95 ± 2.37
FHL	35.59 ± 3.21	37.67 ± 5.04	43.39 ± 2.67	41.13 ± 3.99	35.33 ± 1.46	43.25 ± 3.99
FAW	8.45 ± 1.56	7.88 ± 1.59	9.16 ± 1.72	7.47 ± 1.58	8.03 ± 0.32	10.18 ± 0.86
HAL	17.71 ± 1.46	19.21 ± 2.47	22.52 ± 2.37	21.02 ± 3.01	19.00 ± 1.71	24.80 ± 1.80
F1	9.04 ± 1.35	10.97 ± 1.38	11.90 ± 2.05	12.15 ± 2.41	9.20 ± 0.10	11.33 ± 2.56
F2	6.48 ± 0.86	8.23 ± 1.24	9.12 ± 2.32	11.12 ± 1.44	7.20 ± 1.71	10.50 ± 0.90
F3	10.16 ± 1.33	11.00 ± 1.02	12.51 ± 2.47	13.92 ± 1.14	11.83 ± 0.29	14.03 ± 2.52
F4	6.78 ± 1.09	7.39 ± 1.05	9.00 ± 1.62	10.05 ± 1.07	6.63 ± 1.01	8.65 ± 2.19
HLL	143.85 ± 12.53	159.74 ± 22.37	183.82 ± 16.36	174.38 ± 13.13	137.40 ± 2.55	157.83 ± 6.66
FEL	45.36 ± 5.42	50.05 ± 7.35	57.76 ± 5.49	54.47 ± 6.12	43.07 ± 2.68	51.10 ± 2.54
TIL	45.93 ± 3.64	50.41 ± 5.67	61.64 ± 5.08	57.75 ± 6.77	41.37 ± 1.40	49.53 ± 2.24
TFL	65.85 ± 6.72	73.08 ± 9.39	82.07 ± 7.22	75.85 ± 7.37	58.17 ± 6.42	74.25 ± 1.71
FOL	43.83 ± 4.32	47.81 ± 6.04	52.56 ± 5.20	49.68 ± 4.12	36.60 ± 7.71	49.75 ± 1.28
T1	9.19 ± 1.00	9.89 ± 1.19	11.91 ± 1.99	11.92 ± 1.47	9.23 ± 0.45	13.23 ± 0.17
T2	16.11 ± 1.98	18.20 ± 2.09	19.89 ± 2.28	20.97 ± 1.61	15.50 ± 1.97	19.93 ± 2.14
T3	22.00 ± 2.56	24.68 ± 3.21	27.72 ± 2.56	28.65 ± 2.53	22.00 ± 1.97	25.60 ± 2.41
T4	28.96 ± 2.83	32.81 ± 3.44	36.69 ± 5.16	38.22 ± 2.93	29.83 ± 2.92	34.00 ± 0.92
T5	20.76 ± 2.67	22.60 ± 3.57	27.98 ± 3.41	27.63 ± 3.80	20.97 ± 1.96	25.55 ± 3.67
IMT	4.86 ± 0.78	5.28 ± 0.99	6.86 ± 1.01	6.22 ± 1.92	3.93 ± 0.96	4.73 ± 0.98

Table 3. Body ratios of three species of the genus *Hoplobatrachus*.

	H. litoralis (n = 27)		*H. tigerinus* (n = 15)		*H. rugulosus* (n = 7)	
	Mean	(Min - Max)	Mean	(Min - Max)	Mean	(Min - Max)
HL/SVL	0.378	(0.349 - 0.436)	0.379	(0.346 - 0.425)	0.367	(0.326 - 0.413)
HW/SVL	0.359	(0.316 - 0.400)	0.363	(0.314 - 0.413)	0.386	(0.352 - 0.424)
S-N/SVL	0.062	(0.044 - 0.076)	0.064	(0.053 - 0.077)	0.074	(0.065 - 0.090)
N-N/SVL	0.063	(0.051 - 0.073)	0.059	(0.052 - 0.074)	0.052	(0.037 - 0.069)
N-E/SVL	0.090	(0.065 - 0.108)	0.089	(0.077 - 0.104)	0.079	(0.064 - 0.096)
ED/SVL	0.082	(0.063 - 0.107)	0.073	(0.058 - 0.093)	0.062	(0.049 - 0.076)
E-E/SVL	0.041	(0.029 - 0.054)	0.060	(0.041 - 0.084)	0.052	(0.036 - 0.061)
ELW/SVL	0.075	(0.060 - 0.094)	0.063	(0.048 - 0.077)	0.051	(0.039 - 0.062)
TD/SVL	0.069	(0.051 - 0.083)	0.069	(0.062 - 0.083)	0.053	(0.042 - 0.066)
FLL/SVL	0.541	(0.484 - 0.626)	0.531	(0.434 - 0.605)	0.526	(0.429 - 0.572)
FHL/SVL	0.384	(0.331 - 0.418)	0.374	(0.316 - 0.405)	0.363	(0.336 - 0.402)
FAW/SVL	0.086	(0.059 - 0.121)	0.074	(0.055 - 0.109)	0.085	(0.076 - 0.102)
HAL/SVL	0.193	(0.175 - 0.206)	0.192	(0.171 - 0.223)	0.203	(0.172 - 0.232)
F1/SVL	0.104	(0.081 - 0.131)	0.105	(0.074 - 0.137)	0.095	(0.068 - 0.112)
F2/SVL	0.077	(0.060 - 0.112)	0.088	(0.053 - 0.114)	0.082	(0.056 - 0.100)
F3/SVL	0.111	(0.091 - 0.132)	0.115	(0.076 - 0.154)	0.120	(0.099 - 0.154)
F4/SVL	0.074	(0.053 - 0.093)	0.083	(0.060 - 0.106)	0.071	(0.054 - 0.095)
HLL/SVL	1.586	(1.400 - 1.703)	1.586	(1.389 - 1.845)	1.364	(1.203 - 1.453)
FEL/SVL	0.498	(0.438 - 0.569)	0.497	(0.409 - 0.597)	0.436	(0.376 - 0.478)
TIL/SVL	0.504	(0.469 - 0.531)	0.528	(0.467 - 0.572)	0.421	(0.378 - 0.455)
TFL/SVL	0.726	(0.625 - 0.782)	0.701	(0.578 - 0.836)	0.614	(0.519 - 0.687)
FOL/SVL	0.479	(0.436 - 0.539)	0.453	(0.389 - 0.536)	0.401	(0.280 - 0.464)
T1/SVL	0.100	(0.075 - 0.117)	0.105	(0.080 - 0.146)	0.104	(0.089 - 0.118)
T2/SVL	0.179	(0.149 - 0.218)	0.179	(0.146 - 0.218)	0.640	(0.135 - 0.178)
T3/SVL	0.244	(0.193 - 0.280)	0.248	(0.202 - 0.292)	0.220	(0.199 - 0.239)
T4/SVL	0.323	(0.277 - 0.368)	0.329	(0.264 - 0.412)	0.295	(0.258 - 0.334)
T5/SVL	0.228	(0.177 - 0.280)	0.245	(0.205 - 0.313)	0.215	(0.181 - 0.252)
IMT/SVL	0.053	(0.043 - 0.069)	0.058	(0.041 - 0.076)	0.040	(0.029 - 0.053)
HL/HW	1.055	(0.961 - 1.222)	1.049	(0.919 - 1.193)	0.951	(0.925 - 0.973)
S-N/N-E	0.693	(0.479 - 0.909)	0.722	(0.617 - 0.871)	0.942	(0.795 - 1.066)
ED/E-E	2.054	(1.333 - 2.808)	1.237	(0.827 - 1.818)	1.220	(0.982 - 1.778)
TD/ED	0.846	(0.600 - 1.152)	0.952	(0.733 - 1.178)	0.847	(0.750 - 1.102)
N-N/E-E	1.575	(1.150 - 2.192)	1.002	(0.663 - 1.273)	1.004	(0.868 - 1.129)
ELW/E-E	1.875	(1.234 - 0.615)	1.060	(0.567 - 1.364)	1.021	(0.647 - 1.472)
F1/F2	1.375	(1.011 - 1.632)	1.241	(0.833 - 1.746)	1.179	(0.815 - 1.625)
TIL/FEL	1.015	(0.871 - 1.137)	1.068	(0.957 - 1.257)	0.966	(0.923 - 1.006)
FOL/FEL	0.966	(0.966 - 1.169)	0.913	(0.808 - 1.067)	0.925	(0.614 - 1.052)
TIL/FOL	1.054	(0.973 - 1.148)	1.171	(1.067 - 1.294)	1.071	(0.918 - 1.504)

Table 4. Statistics obtained by Mann-Whitney U-tests for body ratios. *: P<0.05. **P<0.01.

	H. litoralis vs. *H. tigerinus*			*H. litoralis* vs. *H. rugulosus*		
	U	Z	P	U	Z	P
HL/SVL	197	0.144	0.8852	74	0.873	0.3826
HW/SVL	184	0.486	0.6272	41	2.279	0.0227 *
S-N/SVL	185	0.459	0.6460	36	2.492	0.0127 *
N-N/SVL	128	1.956	0.0505	40	2.321	0.0203 *
N-E/SVL	160	1.116	0.2646	32	2.662	0.0078 **
ED/SVL	99	2.717	0.0066 **	17	3.301	0.0010 **
E-E/SVL	19	4.817	0.0000 **	36	2.492	0.0127 *
ELW/SVL	58	3.793	0.0001 **	1	3.983	0.0001 **
TD/SVL	201	0.039	0.9686	17	3.301	0.0010 **
FLL/SVL	187	0.407	0.6841	93	0.064	0.9491
FHL/SVL	159	1.142	0.2535	50	1.895	0.0580
FAW/SVL	105	2.559	0.0105 *	93	0.064	0.9491
HAL/SVL	175	0.722	0.4704	51	1.853	0.0639
F1/SVL	191.5	0.289	0.7728	55	1.682	0.0925
F2/SVL	137	1.719	0.0855	64	1.299	0.1939
F3/SVL	168	0.906	0.3651	66	1.214	0.2248
F4/SVL	134	1.798	0.0722	84	0.447	0.6547
HLL/SVL	193	0.249	0.8031	2	3.940	0.0001 **
FEL/SVL	202	0.013	0.9895	16	3.343	0.0008 **
TIL/SVL	98	2.743	0.0061 **	0	4.025	0.0001 **
TFL/SVL	128	1.956	0.0505	7	3.727	0.0002 **
FOL/SVL	117	2.244	0.0248 *	9	3.642	0.0003 **
T1/SVL	179	0.617	0.5373	75	0.831	0.4062
T2/SVL	199	0.092	0.9268	48	1.981	0.0476 *
T3/SVL	189	0.354	0.7231	28	2.832	0.0046 **
T4/SVL	192	0.276	0.7828	39	2.364	0.0181 *
T5/SVL	146	1.483	0.1380	72	0.958	0.3379
IMT/SVL	141	1.614	0.1064	20	3.173	0.0015 **
HL/HW	193	0.249	0.8031	2	3.940	0.0001 **
S-N/N-E	165	0.984	0.3249	6	3.770	0.0002 **
ED/E-E	10	5.053	0.0000 **	7	3.727	0.0002 **
TD/ED	109	2.455	0.0141 *	88	0.277	0.7819
N-N/E-E	4	5.211	0.0000 **	0	4.025	0.0001 **
ELW/E-E	3	5.238	0.0000 **	5	3.813	0.0001 **
F1/F2	124	2.061	0.0393 *	45	2.108	0.0350 *
TIL/FEL	127	1.982	0.0475 *	42	2.236	0.0253 *
FOL/FEL	129	1.929	0.0537	91.5	0.128	0.8983
TIL/FOL	33	4.449	0.0000 **	65	1.256	0.2090

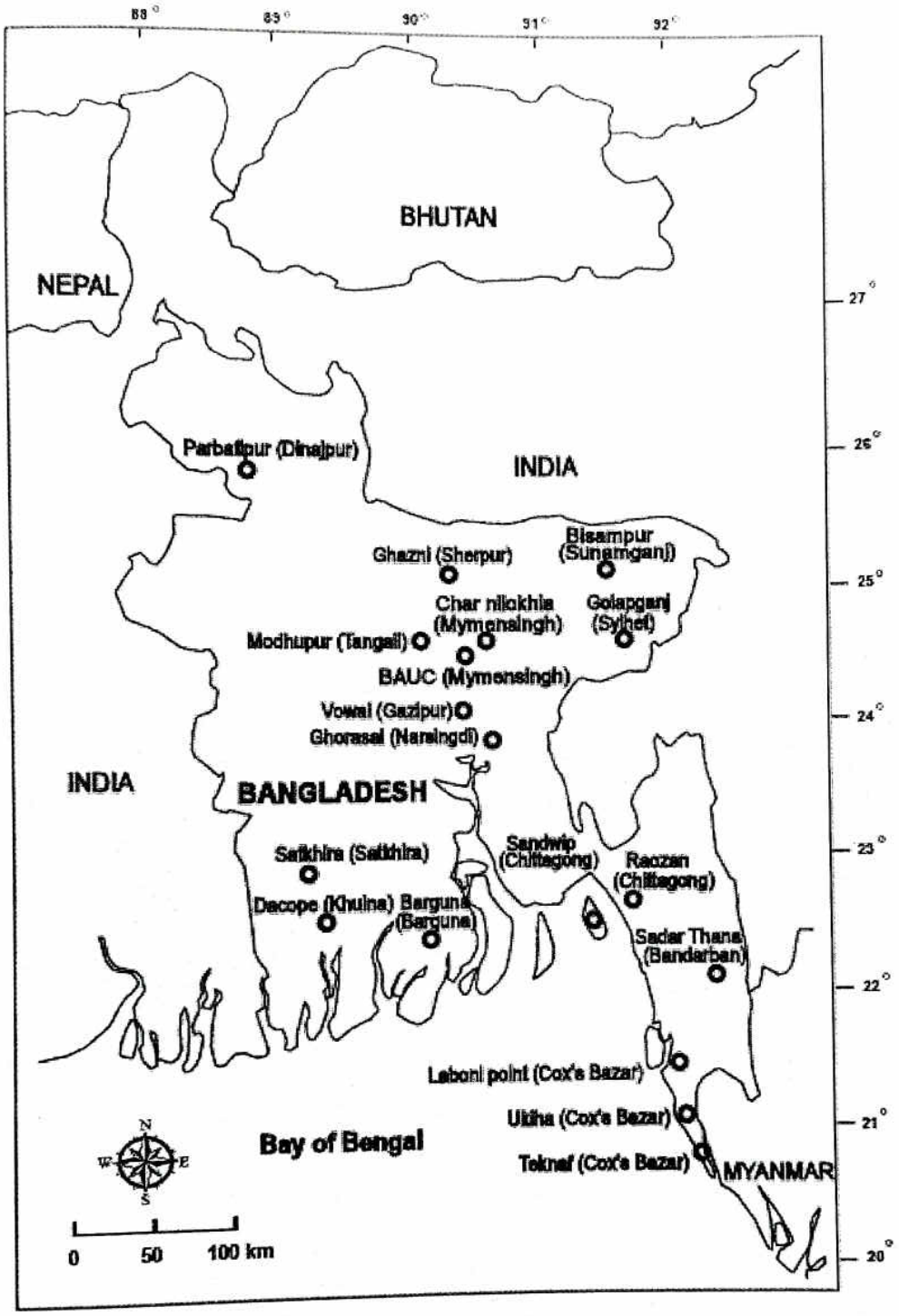


Fig. 1. Map showing the collecting sites of Bangladeshi frogs used for this study. Each black circle represents each sampling sites with locality and district name in parenthesis. Bangladesh neighboring countries are also shown in this map.

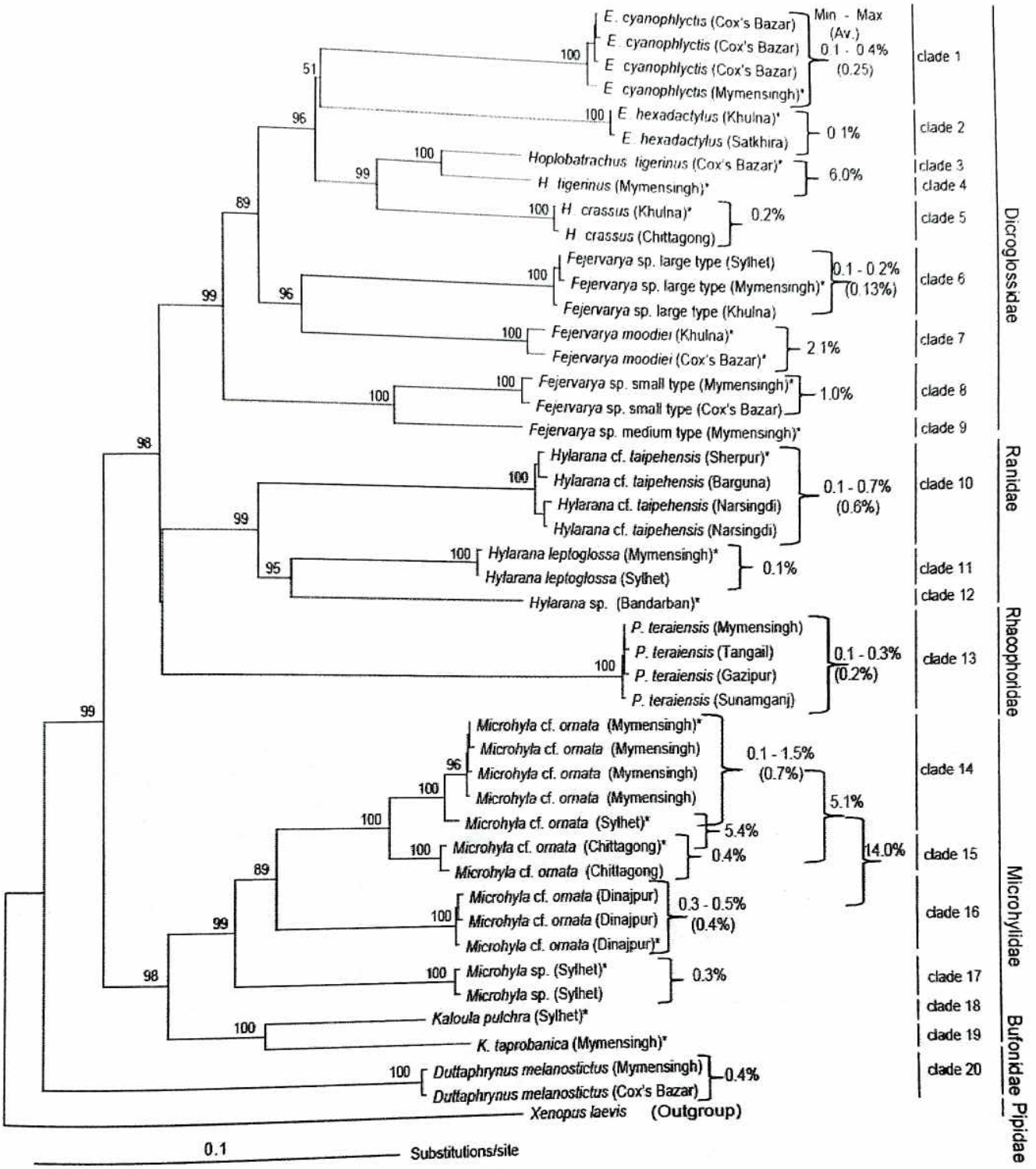


Fig. 2. Neighbor Joining (NJ) tree based on nucleotide sequences of mitochondrial *16S* rRNA gene using the GTR + I + G substitution model from 45 haplotypes with *Xenopus laevis* as an outgroup. The bootstrap support (> 50%) is given above the branches and is based on 1000 replicates. The scale bar represents 0.1 nucleotide substitutions per site for the NJ tree.

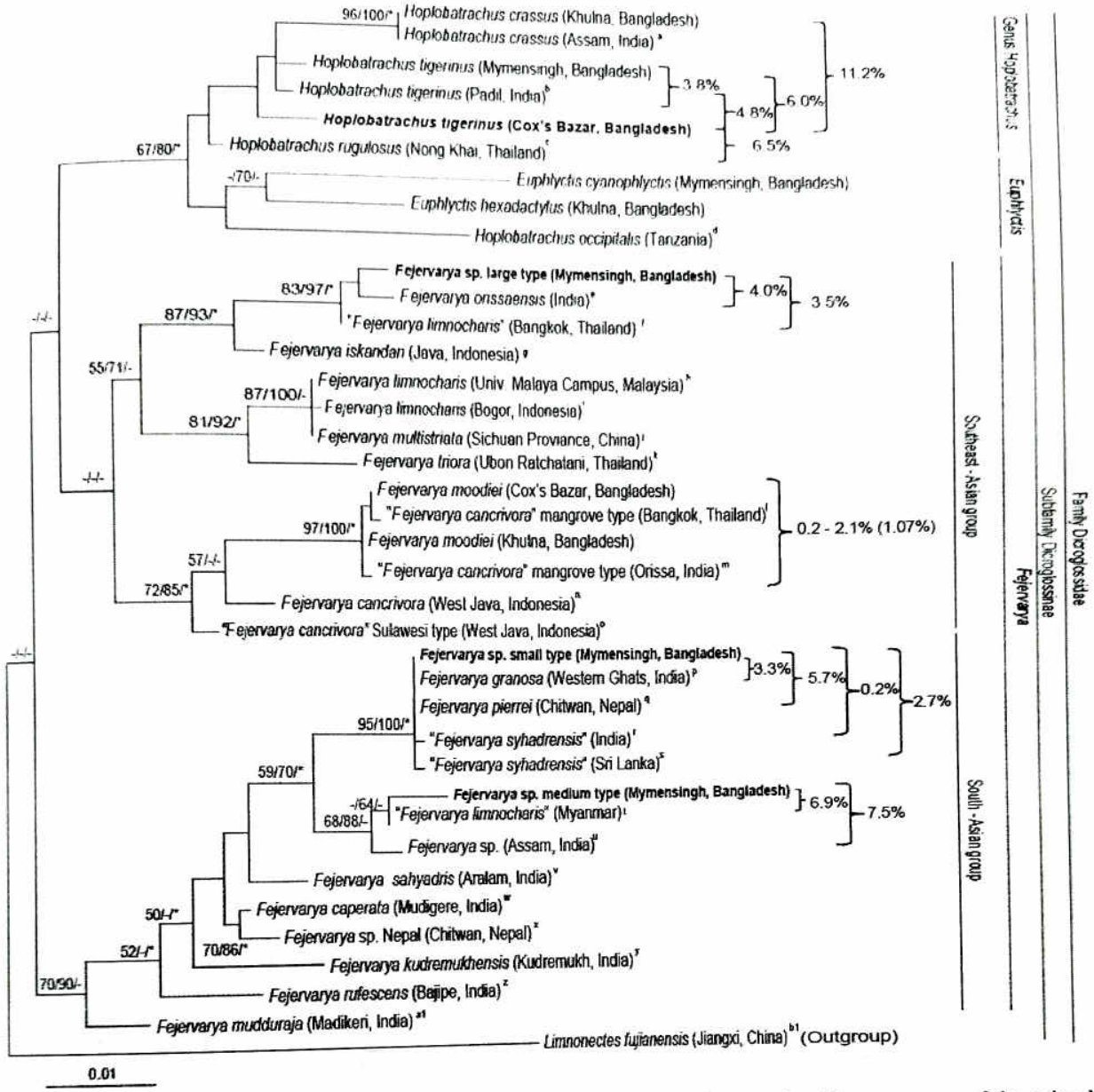


Fig. 3. Maximum Likelihood (ML) tree of dicroglossid frogs based on nucleotide sequences of the mitochondrial *16S* rRNA gene using the SYM + I + G substitution model with *Limnonectes fujianensis* as an outgroup. The bootstrap support (> 50%) is given in order for ML (500) and NJ (1000) replicates. Asterisks represent Bayesian posterior probability (BPP) of ≥ 95%. The scale bar represents 0.01 nucleotide substitutions per site. a) AB290413, Alam et al. (2008); b) AB272594, Alam et al. (2008); c) AB272596, Alam et al. (2008); d) AB272599, Alam et al. (2008); e) AY882957, Tandon et al. (Unpublished); f) AB162444, Sumida et al. (2007); g) AB530613, Hasan et al. (In preparation); h) AB530625, Hasan et al. (In preparation); i) AJ292015, Vieth et al. (2001); j) AB530611, Hasan et al. (In preparation); k) AB488883, Kotaki et al. (2010); l) AB444691, Kurniawan et al. (2010); m) AY841754, Guha et al. (Unpublished); n) AB444689, Kurniawan et al. (2010); o) AB444693, Kurniawan et al. (2010); p) AB167947, Kurabayashi et al. (2005); q) AB488888, Kotaki et al. (2010); r) AY841748, Guha et al. (Unpublished); s) AY141843, Meegaskumbura et al. (2002); t) AF206466, Chen et al. (2005); u) AB488900, Kotaki et al. (2010); v) AB530604, Hasan et al. (In preparation); w) AB530606, Hasan et al. (In preparation); x) AB488889, Kotaki et al. (2010); y) AB530603, Hasan et al. (In preparation); z) AB530601, Hasan et al. (In preparation); a1) AB530607, Hasan et al. (In preparation); and b1) AB526311, Matsui et al. (2010).

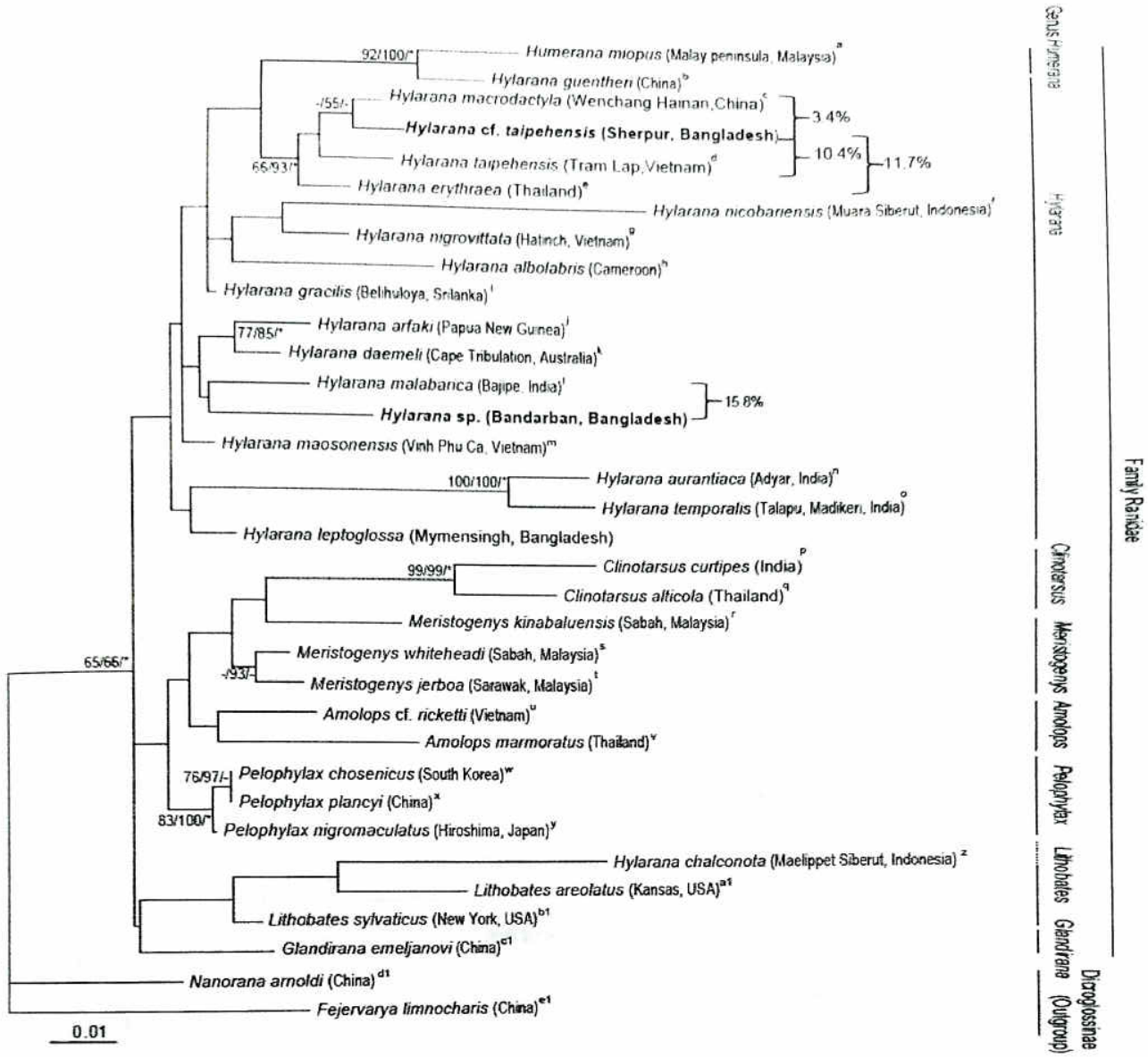


Fig. 4. Maximum Likelihood (ML) tree of ranid frogs based on nucleotide sequences of the mitochondrial *16S* rRNA gene using the GTR + I + G substitutions model with *Nanorana arnoldi* and *Fejervarya limnocharis* as outgroups. The bootstrap support (> 50%) is given in order for ML (500) and NJ (1000) replicates. Asterisks represent Bayesian posterior probability (BPP) of ≥ 95%. The scale bar represents 0.01 nucleotide substitutions per site. a) AB200962, Matsui et al. (2005); b) DQ360001, Che et al. (2007); c) DQ360002, Che et al. (2007); d) AF206495, Chen et al. (2005); e) AB530580, Hasan et al. (In preparation); f) AB530581, Hasan et al. (In preparation); g) DQ283371, Frost et al. (2006); h) DQ283369, Frost et al. (2006); i) AY014376, Kosuch et al. (2001); j) DQ283203, Frost et al. (2006); k) DQ283201, Frost et al. (2006); l) AB530579, Hasan et al. (In preparation), m) DQ283373, Frost et al. (2006); n) AB530574, Hasan et al. (In preparation); o) AB530578, Hasan et al. (In preparation); p) AF249058, Bossuyt & Milinkovitch (2000); q) AB200961, Matsui et al. (2005); r) AB526618, Shimada et al. (2011); s) AB526617, Shimada et al. (2011); t) AB526608, Shimada et al. (2011); u) AY322286, Roelants et al. (2004); v) AB211486, Matsui et al. (2006); w) EU386908, Min et al. (Unpublished); x) EF196679, Nie et al. (Unpublished); y) AB043889, Sumida et al. (2001); z) AB530583, Hasan et al. (In preparation); a1) AY779229, Hillis & Wilcox, (2005); b1) DQ347336, Bossuyt et al. (2006); c1) AY322281, Roelants et al. (2004); d1) EU979836, Che et al. (2009); and e1) AY158705, Liu et al. (2005).

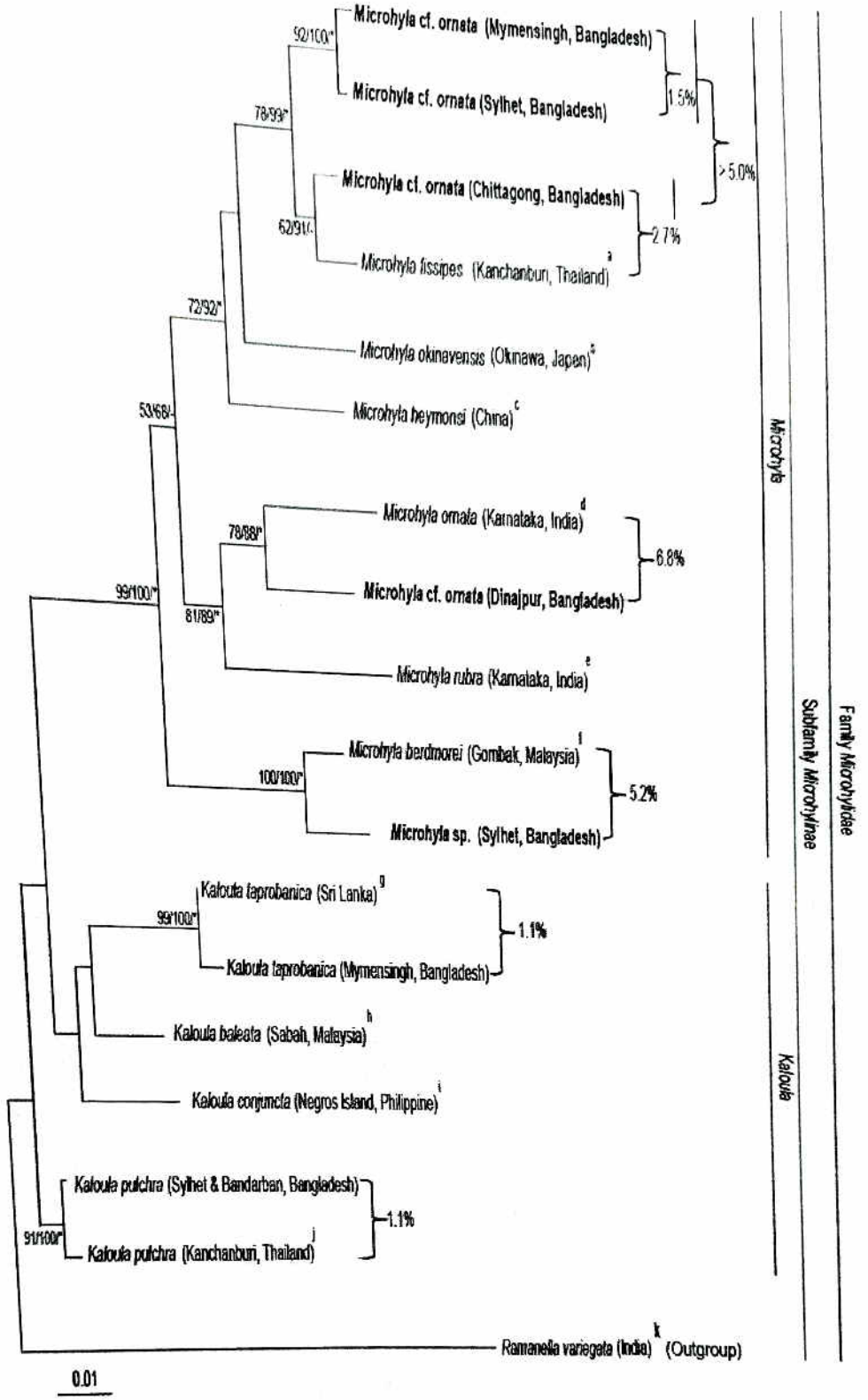


Fig. 5. Maximum Likelihood (ML) tree of microhylid frogs based on nucleotide sequences of the mitochondrial *16S* rRNA gene using the GTR + I + G substitutions model with *Ramanella variegata* as an outgroup. The bootstrap support (> 50%) is given in order for ML (500) and NJ (1000) replicates. Asterisks represent Bayesian posterior probability (BPP) of ≥ 95%). The scale bar represents 0.01 nucleotide substitutions per site. a) AB201186, Matsui et al. (2005); b) AB303950, Igawa et al. (2008); c) AY458596, Zhang et al. (2005); d) AB201188, Matsui et al. (2005); e) AB201192, Matsui et al. (2005); f) AB530638, Hasan et al. (In preparation); g) AF249057, Bossuyt & Milinkovitch, (2000); h) GU154880, Das & Haas, (2010); i) AY326064, Darst & Cannatella, (2004); j) AB201194, Matsui et al. (2005); and k) GU136114, Meenakshi et al. (2009).

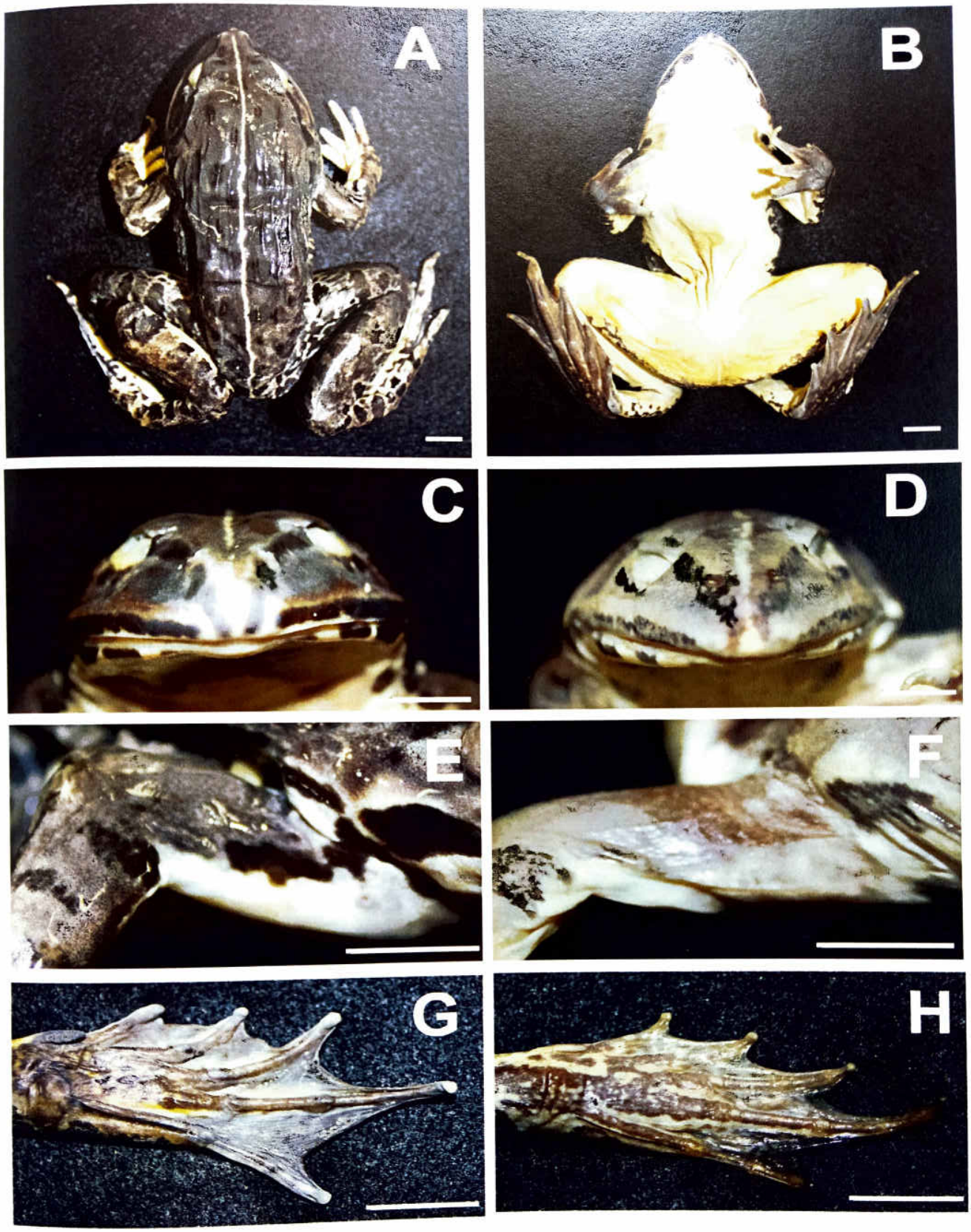


Fig. 6. Holotype (IABHU 3993) of *Hoplobatrachus litoralis* sp. nov. after preservation in alcohol. (A) Dorsal aspect. (B) Ventral aspect. (C) Frontal aspect of head showing distinct black bands, compared with (D) that of *H. tigerinus*. (E) Coloration of upper arm of *H. litoralis* (IABHU 3993), compared with (F) that of *H. tigerinus*. (G) Foot of paratype (IABHU 3980), compared with (H) that of *H. tigerinus*. Scale bar = 10 mm

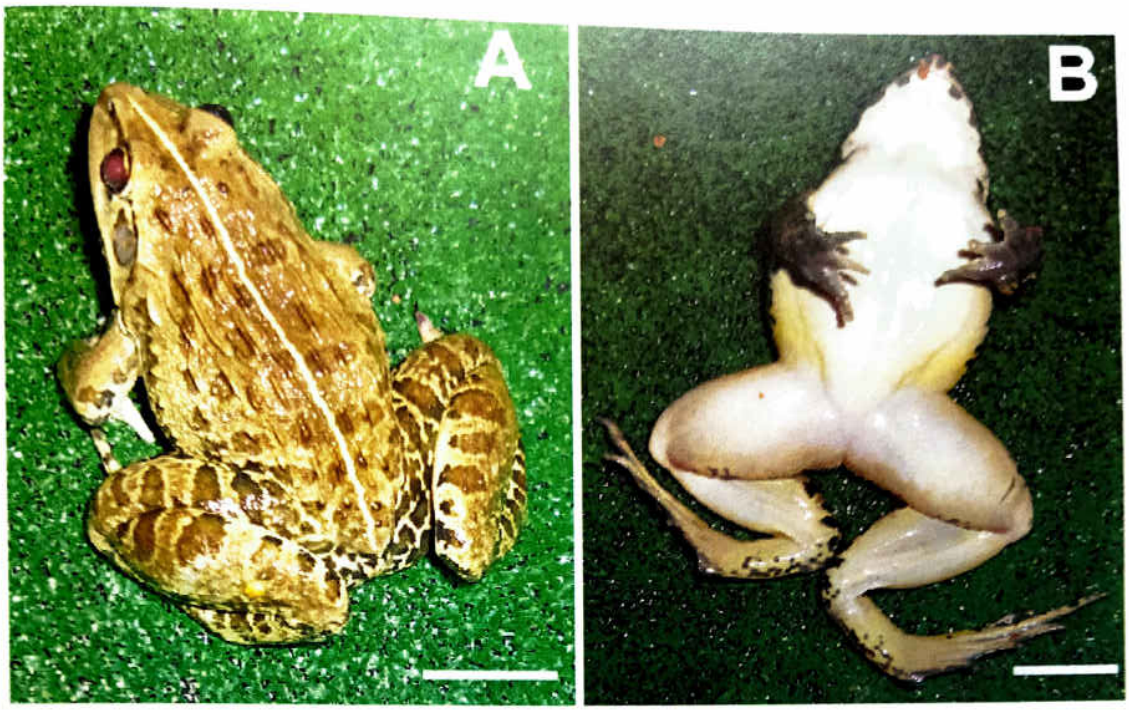


Fig. 7. Holotype (IABHU 3993) of *H. litoralis* sp. nov. in life. (A) Dorsal view. (B) Ventral view. Scale bar = 25 mm.

