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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.