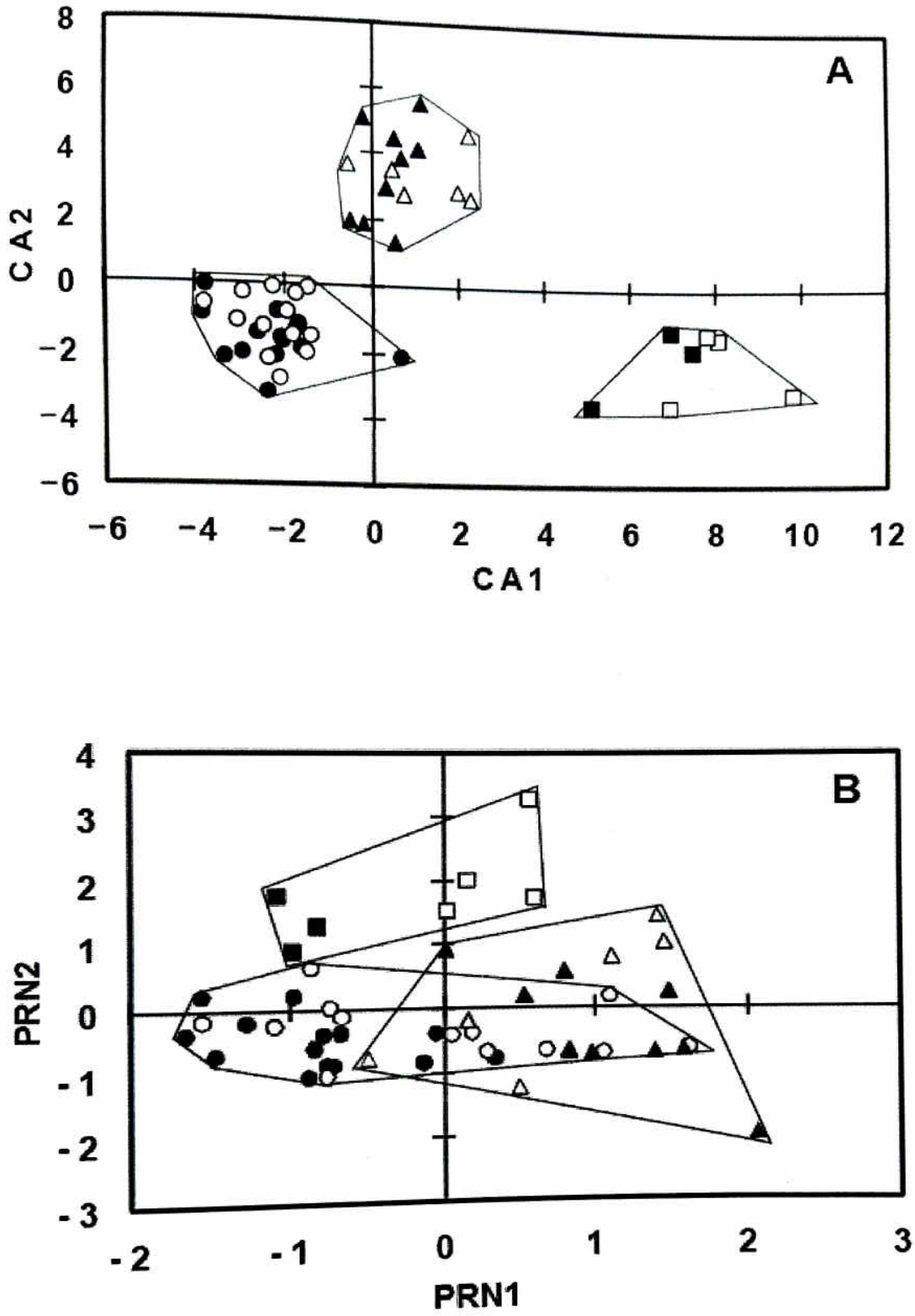


Fig. 8. (A) Scatterplot of individual discriminant scores on the first (CA1) and second canonical axes (CA2) for *H. litoralis* (circle), *H. tigerinus* (triangle), and *H. rugulosus* (rectangle). (B) Scatterplot of principal component 1 (PRN1) versus principal component 2 (PRN2) from the principal component analysis of *H. litoralis* (circle), *H. tigerinus* (triangle), and *H. rugulosus* (rectangle). In each species males were marked as solid and females were marked as open icons.

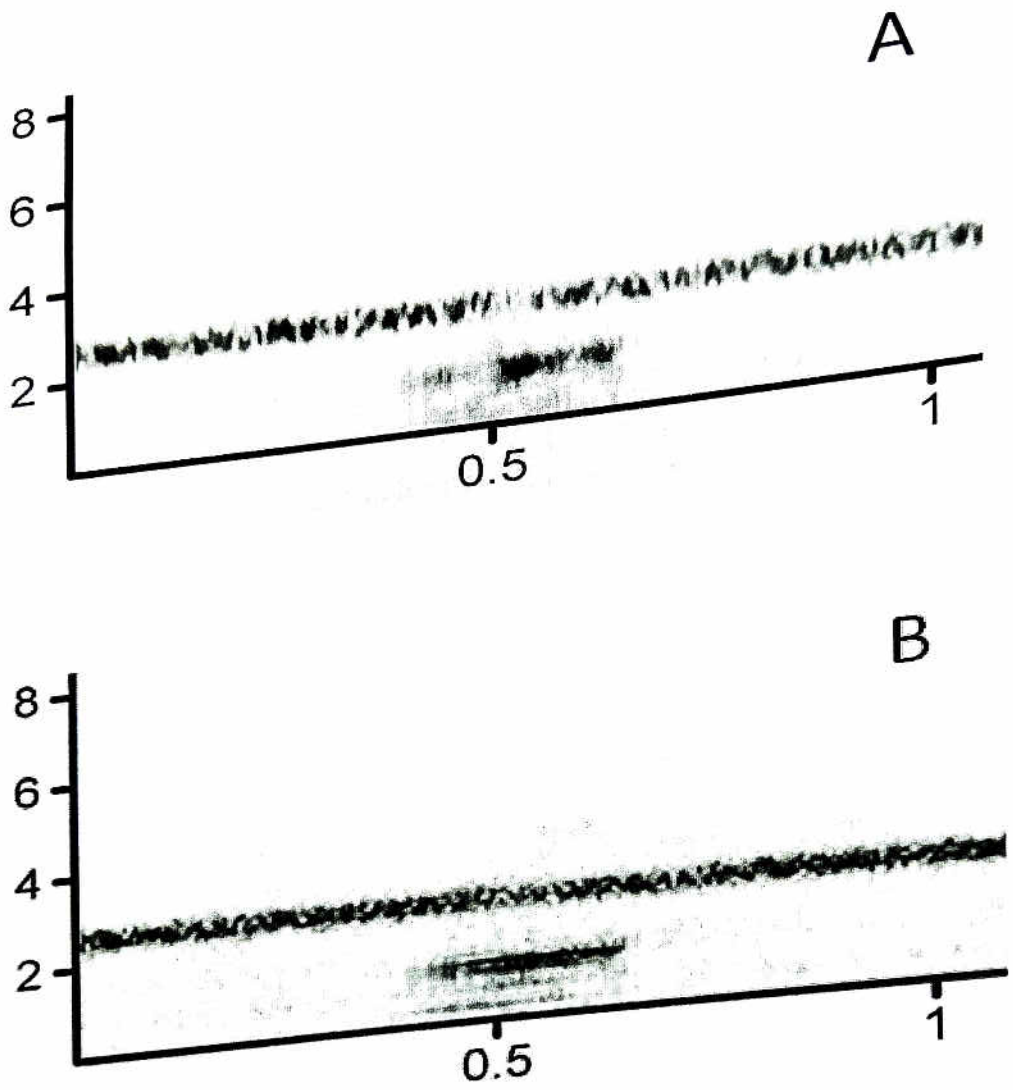


Fig. 9. Sound spectrograms showing advertisement call structure of *H. litoralis*. The same call analyzed by FlatTop (A) and Hamming window (B). Abscissa: time in s. Ordinate: frequency in kHz. The continuous broad band at about 2.5 kHz is chirps of an insect.

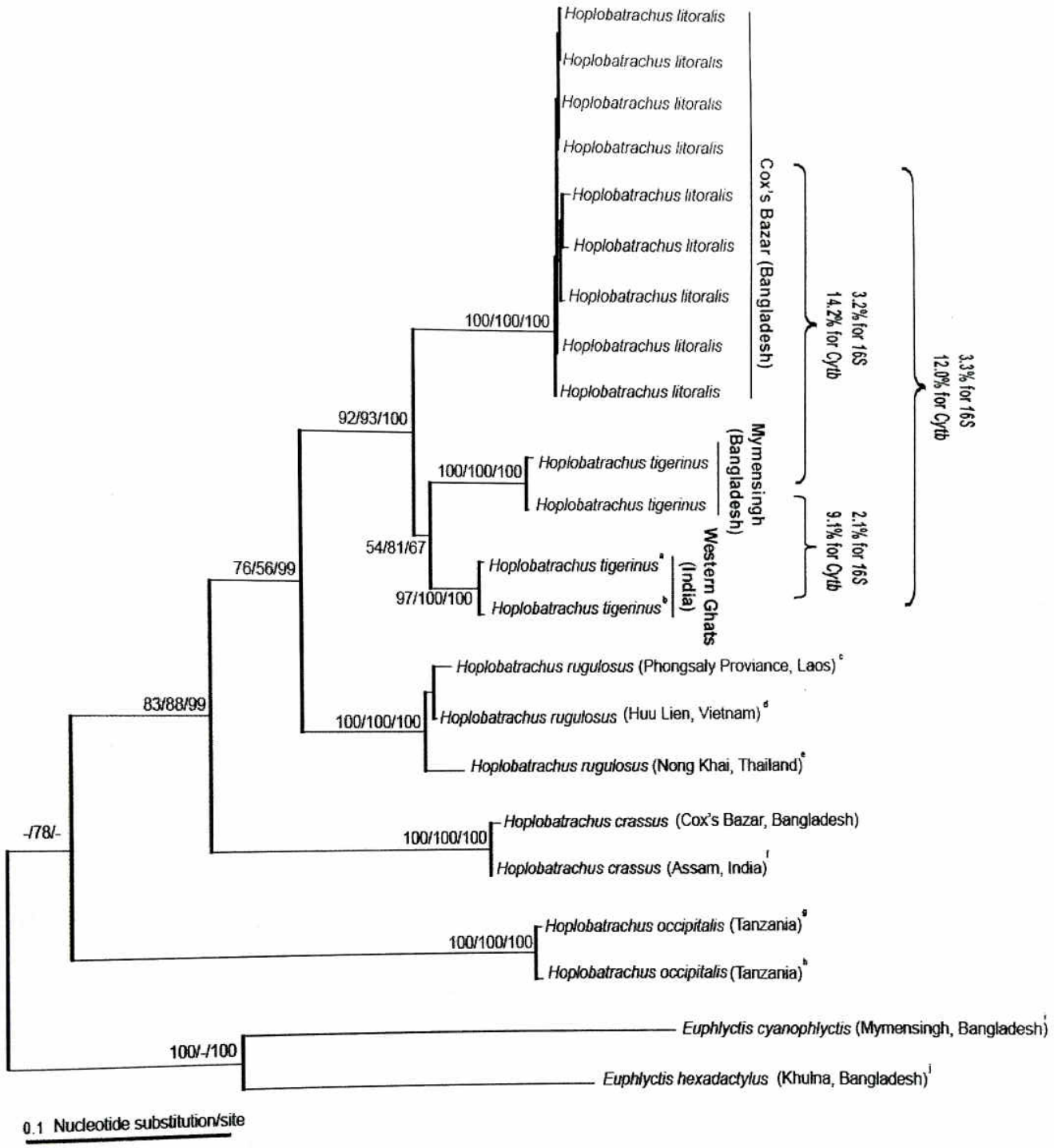


Fig. 10. Maximum likelihood (ML) tree based on the nucleotides sequence of 1078 bp of mitochondrial (*16S* + *Cytb*) genes with *Euphlyctis cyanophlyctis* and *E. hexadactylus* as out groups. The most parsimonious and Bayesian analyses reconstructed the same tree topology. Numbers near branches represent bootstrap support for ML and MP inferences, and Bayesian posterior probability (ML–BPs/MP–BPs/BPP). The scale bar represents 0.1 nucleotide substitutions per site. The superscript letters indicate that the *16S* and *Cytb* data were taken from Alam et al. (2008) for constructing this tree. a) AB272594, AB274137; b) AB290412, AB274139; c) AB290417, AB290601; d) AB290414, AB290603; e) AB272596, AB274144; f) AB290413, AB290597; g) AB272599, AB274148; h) AB272600, AB274150; i) AB272601, AB274151 and j) AB272605, AB274163.

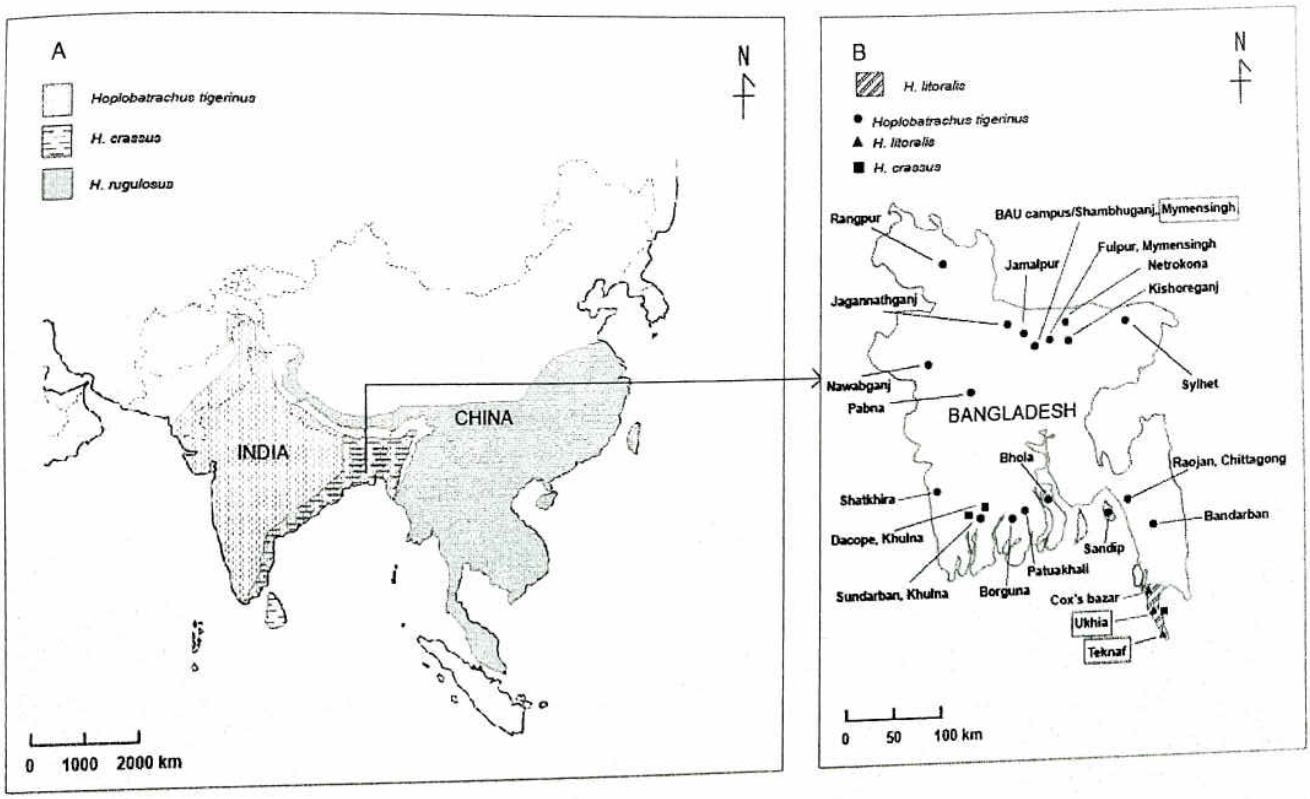


Fig. 11. (A) Map showing the approximate distribution areas of all Asian *Hoplobatrachus* species based on Frost et al. (2011) and Alam et al. (2008). (B) Bangladesh map showing the collecting localities of *H. litoralis* is indicated by ▲ (closed triangle) and, the collecting localities of *H. tigerinus* and *H. crassus* are indicated by ● (closed circle) and ■ (closed square), respectively. Each locality was used for molecular analysis, and the boxed locality was used for morphology.

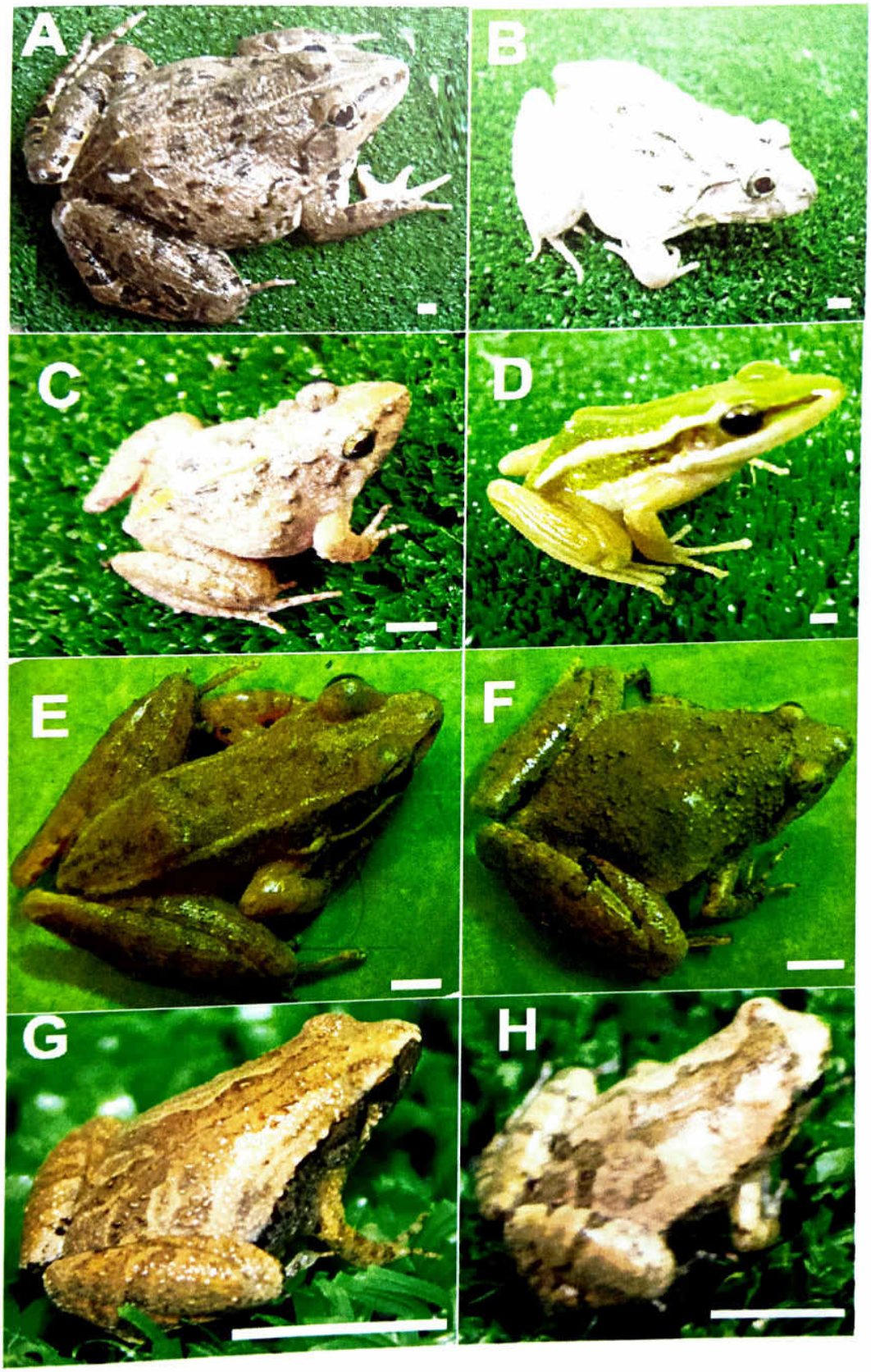
Supplementary Table and Figure

Supplementary Table S1. Conclusive list of samples including GenBank data examined in this study

Family	Genus	Species	Voucher/Tissue Ref.	Locality	Accession No.	Source
Dicroglossidae	*Euphlyctis*	*E. cyanophlyctis*	DFBGBAU Ecya 3-10	Bangladesh: Cox's Bazar	AB530494, AB530496-AB530498	This study
		E. cyanophlyctis	IABHU 3758	Bangladesh: Mymensingh	AB530495	This study
		E. hexadactylus	IABHU F2242 1-3	Bangladesh: Khulna	AB530499	This study
		E. hexadactylus	DFBGBAU Ehex 510	Bangladesh: Satkhira	AB543599	This study
	Hoplobatrachus	*H. tigerinus*	IABHU 3902	Bangladesh: Mymensingh	AB530500	This study
		H. tigerinus	DFBGBAU Htig 405- 406, IABHU 3857	Bangladesh: Cox's Bazar	AB530501, AB530502, AB543600	This study
		H. tigerinus	NA	India: Padil	AB272594	Alam et al., 2008
		H. crassus	DFBGBAU Hrca 1	Bangladesh: Khulna	AB530503	This study
		H. crassus	IABHU 3859	Bangladesh: Chittagong	AB543601	This study
		H. crassus	NA	India: Assam	AB290413	Alam et al., 2008
		H. rugulosus	NA	Thailand: Nong Khai	AB272596	Alam et al., 2008
		H. occipitalis	NA	Tanzania	AB272599	Alam et al., 2008
	Fejervarya	*Fejervarya* sp. large type	IABHU F2246 1-4	Bangladesh: Sylhet	AB530504	This study
		Fejervarya sp. large type	DFBGBAU FspL 313-314	Bangladesh: Mymensingh	AB530505, AB530506	This study
		Fejervarya sp. large type	DFBGBAU FspL 156	Bangladesh: Khulna	AB530507	This study
		F. orissaensis	NA	India	AY882957	Tandon et al., (Unpublished)
		F. limnocharis	NA	Thailand: Bangkok	AB162444	Sumida et al., 2007
		F. limnocharis	IABHU 21015	Malaysia: Univ. Malaya Campus	AB530625	Hasan et al., (In preparation)
		F. limnocharis	NA	Indonesia: Bogor	AJ292015	Veith et al., 2001
		F. limnocharis	USNM 520407	Myanmar	AF206466	Chen et al., 2005
		F. limnocharis	NA	China	AY158705	Liu et al., 2005
		F. iskandari	IABHU 3816	Indonesia: Java	AB530613	Hasan et al., (In preparation)
		F. multristriata	Alive	China: Sichuan Proviance	AB530611	Hasan et al., (In preparation)
		F. triora	NA	Thailand: Ubon Ratchatani	AB488883	Kotaki et al., 2010
		F. moodiei	DFBGBAU Fmod 315	Bangladesh: Khulna	AB530508	This study
		F. moodiei	IABHU 3860	Bangladesh: Cox's Bazar	AB543602	This study
		F. cancrivora	NA	Thailand: Bangkok	AB444691	Kurniawan et al., 2010
		F. cancrivora	NA	India: Orissa	AY841754	Guha et al., (Unpublished)
		F. cancrivora	NA	Indonesia: West Java	AB444689	Kurniawan et al., 2010
		F. cancrivora	NA	Indonesia: West Java	AB444693	Kurniawan et al., 2010
		Fejervarya sp. small type	DFBGBAU FspS 31	Bangladesh: Mymensingh	AB530509	This study
		Fejervarya sp. small type	DFBGBAU FspS 11	Bangladesh: Cox's Bazar	AB530510	This study
		F. granosa	IABHU 20022	India: Western Ghats	AB167947	Kurabayashi et al., 2005
		F. pierrei	NA	Nepal: Chitwan	AB488888	Kotaki et al., 2010
		F. syhadrensis	NA	India	AY841748	Guha et al., (Unpublished)
		F. syhadrensis	WHT2665	Sri Lanka	AY141843	Meegaskumbura et al., 2002
		Fejervarya sp. medium type	DFBGBAU FspM 312	Bangladesh: Mymensingh	AB530511	This study
		Fejervarya sp. Nepal	NA	India: Assam	AB488900	Kotaki et al., 2010
		F. sahyadris	IABHU 20215	India: Aralam	AB530604	Hasan et al., (In preparation)
		F. caperata	IABHU 20612	India: Mudigere	AB530606	Hasan et al., (In preparation)
		Fejervarya sp.	NA	Nepal: Chitwan	AB488889	Kotaki et al., 2010
		F. kudremukhensis	IABHU 20025	India: Kudremukh	AB530603	Hasan et al., (In preparation)
		F. rufescens	IABHU 20013	India: Bajipe	AB530601	Hasan et al., (In preparation)
		F. mudduruja	IABHU 20020	India: Madikeri	AB530607	Hasan et al., (In preparation)
	Limnonectes	*L. fujianensis*	CIB:ZJ 200806223	China: Jiangxi	AB526311	Matsui et al., 2010
Rhacophoridae	*Polypedates*	*P. teraiensis*	DFBGBAU Pter 50-52, 202-211	Bangladesh: Mymensingh	AB530512, AB530513	This study
		P. teraiensis	DFBGBAU Pter 179, 181, 178, 180	Bangladesh: Sunamganj	A B530514, AB530518, AB530519	This study
		P. teraiensis	IABHU F4040 1-3	Bangladesh: Gazipur	AB530515, AB530517	This study
		P. teraiensis	IABHU F4040	Bangladesh: Tangail	AB530516	This study
		P. teraiensis	DFBGBAU Pter 401-402	Bangladesh: Bandarban	AB530520, AB530521	This study
Ranidae	*Hylarana*	*H. cf. taipehensis*	DFBGBAU Htai 216, 225, 229-231	Bangladesh: Sherpur	AB530522	This study
		H. cf. taipehensis	DFBGBAU Htai 228	Bangladesh: Mymensingh	AB530523	This study
		H. cf. taipehensis	IABHU 3893-3894	Bangladesh: Narsingdi	AB530524, AB530525	This study
		H. cf. taipehensis	IABHU 3892	Bangladesh: Barguna	AB543603	This study

		H. taipehensis	ROM 7193	Vietnam: Tram Lap	AF206495	Chen et al., 2005
		H. guentheri	SCUMH002	China	DQ360001	Che et al., 2007
		H. macrodactyla	SCUMH004	Chian: Wenchang Hainan	DQ360002	Che et al., 2007
		H. erythraea	NA	Thailand	AB530580	Hasan et al., (In preparation)
		H. nicobariensis	IABHU 20707	Indonesia: Muara Siberut	AB530581	Hasan et al., (In preparation)
		H. nigrovittata	AMNH A161280	Vietnam: Ha Tinch	DQ283371	Frost et al., 2006
		H. albolabris	UTA A44424	Cameroon	DQ283369	Frost et al., 2006
		H. gracilis	NA	Sri Lanka: Belihuloya	AY014376	Kosuch et al., 2001
		H. arfaki	AMS R114913	Papua New Guinea	DQ283203	Frost et al., 2006
		H. daemeli	SAMA R40355	Australia: Cape Tribulation	DQ283201	Frost et al., 2006
		H. malabarica	IABHU 20335	India: Bajipe	AB530579	Hasan et al., (In preparation)
		Hylarana sp.	IABHU 3865-3866	Bangladesh: Bandarban	AB543604, AB543605	This study
		H. maosonensis	AMNH A161487	Vietnam: Vinh Phu Ca	DQ283373	Frost et al., 2006
		H. aurantiaca	IABHU 20519	India: Adyar	AB530574	Hasan et al., (In preparation)
		H. temporalis	IABHU 20301	India: Talapu, Madikeri	AB530578	Hasan et al., (In preparation)
		H. leptoglossa	IABHU 3897, IABHU F2243 1-2	Bangladesh: Mymensingh	AB530526, AB530527	This study
		H. leptoglossa	IABHU 3784	Bangladesh: Sylhet	AB530528	This study
		H. chalconota	IABHU 20720	Indonesia: Maelippet Siberut	AB530583	Hasan et al., (In preparation)
	Humerana	*H. miopus*	KUHE:15247	Malaysia: Malay Peninsula	AB200962	Matsui et al., 2005
	Clinotarsus	*C. curtipes*	NA	India	AF249058	Bossuyt & Milinkovitch, 2000
		C. alticola	KUHE:19530	Thailand	AB200961	Matsui et al., 2005
	Meristogenys	*M. kinabaluensis*	SPM:SP 21546	Malaysia: Sabah	AB526618	Shimada et al., 2011
		M. whitwheadi	UMS:BORNEENSIS 23010	Malaysia: Sabah	AB526617	Shimada et al., 2011
		M. jerboa	KUHE:12028	Malaysia: Sarawak	AB526608	Shimada et al., 2011
	Amolops	*A.* cf *ricketti*	NA	Vietnam	AY322286	Rolents et al., 2004
		A. marmoratus	KUHE:KUHE19089	Thailand	AB211486	Matsui et al., 2006
	Pelophylax	*P. chosenicus*	NA	South Korea	EU386908	Min et al., (Unpublished)
		P. plancyi	NA	China	EF196679	Nie et al., (Unpublished)
		P. nigromaculatus	NA	Japan: Hiroshima	AB043889	Sumida et al., 2001
	Lithobates	*L. areolatus*	KU 204370	USA: Kansas	AY779229	Hillis & Wilcox, 2005
		L. sylvaticus	NA	USA: New York	DQ347336	Bossuyt et al., 2006
	Glandirana	*G. emeljanovi*	NA	China	AY322281	Roelants et al., 2004
	Nanorana	*N. arnoldi*	SCUM05152123WD	China	EU979836	Che et al., 2009
Microhylidae	*Microhylidae*	*Microhyla* cf. *ornata*	IABHU F5012 1-6, BdMsp 75-76, 81, 70, 72-73, 77-78, DFBGEBAU Msp 306	Bangladesh: Mymensingh	AB530529-AB530536	This study
		Microhyla cf. *ornata*	IABHU 3898-3899	Bangladesh: Sylhet	AB543606, AB543607	This study
		Microhyla cf. *ornata*	IABHU 3879-3880	Bangladesh: Chittagong	AB543608, AB543609	This study
		Microhyla cf. *ornata*	IABHU 22135-22137	Bangladesh: Dinajpur	AB530537-AB530539	This study
		M. ornata	NA	India: Karnataka	AB201188	Matsui et al., 2005
		M. fissipes	KUHE35165	Thailand: Kanchanburi	AB201186	Matsui et al., 2005
		M. okinavensis	IABHU5263	Okinawa: Japan	AB303950	Igawa et al., 2008
		M. heymonsi	NA	Taiwan	AY458596	Zhang et al., 2005
		M. rubra	NA	India: Karnataka	AB201192	Matsui et al., 2005
		Microhyla sp.	DFBGBAU Msp 411-413, 415-416, 418-419, IABHU 3786	Bangladesh: Sylhet	AB530540, AB530542	This study
		Microhyla sp.	DFBGBAU Msp 414, IABHU3864	Bangladesh: Sylhet+Bandarban	AB530541	This study
		M. berdmorei	IABHU 21019	Malaysia: Gombak FSC	AB530638	Hasan et al., (In preparation)
	Kaloula	*K. pulchra*	IABHU 3781-3783	Bangladesh: Sylhet+Bandarban	AB530543, AB530544	This study
		K. pulchra	KUHE35171	Thailand: Kanchanburi	AB201194	Matsui et al., 2005
		K. taprobanica	IABHU F5013	Bangladesh: Mymensingh	AB530545	This study
		K. taprobanica	NA	Sri Lanka	AF249057	Bossuyt & Milinkovitch, 2000
		K. baleata	ZMH A10028	Malaysia: Sabah	GU154880	Das & Haas, 2010
		K. conjuncta	RMB 2252; PNM/CMNH	Philippine: Negros Island	AY326064	Darst & Cannatella, 2004
	Ramanella	*R. variegata*	NA	India	GU136114	Meenakshi et al., 2009
Bufonidae	*Duttaphrynus*	*D. melanostictus*	DFBGBAU Dmel 226	Bangladesh: Mymensingh	AB530546	This study
		D. melanostictus	DFBGBAU Dmel 407	Bangladesh: Cox's Bazar	AB530547	This study

DFBGBAU = Department of Fisheries Biology and Genetics, Bangladesh Agricultural University,
IABHU = Institute for Amphibian Biology, Hiroshima University,
NA = Not available



Supplementary Figure S1.

Cryptic anuran species from Bangladesh. (A) *H. tigerinus* (Cox's Bazar). (B) *Fejervarya* sp. large type (Mymensingh). (C) *Fejervarya* sp. medium type (Mymensingh). (D) *H.* cf. *taipehensis* (Sherpur). (E) *Hylarana* sp. (Bandarban). (F) *Microhyla* sp. (Sylhet). (G) *M.* cf. *ornata* (Mymensingh). (H) *M.* cf. *ornata* (Dinajpur). Scale bars indicate 5 mm.

公表論文

(1) Cryptic anuran biodiversity in Bangladesh revealed by mitochondrial 16S rRNA gene sequences

Mahmudul Hasan, Mohammed Mafizul Islam, Md. Mukhlesur Rahman Khan, Mohammad Shafiqul Alam, Atsushi Kurabayashi, Takeshi Igawa, Mitsuru Kuramoto and Masayuki Sumida

Zoological Science (2012) 29: 162-172

(2) A new species of genus *Hoplobatrachus* (Anura, Dicroglossidae) from the coastal belt of Bangladesh

Mahmudul Hasan, Mitsuru Kuramoto, Md. Mukhlesur Rahman Khan, Mohammed Mafizul Islam and Masayuki Sumida

Zootaxa (2012) 3312: 45-48

ZOOLOGICAL SCIENCE 29: 162–172 (2012)

Cryptic Anuran Biodiversity in Bangladesh Revealed by Mitochondrial 16S rRNA Gene Sequences

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To survey the diversity of anuran species in Bangladesh, we compared mitochondrial 16S rRNA gene sequences (approximately 1.4 kbp) from 107 Bangladesh frog specimens. The results of genetic divergence and phylogenetic analyses incorporating data from related species revealed the occurrence of at least eight cryptic species. *Hoplobatrachus tigerinus* from two districts diverged considerably, indicating the involvement of a cryptic species. Two *Fejervarya* sp. (large and medium types) and *Hylarana* cf. *taipehensis* formed lineages distinct from related species and are probably new species. *Microhyla* cf. *ornata* differed from *M. ornata* with respect to type locality area and involved two distinct species. In addition, we found that *Hylarana* sp. and *Microhyla* sp. did not match congeners examined to date in either morphology or 16S rRNA sequence. The occurrence of *M. fissipes* was tentatively suggested. Consequently, at least, 19 species were found from Bangladesh in this study. These findings revealed a rich anuran biodiversity in Bangladesh, which is unexpected considering the rather simple topographic features of the country.

Key words: biodiversity, cryptic species, 16S rRNA gene, Anura, Bangladesh

INTRODUCTION

Bangladesh is a riverine country nestled between the Indo-Himalayan and Indo-Chinese sub-regions of the Oriental region (Nishat et al., 2002). The country consists predominantly of low plains comprising the Ganges-Brahmaputra River delta, one of the world's largest deltas, and lacks high mountainous regions. In the last decade, more than 60 new anuran species, including the new family Nasikabatrachidae, have been described in the neighboring India (e.g., Biju and Bossuyt, 2003, 2009; Kuramoto et al., 2007). Recently, the abundance of anuran biodiversity in northeast India, which is located adjacent to northern and eastern Bangladesh, has been revealed in several studies. For example, Pawar and Birand (2001) listed 57 anuran species, including several possibly new species, from this area, and Ao et al. (2003) reported 19 new records of frogs from Nagaland, five of which are new to India. Mathew and Sen (2009) described 11 new species from northeast India. Similarly, in Myanmar, the other country bordering the southeastern corner of Bangladesh, three new species have been described (Wogan et al., 2003; Wilkinson et al., 2003, 2005), and more than 10 new species which were described in the last decade from Yunnan, China, and Thailand are presumed to exist in Myanmar (see Frost, 2011) and Wogan et al. (2008) added 12 anuran species to the herpetofauna of Myanmar. Notably, most of these newly added species were found in mountainous regions, including the Western Ghats and Nagaland in India, and only a few species were described from the lowlands. Considering the topographic features in Bangladesh, it can be expected that the anuran biodiversity is relatively low. Recently, Kabir et al. (2009) assembled a list of 34 amphibian species across 20 genera of six families in Bangladesh based on morphology and scattered information from field research. In this list, however, no species endemic to Bangladesh have been recognized.

Recent molecular phylogenetic studies focusing on the family Dicroglossidae have suggested the existence of many cryptic species in Bangladesh. Islam et al. (2008a, b), using mitochondrial gene sequencing and allozyme analyses, identified three *Fejervarya* species that differed from *F. limnocharis* and other known congeners, and designated them as *Fejervarya* sp. large, medium and small types. In addition, Hasan et al. (2008) detected a considerable allozymic divergence among three populations of *Hoplobatrachus tigerinus* in Bangladesh, while Alam et al. (2008) found notable mitochondrial 16S rRNA gene divergence among *Euphlyctis*

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cyanophlyctis and *E. hexadactylus* from Bangladesh and neighboring countries. Together, these studies highlight the current underestimation of anuran biodiversity and necessity for more extensive review of anuran taxonomy in Bangladesh.

Mitochondrial DNA is an effective molecular marker for use in examining genetic divergence and phylogenetic relationships of animal taxa (e.g., Avise, 2000). In South and Southeast Asia, mitochondrial gene information has been used to identify numerous cryptic anuran species (Meegaskumbura et al., 2002; Kurabayashi et al., 2005; Stuart et al., 2006; Kuramoto et al., 2007; Sumida et al., 2007; Alam et al., 2008; Islam et al., 2008b; Inger et al., 2009; Joshy et al., 2009; Kurniawan et al., 2010). In amphibians, the mitochondrial 16S rRNA gene (*16S*) is considered a reliable marker for determining the taxonomic status of frog species (Vences et al., 2005).

In the present study, to survey anuran biodiversity in Bangladesh, we collected frog specimens from throughout Bangladesh and performed molecular phylogenetic analyses using *16S* data. Here, specimens belonging to Ranidae, Rhacophoridae, Microhylidae, and Bufonidae from Bangladesh are examined for the first time. Thus, this study constitutes the first attempt to review the anuran biodiversity in Bangladesh based on molecular data.

MATERIALS AND METHODS

Specimens

Species identification was based mainly on morphological characteristics described by Dutta and Manamendra-Arachchi (1996), Chanda (2002), and Kabir et al. (2009). We followed the species names adopted in the system of Frost (2011), with the exceptions of *Fejervarya sahyadris* (= *Minervarya sahyadris*), which is nested in the South Asian *Fejervarya* clade (Kuramoto et al., 2007; Kotaki et al., 2010), and *F. moodiei*, which is revived from the synonymy of *F. cancrivora* (corresponding to Mangrove type) (Kurniawan et al., 2011). Most dicroglossid specimens in the present study were collected from localities that differ from those of previous studies.

A total of 107 specimens were collected from 18 localities of 14 districts of Bangladesh (Fig. 1). Based on their external morphology and relevant literature, *Euphlyctis cyanophlyctis*, *E. hexadactylus*, *Hoplobatrachus crassus*, *H. tigerinus*, *F. moodiei*, *Hylarana leptoglossa*, *Polypedates teraiensis*, *Kaloula pulchra*, *K. taprobanica*, and *Duttaphrynus melanostictus* were identified. Specimens resembling *Hylarana taipehensis* and *Microhyla ornata* are treated here as *H.* cf. *taipehensis* and *M.* cf. *ornata*, respectively. Specimens belonging to the genera *Hylarana* and *Microhyla*, but not fitting the descriptions of known congeners, are treated here as *Hylarana* sp. and *Microhyla* sp., respectively. The three unnamed *Fejervarya* taxa are referred to as *Fejervarya* sp. large, medium, and small types, following the designation of Islam et al. (2008a).

DNA extraction, PCR, and sequencing

Total genomic DNA was extracted from the clipped toe of each frog specimen using a DNeasy Tissue Kit (Qiagen, Valencia, USA), as per the manufacturer's instructions. The extracted DNA solutions were used as polymerase chain reaction (PCR) templates for amplifying a partial *16S* region corresponding to positions 3093–4467 of the *16S* gene of *Xenopus laevis* (accession no. M10217; Roe et al., 1985).

PCR amplification and sequencing were performed using the primers F51 and R51 (Sumida et al., 2002), 12S_3′ end_Fow1 (5′–AGAAGARGYAAGTCGTAACA–3′), 12S_3′end_Fow2 (5′–GYAAGTCGTAACAYGGTAAG–3′), 16S_R530 (5′–GGCGATGTTTTTGGTAAACAG–3′), and 16S_R723 (5′–GGAGAADDDYDWHTTCTTRTTAC–3′). The length of the resultant *16S* fragments varied from 1332 to 1390 bp between identified haplotypes. PCR mixtures were prepared with the TaKaRa Ex Taq™ Kit (TaKaRa Bio, Inc., Shiga, Japan), as recommended in the manufacturer's protocol. The *16S* fragments were amplified using 35 cycles, with each cycle consisting of denaturation for 10 s at 98°C, annealing for 30 s at 47.5°C (10 cycles), 45.0°C (10 cycles), and 42.5°C (15 cycles), and extension for 1 min 20 s at 72°C. The PCR products were purified using MicroSpin™ S-300 HR columns (GE Healthcare, Buckinghamshire, UK). Both strands of the amplified *16S* fragments were directly sequenced using the BigDye Terminator Cycle Sequencing Kit (ABI) with an automated DNA sequencer (3100-Avant; ABI, Brooklyn, USA). The obtained sequences were deposited in the DNA Data Bank of Japan (DDBJ) database under the accessions numbers AB530494 to AB530547 and AB543599 to AB543609.

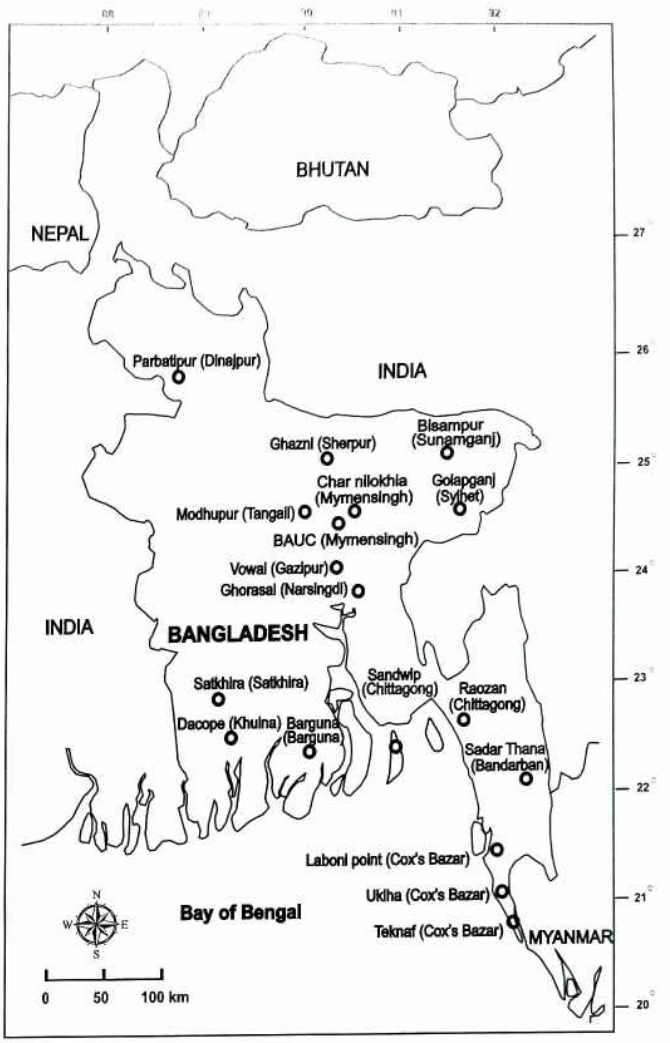

Fig. 1. Map showing the collecting sites of Bangladeshi frogs used for this study. Each black circle represents a sampling site with locality and district name in parenthesis. Bangladesh neighboring countries are also shown in this map.

Alignment data and identified haplotypes

The *16S* sequences from the 107 Bangladeshi frog specimens and *X. laevis* were aligned using the ClustalW program (Thompson et al., 1994). The initial alignment consisted of 1496 nucleotide sites and showed 65 distinct haplotypes. This initial alignment was used for computing the sequence divergence (uncorrected *P* values) among the haplotypes using MEGA Ver. 4.0 (Tamura et al., 2007) with the pairwise-deletion option, in which all alignable sites were

used in the calibration, but indel sites were not counted. The indel and ambiguous alignment sites were then removed using Gblocks Ver. 0.91b (Castresana, 2000) with default parameters, resulting in 1,010 well-aligned sites. After the deletion of indel and ambiguous sites, several of the haplotypes had identical *16S* sequences, and the initial 65 haplotypes were reduced to 45 haplotypes, which were used for constructing a neighbor joining (NJ) tree (see below).

Detailed phylogenetic analyses were performed with respect to the families Dicroglossidae, Ranidae, and Microhylidae using the *16S* data of our specimens and related species in neighboring countries. The *16S* data of related species were obtained from the DDBJ/EMBL/GenBank databases. We selected the related taxa and their *16S* sequences on the basis of (1) BLAST searches, (2) most relevant congeners of Bangladeshi frogs reported by Kabir et al. (2009), and (3) results of our previous studies (Alam et al., 2008). The procedures to construct alignment datasets for each family and to calculate *16S* divergences were identical to those described above. The *16S* sequence lengths of the alignment datasets varied among the three families and were shortened from the initial alignment depending on the lengths of *16S* sequences obtained from DNA databases. The sequence lengths and total number of operational taxonomic units (OTUs) determined from the alignment data were 291 sites of 38 OTUs for dicroglossids, 308 sites of 34 OTUs for ranids, and 457 sites of 18 OTUs for microhylids.

Phylogenetic analyses

We first reconstructed an NJ tree using the alignment data of the 45 haplotypes of Bangladeshi frogs. An appropriate substitution model was estimated using Akaike information criterion (AIC) implemented in Modeltest 3.7 (Posada and Crandall, 1998), and the GTR + I + G model was selected. Support for the nodes of the resultant tree was evaluated by bootstrap probabilities (BPs) calculated from 1000 replicates for NJ analyses. *Xenopus laevis* was used as the outgroup in this analysis.

Further phylogenetic analyses of the families Dicroglossidae, Ranidae, and Microhylidae were performed by the maximum likelihood (ML), NJ, and Bayesian inference (BI) methods. The ML, NJ, and BI analyses were performed using PAUP* 4.0b10 (Swofford, 2003) and MrBayes Ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) software, respectively. Appropriate substitution models were selected using AIC (SYM + I + G, GTR + I + G, and GTR + I + G for the families Dicroglossidae, Ranidae, and Microhylidae, respectively). Node support of the resultant trees was evaluated by BPs calculated from 500 and 1000 replicates for the ML and NJ analy-

Table 1. Specimens used and identified *16S* haplotypes found in this study. District names are used as population names in the text.

Family	Species	Collection station		No. of frogs used	Specimen Voucher No. [b]	16S rRNA gene haplotype		
		Locality	(District)			No.	Kind	Accession Number
Dicroglossidae	*Euphlyctis cyanophlyctis*	Laboni point	(Cox's Bazar)	8	DFBGBAU Ecya 3-10	4	Ecya-Bd1, 3-5	AB530494, AB530496-AB530498
		Char Nilokhia	(Mymensingh)	1	IABHU 3758	1	Ecya-Bd2*	AB530495
	Euphlyctis hexadactylus	Dacope	(Khulna)	3	IABHU F2242 1-3	1	Ehex-Bd1*	AB530499
		Satkhira	(Satkhira)	1	DFBGBAU Ehex 510	1	Ehex-Bd2	AB543599
	Hoplobatrachus tigerinus	BAUC [a]	(Mymensingh)	1	IABHU 3902	1	Htig-Bd1*	AB530500
		Ukhia	(Cox's Bazar)	2	DFBGBAU Htig 405-406	2	Htig-Bd2*-3	AB530501, AB530502
		Teknaf	(Cox's Bazar)	1	IABHU 3857	1	Htig-Bd4	AB543600
	Hoplobatrachus crassus	Dacope	(Khulna)	1	DFBGBAU Hrca 1	1	Hcra-Bd1*	AB530503
		Sandwip	(Chittagong)	1	IABHU 3859	1	Hcra-Bd2	AB543601
	Fejervarya sp. large type	Golapganj	(Sylhet)	4	IABHU F2246 1-4	1	Fsp. L-Bd1	AB530504
		BAUC [a]	(Mymensingh)	2	DFBGBAU FspL 313-314	2	Fsp. L-Bd2*-3	AB530505, AB530506
		Dacope	(Khulna)	1	DFBGBAU FspL 156	1	Fsp. L-Bd4	AB530507
	Fejervarya moodiei	Dacope	(Khulna)	1	DFBGBAU Fmod 315	1	Fmod-Bd1*	AB530508
		Teknaf	(Cox's Bazar)	1	IABHU 3860	1	Fmod-Bd2*	AB543602
	Fejervarya sp. small type	Char Nilokhia	(Mymensingh)	1	DFBGBAU FspS 31	1	Fsp. S-Bd1*	AB530509
		Laboni point	(Cox's Bazar)	1	DFBGBAU FspS 11	1	Fsp.S-Bd2	AB530510
	Fejervarya sp. medium type	BAUC [a]	(Mymensingh)	1	DFBGBAU FspM 312	1	Fsp. M-Bd*	AB530511
Rhacophoridae	*Polypedates teraiensis*	Char Nilokhia	(Mymensingh)	13	DFBGBAU Pter 50-52, 202-211	2	Pter-Bd1-2	AB530512, AB530513
		Bisampur	(Sunamganj)	4	DFBGBAU Pter 179, 181, 178, 180	3	Pter-Bd3, 7-8	A B530514, AB530518, AB530519
		Vowal	(Gazipur)	3	IABHU F4040 1-3	2	Pter-Bd4, 6	AB530515, AB530517
		Modhupur	(Tangail)	1	IABHU F4040	1	Pter-Bd5	AB530516
		Sadar Thana	(Bandarban)	2	DFBGBAU Pter 401-402	2	Pter-Bd9-10	AB530520, AB530521
Ranidae	*Hylarana* cf. *taipehensis*	Ghazni	(Sherpur)	5	DFBGBAU Htai 216, 225, 229-231	1	Htai-Bd1*	AB530522
		BAUC [a]	(Mymensingh)	1	DFBGBAU Htai 228	1	Htai-Bd2	AB530523
		Ghorasal	(Narsingdi)	2	IABHU 3893-3894	2	Htai-Bd3-4	AB530524, AB530525
		Barguna	(Barguna)	1	IABHU 3892	1	Htai-Bd5	AB543603
	Hylarana leptoglossa	Kewatkhali, BAUC [a]	(Mymensingh)	3	IABHU 3897, IABHU F2243 1-2	2	Hlep-Bd1*-2	AB530526, AB530527
		Golapganj	(Sylhet)	1	IABHU 3784	1	Hlep-Bd3	AB530528
	Hylarana sp.	Bandarban	(Bandarban)	2	IABHU 3865-3866	2	Hsp. -Bd1*-2	AB543604, AB543605
Microhylidae	*Microhyla* cf. *ornata*	Char Nilokhia	(Mymensingh)	14	IABHU F5012 1-6, BdMsp 75-76, 81, 70, 72-73, 77-78	7	Morn -Bd1*-7	AB530529-AB530535
		BAUC [a]	(Mymensingh)	1	DFBGBAU Msp 306	1	Morn -Bd8	AB530536
		Golapganj	(Sylhet)	2	IABHU 3898-3899	2	Morn -Bd9*-10	AB543606, AB543607
		Raozan	(Chittagong)	2	IABHU 3879-3880	2	Morn -Bd11*-12	AB543608, AB543609
		Parbatipur	(Dinajpur)	3	IABHU 22135-22137	3	Morn-Bd1*-3	AB530537-AB530539
	Microhyla sp.	Golapganj	(Sylhet)	8	DFBGBAU Msp 411-413, 415-416, 418-419, IABHU 3786	2	Msp.-Bd1*, Msp.-Bd3	AB530540, AB530542
		Golapganj + Bandarban	(Sylhet + Bandarban)	2	DFBGBAU Msp 414, IABHU 3864	1	Msp.-Bd2	AB530541
	Kaloula pulchra	Golapganj + Sadar Thana	(Sylhet + Bandarban)	3	IABHU 3781-3783	2	Kpul-Bd1*-2	AB530543, AB530544
	Kaloula taprobanica	BAUC [a]	(Mymensingh)	1	IABHU F5013	1	Ktap-Bd*	AB530545
Bufonidae	*Duttaphrynus melanostictus*	BAUC [a]	(Mymensingh)	1	DFBGBAU Dmel 226	1	Dmel-Bd1	AB530546
		Ukhia	(Cox's Bazar)	1	DFBGBAU Dmel 407	1	Dmel-Bd2	AB530547
	Total			107		65		

[a] BAUC, Bangladesh Agricultural University Campus.
[b] DFBGBAU, Department of Fisheries Biology and Genetics, Bangladesh Agricultural University; IABHU, Institute for Amphibian Biology, Hiroshima University.
*used for further molecular analyses (ML/NJ/BI) incorporating GenBank data.

ses, respectively. BI analysis was performed with the following settings: Markov chain Monte Carlo of 2×10^6 generations and sampling frequency of 100. The burn-in size was determined by checking the convergence of -log likelihood (-ln*L*) values, and the first 10% generations were discarded. Statistical support of the BI tree was evaluated by Bayesian posterior probability (BPP).

RESULTS

Haplotypes and phylogeny of Bangladesh frogs

Among the *16S* sequences from 107 frog specimens, we identified 65 haplotypes (sequences with ≥ 1 nucleotide change were assigned as different haplotypes). These haplotypes and their DNA database accession numbers are shown in Table 1. The initial 65 haplotypes were reduced to 45 after indel and ambiguous sites were excluded from analysis. For the remaining haplotypes, we constructed an NJ tree (Fig. 2), which showed five well-supported major clades corresponding to the five families involved. Interfamilial relationships and generic level relationships within each family were congruent with nearly all recent molecular phylogenetic studies (e.g., Frost et al., 2006; Roelants et al., 2007). The paraphyletic nature of the genus *Fejervarya* with respect to the genera *Hoplobatrachus* and *Euphlyctys*, which has been suggested in several studies (Frost et al., 2006; Kotaki et al., 2008, 2010), was also supported.

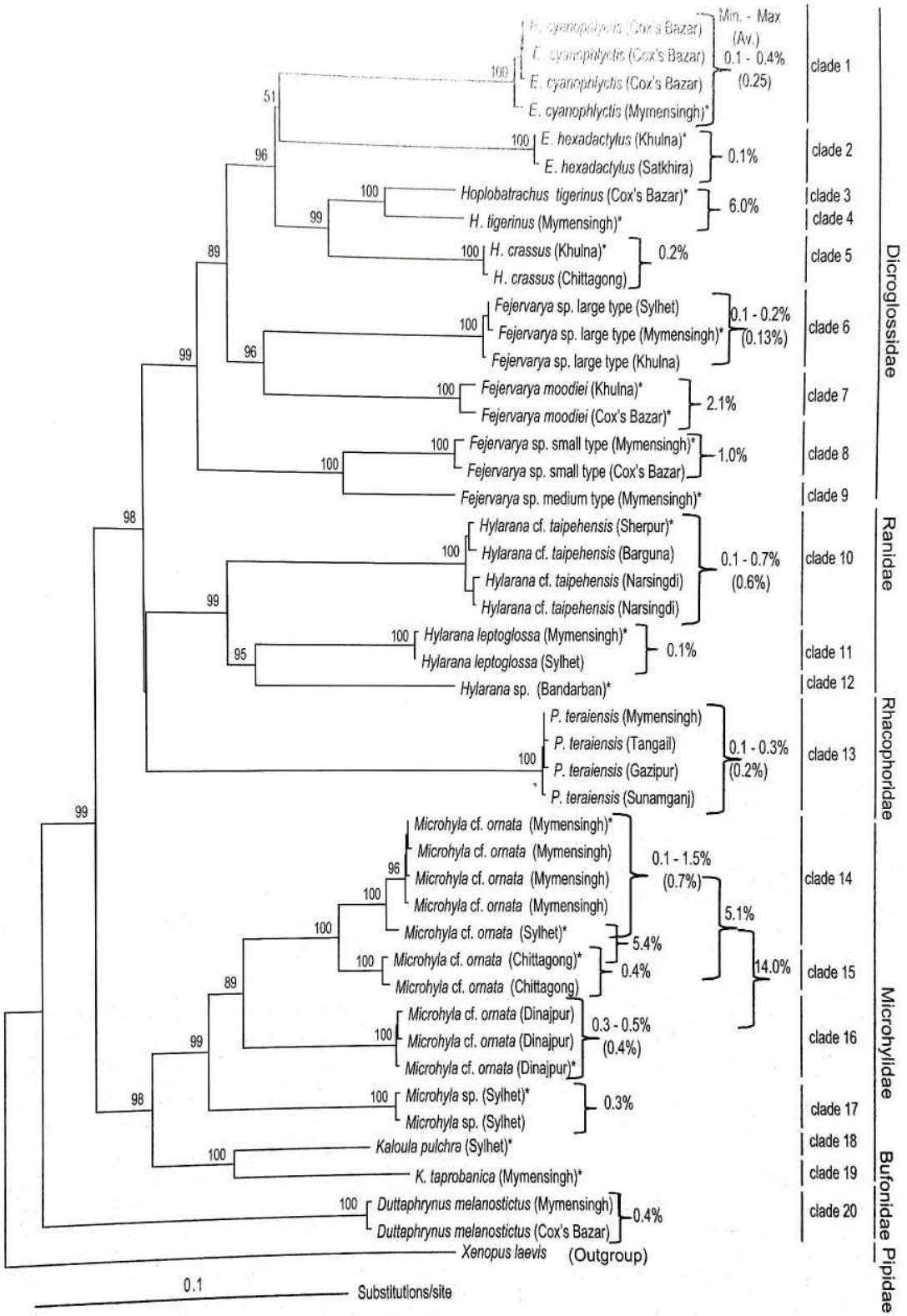

Fig. 2. Neighbor Joining (NJ) tree based on nucleotide sequences of mitochondrial *16S* rRNA gene using the GTR + I + G substitution model from 45 haplotypes with *Xenopus laevis* as an outgroup. The bootstrap support (> 50%) is given above the branches and is based on 1000 replicates. The scale bar represents 0.1 nucleotide substitutions per site for the NJ tree.

As shown in Fig. 2, each species formed a clade, and in many cases, the average *16S* divergence within each species was less than 1.0%. However, slightly divergent haplotypes were detected in *F. moodiei* (2.1%), and the *16S* divergence between *H. tigerinus* from Mymensingh and Cox's Bazar was remarkably high (6.0%). Although the haplotypes of *M.* cf. *ornata* from Mymensingh and those from Sylhet were only slightly divergent (1.5%), markedly high divergence was found between *M.* cf. *ornata* from Chittagong and the above two populations (5.1% and 5.4%, respectively). Furthermore, *M.* cf. *ornata* from Dinajpur constituted a distinct clade from

other *M.* cf. *ornata* specimens and exhibited 14.0% *16S* divergence with respect to the abovemen-tioned populations. The high *16S* divergences among the Chittagong, Dinajpur, and Mymensingh + Sylhet specimens indicated that the *M.* cf. *ornata* specimens with similar external morphology consist of three distinct species. The remaining *Microhyla* sp. from Sylhet formed a sister taxon with respect to the above three taxa in the NJ tree (Fig. 2).

Genetic divergence and phylogenetic position of Bangladeshi frogs with respect to congener species

To clarify the phylogenetic relationships of the taxa in Dicroglossidae, Ranidae, and Microhylidae, we selected 20 representative haplotypes (marked with asterisks in Fig. 2) from the 45 haplotypes initially analyzed and performed further phylogenetic analyses incorporating 28, 31, and 11 *16S* sequences from the DNA database. The resultant ML trees are shown in Figs. 3–5. In these analyses, the majority of nodes were not strongly supported by BP or BPP values. This low statistical support may have been due to the truncated alignment data used. However, in many cases, the sister species recovered in the resultant trees showed the lowest *16S* divergence.

For *P. teraiensis* and *D. melanostictus*, we compared our *16S* data to available sequences in DNA databases, and found that our examined *P. teraiensis* was 3.1% divergent with *P. leucomystax* from the type locality (Java, Indonesia). We could not verify our *16S* data with those of *P. teraiensis* from the type locality (East Nepal) or any other regions due to a lack of available *16S* sequences in

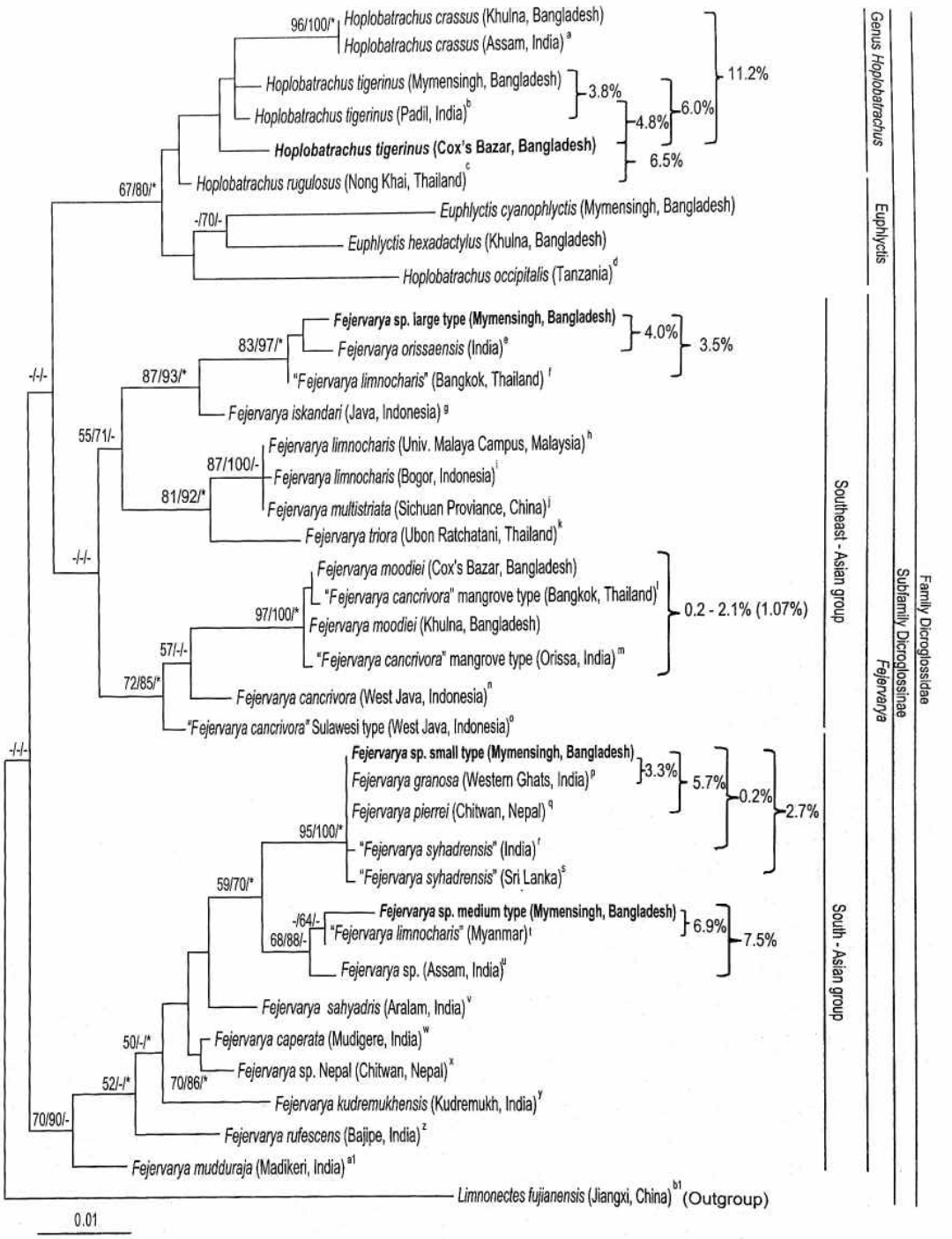

Fig. 3. Maximum Likelihood (ML) tree of dicroglossid frogs based on nucleotide sequences of the mitochondrial *16S* rRNA gene using the SYM + I + G substitution model with *Limnonectes fujianensis* as an outgroup. The bootstrap support (> 50%) is given in order for ML (500) and NJ (1000) replicates. Asterisks represent Bayesian posterior probability (BPP) of ≥ 95%. The scale bar represents 0.01 nucleotide substitutions per site. a) AB290413, Alam et al. (2008); b) AB272594, Alam et al. (2008); c) AB272596, Alam et al. (2008); d) AB272599, Alam et al. (2008); e) AY882957, Tandon et al. (unpublished); f) AB162444, Sumida et al. (2007); g) AB530613, Hasan et al. (in preparation); h) AB530625, Hasan et al. (in preparation); i) AJ292015, Vieth et al. (2001); j) AB530611, Hasan et al. (in preparation); k) AB488883, Kotaki et al. (2010); l) AB444691, Kurniawan et al. (2010); m) AY841754, Guha et al. (unpublished); n) AB444689, Kurniawan et al. (2010); o) AB444693, Kurniawan et al. (2010); p) AB167947, Kurabayashi et al. (2005); q) AB488888, Kotaki et al. (2010); r) AY841748, Guha et al. (unpublished); s) AY141843, Meegaskumbura et al. (2002); t) AF206466, Chen et al. (2005); u) AB488900, Kotaki et al. (2010); v) AB530604, Hasan et al. (in preparation); w) AB530606, Hasan et al. (in preparation); x) AB488889, Kotaki et al. (2010); y) AB530603, Hasan et al. (in preparation); z) AB530601, Hasan et al. (in preparation); a1) AB530607, Hasan et al. (in preparation); and b1) AB526311, Matsui et al. (2010).

DNA databases. In contrast, *16S* divergences of *D. melanostictus* from Bangladesh were compared with publicly available *16S* data, and it was found that our examined specimen was close (*16S* divergence = 1.1%) to one Indian population, but had diverged from the Vietnam and Yunnan (China) populations (*16S* divergence = 2.2% and 2.4%, respectively).

The family Dicroglossidae (Fig. 3)

Euphlyctis cyanophlyctis, *E. hexadactylus*, and *H. crassus* from Bangladesh showed little genetic divergence from those of India. In *H. crassus*, the Khulna (Bangladesh) population showed only 2.9% *16S* divergence from the Assam (India) population. In *H. tigerinus*, two Bangladesh (Mymensingh and Cox's Bazar) populations showed very high *16S* diversity (6.0%). Notably, the Mymensingh and Cox's Bazar (Bangladesh) populations had diverged 3.8% and 4.8%, respectively, from the Padil (India) population.

Fejervarya sp. large type was nested in the Southeast-Asian group of *Fejervarya* and formed a clade with *F. orissaensis* (*16S* divergence = 4.0%), which is a sister group to "*F. limnocharis*" from Bangkok, Thailand (= *Fejervarya* sp. hp2, corresponds to *F. orissaensis* or an undescribed species [Kotaki et al., 2010]). The *16S* divergence between *F.* sp. large type and "*F. limnocharis*" (Thailand) was 3.5%. Three distinct species have been recognized in "*Fejervarya cancrivora*" (designated as large, mangrove, and Sulawesi types). The large type of *F. cancrivora* was designated as the nominal *F. cancrivora* (Kotaki et al., 2010), while the mangrove and Sulawesi types were

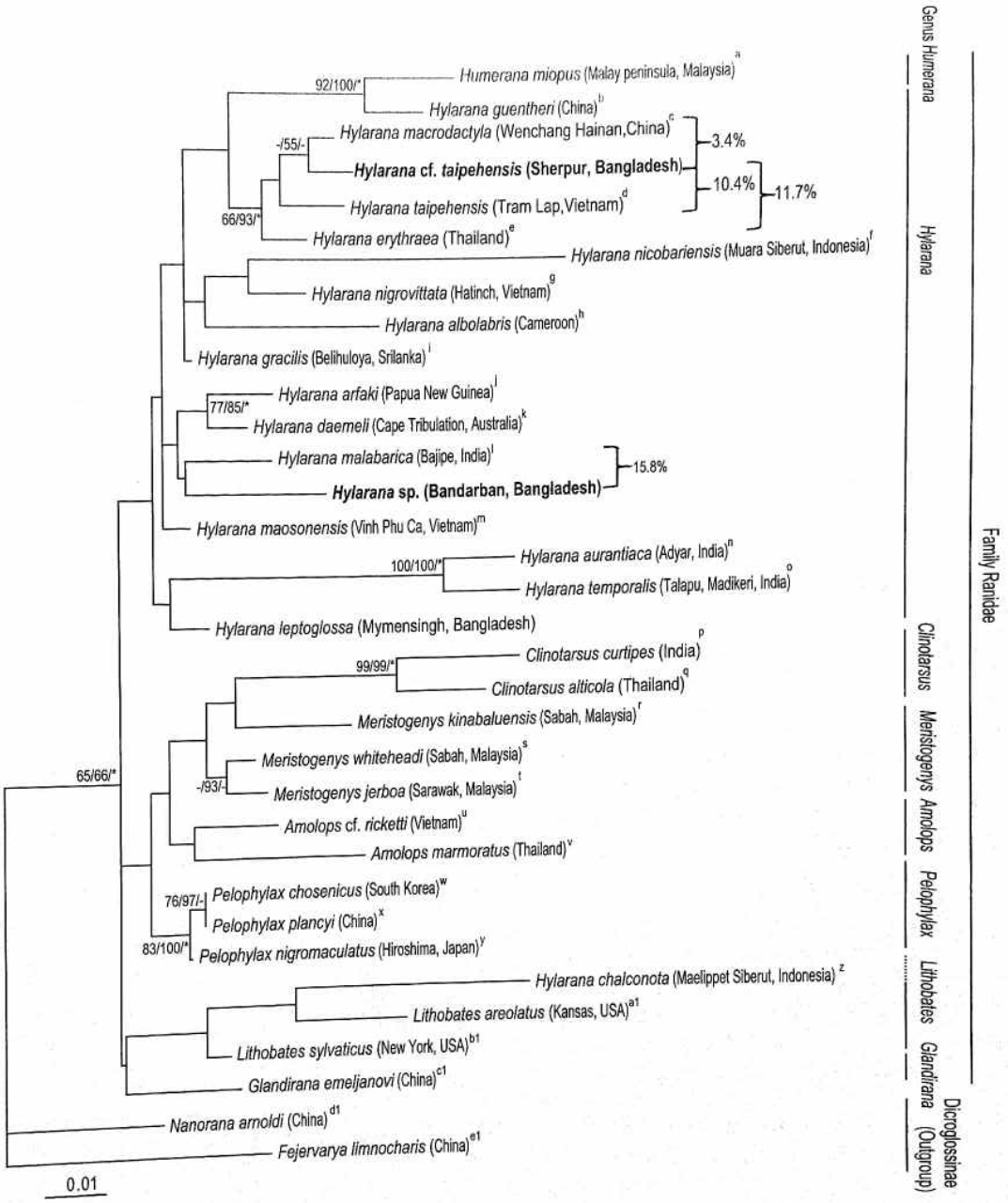

Fig. 4. Maximum Likelihood (ML) tree of ranid frogs based on nucleotide sequences of the mitochondrial *16S* rRNA gene using the GTR + I + G substitutions model with *Nanorana arnoldi* and *Fejervarya limnocharis* as outgroups. The bootstrap support (> 50%) is given in order for ML (500) and NJ (1000) replicates. Asterisks represent Bayesian posterior probability (BPP) of ≥ 95%. The scale bar represents 0.01 nucleotide substitutions per site. a) AB200962, Matsui et al. (2005); b) DQ360001, Che et al. (2007); c) DQ360002, Che et al. (2007); d) AF206495, Chen et al. (2005); e) AB530580, Hasan et al. (in preparation); f) AB530581, Hasan et al. (in preparation); g) DQ283371, Frost et al. (2006); h) DQ283369, Frost et al. (2006); i) AY014376, Kosuch et al. (2001); j) DQ283203, Frost et al. (2006); k) DQ283201, Frost et al. (2006); l) AB530579, Hasan et al. (in preparation); m) DQ283373, Frost et al. (2006); n) AB530574, Hasan et al. (in preparation); o) AB530578, Hasan et al. (In preparation); p) AF249058, Bossuyt & Milinkovitch (2000); q) AB200961, Matsui et al. (2005); r) AB526618, Shimada et al. (2011); s) AB526617, Shimada et al. (2011); t) AB526608, Shimada et al. (2011); u) AY322286, Roelants et al. (2004); v) AB211486, Matsui et al. (2006); w) EU386908, Min et al. (unpublished); x) EF196679, Nie et al. (Unpublished); y) AB043889, Sumida et al. (2001); z) AB530583, Hasan et al. (in preparation); a1) AY779229, Hillis & Wilcox, (2005); b1) DQ347336, Bossuyt et al. (2006); c1) AY322281, Roelants et al. (2004); d1) EU979836, Che et al. (2009); and e1) AY158705, Liu et al. (2005).

designated as *F. moodiei* and an undescribed species, respectively (Kurniawan et al. 2011). *Fejervarya moodiei* from two Bangladeshi populations (Cox's Bazar and Khulna) formed a clade with two *F. cancrivora* mangrove type from Thailand and India (BPs = 97 for ML, 100 for NJ, ≥ 95% for BI, and sequence divergence = 0.2%–2.1%, average 1.07%). This clade became monophyly with *F. cancrivora* (large type) from Indonesia (their average sequence divergence = 9.13%), but the statistical support of this relationship is low (BP = 57 in ML). *Fejervarya* sp. small type formed a clade with *F. granosa* (Western Ghats, India), *F. pierrei* (Chitwan, Nepal), and "*F. syhadrensis*" (India and Sri Lanka) with strong support (BPs = 95 for ML, 100 for NJ, and ≥ 95% for BI). The *16S* divergence among *Fejervarya* sp. small type vs. "*F. syhadrensis*" (India), "*F. syhadrensis*" (Sri Lanka), *F. granosa* (Western Ghats, India), and *F. pierrei* (Chitwan, Nepal) were 0.2%, 2.7%, 3.3%, and 5.7%, respectively. *Fejervarya* sp. medium type formed a clade with "*F. limnocharis*" from Myanmar (BP = 64 for NJ, and *16S* divergence = 6.9%) and the clade was a sister taxon to *Fejervarya* sp. from Assam, India (= *Fejervarya* sp. hp5 in Kotaki et al., 2010). The sequence divergence between *Fejervarya* sp. medium type and *Fejervarya* sp. hp5 was 7.5%.

The family Ranidae (Fig. 4)

Among the Bangladesh ranid specimens examined, *Hylarana leptoglossa* became a sister taxon to the *H. aurantiaca* and *H. temporalis* clade (the latter two species were from Western Ghats, India). *Hylarana* cf. *taipehensis* (Sherpur) formed a clade with *H. macrodactyla* (Wenchang, Hainan, China) with 3.4% sequence divergence. *Hylarana* cf. *taipehensis* and *H. macrodactyla* differ strikingly in many morphological traits. *Hylarana taipehensis* (Tram Lap, Vietnam) was found to be a sister species to the *H.* cf. *taipehensis* + *H. macrodactyla* clade; the *16S* divergence between *H.* cf. *taipehensis* and *H. taipehensis* (Vietnam) was 10.4%. These findings support the distinct specific status of the taxon designated here as *Hylarana* cf. *taipehensis*. *Hylarana* sp. (Bandarban) formed a clade with *H. malabarica* from the Western Ghats and high sequence divergence (15.8%) was found between these two species.

The family Microhylidae (Fig. 5)

In the constructed ML tree, *Mycrohyla* sp. formed a clade with *M. berdmorei* from Gombak, Malaysia, despite a complete difference in morphology and a relatively high *16S* divergence (5.2%). *Microhyla* cf. *ornata* from Dinajpur and *M. ornata* from Karnataka, India, formed a clade, but their sequence divergence was high (6.8%).

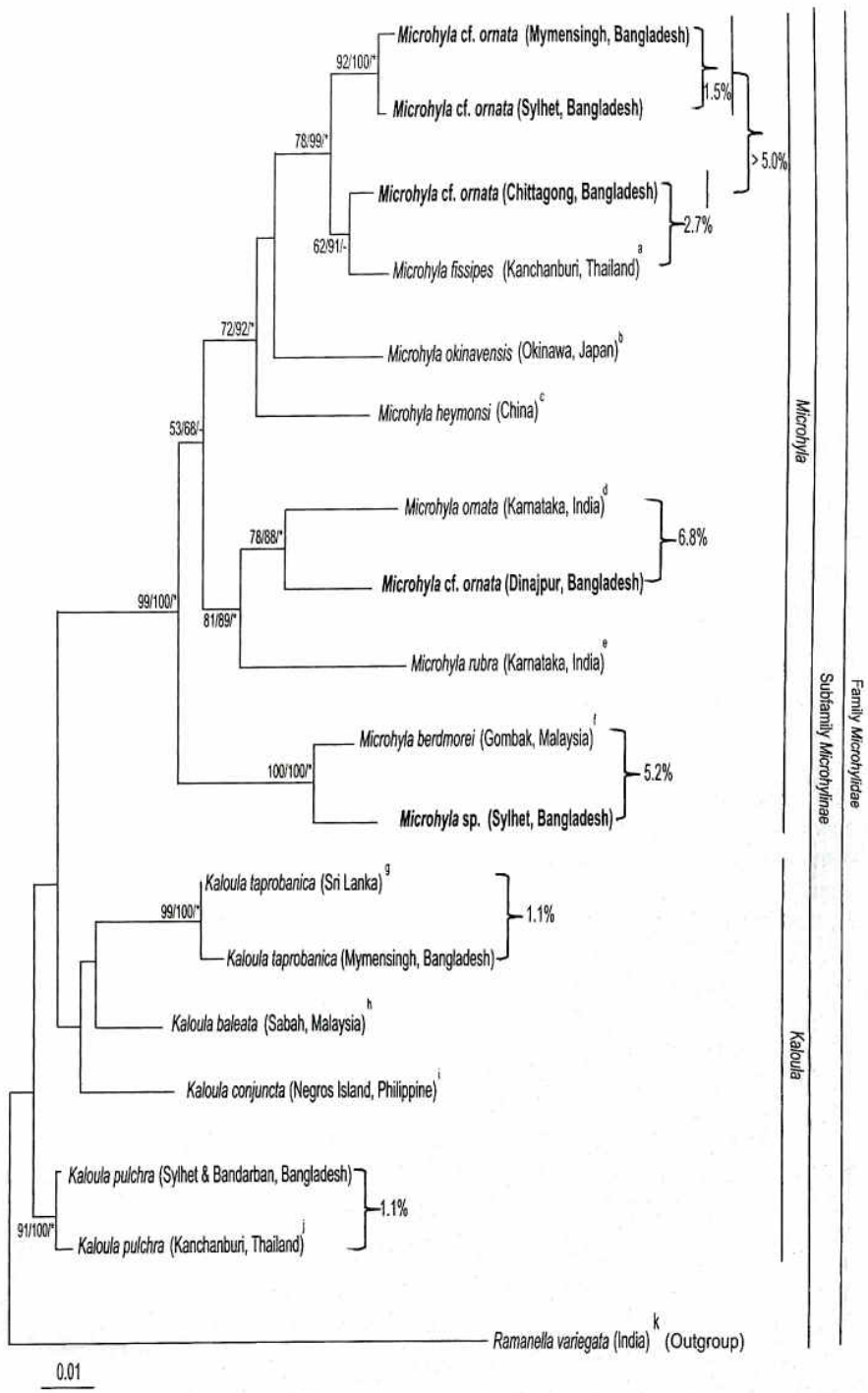

Fig. 5. Maximum Likelihood (ML) tree of microhylid frogs based on nucleotide sequences of the mitochondrial *16S* rRNA gene using the GTR + I + G substitutions model with *Ramanella variegata* as an outgroup. The bootstrap support (> 50%) is given in order for ML (500) and NJ (1000) replicates. Asterisks represent Bayesian posterior probability (BPP) of ≥ 95%). The scale bar represents 0.01 nucleotide substitutions per site. a) AB201186, Matsui et al. (2005); b) AB303950, Igawa et al. (2008); c) AY458596, Zhang et al. (2005); d) AB201188, Matsui et al. (2005); e) AB201192, Matsui et al. (2005); f) AB530638, Hasan et al. (In preparation); g) AF249057, Bossuyt & Milinkovitch, (2000); h) GU154880, Das & Haas, (2010); i) AY326064, Darst & Cannatella, (2004); j) AB201194, Matsui et al. (2005); and k) GU136114, Meenakshi et al. (2009).

Microhyla cf. *ornata* from Chittagong formed a clade with *M. fissipes* from Thailand. The *16S* sequence divergence was only 2.7% between these two species, assuming the existence of *M. fissipes* in Bangladesh. In contrast, *M.* cf.

ornata from Mymensingh and Sylhet was found to be a sister taxon to the *M. fissipes* + *M.* cf. *ornata* (Chittagong) clade. The *16S* divergence between *M.* cf. *ornata* from Chittagong and *M.* cf. *ornata* from Mymensingh and Sylhet was 5.4%. Both *Kaloula pulchra* and *K. taprobanica* formed a clade with the respective conspecific sample from other countries and displayed low *16S* divergence (1.1% for both *K. pulchra* and *K. taprobanica*). In the ML tree, these *Kaloula* species exhibited paraphyly, a finding that is congruent with two recent molecular phylogenetic studies (Van Bocxlaer et al., 2006; Kurabayashi et al., 2011).

DISCUSSION

Recent molecular studies have demonstrated that DNA sequence information, particularly *16S* data, can help to uncover the cryptic biodiversity in anurans. Fouquet et al. (2007) reported that a divergence threshold of 3% in *16S* sequences is useful to identify species of anurans. Vences and Wake (2007) proposed the term "candidate species" for newly discovered units that likely correspond to undescribed species.

In Bangladesh, 35 frog species are currently recognized (Kabir et al., 2009; Howlader, 2011): two bufonids (*Duttaphrynus melanostictus* and *D. stomaticus*), 10 dicroglossids (*Euphlyctis cyanophlyctis*, *E. hexadactylus*, *Fejervarya limnocharis*, *F. syhadrensis*, *F. asmati*, *H. crassus*, *H. tigerinus*, *Occidozyga borealis*, *O. lima*, and *Sphaerotheca breviceps*), two megophryids (*Leptobrachium smithii* and *Xenophrys parva*), seven microhylids (*Kalophrynus interlineatus*, *K. pulchra*, *K. taprobanica*, *Microhyla berdmorei*, *M. ornata*, *M. rubra*, and *Uperodon globulosus*), eight ranids (*Amolops marmoratus*, *Clinotarsus alticola*, *Humarana humeralis*, *Hylarana erythraea*, *H. taipehensis*, *H. tytleri*, *H. leptoglossa*, and *H. nigrovittata*), and six rhacophorids (*Chiromantis simus*, *C. vittatus*, *Polypedates leucomystax*, *P. maculatus*, *Rhacophorus htunwini*, and *R. maximus*). Of these 35 species, 26 have *16S* data available in GenBank. On the basis of the *16S* data obtained in the present study and the available GenBank data, we discuss below the taxonomical status of several unresolved taxa from Bangladesh.

Taxonomic status of dicroglossid frogs from Bangladesh

Four nominal species have been described in the genus *Hoplobatrachus*. Among them, *H. tigerinus* and *H. crassus* have been identified in Bangladesh (Alam et al., 2008). In the present study, it was shown that *H. tigerinus* from Cox's Bazar and *H. tigerinus* from Mymensingh have diverged from each, based on the detected *16S* divergence of 6.0%. As the two populations differ in size and in a few morphological traits (Hasan et al., in preparation), *H. tigerinus* from Cox's Bazar, Bangladesh represents an undescribed cryptic species. However, it remains for future studies to determine which population belongs to the nominal species with the type locality "Bengal" (Frost, 2011).

In *E. cyanophlyctis* and *E. hexadactylus*, whose type localities are Tranquebar and Pondichéry, India, respectively (Bauer, 1998; Frost, 2011), considerable *16S* divergences (4.0–5.9%) were detected between the India and Bangladesh populations (Alam et al., 2008). They (2008) speculated that *E. cyanophlytis* from Bangladesh might be a cryptic species compared with that from Western Ghats (India), and that *E. hexadactylus* from Bangladesh might be "real" *E. hexadactylus* if the Sri Lanka specimens correspond to the nominal species. Thereafter, Joshy et al. (2009) described two species of the genus *Euphlyctis* from Western Ghats (India) as new species: *E. mudigere* and *E. aloysii*. However, at present it is difficult to confirm that the Bangladesh specimens correspond to real *E. cyanophlyctis* and *E. hexadactylus*. Further study involving comparisons with topotypic specimens is necessary for elucidating the taxonomic status of *E. cyanophlyctis* and *E. hexadactylus* from Bangladesh.

The genus *Fejervarya* comprises 31 species that are distributed in South and Southeast Asia (Frost, 2011). Two species (*F. limnocharis* and *F. syhadrensis*) are listed as Bangladeshi *Fejervarya* species in Kabir et al. (2009) and one new species (*F. asmati*) was recently described from Bangladesh by Howlader (2011). Asmat et al. (2003) first reported the occurrence of *F. limnocharis* in Bangladesh, but Rasel et al. (2007) later suggested the presence of *F. nepalensis*, *F. pierrie*, *F. syhadrensis*, and *F. teraiensis*, rather than *F. limnocharis*. Based on morphological, crossing, and molecular analyses, Islam et al. (2008b) claimed that four types of *Fejervarya* exist in Bangladesh: *Fejervarya* sp. large type, *Fejervarya* sp. medium type, *Fejervarya* sp. small type, and "*F. cancrivora*" mangrove type (= *F. moodiei*). In the present study, *F. moodiei* (including the previous "*F. cancrivora*" mangrove type) from Bangradesh (Cox's Bazar and Khulna), India, and Thailand formed a clade, which exhibited less than 3% (0.2–2.1%) *16S* divergence. *Fejervarya* sp. small type shows close relationships with "*F. syhadrensis*" from India and Sri Lanka, *F. pierreri* from Nepal, and *F. granosa* from India. Among these related species, "*F. syhadrensis*" exhibits low *16S* divergence with *Fejervarya* sp. small type (0.2% and 2.7% for India and Sri Lanka specimens, respectively). Thus, our *Fejervarya* sp. small type clearly corresponds to this taxon. However, several *F. syhadrensis*-like species have been identified in South and Southeast Asia (including the India and Sri Lanka populations), and at present, it is unclear which populations correspond to real *F. syhadrensis* (Kuramoto et al., 2007; Kotaki et al., 2010). Thus, although our results suggest that "*F. syhadrensis*" occurs in Bangladesh, final confirmation as to whether "*F. syhadrensis*" in Bangladesh corresponds to bona fide *F. syhadrensis* requires *16S* sequence analysis of the topotypic *F. syhadrensis* specimens (Poona district, India). There is a possibility that "*F. syhadrensis*" from the southeastern part of Bangladesh corresponds to *F. asmati* that was recently described from Chittagong, Bangladesh (Howlader, 2011), but more investigations are needed to confirm this speculation.

Fejervarya sp. large and medium types have been examined in previous studies, which have suggested that these taxa are possibly undescribed species (Islam et al., 2008b). The present results are consistent with the findings of Islam et al. (2008b). *Fejervarya* sp. large type shows a close relationship with *F. orissanensis*, but the *16S* divergence (4%) is larger than the species threshold value. *Fejervarya* sp. medium type constitutes a clade with "*F. limnocharis*" from Myanmar, but their *16S* divergence is high

(6.9%). It was suggested that "*F. limnocharis*" from Myanmar is not real *F. limnocharis* (Islam et al., 2008b), a view that is also supported by our results. Consequently, our study confirmed the occurrence of two possibly undescribed species, namely *Fejervarya* sp. large and medium types, from Bangladesh. Although our sampling areas covered a wide range in Bangladesh, *F. limnocharis* specimens corresponding to the haplotype from the type locality area (Indonesia) were not found. As previous molecular studies also failed to detect *F. limnocharis* in Bangladesh, we propose that the name *F. limnocharis* should be removed from the list of Bangladesh anurans.

The species in the genus *Fejervarya* constitute two distinct groups, the Southeast-Asian and South-Asian groups (Fig. 3), with *F. moodiei* and *Fejervarya* sp. large type belonging to the former, and *Fejervarya* sp. medium and small types belonging to the latter. Thus, the intermingling nature of anuran fauna of Bangladesh is evident. Two species of "*F. limnocharis*" (large and small, which also differ in their habitat) were recognized in Myanmar (Zug et al., 1998), but the relationship between *Fejervarya* taxa of Bangladesh and Myanmar remain to be determined in future studies.

Taxonomic status of ranid frogs from Bangladesh

The genus *Hylarana* consists of 86 nominal species, and 75 *Hylarana* species are distributed in Asia and northern Australia (Frost, 2011). It has been reported that five species of this genus (*H. erythraea*, *H. taipehensis*, *H. leptoglossa*, *H. tytleri*, and *H. nigrovittata*) are distributed in Bangladesh (Kabir et al., 2009). Our present specimens contained *H. leptoglossa* and two unidentified species (*H.* cf. *taipehensis* and *Hylarana* sp.). Among these species, *H.* cf. *taipehensis* has a close affinity with *H. macrodactyla* (Wenchang, Hainan, China), with 3.4% *16S* divergence, but the external morphologies of the two differ completely (Hasan et al., in preparation). In contrast, the *16S* divergence between *H.* cf. *taipehensis* and *H. taipehensis* (Vietnam) is very high (10.4%). Thus, our results show that *H.* cf. *taipehensis* does not correspond to either *H. macrodactyla* or *H. taipehensis*, and likely represents a new cryptic species. Specimens of *H.* cf. *taipehensis* were collected from many regions of Bangladesh and it is probable that this taxon has long been confused with *H. taipehensis*. Thus, the name *H. taipehensis* should be removed from the anuran list of Bangladesh.

Hylarana sp. (Bandarban, Bangladesh) and *H. malabarica* (India) formed a clade and exhibited 15.8% *16S* divergence. Due to the limited number of available *16S* sequences of nominal *Hylarana* species (15 of 86) and lack of *16S* data for *H. tytleri* specimens, our analyses could not verify the taxonomic status of this unidentified *Hylarana* taxon. However, the present phylogenetic analyses, together with morphological comparisons (Hasan et al., in preparation), suggests that *Hylarana* sp. does not correspond to four *Hylarana* species (*H. leptoglossa*, *H. erythraea*, *H. taipehensis*, and *H. nigrovittata*) currently recognized in Bangladesh. Although usable *16S* data is lacking for *H. tytleri*, the morphologies of our *Hylarana* sp. differ from those of the remaining Bangladeshi *Hylarana* species (*H. tytleri*). Detailed morphological comparisons are now in progress.

Taxonomic status of microhylid frogs from Bangladesh

The genus *Microhyla* consists of 31 species that are widely distributed throughout South and Southeast Asia (Frost, 2011). In Bangladesh, only three nominal species (*M. ornata*, *M. berdmorei*, and *M. rubra*) are reported to exist (Kabir et al., 2009). In the present study, we identified four distinct taxa in the genus *Microhyla*. *Microhyla* cf. *ornata* from Chittagong formed a clade with *M. fissipes* (Thailand) and displayed a *16S* divergence of only 2.7%. Thus, we speculated this taxon to *M. fissipes*, which needs further taxonomic study to confirm this idea. *Microhyla fissipes* has long been confused with *M. ornata* (Matsui et al., 2005) and is presumed to occur in Myanmar (Frost, 2011). *Microhyla* cf. *ornata* from Mymensingh and Sylhet showed a considerable genetic divergence (> 5.0%) from these above taxa, although they share similar external morphologies. Thus, it is highly probable that *M.* cf. *ornata* from Mymensingh and Sylhet is a cryptic species. *Microhyla* cf. *ornata* from Dinajpur is morphologically similar to *M. ornata* (Karnataka, India; type locality area), but a relatively high *16S* divergence (6.8%) exists between them. Therefore, this taxon is apparently a new cryptic species, as suggested by Matsui et al. (2005). *Microhyla* sp. from Sylhet has 5.2% *16S* divergence from *M. berdmorei* (Gombak, Malaysia). As these two taxa differ morphologically, *Microhyla* sp. from Sylhet is likely a cryptic species.

In conclusion, the present study revealed the presence of at least eight undescribed frog taxa in Bangladesh. This finding is remarkable in view of the relatively simple topographic features of Bangladesh, which mainly consists of lowlands and lacks high mountainous regions. In addition, our results clearly indicate that anuran biodiversity has been underestimated in Bangladesh and emphasize the necessity for further taxonomic studies of anurans in this country.

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A new species of genus *Hoplobatrachus* (Anura, Dicroglossidae) from the coastal belt of Bangladesh

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Abstract

A new cryptic species of the genus *Hoplobatrachus* from Cox's Bazar district of Bangladesh is described and compared with its relevant congeners both in morphology and mitochondrial gene sequences. The new species differs from its close relative *H. tigerinus* in having a distinct broad black band from the eye, through the nostrils, to the anterior edge of the upper jaw, another black band along the lateral margin of the upper jaw, and a narrow inter-orbital distance relative to eyelid width and inter-nostril distance. Advertisement calls of the new species are similar to those of *H. tigerinus* but differ in dominant frequency and number of pulses. Based on mitochondrial DNA sequence data, this species was proved to genetically divergent from *H. tigerinus* at 3.2% for the 16S rRNA gene and 14.2% for the *Cytb* gene. The known distribution range of the new species is restricted to the southeastern corner of Bangladesh and it seems to be endemic in this coastal belt.

Key words: *Hoplobatrachus litoralis* sp. nov., Dicroglossidae, Morphology, Advertisement call, Mitochondrial DNA, Bangladesh

Introduction

The genus *Hoplobatrachus* comprises large robust frogs with numerous ridges or warts on the back and extensive webbing between toes. Individuals are semi-aquatic and live mostly near water edge of ponds, marshes, rivers, and flooded rice paddies. The following four species are currently recognized (Frost 2011): *H. crassus* in south to east India, Sri Lanka, Nepal, and Bangladesh; *H. occipitalis* in western and central Africa; *H. rugulosus* (=*H. chinensis*, used by some authors [eg., Kosuch *et al.* 2001] as its nomenclature status is unclarified) in Myanmar, southern China, Taiwan, Thailand, and peninsular Malaysia; and *H. tigerinus* in east Afghanistan, north Pakistan, India, Sri Lanka, Nepal, Bangladesh, and Myanmar. All of these species were described during the early to middle 19th century, and no new species has been reported far more than a century thereafter.

In our previous study (Hasan *et al.* 2012), we revealed the existence of two genetically different forms of *H. tigerinus* in Bangladesh. Divergence in mitochondrial 16S rRNA gene sequences was 6.0% between specimens from Mymensingh and Cox's Bazar districts. One of the forms is widely distributed throughout Bangladesh, whereas the other occurs only in the southeastern corner of Bangladesh. The type locality of *H. tigerinus* is "Bengale" (Bengal), India (Frost 2011). The distribution and molecular comparison clearly indicate that the wide-ranging form corresponds to the nomen *H. tigerinus*. Therefore, the other form is described as a new species. Morphological comparisons of the new species were performed with *H. tigerinus* and *H. rugulosus*, and new molecular data are presented.

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Materials and methods

Specimens of the new species were found in Cox's Bazar district, whereas *H. tigerinus* were collected from throughout Bangladesh from 2000 to 2011. Finger tips were cut for DNA analysis, and voucher specimens were deposited in the Institute for Amphibian Biology, Hiroshima University (IABHU).

The following 29 body parts were measured for both sexually matured individuals of male and female using digital calipers to the nearest 0.1 mm. Mature male frogs were identified by their secondary sexual character, i.e., presence of vocal sac, and big sized females were considered as mature, although few smaller female specimens were confirmed for maturity by checking their gonad. 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: horizontal eye diameter; E-E: inter-orbital distance between inner borders of upper eyelids; ELW: eyelid width; TD: horizontal tympanum diameter; FLL: forelimb length; FHL: forearm and hand length; FAW: forearm width; HAL: hand length; F1-F4: length of 1st to 4th finger; HLL: hindlimb length; FEL: femur length; TIL: tibia length; TFL: tarsus and foot length; FOL: foot length; T1-T5: length of 1st to 5th toe; and IMT: inner metatarsal tubercle length. For comparison of morphometric data, we considered 27 mature *H.* sp from Ukhia and Teknaf of Cox's Bazar district; 15 mature *H. tigerinus* as representative samples from a single locality of Mymensingh district, as there was a negligible genetic variation among the *H. tigerinus* distributed all over Bangladesh (Alam *et al.* 2008, Hasan *et al.* 2012 and Islam *et al.* unpublished); 7 mature individuals of *H. rugulosus* from Nong Khai and Chachoengsao of Thailand. Statistic analysis was performed in SPSS (15.0J) software (SPSS Japan Inc., Tokyo, Japan).

Advertisement calls of multiple males of new species were recorded at Ukhia, Cox's Bazar district, with an ICD-UX300F IC recorder (Sony Corp., Tokyo, Japan) on 19 June 2011. These specimens of male were also recorded in our note book and underwent for further molecular and morphological analyses in this study. Sound spectrograms were depicted using Avisoft-SASLab Light software (Avisoft Bioacoustics).

Total DNA was extracted from a clipped toe of each individual in 4 specimens of *H. tigerinus* (= 2 haplotypes) from Mymensingh and in 26 specimens of *H.* sp. (= 9 haplotypes) from Cox's Bazar using the DNeasy Tissue Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. The extracted DNA solutions were used to amplify partial portions of the 16S rRNA gene (*16S*) and Cytb gene (*Cytb*) corresponding to nucleotide position 6,205–6,753 and 16,761–17,372, respectively, in the *Fejervarya limnocharis* complete mtDNA sequence (accession no.: AY158705; Liu *et al.* 2005). PCR amplification and sequencing were performed using the primers F51 (5-CCC GCC TGT TTA CCA AAA ACA T-3) and R51 (5-GGT CTG AAC TCA GAT CAC GTA-3) (Sumida *et al.* 2002) for *16S*, and 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*. Partial *16S* (556 bp) and *Cytb* (616 bp) portions were sequenced, and the obtained sequences were deposited in the DDBJ/EMBL/GenBank database (accessions numbers: AB671173–AB671184 for *16S* and AB671185–AB671196 for *Cytb*).

The resultant nucleotide sequences of the *16S* and *Cytb* genes were separately aligned using the ClustalW program (Thompson *et al.* 1994) with their counterparts from *H. tigerinus* (n = 2) of India, three congener species, *H. crassus* (n = 1), *H. rugulosus* (n = 3), and *H. occipitalis* (n = 2), and two *Euphlyctis* species, *E. cyanophlyctis* (n = 1) and *E. hexadatylus* (n = 1) (Alam *et al.* 2008). From these two alignment data sets, sequence divergences (uncorrected *p* values) were calculated using MEGA Ver. 4.0 (Tamura *et al.* 2007) 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 default parameters. Gap sites in alignments were treated as missing data. Initial two alignments (*16S* and *Cytb*) were combined into one alignment data set and this concatenated alignment data set contained a total of 1036 sites (478 for *16S* and 558 for *Cytb*), 294 of which were parsimoniously informative, and this data set underwent further phylogenetic analyses. Phylogenetic analyses were performed by 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 (AIC) and Bayesian information criterion (BIC) respectively, which 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 analysis with 1,000 replicates. BI analysis was performed using MrBayes Ver. 3.1.2 (Ronquist & 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 was determined by checking the convergence of –log likelihood (–ln*L*) values

and tree length against generation number by using Tracer ver. 1.4 (Rambaut & Drummond 2007). Statistical support of the BI tree was evaluated by Bayesian posterior probability (BPP). MP was performed with 1,000 bootstrap replicates using PAUP* 4.0b10 (Swofford 2003).

Systematics

Hoplobatrachus litoralis sp. nov.

Holotype. IABHU 3993, adult female (SVL: 100.6 mm) collected from Teknaf, Cox's Bazar district (20° 52′ N, 92° 18′ E, > 5 m asl.), Bangladesh on 20 June 2011 by M. Hasan (Figs. 1A, 1B).

Paratypes. IABHU 3974, adult female (SVL: 121.3 mm), IABHU 3979, adult female (SVL: 109.1 mm), and IABHU 3980, adult female (SVL: 119.3 mm) collected from Ukhia, Cox's Bazar district, Bangladesh on 19 June 2011 by M. Hasan. IABHU 3989, adult male (SVL: 89.5 mm), IABHU 3992, adult male (SVL: 83.7 mm), IABHU 3994, adult male (SVL: 84.7 mm) and IABHU 3997, adult female (SVL: 96.1 mm) collected from Teknaf, Cox's Bazar district, Bangladesh on 20 June 2011 by M. Hasan.

Diagnosis. Large frog with SVL of 81.3–102.1 mm in males and 83.2–121.3 mm in females. A broad black band from anterior corner of eye through the nostrils to anterior edge of upper jaw, and another band along the lateral margin of upper jaw (Fig. 1C) are more distinct than in its close relatives *H. tigerinus* and *H. rugulosus* where the band widths are uneven and often discontinuous (Fig. 1D). There is a distinct black margin in the inner side of the upper arm in the new species, but such margin absent in *H. tigernius* (Fig. 1E-F). The inner metatarsal tubercle of the new species is black (Fig. 1G), whereas it is pigmentless in *H. tigerinus* (Fig. 1H). Inter-orbital distance is much narrower than eyelid width and inter-nostril distance in the new species (ELW/E-E = 1.875, N-N/E-E = 1.575, on average), whereas these values are nearly the same in *H. tigerinus* (ELW/E-E = 1.060, N-N/E-E = 1.002) and *H. rugulosus* (ELW/E-E = 1.021, N-N/E-E = 1.004).

Description of holotype (measurements in mm). Vomerine teeth, long oblique lines between choanae. Tongue tip bifurcated. Distinct symphysial knob on anterior edge of lower jaw.

Head longer than wide (HL: 43.9; HW: 40.2), obtusely pointed. Canthus rostralis blunt. Loreal region concave. Nostril nearer to tip of snout than to eye (S-N: 6.6; N-E: 10.7). Tympanum large, slightly smaller than eye (TD: 8.3; ED: 8.9). Inter-orbital space much narrower than eyelid width and inter-nostril space (E-E: 3.9; ELW: 7.2; N-N: 6.5).

Fingers free, finger tips blunt without disk. Finger length F3 > F1 > F2 > F4 (F1: 12.9; F2: 10.3; F3: 13.2; F4: 7.7). Subarticular tubercles moderate. Thenar and palmar tubercles distinct.

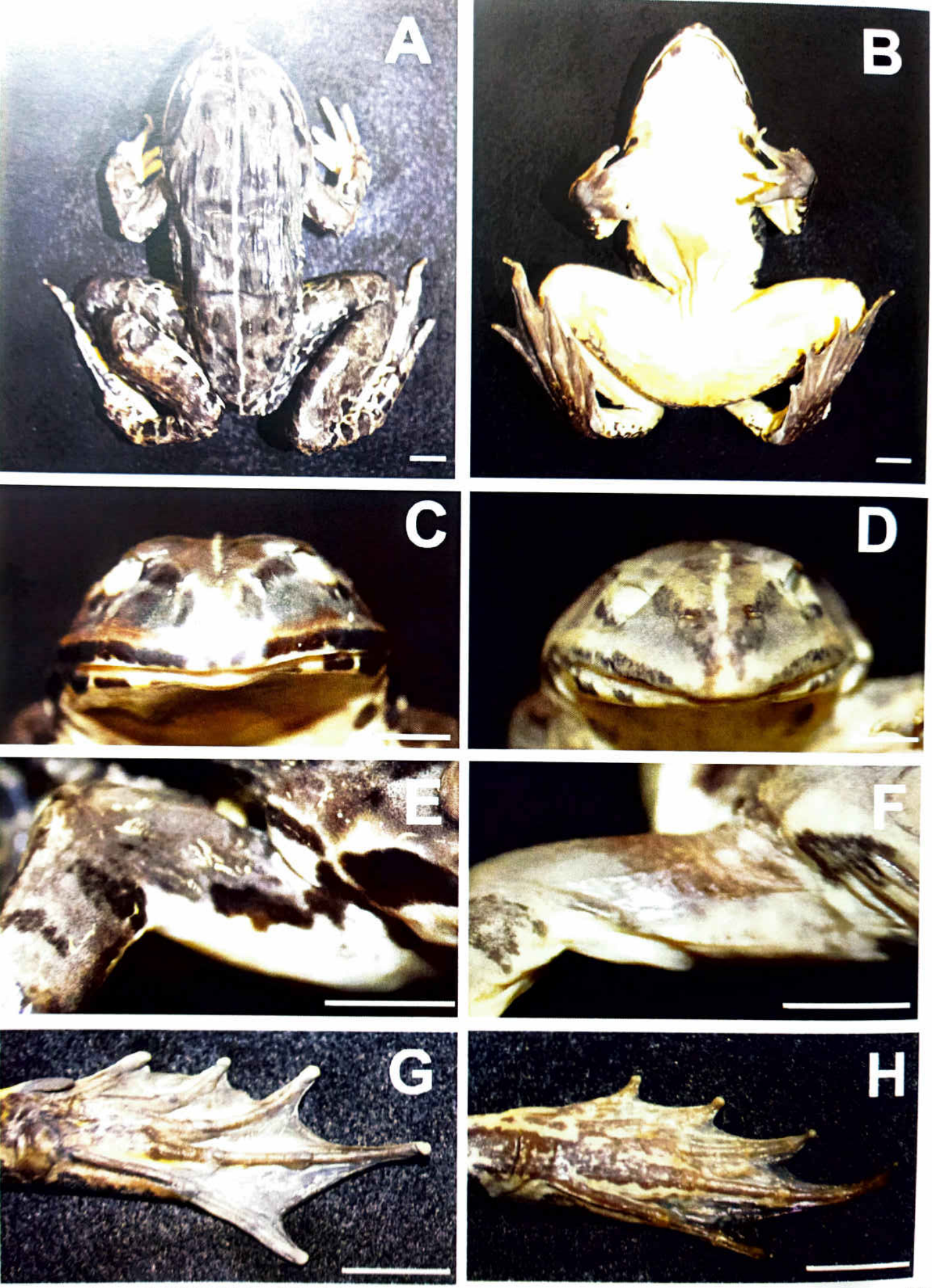
Hindlimb about 1.6 times SVL (HLL: 165.3; SVL: 100.6). Femur length subequal to tibia length (FEL: 51.0; TIL: 51.8). Toe tips blunt, slightly rounded. Toe length T4 > T5 > T3 > T2 > T1 (T1: 11.8; T2: 17.8; T3: 26.2; T4: 34.6; T5: 27.9). Wide web, reaching the base of toe-tip disk (Fig. 1D). Subarticular tubercles were weak. Inner metatarsal tubercle moderate (IMT: 4.6). No outer metatarsal tubercle.

Many thin longitudinal ridges on the dorsum (Fig. 1A). Small round warts over dorsal and lateral side. Supratympanic fold from behind eye to posterior margin of tympanum. Weak tarsal ridge extending from proximal end of inner metatarsal tubercle to heel. Ventral side including thigh and tibia smooth.

Color in alcohol. Dorsum dark gray with many large black spots (Fig. 1A). Thin whitish mid-dorsal stripe from tip of snout to vent. Lateral side with many small black dots. Wide black band from anterior corner of eye through the nostrils to anterior margin of upper jaw (Fig. 1C). Another band on the lateral margin of upper jaw (Fig. 1C). Ventral side immaculate, except for large black blotches along the edge of lower jaw to the base of forelimb (Fig. 1B).

Large transverse black bands on the upper surface of thigh, tibia, and tarsus to the outer edge of foot. Rear side of thigh heavily mottled. Outer side of tarsal ridge dark, whereas inner side yellowish with several dark irregular blotches. Web dark gray, except for whitish upper side of inner web (between toes 1–4).

Color in life. Dorsal ground color varies from yellowish to dark brown with many dark brown to black spots. Large transverse black bands are present on the dorsal surface of the thigh, tibia, and tarsus region (Fig. 2A). Mid-dorsal stripe is yellowish white. Bands running from the anterior part of eye to upper jaw margin and on the lateral



URE 1. Holotype (IABHU 3993) of *Hoplobatrachus litoralis* **sp. nov.** after preservation in alcohol. (A) Dorsal aspect. (B) tral aspect. (C) Frontal aspect of head showing distinct black bands, compared with (D) that of *H. tigerinus* (IABHU 3940). Coloration of upper arm of *H. litoralis* (IABHU 3993), compared with (F) that of *H. tigerinus* (IABHU 3940). (G) Foot of type (IABHU 3980), compared with (H) that of *H. tigerinus* (IABHU 3940). Scale bar = 10 mm.

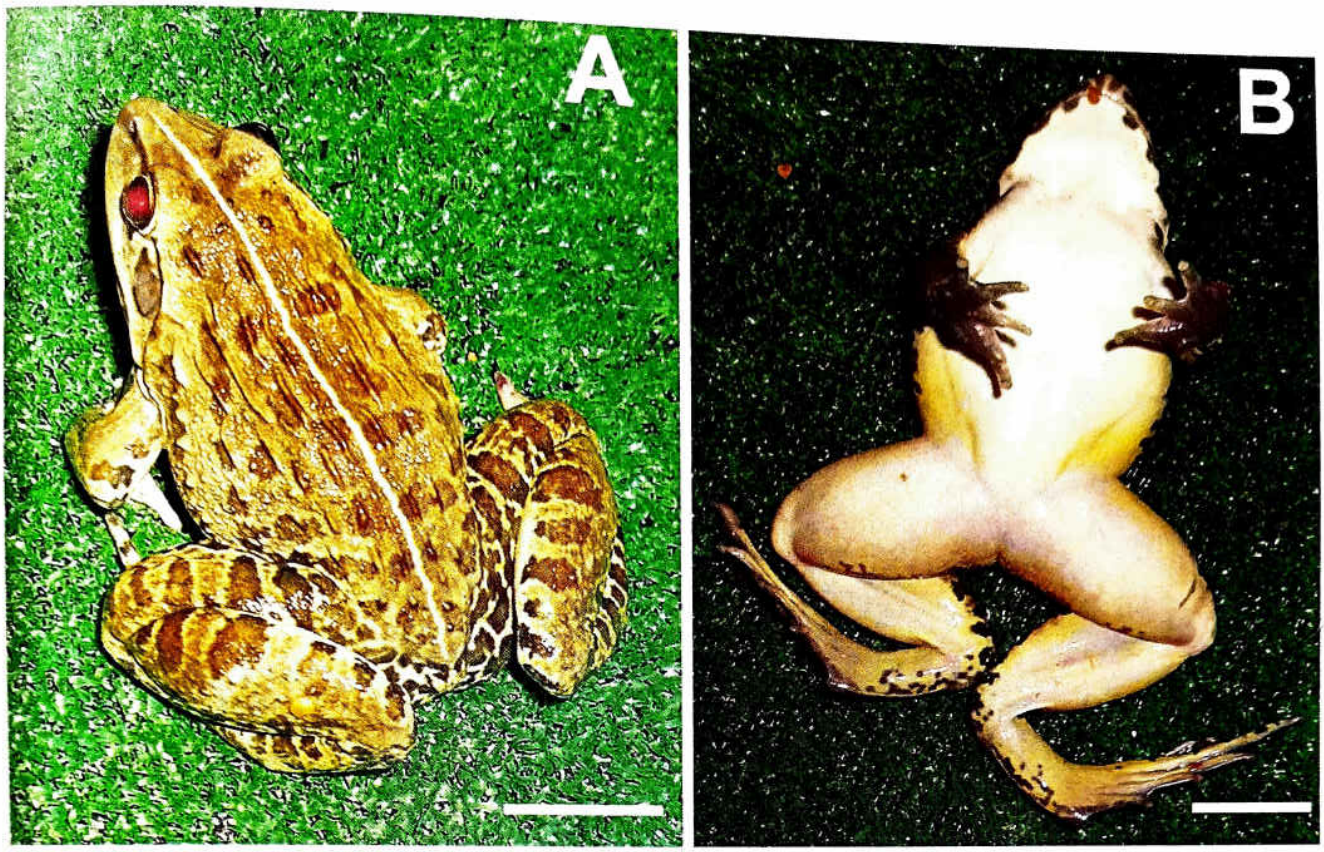


FIGURE 2. Holotype (IABHU 3993) of *H. litoralis* **sp. nov.** in life. (A) Dorsal view. (B) Ventral view. Scale bar = 25 mm.

TABLE 1. Measurements (mean ± S. D., in mm) for 29 body parts of three species of the genus *Hoplobatrachus*.

	Hoplobatrachus litoralis		*Hoplobatrachus tigerinus*		*Hoplobatrachus rugulosus*	
	Male (n = 14)	Female (n = 13)	Male (n = 9)	Female (n = 6)	Male (n = 3)	Female (n = 4)
SVL	89.96 ± 5.92	101.42 ± 12.01	114.04 ± 6.52	113.67 ± 15.43	99.43 ± 0.71	117.33 ± 10.09
HL	34.09 ± 2.77	38.21 ± 5.00	44.13 ± 2.47	41.53 ± 4.68	35.10 ± 1.05	44.13 ± 2.23
HW	32.24 ± 2.93	36.46 ± 4.89	42.46 ± 3.07	39.95 ± 8.37	37.17 ± 1.56	46.20 ± 2.02
S-N	5.76 ± 0.77	6.05 ± 0.55	7.21 ± 0.65	7.38 ± 1.09	7.33 ± 1.36	8.68 ± 0.91
N-N	5.94 ± 0.57	6.06 ± 0.63	7.03 ± 0.95	6.32 ± 0.79	4.53 ± 1.19	6.58 ± 0.90
N-E	8.41 ± 0.90	8.81 ± 1.19	10.07 ± 0.99	10.18 ± 1.28	8.60 ± 0.79	8.58 ± 0.81
ED	7.76 ± 0.90	7.82 ± 0.63	8.71 ± 1.26	7.70 ± 1.19	5.97 ± 0.93	7.38 ± 1.48
E-E	3.89 ± 0.56	3.88 ± 0.75	7.42 ± 1.52	6.05 ± 0.40	4.57 ± 1.19	6.55 ± 0.64
ELW	6.96 ± 0.93	7.32 ± 1.00	7.23 ± 0.90	6.85 ± 0.55	5.50 ± 0.20	5.53 ± 1.13
TD	6.24 ± 0.51	6.89 ± 1.19	7.84 ± 0.79	7.80 ± 1.48	5.20 ± 0.35	6.30 ± 1.06
FLL	49.72 ± 5.79	53.62 ± 6.47	61.57 ± 4.34	58.17 ± 5.65	50.43 ± 6.56	62.95 ± 2.37
FHL	35.59 ± 3.21	37.67 ± 5.04	43.39 ± 2.67	41.13 ± 3.99	35.33 ± 1.46	43.25 ± 3.99
FAW	8.45 ± 1.56	7.88 ± 1.59	9.16 ± 1.72	7.47 ± 1.58	8.03 ± 0.32	10.18 ± 0.86
HAL	17.71 ± 1.46	19.21 ± 2.47	22.52 ± 2.37	21.02 ± 3.01	19.00 ± 1.71	24.80 ± 1.80
F1	9.04 ± 1.35	10.97 ± 1.38	11.90 ± 2.05	12.15 ± 2.41	9.20 ± 0.10	11.33 ± 2.56
F2	6.48 ± 0.86	8.23 ± 1.24	9.12 ± 2.32	11.12 ± 1.44	7.20 ± 1.71	10.50 ± 0.90
F3	10.16 ± 1.33	11.00 ± 1.02	12.51 ± 2.47	13.92 ± 1.14	11.83 ± 0.29	14.03 ± 2.52
F4	6.78 ± 1.09	7.39 ± 1.05	9.00 ± 1.62	10.05 ± 1.07	6.63 ± 1.01	8.65 ± 2.19
HLL	143.85 ± 12.53	159.74 ± 22.37	183.82 ± 16.36	174.38 ± 13.13	137.40 ± 2.55	157.83 ± 6.66
FEL	45.36 ± 5.42	50.05 ± 7.35	57.76 ± 5.49	54.47 ± 6.12	43.07 ± 2.68	51.10 ± 2.54
TIL	45.93 ± 3.64	50.41 ± 5.67	61.64 ± 5.08	57.75 ± 6.77	41.37 ± 1.40	49.53 ± 2.24
TFL	65.85 ± 6.72	73.08 ± 9.39	82.07 ± 7.22	75.85 ± 7.37	58.17 ± 6.42	74.25 ± 1.71
FOL	43.83 ± 4.32	47.81 ± 6.04	52.56 ± 5.20	49.68 ± 4.12	36.60 ± 7.71	49.75 ± 1.28
T1	9.19 ± 1.00	9.89 ± 1.19	11.91 ± 1.99	11.92 ± 1.47	9.23 ± 0.45	13.23 ± 0.17
T2	16.11 ± 1.98	18.20 ± 2.09	19.89 ± 2.28	20.97 ± 1.61	15.50 ± 1.97	19.93 ± 2.14
T3	22.00 ± 2.56	24.68 ± 3.21	27.72 ± 2.56	28.65 ± 2.53	22.00 ± 1.97	25.60 ± 2.41
T4	28.96 ± 2.83	32.81 ± 3.44	36.69 ± 5.16	38.22 ± 2.93	29.83 ± 2.92	34.00 ± 0.92
T5	20.76 ± 2.67	22.60 ± 3.57	27.98 ± 3.41	27.63 ± 3.80	20.97 ± 1.96	25.55 ± 3.67
IMT	4.86 ± 0.78	5.28 ± 0.99	6.86 ± 1.01	6.22 ± 1.92	3.93 ± 0.96	4.73 ± 0.98

margin of upper jaw are black. Many fused spots on the posterior surface of thigh are black with thin yellow reticulations between them. Tympanum is dark gray with pale central circle. A short discontinuous black line is present below the eye. Venter is creamy white with a few black blotches from the margin of lower jaw to base of forelimb (Fig. 2B).

Variation. Males have a pair of gray (in alcohol) subgular vocal sac on the underside of the jaw angle, and a well-developed nuptial pad on the base of 1st finger.

Of the 26 specimens examined, three (12%) lacked a mid-dorsal stripe. A broad mid-dorsal stripe, often found in *Fejervarya* species, was absent. Number and size of dark spots along the margin of the lower jaw varied, and only one specimen lacked these spots. Usually, the lateral upper jaw stripe is continuous, but in 6 specimens the stripe is fragmented into irregular markings.

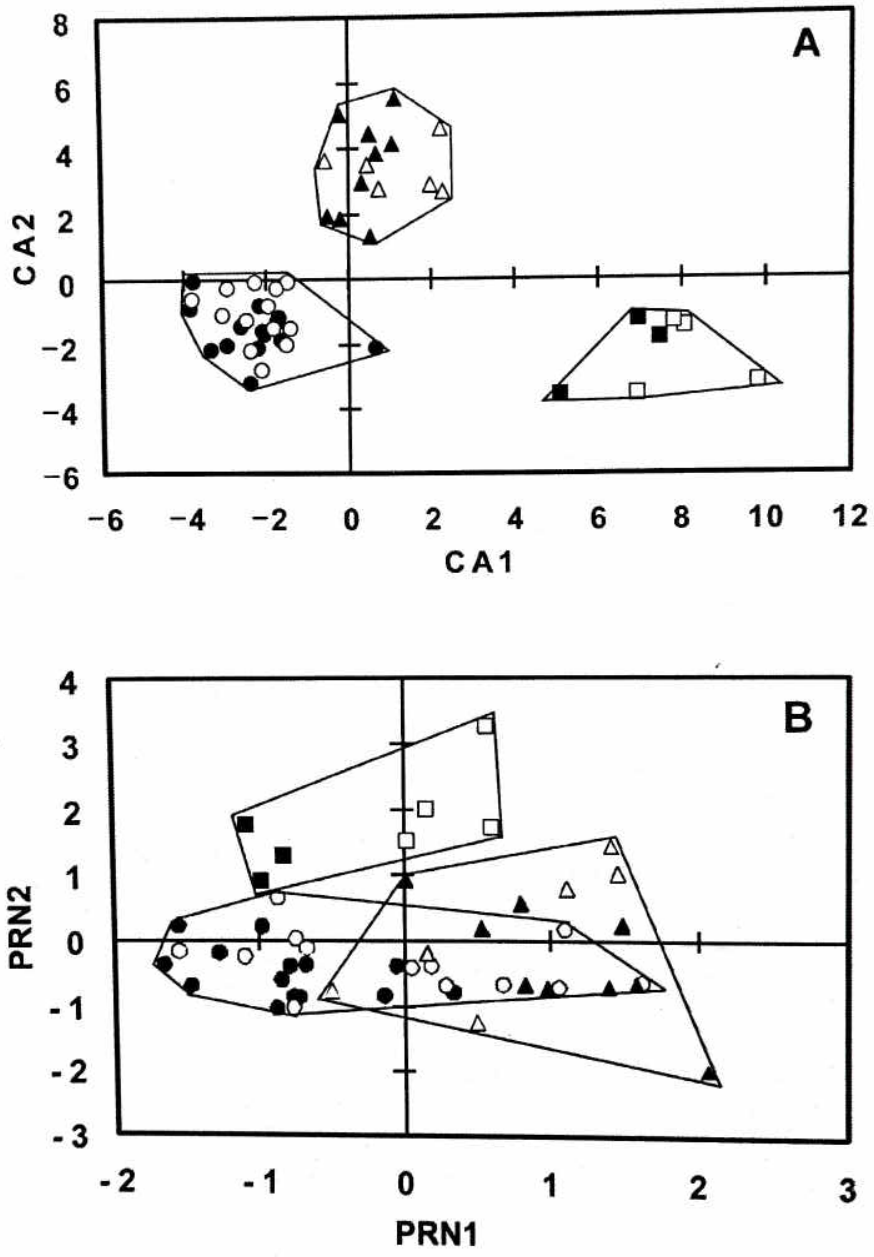


FIGURE 3. (A) Scatterplot of individual discriminant scores on the first (CA1) and second canonical axes (CA2) for *H. litoralis* (circle), *H. tigerinus* (triangle), and *H. rugulosus* (rectangle). (B) Scatterplot of principal component 1 (PRN1) versus principal component 2 (PRN2) from the principal component analysis of *H. litoralis* (circle), *H. tigerinus* (triangle), and *H. rugulosus* (rectangle). In each species males were marked as solid and females were marked as open icons.

Morphological comparisons. Among the three *Hoplobatrachus* species in Bangladesh (*H. litoralis*, *H. tigerinus*, and *H. crassus*), *H. crassus* is easily distinguishable from the other two by large shovel-like inner metatarsal tubercle and usual occurrence of large dark dots in the gular and pectoral region. In the following, we compare the morphological characters of *H. litoralis* with those of *H. tigerinus* and *H. rugulosus*. *Hoplobatrachus rugulosus* is

so similar to *H. tigerinus* in that it was previously regarded as a subspecies of *H. tigerinus*. Because *H. rugulosus* occurs in Myanmar, just adjacent to the distribution range of *H. litoralis*, comparisons of the two are relevant.

All specimens (n = 15) of *H. tigerinus* from Mymensingh, Bangladesh had a thin mid-dorsal stripe. Five specimens (33%) had a thin yellowish stripe along the inner side of the tibia, usually extending over the upper side of the thigh to the base of the thigh. This line was not observed in *H. litoralis*. The underside of the mandible is finely dotted in most specimens. A marginal stripe along the jaw margin is usually fragmented into narrow irregular markings. The stripe from the eye through the nostrils to the anterior edge of the upper jaw is not as broad as in *H. litoralis* and is usually fragmented. In *H. tigerinus*, the broad light line from behind the eye to the groin is more distinct than in *H. litoralis*. Webs are mottled with an irregular dark pattern, in contrast to the uniform gray in *H. litoralis*.

Specimens of *H. rugulosus* from Thailand (n = 7) lacked a mid-dorsal stripe. The snout was rounded rather than pointed. The dorsal dermal ridges were wider and shorter than that of *H. litoralis* and *H. tigerinus*.

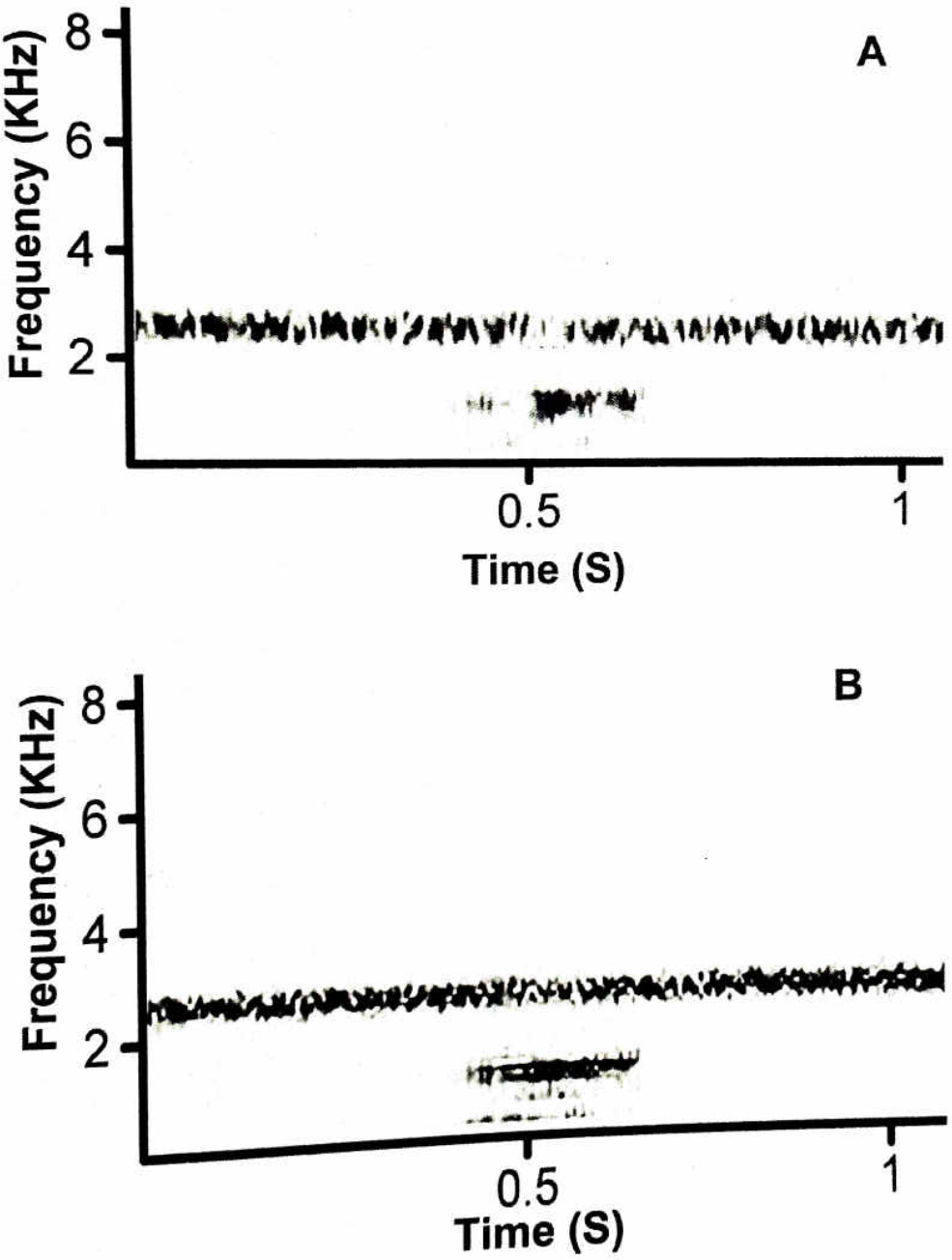


FIGURE 4. Sound spectrograms showing advertisement call structure of *H. litoralis*. The same call analyzed by FlatTop (A) and Hamming window (B). Abscissa: time in s. Ordinate: frequency in kHz. The continuous broad band at about 2.5 kHz is chirps of an insect. The call of several male individuals of *H. litoralis* was recorded from Ukhia, Cox's Bazar district on 19 June, 2011 just after sunset and their voucher specimen numbers are involved in those of molecular analyses, but not specified.

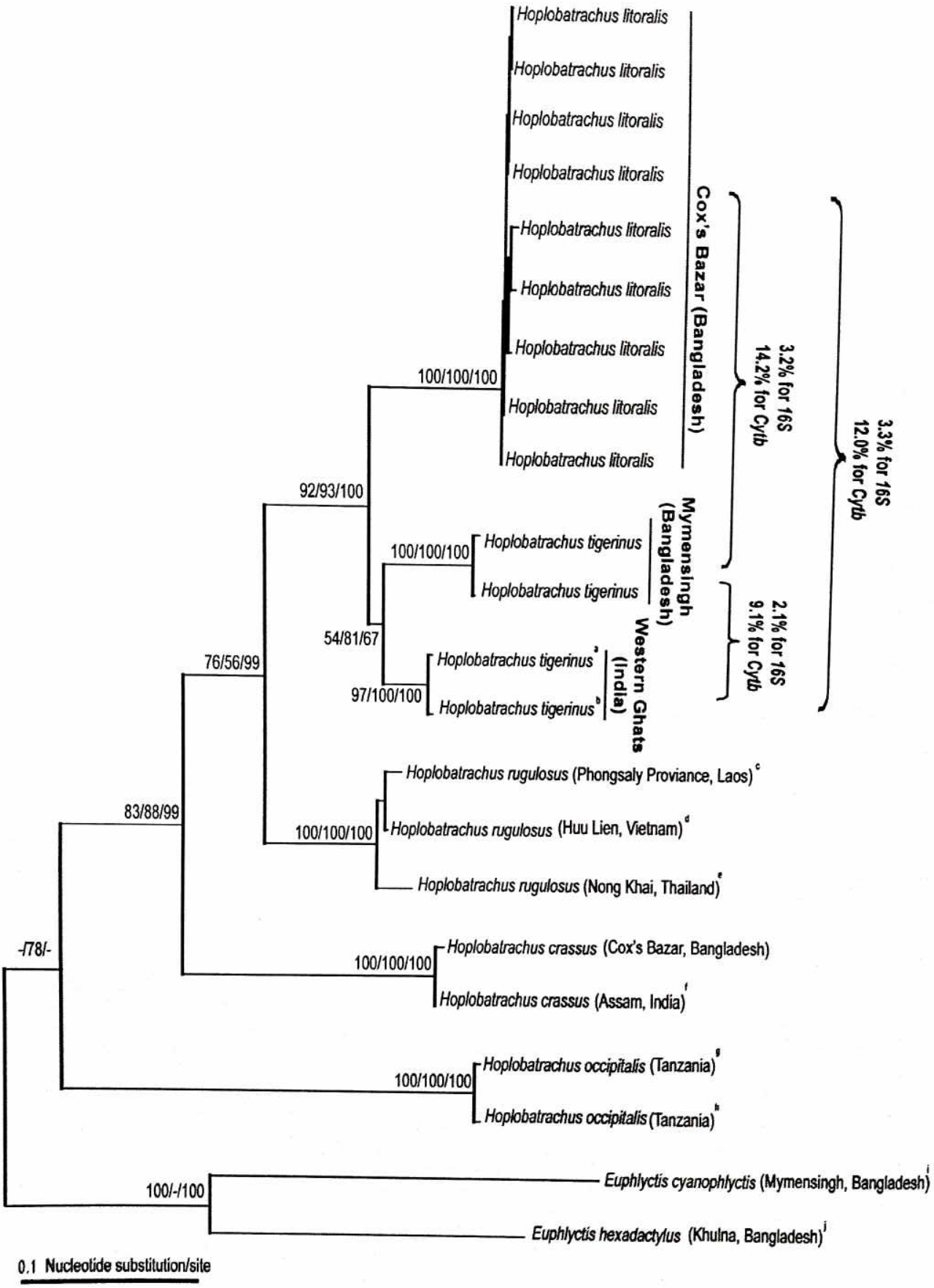


FIGURE 5. Maximum likelihood (ML) tree based on the nucleotides sequence of 1078 bp of mitochondrial (*16S + Cytb*) genes with *Euphlyctis cyanophlyctis* and *E. hexadactylus* as out groups. The most parsimonious and Bayesian analyses reconstructed the same tree topology. Numbers near branches represent bootstrap support for ML and MP inferences, and Bayesian posterior probability (ML–BPs/MP–BPs/BPP). The scale bar represents 0.1 nucleotide substitutions per site. The superscript letters indicate that the *16S* and *Cytb* data were taken from Alam et al. (2008) for constructing this tree. a) AB272594, AB274137; b) AB290412, AB274139; c) AB290417, AB290601; d) AB290414, AB290603; e) AB272596, AB274144; f) AB290413, AB290597; g) AB272599, AB274148; h) AB272600, AB274150; i) AB272601, AB274151 and j) AB272605, AB274163.

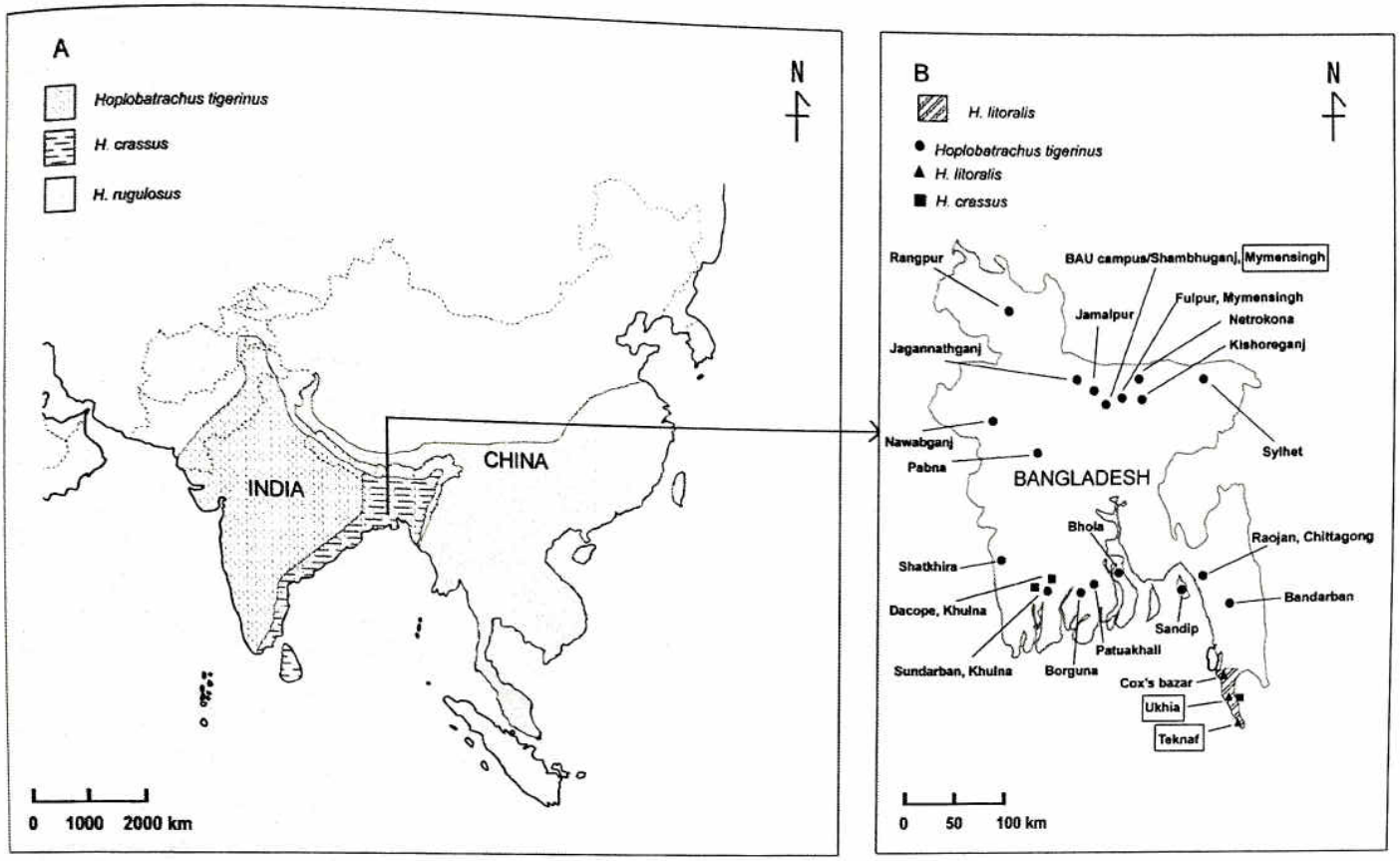


FIGURE 6. (A) Map showing the approximate distribution areas of all Asian *Hoplobatrachus* species based on Frost *et al.* (2011) and Alam *et al.* (2008). (B) Bangladesh map showing the collecting localities of *H. litoralis* is indicated by ▲ (closed triangle) and, the collecting localities of *H. tigerinus* and *H. crassus* are indicated by ● (closed circle) and ■ (closed square), respectively. Each locality was used for molecular analysis, and the boxed locality was used for morphology.

There are several old nomina which have been treated as junior synonyms of currently recognized *Hoplobatrachus* species. *Rana brama* Lesson, 1834, a large frog described from Bengale, was reported as having very smooth surface, depressed dorsum, and short depressed head. Femur is much shorter than tibia. Obviously, *H. litoralis* differs from *R. brama* in these characteristics. *Pyxicephalus frithi* Theobald, 1868 described from Jessore, SW Bangladesh was reported to have quite smooth skin and uniform vinous coloration. These do not fit *H. litoralis*. *Rana burkilli* Annandale, 1910 was described from Tavoy, Myanmar. This species differs from *H. litoralis* in having ventral surface "marked with black, the markings sometimes taking on a reticulate character all over the belly". The inner metatarsal tubercle was described as feebly developed, but *H. litoralis* has well-developed inner metatarsal tubercle. *Rana gracilis* (not of Gravenhorst 1829) Boulenger, 1920, a small frog (SVL 50 mm for male, 41– 64 mm for female) described from Sri Lanka, was disclosed as having an oval inner metatarsal tubercle and smooth skin or feebly granulate above. *Rana gracilis var. pulla* (Stocliczka 1870) described from Penang hill, Malaysia was reported to be a very small frog (SVL is 7/8 inch [< 23 mm] and HLL is 1 1/2 inch [nearly 38.1 mm]). *Hoplobatrachus littoralis* is distinguished from both *R. gracilis* and *R. gracilis var. pulla* considering its very large size (SVL = 89.96 mm and 101.42 mm; and HLL = 143.85 mm and 159.74 mm for male and female, respectively). The skin of *H. litoralis* is rough and its inner metatarsal tubercle is comparatively elongated rather than oval. *Rana picta* Gravenhorst, 1829 whose type locality is unknown (Frost 2011) was reported to have three spots below one eye and there is no trace of tubercle on dorsal skin. These characteristics do not fit with the new species of *H. litoralis*.

Morphometric comparisons. Measurements of 29 body parts of *H. litoralis*, *H. tigerinus*, and *H. rugulosus* are summarized in Table 1. Mean SVL of males differs significantly (U test, $P < 0.05$) between the three species, but that of females does not. Size of *H. litoralis* is slightly smaller than that of the other two species. SVL of females is larger than that of males ($P < 0.05$) in *H. litoralis* and *H. rugulosus*.

The three species are clearly separated by canonical discriminant analysis (Fig. 3A). Eigenvalues are 11.606 for function 1, and 5.684 for function 2. Coefficients for function 1 are large especially in E-E, N-N, N-E, HAL, and ED for function 1 and in E-E, HAL, TD, and TIL for function 2. In principal component analysis (Fig. 3B), *H. litoralis* made completely separate cluster from *H. rugulosus*, but the scores of *H. litoralis* and *H. tigerinus* overlapped considerably.

Body ratios relative to SVL (e.g., HL/SVL and HW/SVL) and 10 other ratios (e.g., HL/HW and S-N/N-E) are shown in Table 2, and the results of the Mann-Whitney U test between ratios of *H. litoralis* vs. *H. tigerinus* and *H. rugulosus* are shown in Table 3. Mean values of ED/SVL, ELW/SVL, ED/E-E, N-N/E-E, and ELW/E-E are significantly large ($P < 0.01$) in *H. litoralis* compared with those in *H. tigerinus* and *H. rugulosus*. These are apparently derived from relatively large ED, ELW, and N-N and relatively small SVL and E-E of *H. litoralis*.

TABLE 2. Body ratios of three species of the genus *Hoplobatrachus*

	H. litoralis (n = 27)	*H. tigerinus* (n = 15)	*H. rugulosus* (n = 7)
	Mean (Min - Max)	Mean (Min - Max)	Mean (Min - Max)
HL/SVL	0.378 (0.349 - 0.436)	0.379 (0.346 - 0.425)	0.367 (0.326 - 0.413)
HW/SVL	0.359 (0.316 - 0.400)	0.363 (0.314 - 0.413)	0.386 (0.352 - 0.424)
S-N/SVL	0.062 (0.044 - 0.076)	0.064 (0.053 - 0.077)	0.074 (0.065 - 0.090)
N-N/SVL	0.063 (0.051 - 0.073)	0.059 (0.052 - 0.074)	0.052 (0.037 - 0.069)
N-E/SVL	0.090 (0.065 - 0.108)	0.089 (0.077 - 0.104)	0.079 (0.064 - 0.096)
ED/SVL	0.082 (0.063 - 0.107)	0.073 (0.058 - 0.093)	0.062 (0.049 - 0.076)
E-E/SVL	0.041 (0.029 - 0.054)	0.060 (0.041 - 0.084)	0.052 (0.036 - 0.061)
ELW/SVL	0.075 (0.060 - 0.094)	0.063 (0.048 - 0.077)	0.051 (0.039 - 0.062)
TD/SVL	0.069 (0.051 - 0.083)	0.069 (0.062 - 0.083)	0.053 (0.042 - 0.066)
FLL/SVL	0.541 (0.484 - 0.626)	0.531 (0.434 - 0.605)	0.526 (0.429 - 0.572)
FHL/SVL	0.384 (0.331 - 0.418)	0.374 (0.316 - 0.405)	0.363 (0.336 - 0.402)
FAW/SVL	0.086 (0.059 - 0.121)	0.074 (0.055 - 0.109)	0.085 (0.076 - 0.102)
HAL/SVL	0.193 (0.175 - 0.206)	0.192 (0.171 - 0.223)	0.203 (0.172 - 0.232)
F1/SVL	0.104 (0.081 - 0.131)	0.105 (0.074 - 0.137)	0.095 (0.068 - 0.112)
F2/SVL	0.077 (0.060 - 0.112)	0.088 (0.053 - 0.114)	0.082 (0.056 - 0.100)
F3/SVL	0.111 (0.091 - 0.132)	0.115 (0.076 - 0.154)	0.120 (0.099 - 0.154)
F4/SVL	0.074 (0.053 - 0.093)	0.083 (0.060 - 0.106)	0.071 (0.054 - 0.095)
HLL/SVL	1.586 (1.400 - 1.703)	1.586 (1.389 - 1.845)	1.364 (1.203 - 1.453)
FEL/SVL	0.498 (0.438 - 0.569)	0.497 (0.409 - 0.597)	0.436 (0.376 - 0.478)
TIL/SVL	0.504 (0.469 - 0.531)	0.528 (0.467 - 0.572)	0.421 (0.378 - 0.455)
TFL/SVL	0.726 (0.625 - 0.782)	0.701 (0.578 - 0.836)	0.614 (0.519 - 0.687)
FOL/SVL	0.479 (0.436 - 0.539)	0.453 (0.389 - 0.536)	0.401 (0.280 - 0.464)
T1/SVL	0.100 (0.075 - 0.117)	0.105 (0.080 - 0.146)	0.104 (0.089 - 0.118)
T2/SVL	0.179 (0.149 - 0.218)	0.179 (0.146 - 0.218)	0.640 (0.135 - 0.178)
T3/SVL	0.244 (0.193 - 0.280)	0.248 (0.202 - 0.292)	0.220 (0.199 - 0.239)
T4/SVL	0.323 (0.277 - 0.368)	0.329 (0.264 - 0.412)	0.295 (0.258 - 0.334)
T5/SVL	0.228 (0.177 - 0.280)	0.245 (0.205 - 0.313)	0.215 (0.181 - 0.252)
IMT/SVL	0.053 (0.043 - 0.069)	0.058 (0.041 - 0.076)	0.040 (0.029 - 0.053)
HL/HW	1.055 (0.961 - 1.222)	1.049 (0.919 - 1.193)	0.951 (0.925 - 0.973)
S-N/N-E	0.693 (0.479 - 0.909)	0.722 (0.617 - 0.871)	0.942 (0.795 - 1.066)
ED/E-E	2.054 (1.333 - 2.808)	1.237 (0.827 - 1.818)	1.220 (0.982 - 1.778)
TD/ED	0.846 (0.600 - 1.152)	0.952 (0.733 - 1.178)	0.847 (0.750 - 1.102)
N-N/E-E	1.575 (1.150 - 2.192)	1.002 (0.663 - 1.273)	1.004 (0.868 - 1.129)
ELW/E-E	1.875 (1.234 - 2.909)	1.060 (0.567 - 1.364)	1.021 (0.647 - 1.472)
F1/F2	1.375 (1.011 - 1.632)	1.241 (0.833 - 1.746)	1.179 (0.815 - 1.625)
TIL/FEL	1.015 (0.871 - 1.137)	1.068 (0.957 - 1.257)	0.966 (0.923 - 1.006)
FOL/FEL	0.966 (0.966 - 1.169)	0.913 (0.808 - 1.067)	0.925 (0.614 - 1.052)
TIL/FOL	1.054 (0.973 - 1.148)	1.171 (1.067 - 1.294)	1.071 (0.918 - 1.504)

TABLE 3. Statistics obtained by Mann-Whitney U-tests for body ratios. *: P<0.05. **P<0.01

	H. litoralis vs. *H. tigerinus*				*H. litoralis* vs. *H. rugulosus*			
	U	Z	P		U	Z	P	
HL/SVL	197	0.144	0.8852		74	0.873	0.3826	
HW/SVL	184	0.486	0.6272		41	2.279	0.0227	*
S-N/SVL	185	0.459	0.6460		36	2.492	0.0127	*
N-N/SVL	128	1.956	0.0505		40	2.321	0.0203	*
N-E/SVL	160	1.116	0.2646		32	2.662	0.0078	**
ED/SVL	99	2.717	0.0066	**	17	3.301	0.0010	**
E-E/SVL	19	4.817	0.0000	**	36	2.492	0.0127	*
ELW/SVL	58	3.793	0.0001	**	1	3.983	0.0001	**
TD/SVL	201	0.039	0.9686		17	3.301	0.0010	**
FLL/SVL	187	0.407	0.6841		93	0.064	0.9491	
FHL/SVL	159	1.142	0.2535		50	1.895	0.0580	
FAW/SVL	105	2.559	0.0105	*	93	0.064	0.9491	
HAL/SVL	175	0.722	0.4704		51	1.853	0.0639	
F1/SVL	191.5	0.289	0.7728		55	1.682	0.0925	
F2/SVL	137	1.719	0.0855		64	1.299	0.1939	
F3/SVL	168	0.906	0.3651		66	1.214	0.2248	
F4/SVL	134	1.798	0.0722		84	0.447	0.6547	
HLL/SVL	193	0.249	0.8031		2	3.940	0.0001	**
FEL/SVL	202	0.013	0.9895		16	3.343	0.0008	**
TIL/SVL	98	2.743	0.0061	**	0	4.025	0.0001	**
TFL/SVL	128	1.956	0.0505		7	3.727	0.0002	**
FOL/SVL	117	2.244	0.0248	*	9	3.642	0.0003	**
T1/SVL	179	0.617	0.5373		75	0.831	0.4062	
T2/SVL	199	0.092	0.9268		48	1.981	0.0476	*
T3/SVL	189	0.354	0.7231		28	2.832	0.0046	**
T4/SVL	192	0.276	0.7828		39	2.364	0.0181	*
T5/SVL	146	1.483	0.1380		72	0.958	0.3379	
IMT/SVL	141	1.614	0.1064		20	3.173	0.0015	**
HL/HW	193	0.249	0.8031		2	3.940	0.0001	**
S-N/N-E	165	0.984	0.3249		6	3.770	0.0002	**
ED/E-E	10	5.053	0.0000	**	7	3.727	0.0002	**
TD/ED	109	2.455	0.0141	*	88	0.277	0.7819	
N-N/E-E	4	5.211	0.0000	**	0	4.025	0.0001	**
ELW/E-E	3	5.238	0.0000	**	5	3.813	0.0001	**
F1/F2	124	2.061	0.0393	*	45	2.108	0.0350	*
TIL/FEL	127	1.982	0.0475	*	42	2.236	0.0253	*
FOL/FEL	129	1.929	0.0537		91.5	0.128	0.8983	
TIL/FOL	33	4.449	0.0000	**	65	1.256	0.2090	

Advertisement calls. Advertisement calls of *H. litoralis* are low-pitched groans emitted at about 4.4 s interval. Mean call duration is 0.28 s (n = 34). The call is composed of about 20 rapidly repeating pulses (Fig. 4A). Fundamental frequency is 0.30 kHz and the 3rd and 4th harmonic bands (at about 1.2 kHz) are dominant (Fig. 4B).

Roy and Elepfandt (1993) and Kanamadi *et al.* (1994) reported the acoustic features of *H. tigerinus* from northeast India (Assam and Meghalaya) and southwest India (Karnataka), respectively. Call durations are reported as 0.30 s (NE India) and 0.22 s (SW India); these values do not differ significantly from those of *H. litoralis*. Dom-

inant frequency bands in *H. tigerinus* are 1.65 kHz and 0.52 kHz in NE India populations and 1.5–2.2 kHz and 0.2–1.2 kHz in SW India populations. These dual-dominant bands are absent in *H. litoralis*. The number of pulses in a call is larger in *H. litoralis* than in *H. tigerinus* (16 in NE India and 12.6 in SE India).

Advertisement calls of *H. crassus* (Kanamadi *et al.* 1992) are different from those of *H. litoralis* and *H. tigerinus* with regard to distinctly few number of pulse groups (2-4 per call) and show many intense harmonic bands.

Divergence in mitochondrial 16S rRNA and Cytb gene sequences. The average sequence divergence for *16S* and *Cytb* was 3.2% and 14.2%, respectively, between *H. litoralis* from Cox's Bazar and *H. tigerinus* from Mymensingh, while these values were only 2.1% and 9.1%, respectively, between the Mymensingh and Western Ghats populations of *H. tigerinus* (Fig. 5). *Hoplobatrachus litoralis* is diverged from *H. tigerinus* of the Western Ghats by 3.3% for *16S* and 12.0% for *Cytb*. *Hoplobatrachus litoralis* clearly differ from *H. tigerinus* from Mymensingh and the Western Ghats (BP: 92 for ML, 93 for MP, and 100 for BI). All *H. litoralis* specimens formed one clade supported by high bootstrap values (BP: 100 for ML, MP, and BI), while the *H. tigerinus* clade from Mymensingh and the Western Ghats was supported by medium bootstrap values (BP: 54 for ML, 81 for MP, and 67 for BI).

Distribution. *Hoplobatrachus litoralis* occurs in the southeastern coastal belt in Ukhia, Teknaf Upazilla (sub-district) and Cox's Bazar town of Cox's Bazar district (21° 45′ N, 91° 97′ E, > 3 m asl.) in Bangladesh. The preferable habitat of this species is a vegetated, marshy ditch/ pond, beside the wetland created by hill stream (locally called "Jiri") and/or sometimes the base of mountains having different soil texture from the mainland Bangladesh. This new species is sympatrically occurs with other anuran species such as *H. crassus*, as well as some *Euphlyctis*, *Fejervarya*, and *Polypedates* species. *Hoplobatracus tigerinus* is widely distributed in mainland Bangladesh (Alam *et al.*, 2008) as well as also in coastal region ranging from southwestern Shatkhira to southeastern Bandarban including Shatkhira, Barguna, Patuakhali, Bhola, Sandwip and Raojan of Chittagong and Bandarban districts (Islam *et al.* unpublished); but no *H. tigerinus* specimens have been obtained from Cox's Bazar district area. Thus we have no confirmation about the overlapping region between this two species, but we speculate that this species is endemic in Cox's Bazar district. The adjacent area of Myanmar (Teknaf) is only separated by the Naaf river, which probably does not constitute a strong barrier to *H. litoralis* migration. Thus it is possible that our new species may also occur in the adjacent coastal geographic region of Myanmar. But as we have no access for sampling in that region, we cannot confirm whether our new species is distributed in that region of Myanmar or not. Approximate distribution of all Asian *Hoplobatrachus* species is shown in Fig. 6A, whereas the detail distribution of the three genetically different *Hoplobatrachus* species in Bangladesh is shown in Fig. 6B.

The Teknaf-Ukhia peninsula is long, narrow and forested area rising up to 300 meters above the sea level and, encompassed by the Bay of Bengal to the west and the Naaf river to the east. This is a diverse habitat for many flora and fauna due to its special characteristics i.e. estuarine habitat and wetland at the base of the mountain. In April 1999, the Department of Environment (DOE) of Bangladesh declared that Teknaf peninisula is a Ecologically Critical Area (ECA) due to adverse change of its ecosystems by human activities. It is an important nesting site for at least four species (*Chelonia mydas*, *Eretmochelys imbricata*, *Lepidochelys olivacea* and *Dermochelys coraicea*) of marine turtle listed as globally threatened by IUCN. A few globally threatened shorebirds (*Eurynorhynchus pygmeus*, *Limnodromus semiplamatus*, *Tringa guttife*r) also prefer this region for their habitat (GoB/GEF/UNDP 1999).

Etymology. The specific name is derived from the Latin *litoralis* meaning coastal, in reference to the distribution range of this species; the Coastal belt of Bangladesh.

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Appendix 1. Examined specimens list

Hoplobatrachus litoralis **(27 specimens):** Institute for Amphibian Biology, Hiroshima University: IABHU 3939, 3974–3999.
Collection localities: Ukhia, Teknaf (Cox's Bazar district, Bangladesh)

Hoplobatrachus tigerinus **(15 specimens):** Institute for Amphibian Biology, Hiroshima University: IABHU 3066–3068, 3087, 3589, 3776, 4000–4003, 20008–20010, 20022, 20027.
Collection locality: Bangladesh Agricultural University Campus (BAUC), Mymensingh district, Bangladesh

Hoplobatrachus rugulosus **(7 specimens):** Institute for Amphibian Biology, Hiroshima University: IABHU 3576–3578, 3583–3584, 3663–3664.
Collection localities: Nong Khai, Chachoengsao (Thailand)

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(1) Genetic divergence and reproductive isolation in the genus *Fejervarya* (Amphibia:Anura) from Bangladesh inferred from morphological observations, crossing experiments, and molecular analyses

Mohammed Mafizul Islam, Naoko Kurose, Md. Mukhlesur Rahman Khan, Toshitaka Nishizawa, Mitsuru Kuramoto, Mohammad Shafiqul Alam, Mahmudul Hasan, Nia Kurniawan, Midori Nishioka and Masayuki Sumida

Zoological Science (2008) 25: 1084-1105

(2) Morphological and genetic variation in three populations of *H. tigerinus* from Bangladesh

Mahmudul Hasan, Md. Mukhlesur Rahman Khan and Masayuki Sumida

Progressive Agriculture (2008) 19: 139-149

(3) Geographic distribution. *Hylarana leptoglossa* (Long-tongued Frog).

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Herptological Review (2012) 43: (in press)

(4) Geographic distribution. *Kaloula taprobanica* (Sri Lankan Bull Frog)

Mahmudul Hasan and Masayuki Sumida

Herptological Review (2012) 43: (in press)

ZOOLOGICAL SCIENCE 25: 1084–1105 (2008)

Genetic Divergence and Reproductive Isolation in the Genus *Fejervarya* (Amphibia: Anura) from Bangladesh Inferred from Morphological Observations, Crossing Experiments, and Molecular Analyses

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In the present study, morphological examinations, crossing experiments and molecular analyses were performed to elucidate the degree of genetic divergence and phylogenetic relationships within the genus *Fejervarya* from Bangladesh and other Asian countries. Morphological characteristics revealed that *Fejervarya* species from Bangladesh were divided into four distinct groups: large, medium, small, and mangrove types. Crossing experiments indicated the involvement of three reproductive isolating mechanisms: gametic isolation between the large type and mangrove type, hybrid inviability between the large type and two other types, and hybrid sterility between the medium and small types. Experimental results also indicated that these four types of frogs merit the status of individual species of *Fejervarya*. Molecular analyses based on mtDNA gene sequences showed that the Bangladesh *Fejervarya* species were largely divided into three groups: the mangrove type, large type, and others, with the last further subdivided into the medium and small types. Comparison with other Asian *Fejervarya* species revealed that the Bangladesh mangrove type (which resembled *F. cancrivora* in morphology) was closely related to *F. cancrivora* from India, Thailand, and the Philippines; the large type belonged to the *F. iskandari* group and closely resembled *F. orissaensis*; the small type was included in the South Asian or Indian group, and was closest to *F. syhadrensis* from India and Sri Lanka, whereas the medium type was most closely related to *F. limnocharis* from Myanmar among all described species of this genus.

Key words: genetic divergence, *Fejervarya*, morphometric variation, reproductive isolation, mtDNA, Bangladesh

INTRODUCTION

Frogs of the genus *Fejervarya* are distributed throughout South and Southeast Asia, from India, Sri Lanka, and Nepal eastwards to Indonesia, China, and Japan (Frost, 2007). Several different species from this genus have been collectively identified as belonging to the *Fejervarya limnocharis* complex. However, the wide distribution and slight morphological differences among the species of the *F. limnocharis* complex have created some confusion and difficulty regarding the taxonomy of this complex. Based on a detailed study of mating calls and morphology, Dubois (1975) reported that no fewer than four distinct species were distributed in Nepal, and had been mistakenly classified together with *F. limnocharis* (the author used the name *Rana limnocharis*). Dubois (1984, 1987, 1992) proposed that the whole group consists of at least 15 species and probably many more in South India. Dutta (1997) reported nine nominal *Fejervarya* species from India (as *Limnonectes*). Frost (2007) listed 31 *Fejervarya* species in his comprehensive checklist, but six additional species were recently described in this genus, including *F. triora* from Thailand (Stuart et al., 2006); *F. mudduraja*, *F. granosa*, *F. caperata*, and *F. kudremukhensis* from the Western Ghats, India (Kuramoto et al., 2007); and *F. sakishimensis* from the Sakishima Islands, Japan (Matsui et al., 2007), bringing the total number of species in this genus to 37. There was some con-

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Fig. 1. Four *Fejervarya* types from Bangladesh. **(A)** Large type ♀. **(B)** Medium type ♀. **(C)** Small type ♀. **(D)** Mangrove type. Scale bar, 5 mm.

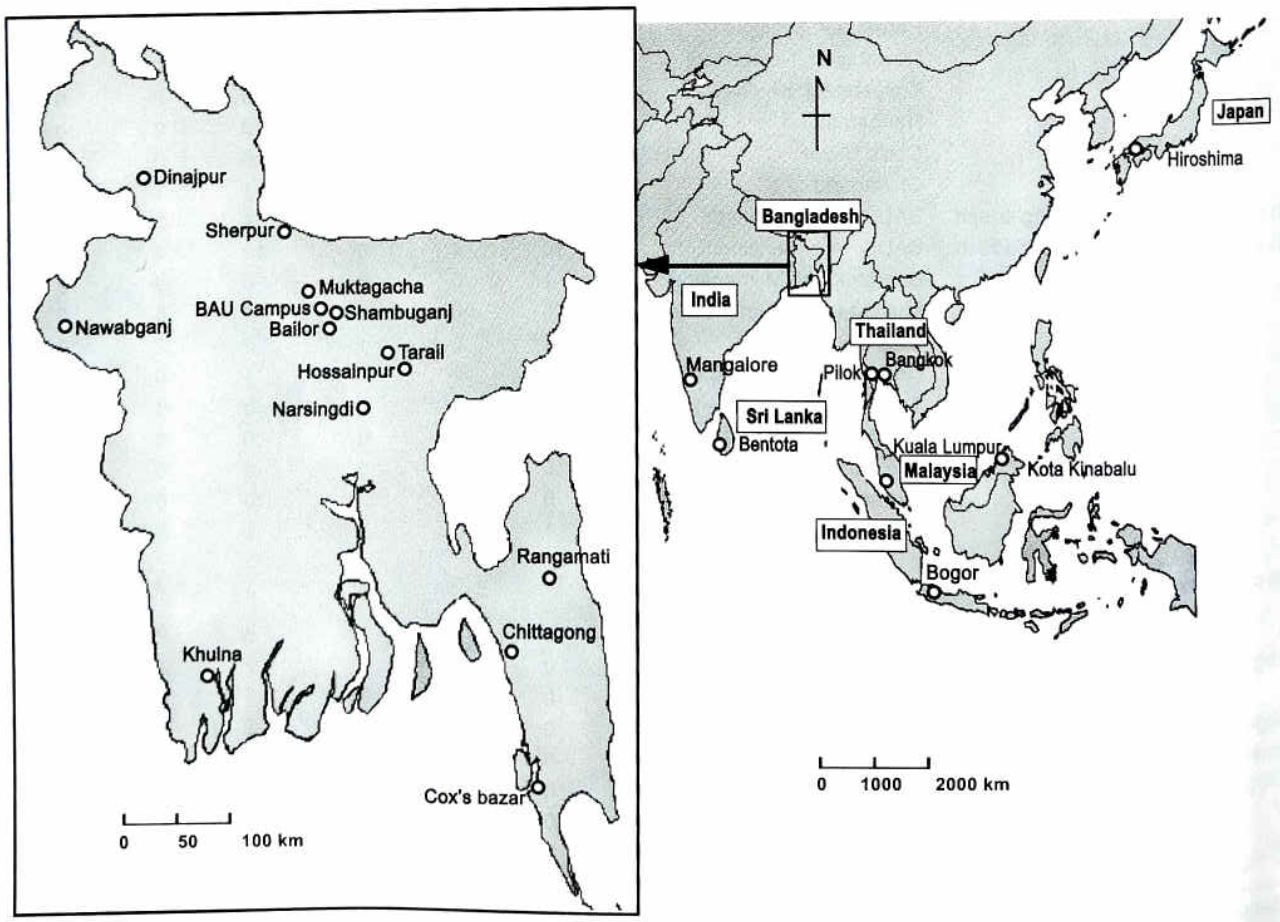

Fig. 2. Maps showing collecting locations in Bangladesh (left) and other Asian countries (right) for frogs used for this study.

mangrove area of the Khulna region of southern Bangladesh, and refer to them here as the mangrove type.

We used morphological observations, crossing experiments, and molecular analyses to evaluate genetic divergence and reproductive isolation among *Fejervarya* frogs from Bangladesh. From the results, we discuss the taxonomic status of these frogs.

MATERIALS AND METHODS

Morphological observations

A total of 101 mature male and female frogs were used for morphological examination (Table 1; Figs. 1, 2). Live frogs were anesthetized with diethylether and 31 characters were measured with digital Vernier calipers to the nearest 0.1 mm (Table 2, Fig. 3). To investigate morphological variation among the different frog types, principal component analysis (PCA) was performed with R2.4.1 software. Differences between the types were examined by means of nonparametric Dunn's multiple comparison test at a significance levels of 5% and 1% by using Statistica software (Statsoft, Tokyo, Japan). Along with the quantitative morphological measurements, some qualitative characters including body color, dermal ridge, median stripe, snout shape, body shape, and thigh region coloration were also observed.

Crossing experiments

Crossing experiments among different types of *Fejervarya* were conducted by artificial insemination according to the methods of Kawamura et al. (1980). Crossings were done in four successive breeding seasons from 2004 to 2007 and involved a total of 52 frogs (22 females and 30 males) (Table 1). Mature female frogs were injected (via the body cavity) with saline solution containing pituitary of *Rana catesbeiana* (*Lithobates catesbeinus* of Frost et al., 2006) at a dose of one pituitary gland per frog. A sperm suspension was made by crushing a testis removed from each male in a small volume of distilled water. After ovulation, eggs were stripped from the females, placed on glass slides, and fertilized with the sperm suspension after confirmation of sperm motility under a microscope. After fertilization, tadpoles were reared first in a glass Petri dish and later on in a cement tank. Tadpoles were fed boiled spinach; after metamorphosis, offspring were housed in a rearing box and fed small crickets, the size of which depended on the frogs' mouth size. Sex was determined by observing the presence of a vocal sac in mature males. In cases where a frog died before maturation, the sex was determined by observing the gonads after biopsy.

Table 2. Morphological parameters used in this study.

Abbreviation	Character
SVL	Snout-vent length
HL	Head length (from back of mandible to tip of snout).
HW	Head width (left side back of mandible to right side back of mandible)
STL	Snout-tympanum length (Tip of snout to front of tympanum)
MSL	Mouth angle-snout length (Tip of snout to end of mouth opening)
NS	Nostril- snout length (Distance from nostril to tip of snout)
SL	Snout length
NTL	Nostril tympanum length (Distance between nostril and front of tympanum)
EN	Distance from front of eyes to nostril
TEL	Tympanum eye length (distance between end of eye to front of tympanum)
TD	Tympanum diameter (Maximum diameter)
MN	Distance from back of mandible to nostril
MFE	Distance from back of mandible to front of eye
MBE	Distance from back of mandible to back of eye
IN	Internarial space (Distance between 2 nostrils)
EL	Eye length (greatest diameter of the eye including upper eyelids)
IOD	Interorbital distance
UEW	Maximum width of upper eyelid
HAL	Hand length (from base of outer palmer tubercle to tip of third finger)
FAL	Fore arm length (from elbow to base of outer palmer tubercle)
LAL	Lower arm length
HLL	Hind limb length
THIGHL	Thigh length
TL	Tibia length
FOL	Foot length (from base of inner metatarsal tubercle to tip of fourth toe)
TFOL	Length of tarsus and foot (from base of tarsus to tip of fourth toe)
3FL	Third finger length
1FL	First finger length
4TL	Fourth toe length
IMTL	Inner metatarsal tubercle length
ITL	Inner toe length

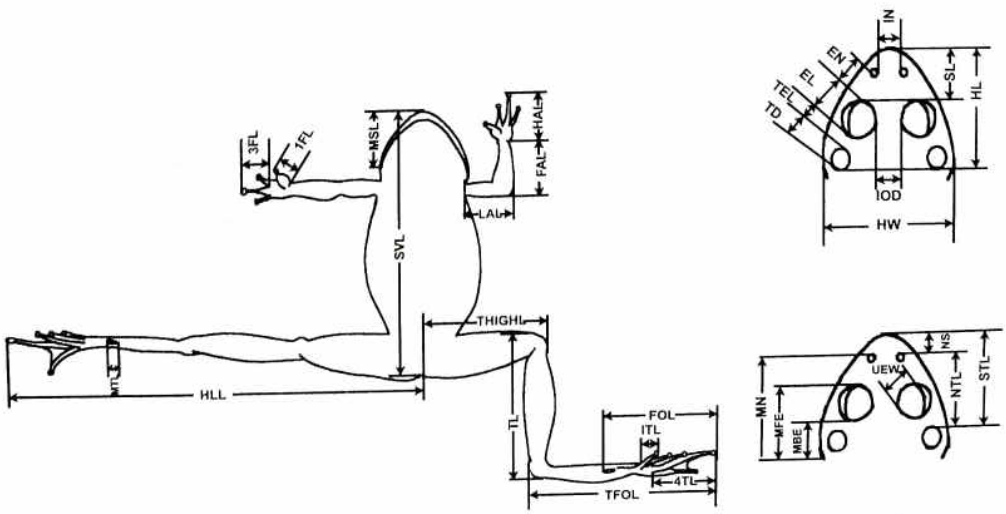

Fig. 3. Morphological parameters for measurements. See Table 2 for abbreviations.

Table 3. Measurements of morphological parameters in male and female individuals of four types of *Fejervarya* frogs from Bangladesh. Data are shown as the mean and standard deviation, followed by the range in parentheses.

Morphological Character	Large type		Medium type	
	♀ n=34 (mm)	♂ n=26 (mm)	♀ n=3 (mm)	♂ n=4 (mm)
SVL	59.6±3.86 (50.9–60.0)	49.2±3.54 (40.0–59.6)	37.4±2.64 (34.8–42.5)	33.1 ±3.62 (30.0–37.7)
HL	21.8±1.57 (19.3–25.3)	19.2±1.04 (16.6–21.6)	14.4±0.97 (13.4–16.0)	12.9 ±1.01 (12.0–14.3)
HW	23.0±2.20 (19.2–28.1)	19.8±1.30 (16.5–23.2)	13.9±1.24 (12.6–15.8)	12.2 ±1.08 (11.2–14.0)
STL	15.4±1.28 (13.0–17.2)	13.5±0.90 (11.3–15.4)	10.3±0.78 (9.7–12.1)	9.2 ±0.83 (8.5–10.6)
MSL	17.5±1.56 (14.9–20.1)	15.2±0.88 (13.5–16.9)	11.5±0.88 (10.7–13.4)	10.4 ±1.07 (9.4–12.4)
NS	3.7±0.39 (3.0–4.6)	3.2±0.36 (2.5–4.1)	2.6±0.21 (2.3–3.0)	2.4 ±0.26 (2.0–2.8)
SL	7.5±0.82 (6.0–9.0)	6.4±0.56 (5.3–7.7)	5.2±0.82 (4.4–7.1)	4.6 ±0.43 (4.1–5.1)
NTL	11.9±0.86 (10.2–13.3)	10.5±1.00 (9.3–14.0)	8.2±0.73 (7.6–9.8)	7.4 ±0.71 (6.5–8.3)
EN	3.9±0.40 (2.9–4.6)	3.2±0.28 (2.3–3.6)	2.8±0.32 (2.3–3.4)	2.2 ±0.14 (2.0–2.4)
TEL	1.5±0.38 (0.7–2.1)	1.2±0.34 (0.6–2.4)	0.9±0.22 (0.7–1.4)	0.7 ±0.12 (0.5–0.8)
TD	3.7±0.38 (2.8–4.3)	3.1±0.29 (2.6–3.6)	2.3±0.35 (1.9–3.1)	2.0 ±0.14 (1.8–2.1)
MN	18.5±1.47 (16.0–20.7)	16.5±0.76 (14.6–18.0)	12.7±1.23 (11.6–15.3)	10.8 ±0.98 (9.6–11.9)
MFE	15.3±1.51 (12.7–18.3)	13.3±0.62 (11.4–14.8)	10.0±0.85 (9.0–11.7)	8.97±0.95 (8.0–10.1)
MBE	9.8±1.17 (7.9–11.8)	8.3±0.59 (7.2–9.6)	6.0±0.63 (5.1–7.2)	4.8 ±0.86 (3.6–5.7)
IN	3.9±0.50 (2.3–4.7)	3.6±0.31 (2.9–4.2)	2.9±0.29 (2.7–3.6)	2.7 ±0.32 (2.4–3.3)
EL	7.3±0.60 (6.1–8.4)	6.6±0.44 (5.3–7.4)	5.2±0.48 (4.6–5.9)	4.8 ±0.42 (4.4–5.4)
IOD	2.6±0.47 (1.5–3.1)	2.2±0.38 (1.5–3.0)	2.1±0.18 (1.8–2.4)	2.0 ±0.13 (1.9–2.2)
UEW	4.8±0.41 (3.7–5.4)	4.1±0.33 (3.4–4.7)	2.9±0.23 (2.5–3.2)	2.7 ±0.27 (2.3–3.0)
HAL	11.2±1.14 (9.0–12.8)	9.3±0.79 (7.7–10.8)	7.9±1.07 (6.6–9.9)	6.9 ±0.31 (6.5–7.2)
FAL	11.9±1.11 (9.7–14.2)	9.9±0.75 (8.5–11.4)	8.2±0.76 (7.2–9.6)	6.9 ±0.75 (6.3–8.0)
LAL	9.0±0.86 (6.8–10.4)	7.8±0.57 (7.0–9.2)	6.4±0.34 (5.9–6.9)	4.9 ±0.83 (3.7–5.8)
HLL	81.5±7.38 (66.1–93)	66.6±4.31 (58.0–75.5)	57.6±6.22 (51.5–71.7)	49.7 ±4.49 (45.0–55.2)
THIGHL	24.2±2.39 (19.3–28.1)	20.3±1.46 (18.2–24)	16.7±2.06 (14.7–20.9)	14.7 ±1.71 (12.5–17.3)
TL	26.0±2.70 (19.9–30.0)	21.2±1.60 (18.2–24)	18.6±1.60 (17.4–22.3)	15.9 ±1.36 (14.3–17.6)
FOL	25.1±2.99 (19.2–30.8)	20.8±1.92 (17.4–24.5)	18.5±1.66 (16.9–22.0)	16.1 ±1.30 (14.6–17.5)
TFOL	37.1±3.79 (29.0–43.6)	29.8±2.40 (25.4–34.1)	26.5±2.82 (23.9–32.4)	22.5 ±2.19 (20.0–25.0)
3FL	6.9±0.70 (5.8–8.2)	5.6±0.48 (4.7–6.5)	4.7±0.40 (4.3–5.5)	4.1 ±0.40 (3.5–4.5)
1FL	6.7±1.12 (5.1–8.8)	5.7±0.79 (3.6–6.8)	4.3±0.45 (3.6–5.2)	3.7 ±0.39 (3.2–4.2)
4TL	16.5±2.18 (12.5–20.5)	13.4±1.30 (9.5–16.7)	12.7±1.39 (11.4–15.6)	11.18±1.18 (9.8–12.5)
IMTL	3.2±0.48 (2.2–4.1)	2.6±0.26 (2.2–3.1)	1.8±0.29 (1.5–2.3)	1.4 ±0.18 (1.3–1.7)
ITL	4.7±0.79 (3.1–6.2)	4.1±0.57 (2.6–5.0)	3.8±0.69 (2.6–5.0)	3.7 ±0.71 (2.7–4.7)

Continued

Morphological Character	Small type		Mangrove type	
	♀ n=21 (mm)	♂ n=9 (mm)	♀ n=6 (mm)	♂ n=7 (mm)
SVL	43.9±0.28 (43.7–44.1)	34.1±1.76 (32.5–36.0)	70.4±12.95(46.6–83.6)	54.5±5.40(46.6–60.6)
HL	16.4±0.64 (15.9–16.8)	13.6±0.50 (13.1–14.1)	23.2±3.89(15.8–26.2)	18.3±1.48(16.6–20.0)
HW	15.6±0.64 (15.1–16.0)	12.5±0.81 (11.8–13.4)	24.8±4.65(15.8–28.2)	18.4±1.6(15.8–20.9)
STL	12.7±0.21 (12.5–12.8)	10.1±0.35 (9.9–10.5)	18.3±3.62(11.7–21.4)	14.5±1.26(12.1–15.5)
MSL	14.7±0.42 (14.4–15)	11.8±0.17 (11.7–12.0)	19.5±4.15(12.8–25.0)	16.7±1.65(14.2–19.2)
NS	3.0±0.21 (2.8–3.1)	2.6±0.00 (2.6–2.6)	3.7±0.63(2.7–4.3)	3.7±0.43(3.1–4.1)
SL	6.4±0.21 (6.2–6.5)	5.4±0.21 (5.2–5.6)	9.6±1.84(6.7–12.3)	7.7±0.78(6.7–8.7)
NTL	9.5±0.00 (9.5–9.5)	7.3±0.35 (7.0–7.7)	15.1±2.20(11.0–16.8)	12.2±0.90(11.0–13.4)
EN	2.7±0.35 (2.4–2.9)	2.2±0.36 (1.8–2.5)	6.6±1.25(4.5–8.0)	5.9±0.91(5.3–7.9)
TEL	1.2±0.07 (1.1–1.2)	0.7±0.17 (0.6–0.9)	3.5±0.60(2.5–4.0)	2.6±0.29(2.2–2.9)
TD	3.1±0.21 (2.9–3.2)	2.8±0.10 (2.7–2.9)	4.7±0.89(3.3–5.8)	4.1±0.47(3.3–4.6)
MN	14.3±0.49 (13.9–14.6)	12.2±0.89 (11.5–13.2)	21.5±3.87(14.9–24.8)	18.1±2.11(14.9–20.5)
MFE	11.1±0.85 (10.5–11.7)	9.5±0.66 (8.9–10.2)	16.6±3.35(11.2–19.6)	13.8±1.64(11.2–15.8)
MBE	6.0±0.28 (5.8–6.2)	5.3±0.81 (4.4–5.9)	10.2±2.23(7.2–12.7)	8.0±1.26(6.3–9.4)
IN	3.6±0.21 (3.4–3.7)	3.1±0.06 (3.0–3.1)	4.3±0.64(3.2–5.1)	3.6±0.24(3.2–4.0)
EL	5.8±0.35 (5.5–6.0)	5.2±0.10 (5.1–5.3)	6.9±1.10(4.7–7.8)	6.5±0.95(4.7–9.5)
IOD	1.7±0.00 (1.7–1.7)	1.7±0.15 (1.6–1.9)	4.5±0.86(3.0–5.3)	3.3±0.55(2.3–4.0)
UEW	3.2±0.00 (3.2–3.2)	3.0±0.10 (2.9–3.1)	4.2±0.66(3.0–5.0)	4.3±0.61(3.5–5.1)
HAL	10.1±0.14 (10.0–10.2)	8.5±0.50 (8.0–9.0)	14.5±2.25(10.5–16.9)	11.8±0.93(10.5–13.0)
FAL	8.0±0.21 (7.8–8.1)	7.1±0.17 (7.0–7.3)	15.9±2.38(11.2–17.8)	13.4±1.65(11.2–15.3)
LAL	6.4±0.21 (6.2–6.5)	5.5±0.21 (5.3–5.7)	18.1±3.53(12.5–22.0)	15.8±2.57(12.4–19.4)
HLL	77.1±0.78 (76.5–77.6)	52.6±2.13 (50.7–54.9)	97.7±18.95(64.0–113.7)	77.2±7.29(64.0–85.9)
THIGHL	21.8±1.84 (20.5–23.1)	16.5±0.49 (16.2–17.1)	29.4±6.11(18.1–33.7)	23.3±2.21(20.1–25.7)
TL	24.8±0.07 (24.7–24.8)	17.9±1.61 (16.6–19.7)	32.5±7.16(21.8–40.8)	25.1±1.74(21.8–27.3)
FOL	25.5±0.64 (25.0–25.9)	16.0±1.47 (14.4–17.3)	34.4±7.44(21.4–40.6)	25.5±3.41(21.4–29.5)
TFOL	34.4±0.85 (33.8–35.0)	23.9±0.61 (23.5–24.6)	51.1±10.49(32.4–60.4)	36.3±4.40(30.2–41.4)
3FL	5.5±0.35 (5.2–5.7)	4.9±0.26 (4.6–5.1)	8.8±1.94(5.6–11.1)	7.3±0.71(6.4–8.3)
1FL	5.2±0.42 (4.9–5.5)	3.3±0.11 (3.2–3.4)	8.5±2.40(5.0–10.6)	6.0±0.68(5.0–7.2)
4TL	18.3±0.35 (18.0–18.5	11.0±1.66 (9.3–12.6)	23.0±3.40(16.5–26.1)	19.1±1.96(15.9–21.3)
IMTL	4.6±0.07 (4.5–4.6)	2.9±0.25 (2.6–3.1)	4.1±0.68(3.0–4.7)	3.2±0.24(3.0–3.5)
ITL	2.2±0.00 (2.2–2.2)	2.0±0.06 (1.9–2.0)	7.6±1.11(5.3–8.1)	7.0±1.31(5.6–8.9)

Table 4. Comparisons among adult males of four types of *Fejervarya* frogs from Bangladesh by Dunn's multiple comparision test. For each entry, differences in mean measurement values are in the upper row and critical values are in the lower row.

	Large-Small	Large-Medium	Large-Mangrove	Small-Medium	Small-Mangrove	Medium-Mangrove
SVL	18.854	15.064	−5.331	−3.790	−24.186	−20.395
	4.768 **	6.927 **	4.768 **	7.946	6.155 **	7.946 **
HL	6.871	5.638	0.957	−1.233	−5.914	−4.681
	1.444 **	2.099 **	1.444	2.407	1.865 **	2.407 **
HW	8.349	7.330	1.420	−1.019	−6.929	−5.910
	1.743 **	2.532 **	1.743	2.905	2.250 **	2.905 **
STL	4.660	3.446	−0.911	−1.214	−5.571	−4.357
	1.219 **	1.771 **	1.219	2.031	1.573 **	2.031 **
MSL	5.706	3.577	−1.351	−2.129	−7.057	−4.929
	1.413 **	2.052 **	1.413 *	2.354 *	1.824 **	2.354 **
NS	0.971	0.557	−0.514	−0.414	−1.486	−1.071
	0.461 **	0.670 *	0.461 **	0.769	0.596 **	0.769 **
SL	1.891	1.168	−1.209	−0.724	−3.100	−2.376
	0.841 **	1.223 *	0.841 **	1.402	1.086 **	1.402 **
NTL	3.597	3.250	−1.660	−0.348	−5.257	−4.910
	1.235 **	1.795 **	1.235 **	2.059	1.595 **	2.059 **
EN	0.831	1.131	−2.554	0.300	−3.386	−3.686
	0.649 **	0.943 **	0.649 **	1.082	0.838 **	1.082 **
TEL	0.477	0.606	−1.266	0.129	−1.743	−1.871
	0.498 *	0.724 *	0.498 **	0.831	0.644 **	0.831 **
TD	1.371	0.329	−0.971	−1.043	−2.343	−1.300
	0.423 **	0.614	0.423 **	0.705 **	0.546 **	0.705 **
MN	6.186	4.329	−1.629	−1.857	−7.814	−5.957
	1.411 **	2.051 **	1.411 **	2.352	1.822 **	2.352 **
MFE	5.043	3.814	−0.471	−1.229	−5.514	−4.286
	1.116 **	1.621 **	1.116	1.860	1.441 **	1.860 **
MBE	3.629	3.024	0.357	−0.605	−3.271	−2.667
	1.014 **	1.474 **	1.014	1.690	1.309 **	1.690 **
IN	0.980	0.542	0.051	−0.438	−0.929	−0.490
	0.376 **	0.546 *	0.376	0.627	0.485 **	0.627
EL	2.169	1.383	0.040	−0.786	−2.129	−1.343
	0.699 **	1.016 **	0.699	1.166	0.903 **	1.166 **
IOD	0.177	0.530	−1.066	0.352	−1.243	−1.595
	0.624	0.906	0.624 **	1.039	0.805 **	1.039 **
UEW	1.557	1.143	−0.200	−0.414	−1.757	−1.343
	0.482 **	0.701 **	0.482	0.804	0.623 **	0.804 **
HAL	2.551	0.942	−2.349	−1.610	−4.900	−3.290
	1.097 **	1.593	1.097 **	1.828 *	1.416 **	1.828 **
FAL	3.357	2.857	−3.400	−0.500	−6.757	−6.257
	1.188 **	1.726 **	1.188 **	1.980	1.533 **	1.980 **
LAL	2.874	2.341	−7.940	−0.533	−10.814	−10.281
	1.501 **	2.180 **	1.501 **	2.501	1.937 **	2.501 **
HLL	20.680	14.723	−9.849	−5.957	−30.529	−24.571
	6.897 **	10.021 **	6.897 **	11.495	8.904 **	11.495 **
THIGHL	6.580	3.990	−2.791	−2.590	−9.371	−6.781
	2.083 **	3.026 **	2.083 **	3.471	2.689 **	3.471 **
TL	6.046	3.603	−3.569	−2.443	−9.614	−7.171
	2.395 **	3.480 **	2.395 **	3.992	3.092 **	3.992 **
FOL	5.520	4.991	−4.523	−0.529	−10.043	−9.514
	2.937 **	4.267 **	2.937 **	4.895	3.791 **	4.895 **
TFOL	8.517	6.260	−6.140	−2.257	−14.657	−12.400
	3.898 **	5.664 **	3.898 **	6.497	5.032 **	6.497 **
3FL	1.617	0.817	−1.554	−0.800	−3.171	−2.371
	0.712 **	1.034	0.712 **	1.187	0.919 **	1.187 **
1FL	2.217	2.479	−0.297	0.262	−2.514	−2.776
	0.996 **	1.447 **	0.996	1.659	1.285 **	1.659 **
4TL	2.709	2.604	−5.520	−0.105	−8.229	−8.124
	2.030 **	2.950 *	2.030 **	3.384	2.621 **	3.384 **
IMTL	1.183	0.673	−0.589	−0.510	−1.771	−1.262
	0.337 **	0.489 **	0.337 **	0.561 *	0.435 **	0.561 **
ITL	1.109	1.256	−2.906	0.148	−4.014	−4.162
	1.008 **	1.465 *	1.008 **	1.680	1.301 **	1.680 **

*significance level $p < 0.05$; ** significance level $p < 0.01$.

Table 5. Comparisons among adult females of four types of *Fejervarya* frogs from Bangladesh by Dunn's multiple comparision test. For each entry, differences in mean measurement values are in the upper row and critical values are in the lower row.

	Large-Small	Large-Medium	Large-Mangrove	Small-Medium	Small-Mangrove	Medium-Mangrove
SVL	23.468	18.102	−11.356	−5.366	−34.824	−29.458
	6.720 **	10.035 **	8.462 **	10.909	9.482 **	12.060 **
HL	7.426	6.110	−1.432	−1.316	−8.858	−7.542
	2.195 **	3.278 **	2.764	3.564	3.098 **	3.940 **
HW	9.616	7.837	−1.905	−1.780	−11.521	−9.742
	2.801 **	4.183 **	3.528	4.548	3.953 **	5.028 **
STL	1.276	4.985	0.894	3.709	−0.382	−4.091
	1.863	2.782 **	1.863	3.024 **	2.208	3.024 **
MSL	19.601	10.496	28.196	−9.105	8.595	17.700
	4.382 **	6.544 **	5.518 **	7.114 **	6.184 **	7.865 **
NS	2.615	1.415	5.849	−1.200	3.233	4.433
	0.956 **	1.427 *	1.203 **	1.551	1.348 **	1.715 **
SL	10.736	7.277	19.310	−3.459	8.574	12.033
	3.145 **	4.697 **	3.961 **	5.106	4.438 **	5.645 **
NTL	3.734	2.740	−3.285	−0.993	−7.018	−6.025
	1.225 **	1.829 **	1.542 **	1.988	1.728 **	2.198 **
EN	0.953	0.958	−2.676	0.005	−3.629	−3.633
	0.694 **	1.036 *	0.873 **	1.126	0.979 **	1.245 **
TEL	0.451	0.169	−1.914	−0.282	−2.365	−2.083
	0.501 *	0.749	0.631 **	0.814	0.707 **	0.900 **
TD	1.369	0.873	−1.044	−0.495	−2.412	−1.917
	0.565 **	0.844 **	0.712 **	0.918	0.798 **	1.015 **
MN	6.141	5.823	−3.077	−0.318	−9.218	−8.900
	2.178 **	3.253 **	2.743 **	3.536	3.074 **	3.910 **
MFE	5.571	4.235	−1.382	−1.336	−6.953	−5.617
	1.955 **	2.919 **	2.462	3.173	2.758 **	3.508 **
MBE	3.845	3.504	−0.413	−0.341	−4.258	−3.917
	1.404 **	2.097 **	1.768	2.280	1.982 **	2.520 **
IN	1.071	0.483	−0.376	−0.589	−1.447	−0.858
	0.526 **	0.785	0.662	0.853	0.742 **	0.943 *
EL	2.448	2.012	0.362	−0.436	−2.086	−1.650
	0.802 **	1.197 **	1.010	1.302	1.131 **	1.439 **
IOD	0.266	0.638	−1.862	0.373	−2.127	−2.500
	0.668	0.998	0.841 **	1.085	0.943 **	1.199 **
UEW	1.823	1.450	0.600	−0.373	−1.223	−0.850
	0.476 **	0.711 **	0.600 **	0.773	0.672 **	0.855 *
HAL	3.356	1.944	−3.381	−1.411	−6.736	−5.325
	1.481 **	2.211 *	1.865 **	2.404	2.089 **	2.658 **
FLL	4.014	3.698	−4.044	−0.316	−8.058	−7.742
	1.467 **	2.190 **	1.847 **	2.381	2.070 **	2.632 **
LAL	2.651	2.494	−9.064	−0.157	−11.715	−11.558
	1.652 **	2.467 **	2.080 **	2.682	2.331 **	2.965 **
HLL	25.790	11.383	−16.726	−14.407	−42.515	−28.108
	10.940 **	16.336	13.775 **	17.759	15.436 **	19.633 **
THIGHL	7.672	4.202	−5.290	−3.470	−12.962	−9.492
	3.539 **	5.284	4.456 **	5.744	4.993 **	6.351 **
TL	7.976	3.935	−6.599	−4.041	−14.574	−10.533
	4.012 **	5.991	5.052 **	6.512	5.661 **	7.200 **
FOL	7.286	2.250	−9.383	−5.036	−16.670	−11.633
	4.214 **	6.292	5.306 **	6.840	5.946 **	7.562 **
TFOL	11.541	5.300	−14.267	−6.241	−25.808	−19.567
	5.652 **	8.440	7.118 **	9.176	7.976 **	10.144 **
3FL	2.103	1.583	−1.926	−0.520	−4.029	−3.508
	1.061 **	1.585 *	1.336 **	1.723	1.498 **	1.905 **
1FL	2.356	1.940	−1.801	−0.416	−4.158	−3.742
	1.340 **	2.001 *	1.687 **	2.175	1.891 **	2.405 **
4TL	4.078	−0.454	−6.354	−4.532	−10.432	−5.900
	2.481 **	3.705	3.124 **	4.028 **	3.501 **	4.453 **
IMTL	1.367	0.908	−0.909	−0.459	−2.276	−1.817
	0.522 **	0.780 **	0.658 **	0.848	0.737 **	0.937 **
ITL	1.105	0.448	−2.827	−0.657	−3.932	−3.275
	0.921 **	1.376	1.160 **	1.496	1.300 **	1.653 **

*significance level $p < 0.05$; ** significance level $p < 0.01$.

Molecular methods and phylogenetic analyses

In total, 136 frogs comprising 82 of the large type, 10 of the medium type, 39 of the small type, and five of the mangrove type were used for molecular work (Table 1, Fig. 1). The frogs were collected from 14 populations in Bangladesh (Fig. 2).

Total genomic DNA was extracted from clipped toe tips by using a DNA extraction kit (DNeasy Tissue Kit, QIAGEN) according to the manufacturer's instructions. Extracted DNA was used for amplification by polymerase chain reaction (PCR) of fragments (516 bp, 807 bp and 516 bp, respectively) of the 16S rRNA, 12S rRNA, and Cyt *b* genes. These segments correspond to sites 3780 to 4179, 1908 to 2722, and 16671 to 16812, respectively, in the complete mtDNA sequence of *F. limnocharis* (Liu et al., 2005). Primers used for amplifications were F51 and R51 (Sumida et al., 2002) for 16S; FS01, R16, and RFR 60 (Sumida et al., 2002) for 12S; and Fow 1-1 (Sano et al., 2005), Rev 1-1, and Rev-1 (Kurabayashi et al., unpublished) for Cyt *b*. The PCR mixture was prepared by using a TaKaRa Ex Taq Kit as recommended by the manufacturer. PCR conditions were 35 cycles of [10 sec at 98°C; 30 sec at 47.5°C (10 cycles), 45°C (10 cycles), and 42.5°C (15 cycles); and 1 min 20 sec at 72°C]. PCR products were purified by ethanol precipitation, and both strands were directly sequenced by using the Big Dye Terminator Cycle Sequencing Kit (ABI) and a 3100-Avant automated DNA sequencer (ABI). The resultant sequences were deposited in the DDBJ database under accession nos. AB372009–AB372018 for 16S, AB372019 and AB372072–AB372084 for 12S, and AB372046–AB372071 for Cyt *b* (Table 9).

Nucleotide sequences were aligned with ClustalW (Thompson et al., 1994), and ambiguous sites were manually eliminated. Based on the aligned data, phylogenetic analyses were performed with the maximum likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ) methods implemented in PAUP Version 4.10b (Swofford, 2002). For each frog type and each gene, sequences with at least one nucleotide change were considered to be different haplotypes and were included in the analyses. In all cases, *Limnonectes fujianensis* (Liu et al., 2005) was used as the outgroup. For the ML and NJ analyses, best-fit substitution models were chosen by using the Akaike information criterion implemented in MODEL TEST Ver 3.06 (Posada and Crandall, 1998). Support for the resulting trees was evaluated by calculating bootstrap probabilities (BP). BP values were calculated from 100 replicates for ML and 1000 replicates for MP and NJ analyses. A 16S ML analysis was also performed using the present sequences and the sequences from Asian *Fejervarya* species available from the DDBJ/GenBank database (Appendix). In this case, 396 base-pair positions were alignable and were used in the analysis. Sequence diver-

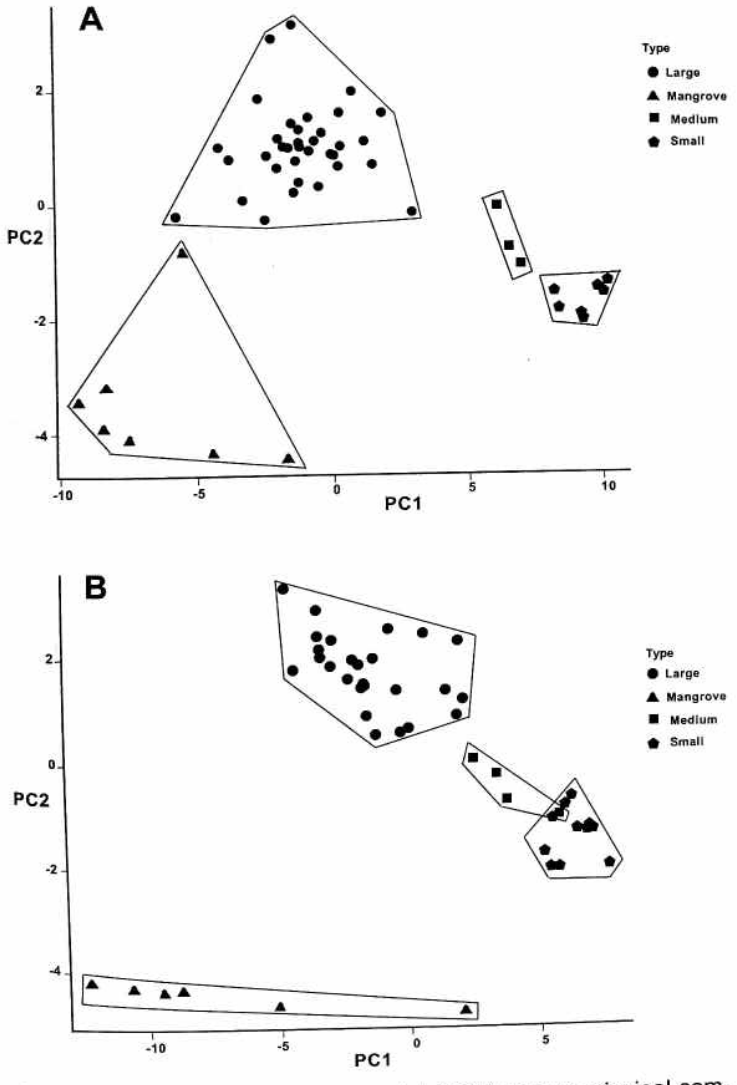

Fig. 4. Plot of principal component 1 (PC1) versus principal component 2 (PC2) from the principal component analysis of four *Fejervarya* types in Bangladesh. **(A)** Males. **(B)** Females.

Table 6. Factor loading on the first two principal components extracted from a correlation matrix of 31 morphological parameters for male and female individuals of *Fejervarya* frogs from Bangladesh.

Character	Male		Female	
	PC1	PC2	PC1	PC2
SVL	−0.196	0.113	−0.201	0.060
HL	−0.177	0.245	−0.195	0.124
HW	−0.170	0.266	−0.193	0.128
STL	−0.195	0.133	−0.201	0.037
MSL	−0.197	0.099	−0.042	0.425
NS	−0.172	0.034	0.010	0.434
SL	−0.189	−0.018	−0.018	0.425
NTL	−0.194	0.031	−0.203	−0.025
EN	−0.154	−0.307	−0.172	−0.200
TEL	−0.145	−0.255	−0.160	−0.240
TD	−0.187	−0.053	−0.193	0.013
MN	−0.198	0.103	−0.197	0.054
MFE	−0.187	0.194	−0.195	0.114
MBE	−0.167	0.248	−0.184	0.141
IN	−0.161	0.227	−0.182	0.091
EL	−0.175	0.241	−0.168	0.214
IOD	−0.125	−0.314	−0.152	−0.237
UEW	−0.182	0.169	−0.152	0.252
HAL	−0.190	−0.120	−0.200	−0.040
FAL	−0.188	−0.125	−0.198	−0.039
LAL	−0.161	−0.287	−0.177	−0.215
HLL	−0.201	−0.006	−0.201	0.022
THIGHL	−0.195	0.022	−0.197	0.016
TL	−0.198	−0.037	−0.200	−0.009
FOL	−0.187	−0.112	−0.196	−0.072
TFOL	−0.191	−0.100	−0.200	−0.070
3FL	−0.177	−0.128	−0.200	−0.024
1FL	−0.169	0.096	−0.196	0.003
4TL	−0.168	−0.275	−0.185	−0.113
IMTL	−0.190	−0.031	−0.200	0.017
ITL	−0.156	−0.279	−0.170	−0.177
Eigen values	23.312	3.319	23.587	5.000
Varience explained (%)	75.200	10.710	76.090	16.130
Cumulative explained (%)	75.200	85.910	76.090	92.210

gence was calculated among the different types of *Fejervarya* from Bangladesh and the outgroup, at different taxonomic levels.

RESULTS

Morphometry

All measurement data on the four types of *Fejervarya* from Bangladesh are shown in Table 3. Comparisons for male and female adults are shown in Tables 4 and 5, respectively. In comparisons among the types based on the Dunn's multiple comparison test, the large and medium types differed significantly (at the 1% level) for 21 parameters in males and 16 parameters in females; the large and small types showed significant differences for 29 parameters in males and 28 in females; the large and mangrove types showed significant differences for 21 parameters in males and 24 in females; the medium and mangrove types showed significant differences for 30 parameters in males and 29 in females; and the small and mangrove types showed differences for all 31 parameters in males and 30 in females. In contrast, the medium and small types showed differences for only one parameter in males and three in females (Tables 4, 5).

A principal component analysis (PCA) based on the 31 morphometric parameters showed clear differentiation among the four types for both males and females, although the small and medium types were comparatively close (Fig.

Table 7. Developmental capacity of hybrid and control offspring from crosses among *Fejervarya* frogs from Bangladesh. BD, Bangladesh; L, large type; M, medium type; S, small type; C, mangrove type.

Parents		No. of eggs	No. of normally cleaved eggs (%)	No. of normal tail-bud embryos (%)	No. of normally hatched tadpoles (%)	No. of normally feeding tadpoles (%)	No. of 30-day-old tadpoles (%)	No. of metamorphos-ed frogs (%)
Female	Male							
BD (L) 1	BD (L) 1	130	125 (96.2)	119 (91.5)	116 (89.2)	107 (82.3)	107 (82.3)	105 (80.8)
BD (L) 2	BD (L) 1	36	32 (88.9)	26 (72.2)	26 (72.2)	26 (72.2)	25 (69.4)	25 (69.4)
BD (L) 3	BD (L) 1	46	40 (87.0)	37 (80.4)	36 (78.3)	36 (78.3)	32 (69.6)	32 (69.6)
BD (L) 5	BD (L) 2	379	368 (97.1)	362 (95.5)	356 (93.9)	352 (92.9)	349 (92.1)	349 (92.1)
BD (L) 6	BD (L) 3	335	301 (89.8)	301 (89.8)	296 (88.3)	225 (67.2)	221 (66.0)	221 (66.0)
BD (L) 7	BD (L) 5	717	683 (95.3)	420 (58.6)	361 (50.3)	337 (47.0)	321 (44.8)	272 (37.9)
BD (L) 8	BD (L) 4	281	238 (84.7)	231 (82.2)	231 (82.2)	227 (80.8)	201 (71.5)	151 (53.7)
Total		1924	1787 (92.9)	1496 (77.8)	1422 (73.9)	1310 (68.1)	1256 (65.3)	1155 (60.0)
BD (L) 1	BD (S) 1	165	120 (72.7)	30 (18.2)	0 (0)			
BD (L) 2	BD (S) 1	204	162 (79.4)	58 (28.4)	0 (0)			
BD (L) 3	BD (S) 1	200	180 (90.0)	62 (31.0)	0 (0)			
BD (L) 5	BD (S) 2	149	121 (81.2)	33 (22.1)	0 (0)			
BD (L) 7	BD (S) 6	660	591 (89.5)	30 (4.5)	0 (0)			
Total		1378	1174 (85.2)	213 (15.5)	0 (0)			
BD (L) 1	BD (M) 1	93	68 (73.1)	48 (51.6)	0 (0)			
BD (L) 2	BD (M) 1	102	70 (68.6)	46 (45.1)	0 (0)			
BD (L) 3	BD (M) 1	112	103 (92.0)	77 (68.8)	8 (7.1)	0 (0)		
BD (L) 6	BD (M) 2	158	14 (8.9)	3 (1.9)	0 (0)			
Total		365	255 (69.9)	174 (47.7)	8 (2.2)	0 (0)		
BD (L)8	BD (C)	181	0 (0)					
BD (S) 1	BD (L) 1	24	23 (95.8)	0 (0)	0 (0)			
BD (S) 4	BD (L) 2	178	165 (92.7)	0 (0)				
BD (S) 5	BD (L) 2	188	179 (91.9)	1 (0.5)	0 (0)			
BD (S) 7	BD (L) 4	304	270 (88.8)	13 (4.3)	2 (0.7)			
BD (S) 6	BD (L) 4	259	229 (88.4)	7 (2.7)	0 (0)			
Total		953	866 (90.9)	21 (2.2)	2 (0.2)	0 (0)		
BD (S) 1	BD (M) 2	62	56 (90.0)	52 (83.9)	52 (83.9)	44 (71.0)	38 (61.3)	35 (56.5)
BD (S) 3	BD (M) 2	28	18 (64.3)	13 (46.4)	13 (46.4)	6 (21.4)	6 (21.4)	5 (17.9)
BD (S) 4	BD (M) 2	159	120 (75.5)	14 (8.8)	14 (8.8)	14 (8.8)	13 (8.2)	10 (6.3)
BD (S) 5	BD (M) 2	258	246 (95.3)	47 (18.2)	31 (12.0)	24 (9.3)	17 (6.6)	17 (6.6)
BD (S) 6	BD (M) 2	374	359 (96.0)	59 (15.8)	25 (6.7)	19 (5.1)	11 (2.9)	7 (1.9)
Total		881	799 (90.7)	185 (21.0)	135 (15.3)	107 (12.1)	85 (9.6)	74 (8.4)
BD (S) 1	BD (S) 1	28	21 (75.0)	16 (57.1)	14 (50.0)	13 (46.4)	11 (39.2)	3 (10.7)
BD (S) 4	BD (S) 2	89	78 (87.6)	73 (82.0)	70 (78.7)	67 (75.3)	62 (69.7)	59 (66.3)
BD (S) 5	BD (S) 2	188	179 (95.2)	174 (92.6)	169 (89.9)	160 (85.1)	153 (81.4)	117 (62.2)
BD (S) 7	BD (S) 6	284	281 (98.9)	129 (45.4)	109 (38.4)	81 (28.5)	68 (23.9)	54 (19.0)
Total		589	559 (94.9)	392 (66.6)	362 (61.4)	321 (54.5)	294 (49.9)	233 (39.6)
BD (M) 1	BD (L) 3	82	32 (39.0)	1 (1.2)	0 (0)			
BD (M) 2	BD (L) 6	158	14 (8.9)	3 (1.9)	0 (0)			
Total		240	46 (19.2)	4 (1.7)	0 (0)			
BD (M) 2	BD (S) 1	28	10 (35.7)	3 (10.7)	3 (10.7)	3 (10.7)	2 (7.1)	1 (3.6)
BD (M) 2	BD (S) 4	241	214 (88.8)	148 (61.4)	129 (53.5)	85 (35.3)	47 (19.5)	44 (18.3)
Total		269	224 (83.3)	151 (56.1)	132 (49.1)	88 (32.7)	49 (18.2)	45 (16.7)

4; Table 6). Two components extracted with eigenvalues greater than 1 explained 75.20% and 76.09% (first component) and 10.71% and 16.13% (second component) of all morphometric variation in males and females, respectively (Table 6). For the first component, almost all the variables showed similar negative loading, except for MSL, NS, and SL in females (Table 6). For the second component, EN and IOD (negative) in males, and MSL, NS, and SL (positive), showed relatively large loading (Table 6).

We also found several external differences among the four types (Fig. 1). The mature mangrove type was the largest in size, followed by the large, medium, and small types. The body of the mangrove type was exceptionally smooth compared to the rough body of the other types. The short ridges on the dorsum were longest in the large type, moderately long in the medium type, dot-shaped in the small type, and lacking in the mangrove type. The snout was most pointed in the medium type, moderately pointed in the large type, but rounded in the small and mangrove types. Body color also differed among the four types. The large type was grayish, with irregular black stripes dorsally, especially in the leg, hand, and mouth regions, and a median stripe was absent in some cases and narrow where present; the medium type was brownish to grayish, with yellow and black spots, and in most cases had a broad, prominent median stripe; the small type was grayish to brownish, with irregular black spots; and the mangrove type was pale brown, with irregular black spots and no median stripe.

Crossing experiments

The results of crossing experiments conducted among the four *Fejervarya* types and other Asian *Fejervarya* are shown in Tables 7 and 8. The developmental capacity of hybrids and controls is shown in Figs. 5 and 6, and that of

Table 8. Developmental capacity of hybrid and control offspring from crosses among *Fejervarya* frogs from Bangladesh and other Asian countries. BD, Bangladesh; L, large type; M, medium type; S, small type; Thai, Thailand; Malay, Malaysia; U, University of Malaya Campus; K, Kota Kinabalu.

Parents		No. of eggs	No. of normally cleaved eggs (%)	No. of normal tail-bud embryos (%)	No. of normally hatched tadpoles (%)	No. of normally feeding tadpoles (%)	No. of 30-day-old tadpoles (%)	No. of metamorph-osed frogs (%)
Female	Male							
BD (L) 1	Japan.1	112	89 (79.5)	83 (74.1)	79 (70.5)	68 (60.7)	49 (43.8)	0 (0)
BD (L) 2	Japan.1	83	65 (78.3)	58 (69.9)	49 (59.0)	47 (56.6)	2 (2.4)	0 (0)
BD (L) 3	Japan.1	77	72 (93.5)	67 (87.0)	64 (83.1)	57 (74.0)	20 (26.0)	0 (0)
BD (L) 7	Japan.2	228	186 (81.6)	39 (17.1)	31 (13.6)	22 (9.7)	17 (7.5)	2 (1)
BD (L) 7	Japan.3	1150	860 (74.8)	272 (23.7)	219 (19.0)	208 (18.1)	169 (14.7)	20 (1.7)
	Total	1650	1272 (77)	519 (31.5)	442 (26.8)	402 (24.3)	257 (15.6)	22 (1.3)
BD (L) 1	Thai (L)	138	131 (94.9)	129 (93.5)	127 (92.0)	125 (90.6)	122 (88.4)	120 (87.0)
BD (L) 2	Thai (L)	111	105 (94.6)	96 (86.5)	99 (89.2)	98 (88.3)	93 (83.8)	92 (82.9)
BD (L) 3	Thai (L)	86	80 (93.0)	73 (84.9)	73 (84.9)	72 (83.7)	68 (79.1)	68 (79.1)
	Total	335	316 (93.3)	298 (89.2)	299 (89.2)	295 (88.1)	283 (84.5)	280 (83.6)
BD (L) 1	Malay (K)	37	32 (86.5)	29 (78.4)	28 (75.7)	28 (75.7)	25 (67.6)	19 (51.4)
BD (L) 2	Malay (K)	74	70 (94.6)	62 (83.8)	62 (83.8)	57 (77.0)	51 (68.9)	36 (48.6)
BD (L) 3	Malay (K)	92	85 (92.4)	80 (87.0)	68 (73.9)	64 (69.6)	48 (52.2)	28 (30.4)
	Total	203	187 (92.1)	171 (84.2)	158 (77.8)	149 (73.4)	124 (61.1)	83 (40.9)
BD (L) 7	Malay (U)	628	600 (95.5)	348 (55.4)	195 (31.1)	188 (29.9)	171 (27.2)	7 (1.1)
BD (L) 6	*F. iskandari*.1	394	331 (84.0)	262 (66.5)	209 (53.0)	128 (32.5)	121 (30.7)	110 (27.9)
BD (L) 5	Sri Lanka.1	159	138 (86.8)	11 (6.9)	2 (1.3)	0 (0)	0 (0)	0 (0)
BD (M) 2	Japan. 4	180	9 (5.0)	2 (1.1)	0 (0)	–	–	–
BD (M) 2	Malay(U)	200	2 (1.0)	0 (0)	–	–	–	–
BD (M) 2	*F. iskandari*.1	57	4 (7.0)	0 (0)	–	–	–	–
BD (M) 2	India.2	209	157 (75.1)	78 (37.3)	76 (36.3)	59 (28.2)	52 (24.9)	47 (22.5)
BD (S) 4	Sri Lanka.1	201	188 (93.5)	181 (90.0)	177 (88.1)	152 (75.6)	134 (66.7)	97 (48.3)
BD (S) 5	Sri Lanka.1	132	120 (90.9)	115 (87.1)	113 (85.6)	112 (84.8)	110 (83.3)	106 (80.3)
	Total	333	308 (92.5)	296 (88.9)	290 (87.1)	264 (79.3)	244 (73.3)	203 (61.0)
India.1	India.1	492	485 (98.6)	447 (90.9)	426 (86.6)	340 (69.1)	335 (68.1)	333 (67.7)
India.1	BD (S) 3	478	438 (91.6)	382 (79.9)	372 (77.8)	316 (66.1)	304 (63.6)	265 (55.4)
Thai (L)	Thai (L)	231	205 (88.7)	189 (81.8)	158 (68.4)	83 (35.9)	*	52 (25.4)
Thai (L)	BD (L) 1	226	165 (73.0)	142 (62.8)	119 (52.7)	72 (31.9)	*	70 (31.0)
Thai (S)	BD (M) 2	102	100 (98.0)	0 (0)	–	–	–	–
Thai (S)	BD (L) 8	91	72 (79.1)	0 (0)	–	–	–	–
Thai (S)	BD (S) 6	121	115 (95.0)	0 (0)	–	–	–	–
Malay (U)	Malay (U)	355	315 (88.7)	185 (52.1)	172 (48.5)	135 (38.0)	128 (36.1)	126 (35.5)
Malay (U)	BD (L) 9	290	282 (97.2)	179 (61.7)	173 (59.7)	144 (50.0)	115 (40.0)	11 (3.7)
Malay (U)	BD (S) 7	282	4 (1.4)	0 (0)	–	–	–	
Japan. 1	Japan 1	123	111 (90.0)	101 (82.1)	94 (76.4)	86 (69.9)	*	83 (67.5)
Japan. 1	BD (L) 1	180	168 (93.3)	158 (87.8)	156 (86.7)	154 (85.6)	*	23 (12.8)
Japan. 3	BD (S) 1	273	270 (98.9)	242 (88.6)	91 (33.3)	2 (0.7)	0 (0)	–

tadpoles of hybrids and controls at various developmental stages is shown in Fig. 7. The crossing results indicated that the Bangladesh large-type female was completely isolated from the mangrove type male by gametic isolation, i.e., no fertilization occurred in this cross (Table 7, Fig. 5). In reciprocal hybrids between the Bangladesh large and medium types, isolation was by complete hybrid inviability at the embryonic stage (Table 7; Figs. 5, 7). Similar results were found in reciprocal hybrids between the Bangladesh large and small types (Table 7: Figs. 5, 7). The Bangladesh small type was not isolated from the medium type by hybrid inviability (Table 7 and Fig. 5), but instead by complete hybrid sterility due to a very high degree of abnormality in spermatogenesis and also by the absence of sperm with normal motility (our preliminary data) (Fig. 13).

In crosses between the four types from Bangladesh and other Asian *Fejervarya*, the Bangladesh large type produced viable hybrids with the Thailand large type from Bangkok, with Malaysian frogs from Kota Kinabalu, and with *F. iskandari* from Indonesia; it was completely isolated from Sri Lankan frogs by complete hybrid inviability at the embryonic stage, and from Japanese frogs from Hiroshima and Malaysian frogs from Kuala Lumpur by incomplete hybrid inviability at the tadpole stage (Table 8, Figs. 5–7). Reciprocal hybrids between the Bangladesh large type and Thailand large type also showed normal viability, whereas those between the Bangladesh large type and both Japanese frogs and Malaysian frogs from Kuala Lumpur showed incomplete hybrid inviability at the tadpole stage. Hybrids between the Bangladesh large type and Thailand small type showed complete hybrid inviability at the embryonic stage (Table 8, Figs. 5–7).

The Bangladesh medium type produced viable hybrids only with a frog from India, but not with Malaysian frogs from Kuala Lumpur, Japanese frogs, the Thailand small type, or *F. iskandari* (Table 8; Figs. 5, 6). The Bangladesh small type produced viable hybrids with the Sri Lankan and Indian frogs, but was completely isolated from Malaysian frogs from Kuala Lumpur by hybrid inviability at the embryonic stage (Table 8).

Haplotypes and sequence divergence

The concatenated data set used in our analyses was 1839 base pairs long and consisted of 516 bp for 16S, 807

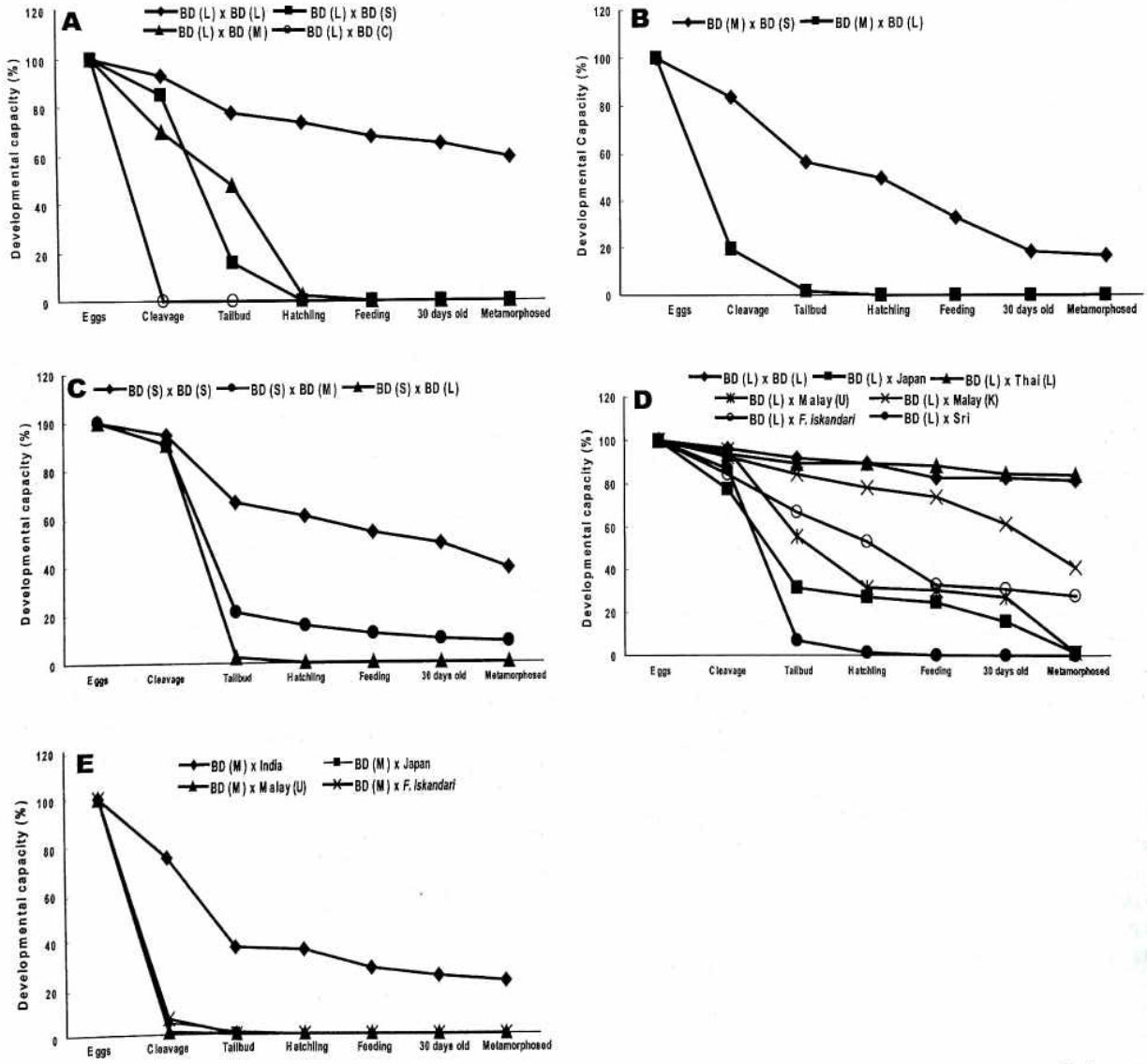

Fig. 5. Graphs (A–E) showing the developmental capacity of hybrid and control offspring from crosses among *Fejervarya* frogs from Bangladesh and other Asian countries. BD, Bangladesh; L, large type; M, medium type; S, small type; C, mangrove type; Thai, Thailand; Malay, Malaysia; U, University of Malaya Campus; K, Kota Kinabalu; Sri, Sri Lanka.

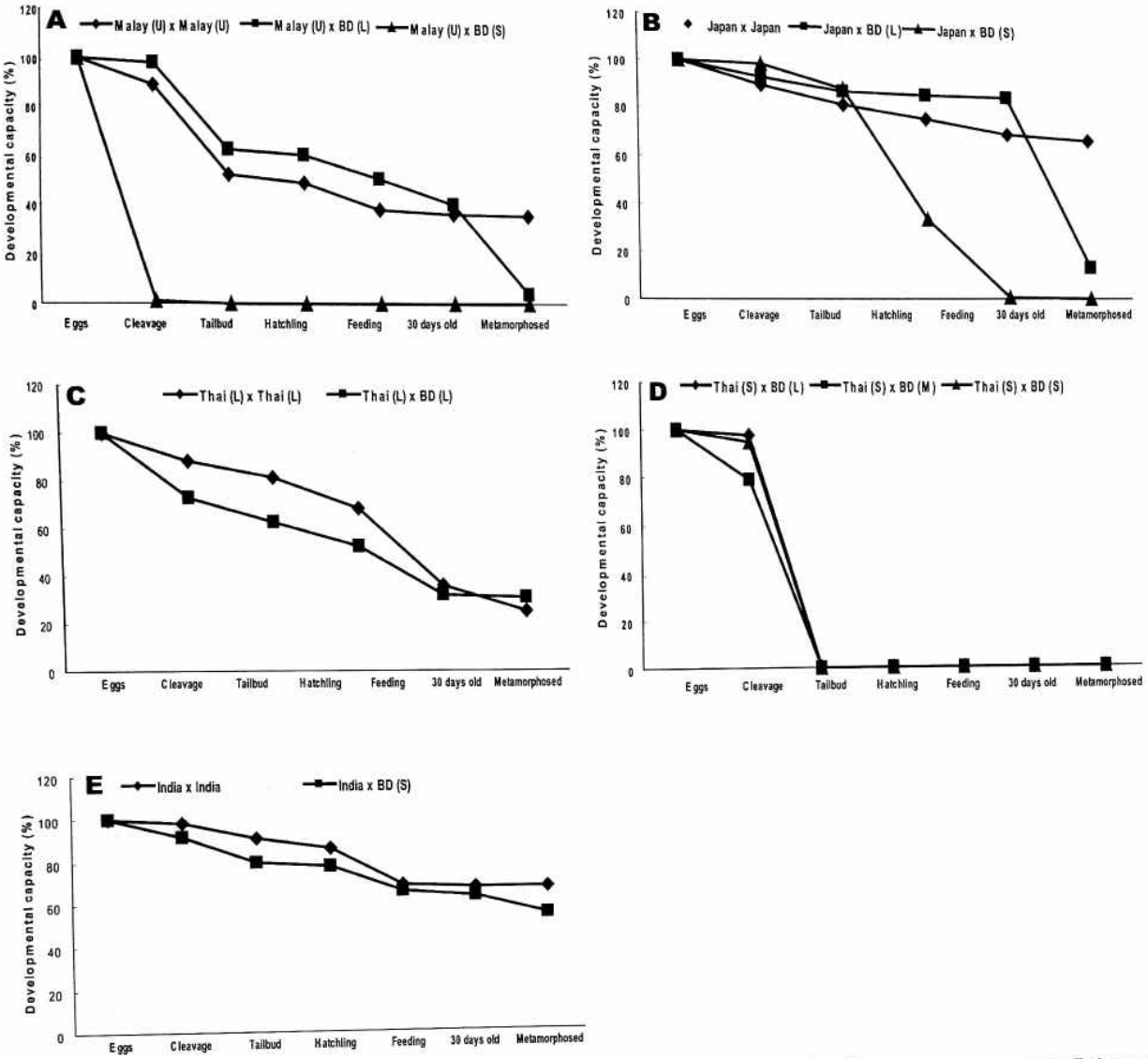

Fig. 6. Graphs (A–E) showing the developmental capacity of hybrid and control offspring from crosses among *Fejervarya* frogs from Bangladesh and other Asian countries. BD, Bangladesh; L, large type; M, medium type; S, small type; Thai, Thailand; Malay, Malaysia; U, University of Malaya Campus; Sri, Sri Lanka.

bp for 12S, and 516 bp for Cyt *b*. The 16S alignment contained 136 polymorphic sites, of which 87 were parsimony informative; the 12S alignment contained 245 polymorphic sites, of which 177 were parsimony informative; and the Cyt *b* alignment contained 212 polymorphic sites, of which 191 were parsimony informative. In total, 51 haplotypes were found for the three genes, including 10 haplotypes for 16S, 14 for 12S, and 27 for Cyt *b*. A complete list of the haplotypes and their DDBJ accession numbers are shown in Table 9.

Sequence divergences among the four *Fejervarya* types from Bangladesh and the outgroup are shown in Table 10 and Fig. 8. In pairwise comparisons among all taxa, Cyt *b* had the greatest divergence, followed by 12S and 16S. Divergence was greatest between the small and mangrove types, and smallest between the medium and small types. All four types showed high divergence from the outgroup, *Limnonectes fujianensis* (Table 10).

Phylogenetic analyses

Phylogenetic trees constructed with different methods (ML, MP, and NJ) were identical in topology, except the 12S ML tree. The only difference was in the grouping of the large and mangrove types, which was not supported by high bootstrap values in any trees (Figs. 9–11). In the ML trees for all three genes, the haplotypes of the Bangladesh frogs formed four clades corresponding to the four types (Figs. 9–11).

In phylogenetic trees that included the four types from Bangladesh and other Asian *Fejervarya* for which sequences were available from the DDBJ/GenBank database, the mangrove type from Bangladesh formed a clade with *F. cancrivora* from India, the Philippines, and China, with bootstrap values of 100 for the ML, MP, and NJ analyses (Fig. 12). The Bangladesh large type was most closely related to two haplotypes of *F. orissaensis* from India, followed by the *Fejervarya* frog from Bangkok (Thailand), and finally formed a clade with *F. iskandari* supported by high bootstrap values (91 for ML and MP; 97 for NJ) (Appendix). *Fejervarya* frogs from East and Southeast Asian countries, including Japan, Taiwan, Indonesia, Malaysia, China, Vietnam, and Laos, formed a single clade with high bootstrap values (100 for ML, MP, and NJ). The Bangladesh medium and small types formed a clade with Indian *Fejervarya* group (a *Fejervarya* species group consisting of *F. syhadrensis, F rufescens*,

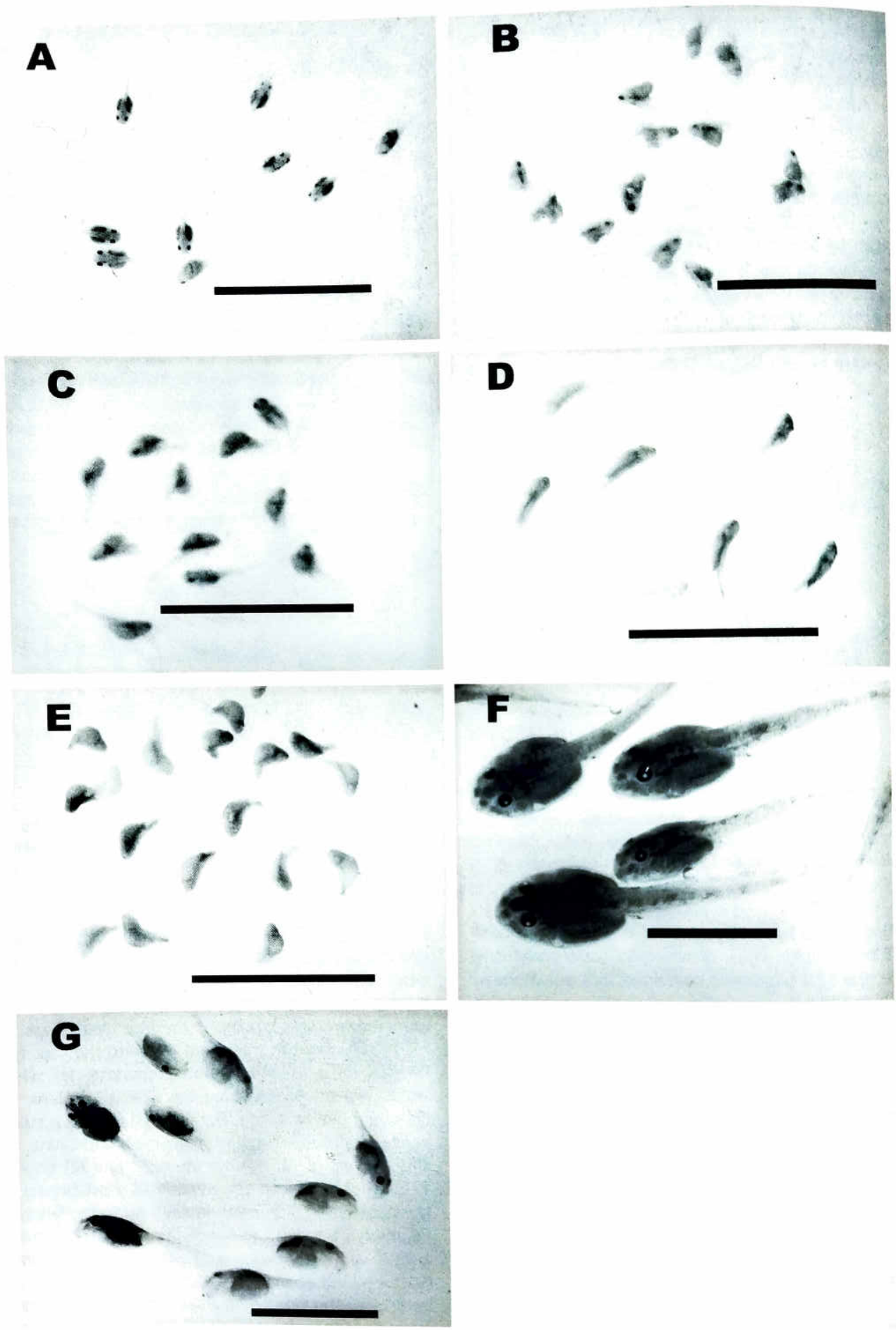

Fig. 7 Feeding-stage tadpoles **(A–C)**, hatching-stage tadpoles **(D, E)**, and 30-day-old tadpoles **(F, G)** of hybrid and control offspring. Scale bar, 1.0 cm. **(A)** Bangladesh (L) ♀× Bangladesh (L) ♂. **(B)** Bangladesh (L) ♀× Bangladesh (M) ♂. **(C)** Bangladesh (L) ♀× Bangladesh (S) ♂. **(D)** Bangladesh (L) ♀× Bangladesh (L) ♂. **(E)** Bangladesh (S) ♀× Malaysia (U) ♂. **(F)** Bangladesh (L) ♀× Bangladesh (L) ♂. **(G)** Bangladesh (L) ♀× Japan (Hiroshima) ♂.

Table 9. Number of haplotypes found in the different populations of four types of *Fejervarya* frogs from Bangladesh, haplotype designations, and accession numbers in DDBJ.

Type	Station	Haplotype								
		Cyt *b*			16S rRNA			12S rRNA		
		No.	Kind	Accession No.	No.	Kind	Accession No.	No.	Kind	Accession No.
Large	BAUC, Mymensingh	3	BFL-Cyt*b*-1~3	AB372046~8	2	BFL-16S-1~2	AB372009, AB372010	4	BFL-12S-1~4	AB372019, AB372072, AB372073, AB372074
	Shambuganj, Mymensingh	1	BFL-Cyt*b*-1	AB372046	1	BFL-16S-1	AB372009	1	BFL-12S-1	AB372019
	Muktagacha, Mymensingh	3	BFL-Cyt*b*-2,4~5	AB372047, AB372049, AB372050	1	BFL-16S-1	AB372009	3	BFL-12S-1, BFL-12S-4,5	AB372019. AB372074, AB372075
	Gazni, Sherpur	1	BFL-Cyt*b*-2	AB372047	1	BFL-16S-1	AB372009	1	BFL-12S-1	AB372019
	Hossainpur, Kishoreganj	2	BFL-Cyt*b*-2,6	AB372047, AB372051	1	BFL-16S-1	AB372009	1	BFL-12S-1	AB372019
	Tarail, Kishoreganj	1	BFL-Cyt*b*-2	AB372047	1	BFL-16S-1	AB372009	2	BFL-12S-1,5	AB372019, AB372075
	Ghorasal, Narsingdi	1	BFL-Cyt*b*-1	AB372046	1	BFL-16S-1	AB372009	1	BFL-12S-1	AB372019
	Parbatipur, Dinajpur	2	BFL-Cyt*b*-2,7	AB372047, AB372052	1	BFL-16S-1	AB372009	1	BFL-12S-1	AB372019
	Nawabganj, Nawabganj	2	BFL-Cyt*b*-2,8	AB372047, AB372053	1	BFL-16S-1	AB372009	1	BFL-12S-1	AB372019
	Koilashganj, Khulna	1	BFL-Cyt*b*-2	AB372047	1	BFL-16S-1	AB372009	1	BFL-12S-1	AB372019
	Rangamati	1	BFL-Cyt*b*-8	AB372053	1	BFL-16S-1	AB372009	1	BFL-12S-6	AB372076
	Cox's Bazar	1	BFL-Cyt*b*-9	AB372054	1	BFL-16S-1	AB372009	1	BFL-12S-5	AB372075
	Chittagong	1	BFL-Cyt*b*-8	AB372053	1	BFL-16S-1	AB372009	1	BFL-12S-4	AB372074
Medium	BAUC, Mymensingh	4	BFM-Cyt*b*-1~4	AB372054, AB372055,	1	BFM-16S-1	AB372011	2	BFM-12S-1,2	AB372077, AB372078
Small	BAUC, Mymensingh	7	BFS-Cyt*b*-1~7	AB372058~ AB372064	3	BFS-16S-1~3	AB372012~ AB372014	3	BFS-12S-1~3	AB372079~ AB372081
	Shambuganj, Mymensingh	1	BFS-Cyt*b*-8	AB372065	1	BFS-16S-2	AB372013	1	BFS-12S-1	AB372079
	Muktagacha, Mymensingh	1	BFS-Cyt*b*-2	AB372059	1	BFS-16S-2	AB372013	1	BFS-12S-3	AB372081
	Gazni, Sherpur	1	BFS-Cyt*b*-2	AB372059	1	BFS-16S-2	AB372013	1	BFS-12S-1	AB372079
	Hossainpur, Kishoreganj	1	BFS-Cyt*b*-2	AB372059	1	BFS-16S-2	AB372013	1	BFS-12S-1	AB372079
	Tarail, Kishoreganj	1	BFS-Cyt*b*-1	AB372058	1	BFS-16S-2	AB372013	1	BFS-12S-1	AB372079
	Chittagong	1	BFS-Cyt*b*-11	AB372068	2	BFS-16S-2, 4	AB372013, AB372015	1	BFS-12S-1	AB372079
	Cox's Bazar	1	BFS-Cyt*b*-10	AB372067	2	BFS-16S-5~6	AB372016, AB372017	1	BFS-12S-1	AB372079
	Parbatipur, Dinajpur	1	BFS-Cyt*b*-2	AB372059	1	BFS-16S-2	AB372013	1	BFS-12S-1	AB372079
	Koilashganj, Khulna	1	BFS-Cyt*b*-9	AB372066	1	BFS-16S-2	AB372013	1	BFS-12S-1	AB372079
Mangrove	Koilashganj, Khulna	3	BFC-Cyt*b*-1~3	AB372069, AB372070, AB372071	1	BFC-16S-1	AB372018	3	BFC-12S-1~3	AB372082~ AB372084
	Total	27	BFL-Cyt*b*-1~9, BFM-Cyt*b*-1~4, BFS-Cyt*b*-1~11, BFC-Cyt*b*1~3		10	BFL-16S-1~2, BFM-16S-1, BFS-16S-1~6, BFC-16S-1		14	BFL-12S-1~6, BFM-12S-1~2, BFS-12S-1~3, BFC-12S-1~3	

Table 10. Percent sequence divergence (uncorrected p distance) for 16S rRNA, 12S rRNA, and Cyt *b* gene sequences among four *Fejervarya* types from Bangladesh and the outgroup, *Limnonectes fujianensis*. Ranges are in parentheses. L, Large type; M, Medium type; S, Small type; C, Mangrove type.

Combination	16S rRNA	12S rRNA	Cyt *b*
BD (L)- BD (M)	17.0 (16.9–17.1)	15.9 (15.8–16.0)	24.7 (24.2–25.0)
BD (L)- BD (S)	15.9 (15.7–16.1)	16.2 (15.8–16.5)	24.4 (23.3–25.0)
BD (L)- BD (C)	13.1 (13.0–13.2)	12.0 (11.9–12.2)	20.7 (20.3–21.1)
BD (M)- BD (S)	5.7 (5.5–5.7)	7.6 (7.4–7.8)	18.4 (18.0–19.0)
BD (M)- BD (C)	14.0 (14.0–14.0)	17.2 (17.2–17.3)	26.0 (25.6–26.29
BD (S)- BD (C)	14.5 (14.2–14.8)	17.1 (16.9–17.3)	25.6 (25.0–26.2)
BD (L)- *L. fujianensis*	18.4 (18.3–18.5)	20.4 (20.4–20.6)	21.7 (21.5–21.9)
BD (M)- *L. fujianensis*	17.5 (17.5–17.5)	22.9 (22.8-22.9)	25.9 (25.8–26.0)
BD (S)- *L. fujianensis*	17.2 (16.9–17.5)	23.0 (22.7-23.2)	23.3 (22.9–23.6)
BD (C)- *L. fujianensis*	17.7 (17.7–17.7)	20.9 (20.9–20.9)	23.1 (23.1–23.3)

Ranges in parenthesis
L, Large type; M, Medium type; S, Small type; C, Mangrove type

and some other frogs mainly distributed on the Indian subcontinent and Sri Lanka). The medium type was most closely related to a *Fejervarya* frog from Myanmar, with high bootstrap values (88 for ML; 93 for MP; 98 for NJ). The Bangladesh small type formed a clade with *F. syhadrensis* from India and Sri Lanka and *F. granosa* from India, with

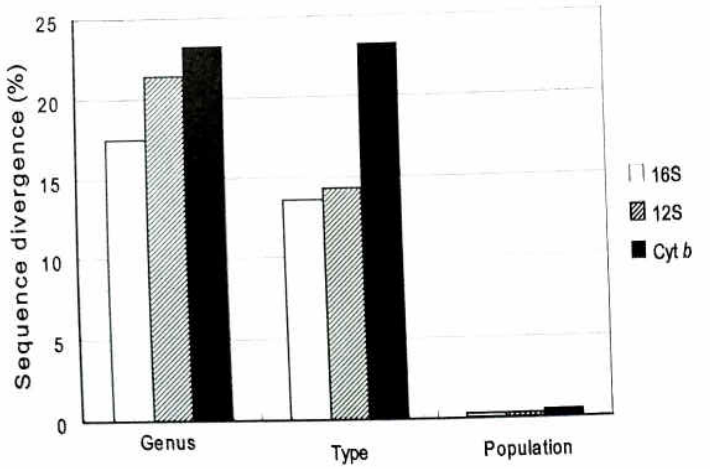

Fig. 8. Sequence divergence in the 16S rRNA, 12S rRNA, and Cyt *b* genes at the levels of genus, type, and population among *Fejervarya* frogs from Bangladesh and the outgroup, *Limnonectes fujianensis*.

high bootstrap values (88 for ML; 99 for MP; 100 for NJ) (Fig. 12).

DISCUSSION

Morphological divergence among species of *Fejervarya* is very small and mainly involves differences in body proportions (Veith et al., 2001; Djong et al., 2007b; Kuramoto et al., 2007). Djong et al. 2007b) compared populations of *F. limnocharis* from Indonesia, Japan, and Malaysia, and found significant differences between those from Japan and Indonesia, suggesting that these are different species. Kuramoto et al. (2007) found significant differences among six taxa of *Fejervarya* from the central Western Ghats, southwestern India, and described four of them as new species. The present morphometric analyses showed significant divergence among the four types of *Fejervarya* from Bangladesh. Among them, the large and small types were sympatrically

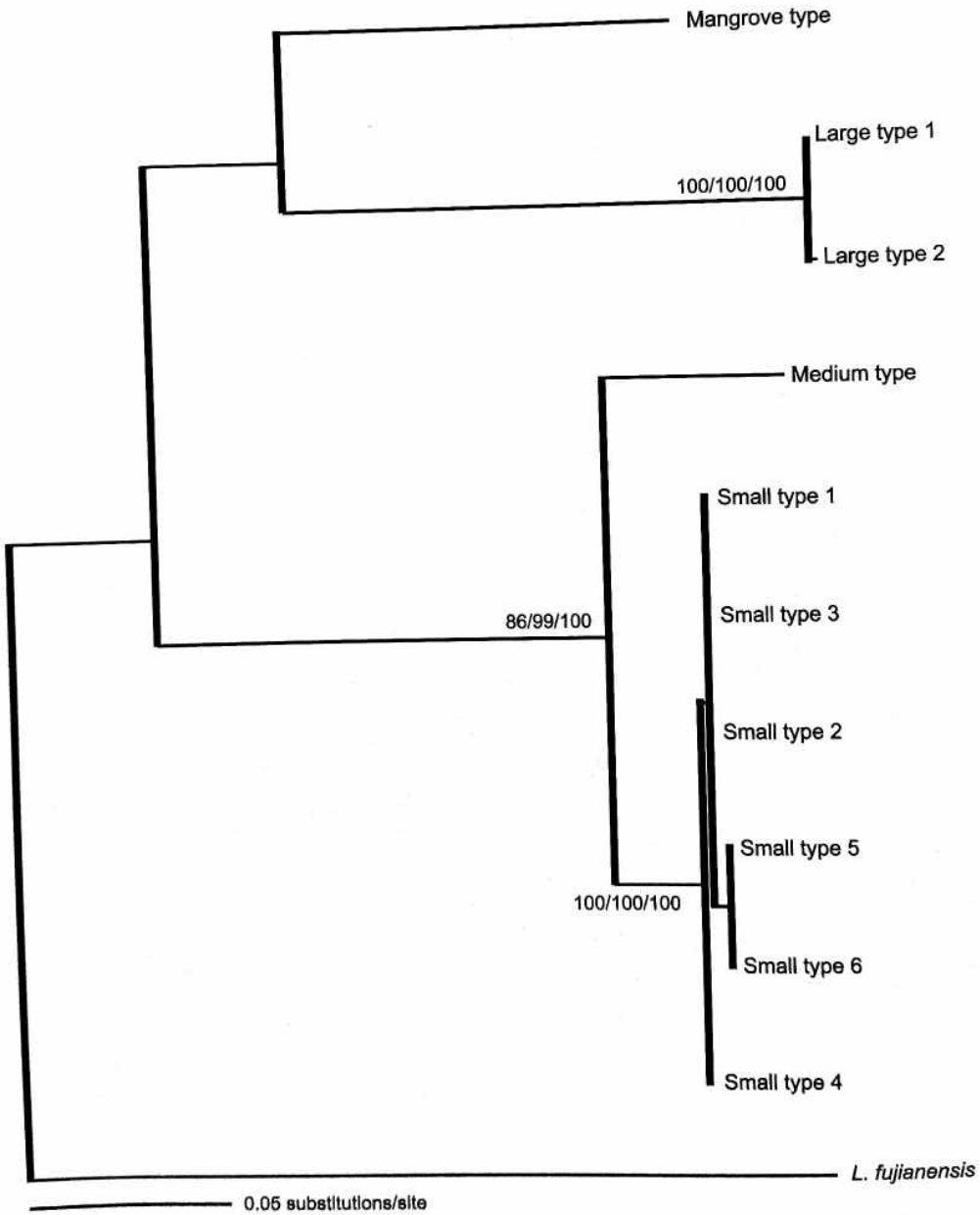

Fig. 9. Maximum-likelihood tree based on the nucleotide sequences of a 516-bp segment of the 16S rRNA gene from four *Fejervarya* types among 14 different populations in Bangladesh and the outgroup, *Limnonectes fujianensis*. Numbers near nodes are bootstrap values (>50%) for ML, MP, and NJ analyses, respectively. The scale bar represents branch length in nucleotide substitutions per site.

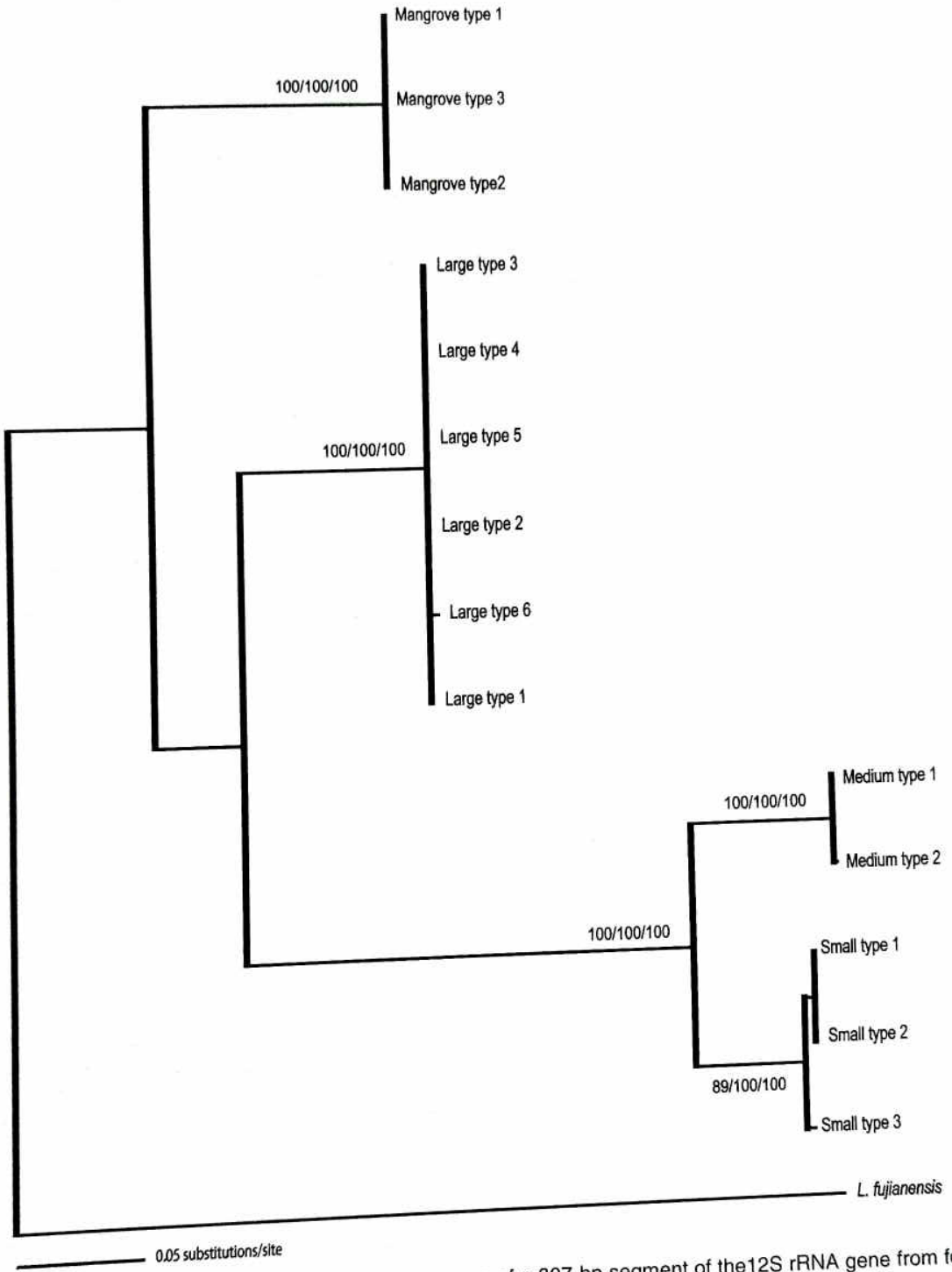

Fig. 10. Maximum-likelihood tree based on the nucleotide sequences of a 807-bp segment of the12S rRNA gene from four *Fejervarya* types among 14 different populations in Bangladesh and the outgroup, *Limnonectes fujianensis*. Numbers near nodes are bootstrap values (>50%) for ML, MP, and NJ analyses, respectively. The scale bar represents branch length in nucleotide substitutions per site.

distributed throughout the country, the medium type was found at a single locality in the central part of Bangladesh, and the mangrove type was found at a single locality, the mangrove region of southern Bangladesh. Even though they are sympatric in similar narrow habitats, the morphological distinctiveness of these four types suggests that they are separate biological entities.

Along with morphometric comparisons, degree of reproductive isolation revealed by crossing experiments provides strong evidence with which to determine the taxonomic position of frogs. Mayr (1940) defined species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups". If two taxa are reproductively isolated from each other and cannot interbreed, they should be considered as different species. Sumida et al. (2007) and Djong et al. (2007a, b) conducted crossing experiments among different types of *Fejervarya* frogs and found that the degree of reproductive isolation is useful for clarifying taxonomic position. We performed crossing experiments among different types of *Fejervarya* to evaluate the reproductive isolation among them (Fig 13). Our results indicated that the large type and mangrove type may each be a separate species, and distinct from the others. The small and medium types were isolated

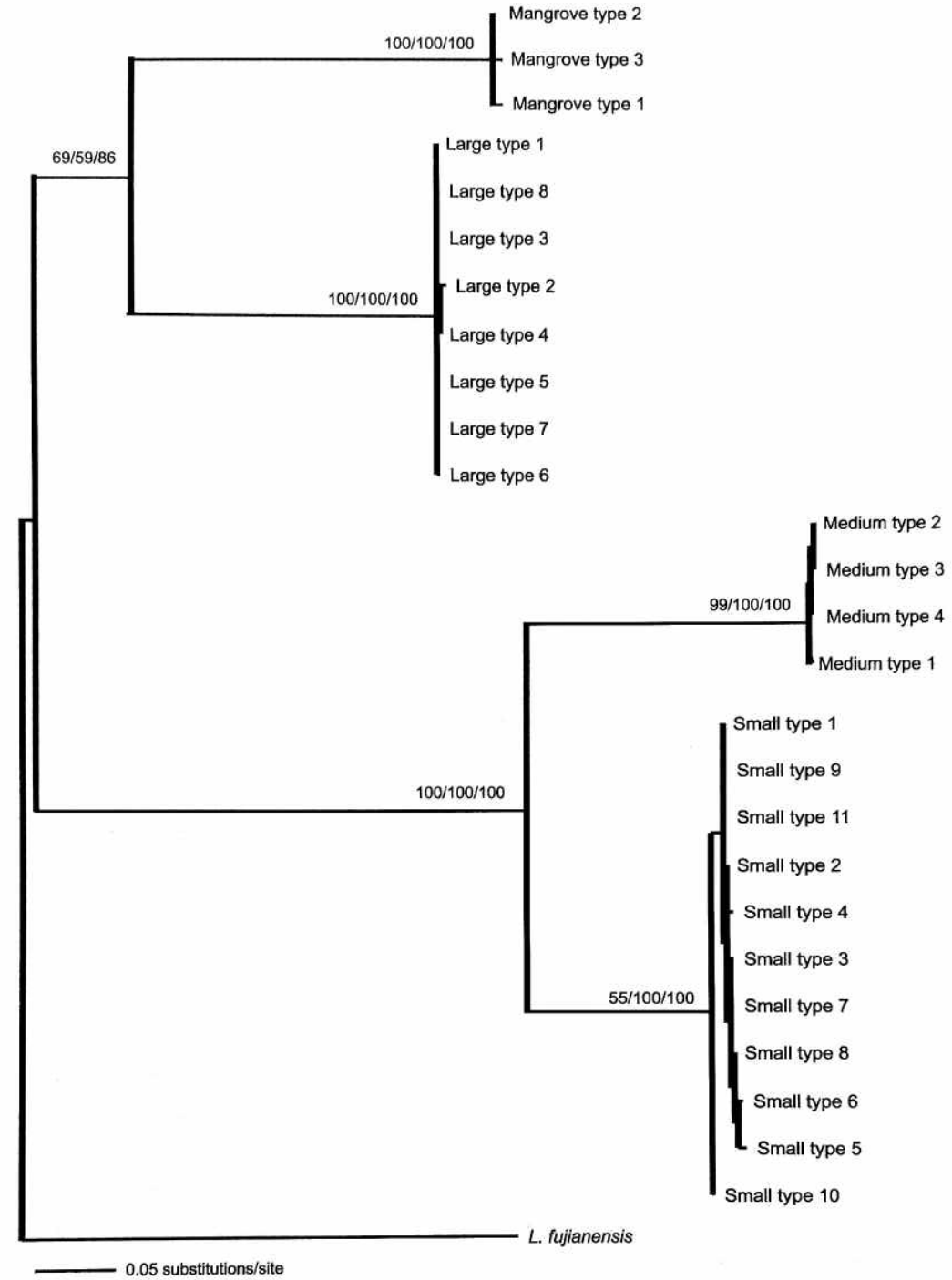

Fig. 11. Maximum-likelihood tree based on the nucleotide sequences of a 516-bp segment of the Cyt *b* gene from four *Fejervarya* types among 14 different populations in Bangladesh and the outgroup, *Limnonectes fujianensis*. Numbers near nodes are bootstrap values (>50%) for ML, MP, and NJ analyses, respectively. The scale bar represents branch length in nucleotide substitutions per site.

from each other not by hybrid inviability, but by complete hybrid sterility (Islam et al., 2006). Thus, we conclude that the four types of *Fejervarya* from Bangladesh should be treated as four different species.

Biochemical and molecular techniques have proven to be very useful for species identification. Veith et al. (2001) distinguished cryptic species *F. iskandari* from *F. limnocharis*, and Nishioka and Sumida (1990) and Sumida et al. (2002) clearly separated *F. cancrivora* from *F. limnocharis* with these techniques. We found high sequence divergences among the four types of *Fejervarya* from Bangladesh (16S, 5.7–17.0%, mean 13.4%; 12S, 7.6–17.2%, mean 14.3%; Cyt *b*, 18.6–26.0%, mean 23.2%). Vences et al. (2002) mentioned that 16S sequence divergences were 5–13% among species of Malagasy tree frogs with strong differences in advertisement calls (whether syntopic, potentially syntopic, or allopatric). At the species level in *Fejervarya*, 16S sequence divergence was reported as 3.0% (Kurabayashi et al., 2005; Sumida et al., 2007; Kuramoto et al., 2007; Djong et al., 2007b). Sumida et al. (2000a, b) reported that the 12S sequence divergence was 10.1% (8.7–11.2%) between European and Far Eastern pond frog species, and 12.2% (11.9–12.4%) between *Lithobates catesbeianus* (as *Rana catesbeiana*) and the Japanese

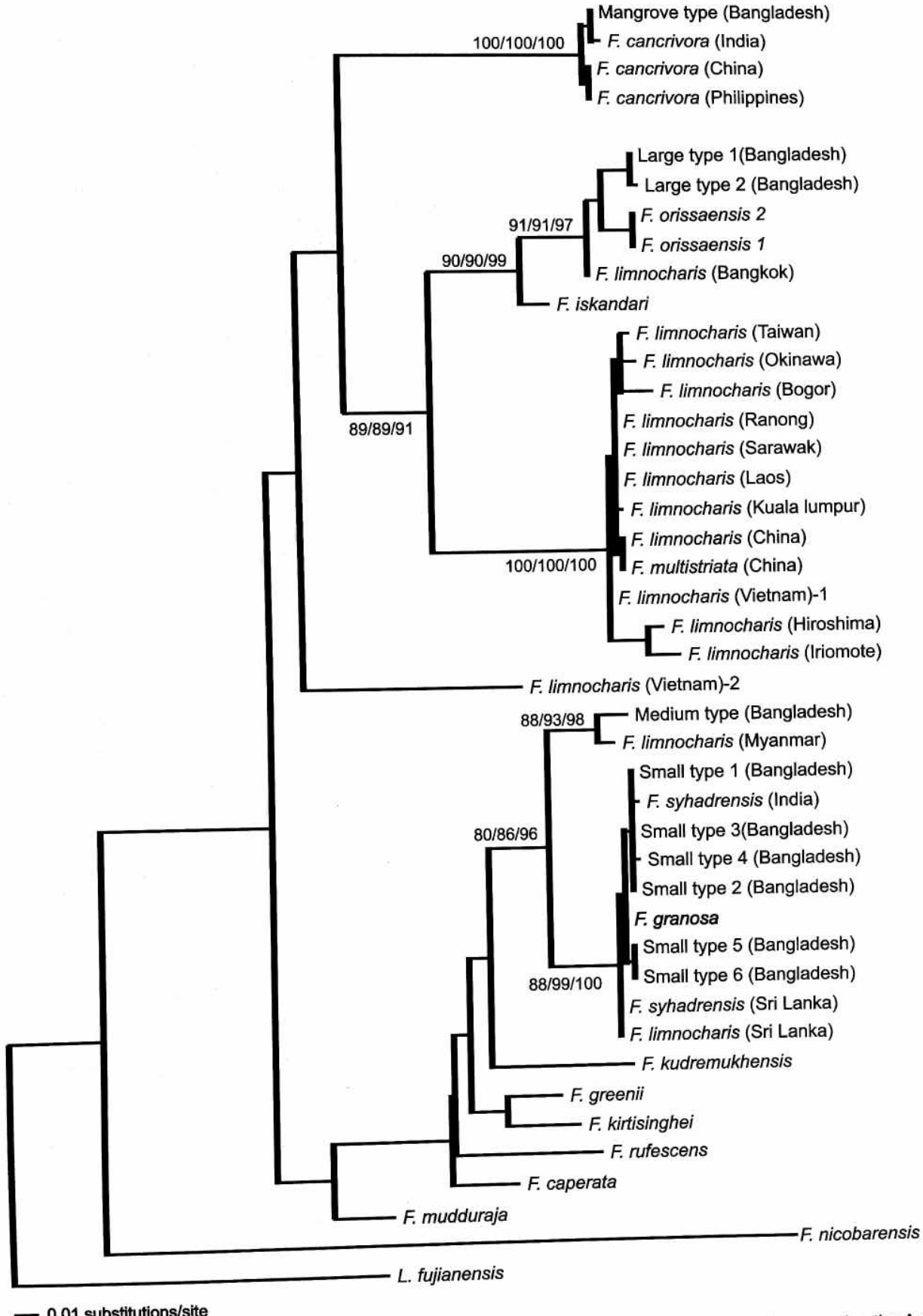

Fig. 12. Maximum-likelihood tree based on the nucleotide sequences of a 334-bp segment of the 16S rRNA gene using the Asian *Fejervarya* species available from the DDBJ/GenBank database (shown in Appendix) including four *Fejervarya* types from Bangladesh with *Limnonectes fujianensis* as the outgroup. Numbers near nodes are bootstrap values (>50%) for ML, MP, and NJ analyses, respectively. The scale bar represents branch length in nucleotide substitutions per site.

pond frogs. Bradly and Baker (2001) noted that Cyt *b* sequence divergences of 2.0–11.0% merit further study of species' status, and that values greater than 11% would indicate separate species. After reviewing 24,000 vertebrate and invertebrate species, Kartavstev and Lee (2006) found that the average Cyt *b* sequence divergence among species

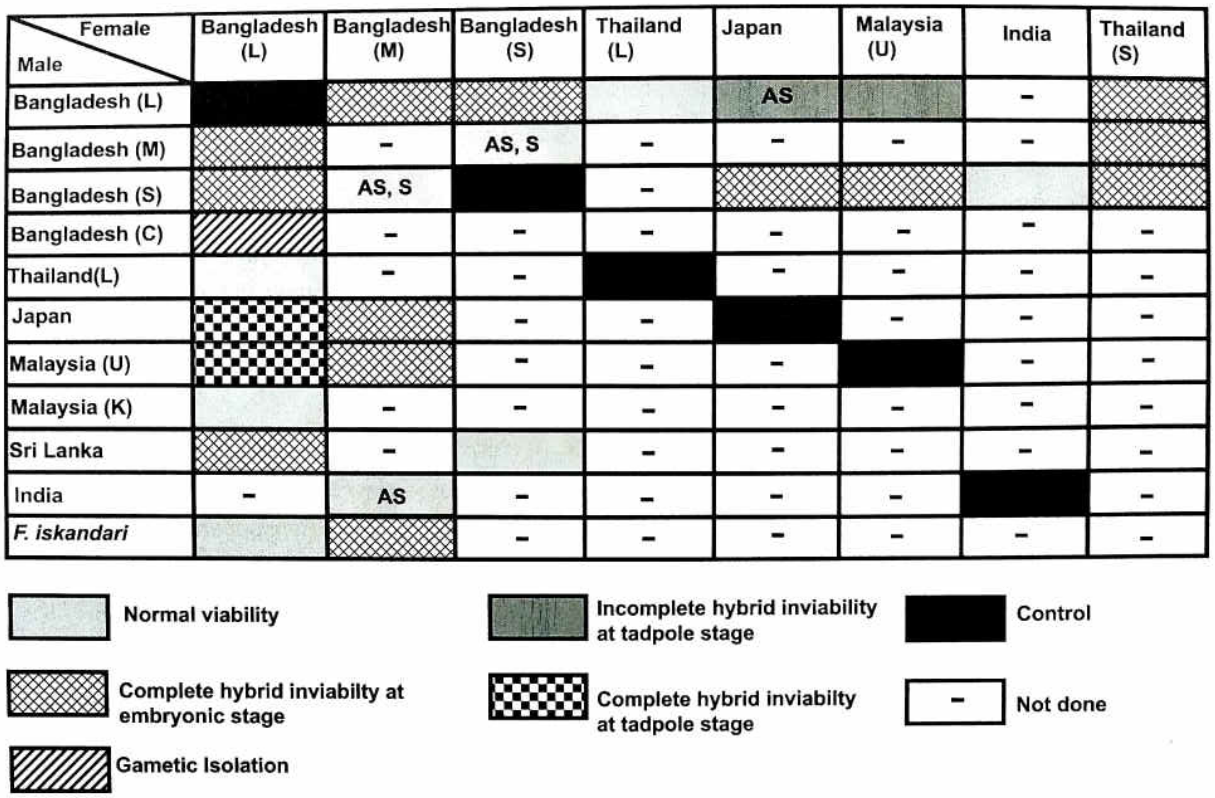

Fig. 13. Reproductive isolation mechanisms detected in various crosses among *Fejervarya* frogs from Bangladesh and other Asian countries. L, large type; M, medium type; S, small type; C, mangrove type; U, University of Malaya campus; K, Kota Kinabalu; AS, abnormal spermatogenesis; S, sterile.

in a genus is 10.7±1.3%. After comparing the sequence divergences in 16S, 12S, and Cyt *b* found in the present study with those mentioned above, we conclude that each type of *Fejervarya* in Bangladesh merits at least distinct species status.

Taxonomic status of the Bangladesh large type

Morphologically, the Bangladesh large type differed significantly from the other types of *Fejervarya* in Bangladesh. This type was characterized by large size (males 40.0–50.6 mm, females 50.9–60.0 mm in SVL); rough dorsum with numerous elongated dermal ridges; smooth venter; lips with irregular white and black vertical bars; and thighs, legs, and arms speckled with black or dark brown bars. Crossing experiments showed that this type was completely isolated from other South Asian *Fejervarya* groups by hybrid inviability, but was not isolated from the East Asian group (species of *Fejervarya* belonging to the *F. limnocharis* complex). The 16S data showed that the Bangladesh large type belongs to the *F. iskandari* group (a group of species within the *F. limnocharis* complex consisting of *F. iskandari, F. orissaensis,* a *Fejervarya* frog from Thailand, and related species), and is most closely related to *F. orissaensis* among this group. Dutta (1997) originally described *F. orissaensis* from Orissa, eastern India, which is close to Bangladesh and is not isolated by any geographical barriers. He described it as a medium-sized ranid (males 36.2–47.2 mm and females 34.2–53.8 mm in SVL) with interrupted longitudinal folds on the dorsum, a smooth venter, a wide inner metatarsal tubercle, and a relatively pointed snout. In the present study, we used the same specimens of the *Fejervarya* frog from Bangkok (Thailand) as those used by Sumida et al. (2007), who found that this form is isolated by incomplete hybrid inviability from the *F. limnocharis* group (a group of species within the *F. limnocharis* complex consisting of *F. limnocharis, F. multistriata, F. sakishimensis,* and related *Fejervarya* frogs from Japan and other East Asian countries). These authors also suggested that the Thailand population is connected to *F. iskandari* from Indonesia, and is most closely related to *F. orissaensis.* Djong et al. (2007a) found that the Bangladesh large type is not reproductively isolated from *F. iskandari* of Indonesia and considered that this type should be included in the *F. iskandari* group. The Bangladesh large type belongs to the *F. iskandari* group, along with *F. orissaensis* and the Thailand large type of *Fejervarya*, but further extensive studies are necessary to clarify its taxonomic position.

Taxonomic status of the Bangladesh medium type

The medium type showed a relatively close relationship to the Bangladesh small type in all aspects of morphological features, hybrid viability and nucleotide sequence divergence, yet the abnormal spermatogenesis in hybrids, nucleotide sequence divergence, and morphological dissimilarity were sufficient to rank it as different species. In the present study, the medium type was found to have a body length (SVL) of 30.0–37.7 mm in males and 34.8–42.5 mm in females, a relatively pointed snout compared with other *Fejervarya* species from Bangladesh, yellow coloration in the thigh region, a moderately long dermal ridge on the rough dorsum, and a smooth venter. In the phylogenetic tree constructed with all available Asian *Fejervarya*, the medium type grouped separately from the Bangladesh small type and was closely related to the *Fejervarya* frog (treated by

Chen et al. [2005], as *F. limnocharis*) from Myanmar, for which the exact taxonomic status was not mentioned. The medium type also produced a viable hybrid with *F. caperata* (one of the four new species described by Kuramoto et al. [2007] from the Western Ghats), but differed significantly from the latter in nucleotide sequence divergence and morphology. Several species of the Indian *Fejervarya* group occur in adjacent countries, including Nepal and India (Dubois, 1975, 1984, 1987, 1992; Kuramoto et al., 2007). Additional morphological, acoustic, and nuclear gene sequence data can confirm whether it is a previously described species, or an undescribed species.

Taxonomic status of the Bangladesh small type

Among *Fejervarya* frogs, several species in South Asia are smaller in size than topotypic *F. limnocharis* from Indonesia; we refer to these here as the South Asian (Indian) group. Among the Indian group, there is some variation in the shape of snout and dermal ridge, and the Bangladesh small type has globular-shaped ridges or a small, rounded tubercle, and a comparatively blunt or obtusely pointed snout; the head is broader than long. Our molecular data showed this type to cluster with *F. granosa,* although there is some divergence between these two types. Kurabayashi et al. (2005) studied the relationships among several species of *Fejervarya* and found that eight species formed a cluster, which was mentioned as the India and Sri Lanka group. These authors found that *F. syhadrensis* from Sri Lanka and hpA from India (later described as *F. granosa* by Kuramoto et al., 2007) were most closely related. Our findings confirmed that the Bangladesh small type groups with these two species. It is noteworthy that, based on the 16S, 12S, and Cyt *b* data, the Bangladesh small type is largely divided into two groups, one distributed throughout the country except in the eastern (Chittagong) region and the other distributed in the Chittagong region. Sequence divergence among the different populations from Bangladesh, excluding the eastern populations, was very low compared to that with the eastern populations (0–0.2% and 0.4–0.6% for 16S; 0.1% and 0.7–0.8% for 12S; 0.7% and 1.4% for Cyt *b*, respectively). These results show clearly that the eastern population is diverged from the other populations, but this divergence is too small to consider these populations as different species.

On the basis of the crossing experiments and molecular data, the Bangladesh small type is closely related to the *Fejervarya* frog from Sri Lanka. Sumida et al. (2007) found that this Sri Lankan type is small in size, with a SVL of 27.0–29.8 mm for males and 31.5 mm for females, with rounded tubercles on the dorsal surface and showed that this Sri Lankan frog is closely related to *F. syhadrensis* from Sri Lanka (used by Meegaskumbara and Manamendra-Aracchi, 2005). Tandon et al. (unpublished) also reported that Indian *F. syhadrensis* grouped with this Sri Lankan type, and Sumida et al. (2007) subsequently stated that it was reasonable to consider this type as *F. syhadrensis.* According to the original description of *F. syhadrensis* by Annandale (1917), who examined this species from the Poona and Nasik districts of India, "the snout-vent length does not exceed 35 mm (27 mm in males and 31.5 mm in females), and the dorsal surface is gray with black spots and sometimes with reddish suffusion; a narrow mid-dorsal line is often present; the ventral surface is white with the whole of the throat black in the adult male". These morphological aspects are compatible with those of the small type. According to Frost (2007), *F. syhadrensis* is distributed in eastern and western India, Pakistan, and Nepal at low to moderate elevations. As this species has also been reported from eastern India, which is adjacent to Bangladesh and not isolated by any geographic barriers, it is likely that this species also occurs in Bangladesh. Chowdhury (1996) and Sarker and Sarker (1988) also proposed Bangladesh as within the distribution area of *F. syhadrensis*. We consider the Bangladesh small type to be conspecific with *F. syhadrensis*, or to be a closely related species.

Taxonomic status of the Bangladesh mangrove type

The Bangladesh mangrove type is characterized by well-developed webbing and large body size, which are diagnostic features of *F. cancrivora. Fejervarya cancrivora* Gravenhorst, 1829 is one of the largest *Fejervarya* frogs and occurs in low-elevation swamps, ponds, flooded rice fields, and ditches in Southeast Asia (Frost, 2007). Although there are significant differences in some morphological parameters between the Bangladesh mangrove type and *F. cancrivora* from the type locality, Indonesia (specified by Dubois and Ohler, 2000), the former might be a species related to the nominate population of *F. cancrivora*, based on allozyme variation and nucleotide sequence divergence (Kurniawan et al., 2008). We thus designate this type as *F.* cf. *cancrivora*. This is the first record of a putative *F. cancrivora* population from Bangladesh. *Fejervarya cancrivora* was previously reported only from the Andaman and Nicobar Islands, but not from mainland India or even the Indian subcontinent. Dutta (2007) recently reported the presence of *F. cancrivora* on mainland India, from the Dhamra Port area of Orissa State near Bangladesh. Islam et al. (2008) reported the occurrence of *Fejervarya* frogs in southern Bangladesh, and Reza et al. (2000) confirmed the existence of eight anuran species, including *F. limnocharis* (as *Limnonectes limnocharis* Boie, 1835), through a preliminary survey in the Sundarban Mangrove Forest, Bangladesh. For a long time, tiger shrimp and several fish species were regularly imported, mainly from India but also from Thailand and the Philippines, for cultivation in brackishwater areas. Thus, it is possible that this species was transported to this region with shrimp and fish via the aquacultural trade. Christy et al. (2007) also reported *F. cancrivora* in Guam, transported from East Asia. Further extensive studies on *F.* cf. *cancrivora* from Bangladesh will be necessary to confirm whether it is the same species as the topotypic *F. cancrivora* from Indonesia and to clarify its taxonomic position.

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Appendix

Accession number of 16S rRNA gene sequences of Asian *Fejervarya* frogs from DDBJ/GenBank database used in this study.
F. muddurajа (India), AB167946; *F. cancrivora* (China), DQ458252; *F. cancrivora* (India), AY841754; *F. cancrivora* (Philippines), AF206473; *F. iskandari* (Bogor, Indonesia), AJ292017; *F. greenii* (Sri Lanka), AY014378; *F. granosa* (India), AB167947; *F. caperata* (India), AB167951; *F. limnocharis* (Bangkok, Thailand), AB162444; *F. limnocharis* (Bogor, Indonesia), AJ292015; *F. limnokirtisinghei* (Sri Lanka), AY014380; *F. limnocharis* (Hiroshima, Japan), AB070732; *F. limnocharis* (Iriomote, Japan), AB070736; *F. limnocharis* (China), AF315139; *F. limnocharis* (Kuala Lumpur, Malaysia), AB296097; *F. limnocharis* (Myanmar), AF206466; *F. limnocharis* (Okinawa, Japan), AB070733; *F. limnocharis* (Ranong, Thailand), AB162445; *F. limnocharis* (Sarawak, Malaysia), AB296099; *F. limnocharis* (Sri Lanka), AB162446; *F. limnocharis* (Taiwan), AB070737; *F. limnocharis* (Vietnam 1), AF285211; *F. limnocharis* (Vietnam 2), AF206465; *F. limnocharis* (Laos), AF215416; *F. multistriata* (China), AB296101; *F. nicobariensis* (Borneo, Malaysia), DQ810283; *F. kudremukhensis* (India), AB167949; *F. orissaensis* (India 1), AY882957; *F. orissaensis* (India 2), AY882957; *F. rufescens* (India), AB167945; *F. syhadrensis* (India), AY841748; *F. syhadrensis* (Sri Lanka), AY141843.

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MORPHOLOGICAL AND GENETIC VARIATION IN THREE POPULATIONS OF *Hoplobatrachus tigerinus* FROM BANGLADESH

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ABSTRACT

In order to investigate the morphological and genetic variation of Indian bullfrog *Hoplobatrachus tigerinus*, live samples were collected from three populations located in Khulna (southern part), Char Nilokhia and Netrokona (central part) in Bangladesh. The proportions of head length and head width (HL : HW), eye length and tympanum diameter (EL:TD), head length and snout length (HL : SL), internarial space and interorbital distance (IN:IOD) and internarial space and distance from nostril to tip of snout (IN:NS) of the Khulna population were significantly higher ($p<0.05$) than those of the other two populations. For allozyme study, 4 enzymes were screened, where 5 presumptive scorable loci were identified. The highest values of mean proportion of polymorphic loci (<0.95), mean number of alleles per locus and mean number of heterozygous loci per individual were observed in the Khulna population (60%, 1.60 and 14.0% respectively). The pairwise genetic differentiation (F_{ST} and Nei's D) was the highest (0.279 and 0.157, respectively) between the Khulna and Netrokona populations. The UPGMA dendrogram showed two clusters i.e. the Char Nilokhia and Netrokona populations formed one cluster, whereas the Khulna population alone made another cluster. These results suggested that the Khulna population diverged morphologically and genetically from the other two populations probably due to its geographical distance. Further extensive study will be necessary for elucidating the whole aspects of differentiation in *H. tigerinus* from Bangladesh.

Key words : Morphology, Allozyme analysis, Geographical distance, *H. tigerinus*

INTRODUCTION

Bangladesh is enriched with aquatic biodiversity (DoF, 2007), and contains a number of diverse ecosystems supporting roughly 113 species of mammals, 628 species of birds, 126 species of reptiles, 22 species of amphibians, 708 species of freshwater and marine fish, about 400 species of mollusks, and 140 species of bees and wasps, but many of them are on the nation's list of threatened animals (Reza, 2007). Among these amphibian species, *Hoplobatrachus tigerinus* (previously called it *Rana tigerina*) was an important group, and it is available in many Asian countries such as India, Pakistan, Nepal, Bhutan, Myanmar,

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Thailand, Malaysia and Indonesia (Frost, 2007). Once *H. tigerinus* species was available in the diversified water bodies of Bangladesh. Its leg is an important delicious food item to many people especially tribal people in Europe and Asia. Some people of Bangladesh used to catch and export frog legs to abroad, but indiscriminate harvesting of frogs had brought a bad impact on the natural ecosystem which pursued the government of Bangladesh to impose a ban on this frog collection, harvesting and processing. It plays an important role in controlling ecosystem, so, it is important to protect the species. Thus, morphological and genetic variation of different natural stocks of the population must be studied. In India, the adverse impact of uncontrolled harvesting of frogs, specifically *H. tigerinus*, from rice-fields and wet-lands, where they protect crops by devouring damaging insects, led to a ban on collecting frogs during the breeding season (Husain and Rahman, 1978).

Research work on frogs of Bangladesh has started in last decade (Islam, 2000 ; Khan *et al.*, 2002; Islam *et al.*, 2008a, b; Alam *et al.*, 2008). The culture, breeding and genetics of frogs have been observed in many frog species. Using allozyme technique, many studies on anuran distributed in Japan and adjacent countries have been reported by Nishioka and her collaborators. The genetic differences in *R. limnocharis* (= *F. limnocharis*) (Nishioka and Sumida, 1990), *Hyla japonica* (Nishioka *et al.*, 1990), *R. japonica* (Sumida and Nishioka, 1994), *R. nigromaculata* and *R. brevipoda* (Nishioka *et al.*, 1992), and *R. rugosa* (Nishioka *et al.*, 1993) have been reported by allozyme techniques. The purpose of this study was to identify the morphological and genetic variation of this particular amphibian species (*H. tigerinus*) in three regions viz. Khulna (southern part), Char Nilokhia and Netrokona (central part) of Bangladesh by allozyme analysis.

MATERIALS AND METHODS

Live frogs of *H. tigerinus* were collected from three different geographical localities, i.e. Khulna, Char Nilokhia and Netrokona during the months of March to May, 2007. A total of 10 specimens were collected from each locality. The distance between Khulna and Char Nilokhia was 200 km, whereas Netrokona and Char Nilokhia was only 30 km. The muscle and liver samples were taken from each individual and stored at -21°C until use. Details of the sampling localities, number of specimen and date of collection are given in Table 1.

Table 1. Collecting locality, sample size and date of collection of three populations of *H. tigerinus*

Population No.	Locality	No. of individual	Date of collection
1	Khulna	10	March 13, 2007
2	Char Nilokhia	10	April 14, 2007
3	Netrokona	10	May 12, 2007

The morphometric data were recorded using normal centimeter scale, divider and forceps, recorded to the nearest centimeter. The morphometric characters viz., snout-vent length (SVL), hind limb length (HLL), head length (HL), head width (HW), thigh length (THIGHL), tibia length (TL), internarial space (IN), inter orbital distance (IOD), eye length (EL), tympanum diameter (TD), snout length (SL), distance from nostril to tip of snout (NS), distance from back of mandible to back of eye (MFE), distance from back of mandible to front of eye (MBE), snout tympanum length (STL), mouth angle snout length (MSL), nostril tympanum length (NTL), distance from front of eyes to nostril (EN), tympanum eye length (TEL), distance from back of mandible to nostril (MN), maximum width of upper eyelid (UEW), hand length (HAL), forelimb length (FLL), lower arm length (LAL), foot length (FOL), length of tarsus and foot (TFOL), third finger length (3FL), first finger length (1FL), fourth toe length (4TL), inner metatarsal tubercle length (IMTL) and inner toe length (ITL). The thirty-one morphometric characters were measured following the conventional methods described by Hubbs and Lagler (1958) with the help of a slide caliper.

In this experiment, horizontal starch gel electrophoresis was done following the methods by Shaw and Prasad (1970). Electrophoresis was conducted using amine-citrate buffer (C.A. 6) (Clayton and Tretaik, 1972). After electrophoresis, the gel slices (about 1 mm thickness) were histochemically stained for different enzymes as described in Aebersold *et al.* (1987). Non-parametric statistical analyses were used in all the comparisons (Zar, 1996). Differences in morphometric characters among populations were analyzed using the Kruskal-Wallis non-parametric analysis of variance (ANOVA). For instances, where significant differences between populations were detected, a non-parametric post hoc test (Zar, 1996) was conducted. Allelic frequencies were calculated directly from observed genotypes. The distribution of observed genotypes was calculated from the Hardy-Weinberg equilibrium using a chi-square (χ^2) test for allozyme system. When the most common allele existed in a frequency of less than 0.95 at a given locus, it was regarded as polymorphic. The mean proportion of heterozygous loci per individual, mean proportion of polymorphic loci per population, and average number of alleles per locus were calculated so as to show the extent of genetic variability for each population (Lewontin, 1974). Expected heterozygosity (*He*) and observed heterozygosity (*Ho*) were examined according to Nei and Roychoudhury (1973). Genetic distance (*D*) value was calculated using Nei's formula (Nei, 1972).

RESULTS

Thirty one morphometric characters recorded from the samples of three populations of *H. tigerinus* are shown in Table 2. Average values of morphometric characters showed slight variation among three populations. Different proportions of morphometric characteristics (SVL : HLL, HL : HW, THIGH : TL, IN : IOD, EL : TD, HL : SL, IN : NS and MFE : MBE) of *H. tigerinus* are given in Table 3. The proportions of seven morphometric characters (HL : HW, THIGH : TL, IN : IOD, EL : TD, HL : SL, IN : NS and MFE : MBE) of all populations of *H. tigerinus* were significantly different ($p<0.05$) from each other. The proportion of head length-head width (HL : HW), eye length-tympanum diameter (EL :

TD), head length-snout length (HL : SL), internarial space-interorbital distance (IN : IOD) and internarial space-distance from nostril to tip of snout (IN : NS) of the Khulna population was significantly higher ($p<0.05$) than those of the rest two populations. Thigh length-tibia length (THIGH : TL) of the Char Nilokhia population was higher than those of the other populations.

Table 2. Mean measurement values of thirty-one morphometric characters in three populations of *H. tigerinus*. n=10 for each population.

Morphometric character	Khulna (Mean ± SD)	Char Nilokhia (Mean ± SD)	Netrokona (Mean ± SD)
SVL	10.59 ± 1.47	12.33 ± 0.79	11.55 ± 1.35
HL	4.68 ± 1.82	4.44 ± 0.26	4.26 ± 0.29
HW	4.14 ± 0.48	4.24 ± 0.58	4.53 ± 0.32
STL	2.81 ± 0.14	3.26 ± 0.21	3.00 ± 0.00
MSL	4.22 ± 1.95	3.76 ± 0.79	3.68 ± 0.11
NS	0.52 ± 0.11	0.72 ± 0.05	0.67 ± 0.05
SL	1.61 ± 0.36	1.94 ± 0.12	1.72 ± 0.09
NTL	1.75 ± 1.05	2.94 ± 0.25	2.73 ± 0.52
EN	0.96 ± 0.06	1.15 ± 0.16	1.01 ± 0.04
TEL	0.51 ± 0.05	0.52 ± 0.08	0.50 ± 0.00
MN	2.92 ± 1.06	3.65 ± 0.29	3.81 ± 0.33
MFE	2.67 ± 0.26	2.95 ± 0.15	2.96 ± 0.08
MBE	2.21 ± 0.22	2.08 ± 0.19	2.05 ± 0.08
IN	0.59 ± 0.05	0.52 ± 0.13	0.68 ± 0.04
EL	0.99 ± 0.03	1.02 ± 0.07	1.01 ± 0.04
IOD	0.63 ± 0.15	0.66 ± 0.14	1.05 ± 0.08
UEW	0.48 ± 0.31	0.67 ± 0.04	0.52 ± 0.04
HAL	2.03 ± 0.09	2.95 ± 0.94	2.18 ± 0.17
FLL	2.05 ± 0.15	2.59 ± 0.29	2.05 ± 0.15
LAL	2.42 ± 0.16	2.67 ± 0.24	2.28 ± 0.12
HLL	15.52 ± 2.80	18.41 ± 0.83	17.26 ± 1.43
THIGH	4.28 ± 0.78	5.22 ± 0.28	4.38 ± 0.44
TL	5.23 ± 0.61	6.06 ± 0.36	5.46 ± 0.44
TFOL	7.21 ± 0.46	8.03 ± 0.06	8.16 ± 0.51
3FL	1.10 ± 0.20	1.48 ± 0.12	1.35 ± 0.22
1FL	0.61 ± 0.16	1.00 ± 0.04	0.90 ± 0.22
4TL	3.13 ± 1.05	4.06 ± 0.67	4.00 ± 0.17
IMTL	0.50 ± 0.00	0.49 ± 0.02	0.50 ± 0.06
FOL	00	00	00

Table 3. Morphometric proportions in three populations of *H. tigerinus*. Values of the parameter in each column with different superscripts (a, b & c) differed significantly ($p<0.05$)

Population	SVL:HLL	HL:HW	THIGH:TL	IN:IOD	EL:TD	HL:SL	IN:NS	MFE:MBE
Char Nilokhia	0.670±0.01^a	1.047±0.01^b	0.861±0.01^a	0.788±0.01^b	1.109±0.01^c	2.289±0.01^c	0.722±0.01^c	1.418±0.01^b
Netrokona	0.669±0.01^a	0.940±0.01^c	0.802±0.01^b	0.648±0.00^c	1.247±0.01^b	2.480±0.01^b	1.010±0.01^b	1.444±0.01^a
Khulna	0.682±0.01^a	1.130±0.01^a	0.818±0.01^b	0.937±0.00^a	1.800±0.01^a	2.900±0.01^a	1.130±0.01^a	1.208±0.01^c

The enzyme analyzed, E.C. number, abbreviation of enzyme and the buffer system used for horizontal starch-gel electrophoresis are shown in Table 4. Allele frequencies were calculated directly from genotypes at 5 loci for each population. Among the five loci observed, three loci (*Gpi-2*, *Mdh-1*, and *Pgm*) were polymorphic, and the *Ldh-1* and *Ldh-2* loci were invariant. The *Gpi-2*, *Mdh-1* and *Pgm* loci showed allelic frequencies of $a = 0.250$ and $b = 0.750$, $a = 0.900$ and $b = 0.100$, and $a = 0.850$ and $b = 0.150$ for the Khulna population, $a = 0.700$ and $b = 0.300$, $a = 1.000$, $a = 0.800$ and $b = 0.200$ for the Char Nilokhin population, and $a = 1.000$, $a = 0.800$ and $b = 0.200$, and $a = 0.650$ and $b = 0.350$ for the Netrokona population, respectively (Table 5, Figs. 1 and 2). The chi-square test was done in all the cases of polymorphic loci between observed genotypes and expected genotypes, based on Hardy-Weinberg equilibrium. In case of three populations of *H. tigerinus*, the *Gpi-2* locus was shown to be significant ($p\leq0.01$) in fitness test of Hardy-Weinberg equilibrium. The observed number of alleles ($Na = 1.60$), the proportion of heterozygous loci per population (14.0%) and the proportion of polymorphic loci per population (60.0%) were higher in the Khulna population than those of the other two populations (Table 6). The fixation index (F_{ST}) and gene flow (N_m) are given in Table 7. The fixation index (F_{ST}) and the gene flow (N_m) overall three populations are 0.219 and 0.889, respectively. In the pair-wise analysis, the highest F_{ST} value and the lowest N_m value were estimated to be 0.279 and 0.647, respectively, between the Khulna and Netrokona populations (Table 7).

Table 4. Enzyme and buffer system used for allozyme analysis

Enzyme (Abbreviation)	Locus	Enzyme pattern	E.C. Number	Buffer system*
Glucose-6-phosphate isomerase (GPI)	*Gpi-2*	Dimer	5.3.1.9	C.A. 6
Lactate dehydrogenase (LDH)	*Ldh-1*	Tetramer	1.1.1.27	C.A. 6
	Ldh-2			
Malate dehydrogenase (MDH)	*Mdh-1*	Dimer	1.1.1.37	C.A. 6
Phosphoglucomutase (PGM)	*Pgm*	Monomer	5.4.2.2	C.A. 6

* C.A. 6 represents the amine-citrate pH 6 buffer

Table 5. Allele frequency at five presumptive loci of three *H. tigerinus* populations

Locus	Allele	Allele frequency		
		Khulna	Char Nilokhia	Netrokona
Gpi-2	*a*	0.250	0.700	1.000
	b	0.750	0.300	-
P		0.009*	0.001*	-
Ldh-1	*a*	1.000	1.000	1.000
Ldh-2	*a*	1.000	1.000	1.000
Mdh-1	*a*	0.900	1.000	0.800
	b	0.100	-	0.200
P		0.808^{NS}	-	0.146^{NS}
Pgm	*a*	0.850	0.800	0.650
	b	0.150	0.200	0.350
P		0.655^{NS}	0.500^{NS}	0.880^{NS}

p : Probability of Chi-square value. * Significant level : p≤ 0.01. ^{NS} Non-significant p≤ 0.1

Table 6. Genetic variabilities at 5 loci of three *H. tigerinus* populations

Population	Mean proportion of polymorphic loci * (%)	Mean number of alleles per locus (*Na*)	Mean proportion of heterozygous loci per individual (%)	Heterozygosity		
				H_o	H_e	H_o/H_e
Khulna	60.0	1.60	14.0	0.12	0.17	0.71
Netrokona	40.0	1.40	12.0	0.08	0.16	0.52
Char Nilokhia	40.0	1.40	14.0	0.140	0.16	0.88
Average	46.7	1.47	13.3	0.11	0.16	0.67

* p≤0.95

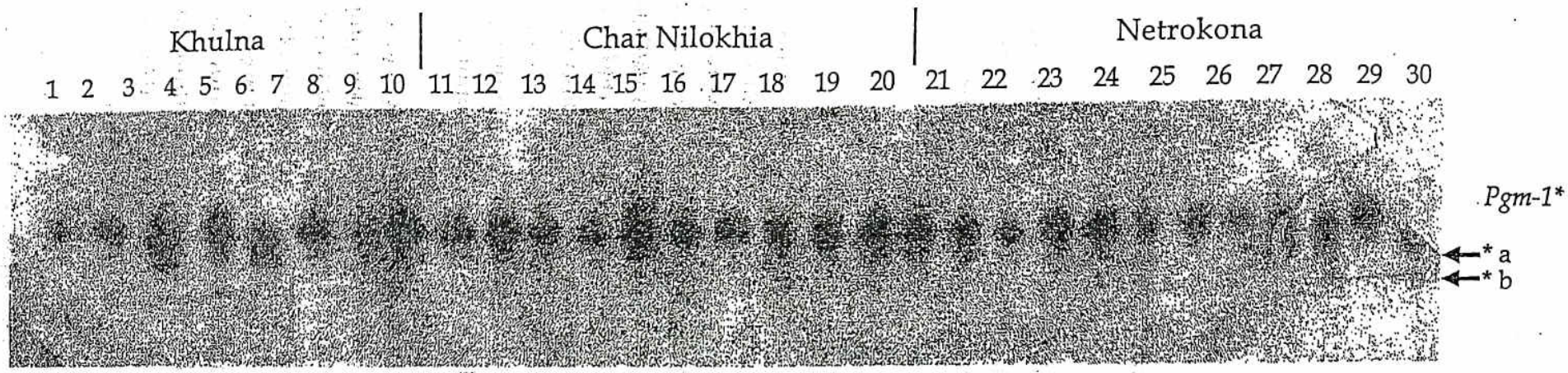
Table 7. Fixation index (F_{ST}) and gene flow (N_m) in three *H. tigerinus* populations

Pairwise	F_{ST}	N_m*
Khulna-Char Nilokhia	0.122	1.802
Char Nilokhia-Netrokona	0.092	2.484
Khulna-Netrokona	0.279	0. 647
Overall	0.219	0.890

*N_m = Gene flow estimated from $F_{ST} = 0.25 (1- F_{ST})/ F_{ST}$

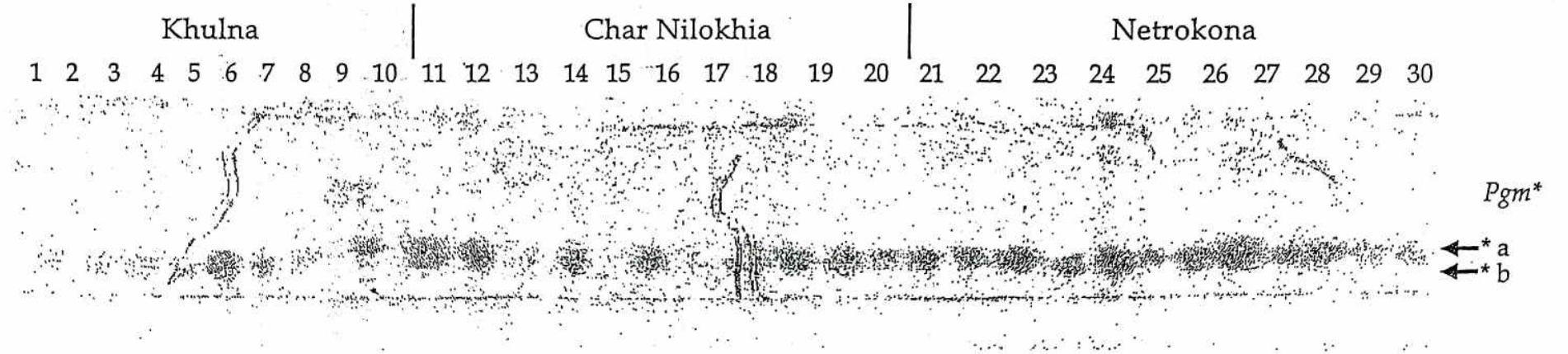
Table 8. Nei's (1972) original measures of genetic identity (above diagonal) and genetic distance (below diagonal) estimated among three *H. tigerinus* populations based on 5 loci

Population	Khulna	Char Nilokhia	Netrokona
Khulna	—	0.94	0.854
Char Nilokhia	0.052	—	0.964
Netrokona	0.157	0.036	—



*Mdh-1**: *aa *aa *ab *aa *ab *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa *aa*aa *aa *aa *ab *ab *aa *bb

Fig. 1. Electrophoregram of enzyme malate dehydrogenase (MDH) of *Mdh-1** locus in three populations of *H. tigerinus*.



*Pgm**: *aa *aa *aa *aa *ab *aa *aa *aa *ab *ab *ab *ab *ab *aa *aa *aa *ab *aa *aa *aa*aa *bb *aa *aa *aa *ab *bb *ab*ab*ab

Fig. 2. Electrophoregram of enzyme (PGM) of *Pgm** locus in three populations of *H. tigerinus*.

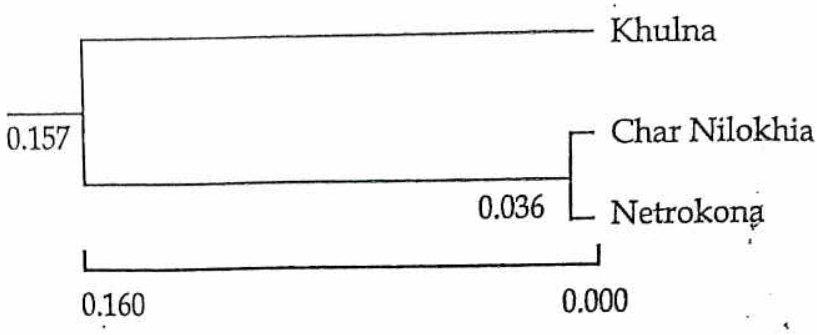


Fig. 3. Nei's (1972) UPGMA dendrogram showing the genetic distance (*D*) among three populations of *H. tigerinus*

The genetic distance (*D*) (Nei, 1972) varied from 0.036 to 0.157 among three populations. The minimum value (0.036) was observed between the Char Nilokhia and Netrokona populations, whereas the highest value (0.157) was observed between the Khulna and Netrokona populations (Table 8, Fig. 3).

DISCUSSION

Mean snout-vent length of the species recorded as 12.33 cm in the Char Nilokhia population, 11.55±1.35 cm in the Netrokona population, and 10.59±1.47 cm in the Khulna population may be explained as the environmental effects. These may be due to the influence of photoperiod, day-length and feeding schedule on tadpole growth and development (Wright *et al.*, 1988). The longest head length was in the Khulna population with the value of 4.68±1.82 cm, whereas the shortest was 4.26±0.29 cm in the Netrokona population. Notwithstanding, these deviations were found due to the anthropological and other genetic factors affected on their growth, which was supported by Hirata *et al.* (1991). Bruneau and Magnin (1980) observed that *Rana catesbeiana* might attain a length of 10.50 cm and its growth depends on the temperature, availability of food and fecundity.

Among five presumptive loci, only three loci (*Gpi-2*, *Mdh-1* and *Pgm*) were found to be polymorphic in all populations. The polymorphic loci for all populations had two alleles (*a* and *b*). Nishioka *et al.* (1993) found that the mean proportion of polymorphic loci in 40 populations of *R. rugosa* was 31.1%, which was much lower than the value (46.7%) obtained in the present study. The *H. tigerinus* populations clearly showed a higher level of polymorphism than the above-mentioned *R. rugosa* populations. The mean number of alleles per locus ranged from 1.40 to 1.60 (average 1.47), which was a little bit higher than the value (1.42) obtained by Nishioka *et al.* (1993) in *R. rugosa*. In contrast, this value (1.47) was lower than that (2.00) obtained by Nishioka *et al.* (1987) in three genera *Buergeria*, *Rhacophorus* and *Polypedates*, and by Khan *et al.* (2002) in *H. tigerinus*. These variations might be due to different sampling size and the difference in the number of loci observed. The mean proportion of heterozygous loci per individual ranged from 12.0% to 14.0% (average 13.3%), which was similar to those obtained by Sumida and Nishikoka (1996). The mean proportion of polymorphic loci of *R. ornativentris* and *R.*

rugosa were 14.4% and 11.3%, respectively. The observed heterozygosity (H_o) ranged from 0.08 to 0.14 (average 0.11) was higher than that (0.10) reported by Raginski and Babik (2000) in *R. arvalis*. The expected heterozygosity (H_e) was found to be 0.16 in the present study, which was similar to those obtained by Rafiniski and Babik (2000) and Khan *et al.* (2002). However, the present value was much higher than that obtained by Formas and Breva (2000). The level of heterozygosity was related with the size of populations within a species, although higher heterozygosity (H_o) value is necessary for breeding program. The highest observed and expected heterozygosities (H_o = 0.14 and H_e = 0.17) were observed in the Char Nilokhia population and the Khulna population, respectively.

The co-efficient of gene differentiation (F_{ST}) in all three *H. tigerinus* populations were examined (Nei, 1972) and F_{ST} was found to be 0.219, which indicated that the population contained slight genetic differentiation, and that the number of migrated individuals from one population to another was high (N_m = 0.890). The overall F_{ST} value (0.219) obtained in *H. tigerinus* populations was lower than that (0.306) obtained in *R. ornativentris* populations (Sumida and Nishioka, 1996). The observed genetic distance between the Khulna population and the other populations might be due to the geographical distances. Based on Nei's (1972) genetic distance (*D*-value), the UPGMA dendrogram showed that the three populations could be grouped into two clusters as shown in the dendrogram (Fig. 3). First cluster contained the Khulna population alone, and separated from second one by D = 0.157, whereas the second cluster comprised of the Char Nilokhia and Netrokona populations. In the second cluster, the Char Nilokhia population differentiated from the Netrokona population by D = 0.036. Islam *et al.* (2008a, b) reported that the mean genetic distance between the medium and small types of the *Fejervarya limnocharis* complex was 0.782, and suggested that these two types are distinct species. The genetic distance (0.157) observed between two clusters (the Khulna population and the Char Nilokhia and Netrokona populations) was smaller than that between two types of the *Fejervary limnocharis* complex mentioned above. Among the *Dicroglossus* species from the Mediterranean area, genetic distances were reported to be 0.17 - 1.04 (Zangari *et al.*, 2006). These differentiations might be due to different species or geographical variations. The genetic distance (*D*) ranged from 0.036 to 0.157 among the three populations of *H. tigerinus*. The minimum genetic distance (0.036) was observed between the Char Nilokhia and Netrokona populations, while the maximum value (0.157) was found between the Khulna and Netrokona populations (Table 8). Nei (1972) stated that values of *D* for inter-species comparison, sub-species and local races are 1.0, 0.1 and 0.01, respectively, while Ayala (1975) proposed that the *D* value for sub-species is 0.20. The present findings assumed that *H. tigerinus* populations might be under local races. Extensive genetic divergence might establish reproductive isolation that reflects further accumulation of genetic differentiation at many loci over a long period of time (Ferguson, 2002; Sites and Marshall, 2003, 2004). However, further extensive research works on genetic variability of the frog populations of many localities will be necessary for elucidating the whole aspects of differentiation of *H. tigerinus* in Bangladesh using different molecular markers i.e. RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), microsatellite DNA markers etc.

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HYLARANA LEPTOGLOSSA (Long-tongued Frog).

BANGLADESH: SYLHET DIVISION: Golapganj (24.45° N, 90.24° E, > 200 m elev.). 01 June 2009. Dr. Md. Mukhlesur Rahman Khan. Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh, Bangladesh. Specimen deposited at Institute for Amphibian Biology, Hiroshima University, Japan (Voucher No. IABHU 3784). Verified by Prof. Mitsuru Kuramoto. First locality record for Sylhet Division and Northeastern record in Bangladesh. Other records from Madhupur National Park, Mymensingh Division (Ca. 210 km to W) (Mahony and Reza, 2007. Herpetol. Rev., 38 [3]: 350) and Chittagong Division (Ca. 285 km to N) with no locality details and specimen photograph or voucher number (Asmat et al., 2003. Univ. J. Zool., Univ. Rajshahi, Bangladesh 22: 141 – 143). Supported by Grant-in-Aids for Scientific Research (C) (Nos. 17570082 and 20510216) to M. Sumida from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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KALOULA TAPROBANICA (Sri Lankan Bull Frog).

BANGLADESH: DHAKA DIVISION: Bangladesh Agricultural University Campus (24.7196° N, 90.4267° E, > 18 m asl.). 11 June 2008. Mahmudul Hasan. Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh, Bangladesh. Specimen deposited at Institute for Amphibian Biology, Hiroshima University, Japan (Voucher No. IABHU F5013). Verified by Mitsuru Kuramoto. First locality record for Mymensingh District in Bangladesh. The nearest population reported from Madhupur National Park, Tangail District (Ca. 38 km to W) (Reza and Mahony, 2007. Herpetol. Rev., 38: 348). Other records from Assam, India, > 200 km to NE and Kolkata, West Bengal, India > 300 km to SW of this locality (Dutta, 1997. Amphibians of India and Sri Lanka [Checklist and Bibliography]. Odyssey Publishing House, Bhubaneswar. Xiii + 343 + xxii pp.). Supported by Grant-in-Aids for Scientific Research (C) (Nos. 17570082 and 20510216) to M. Sumida from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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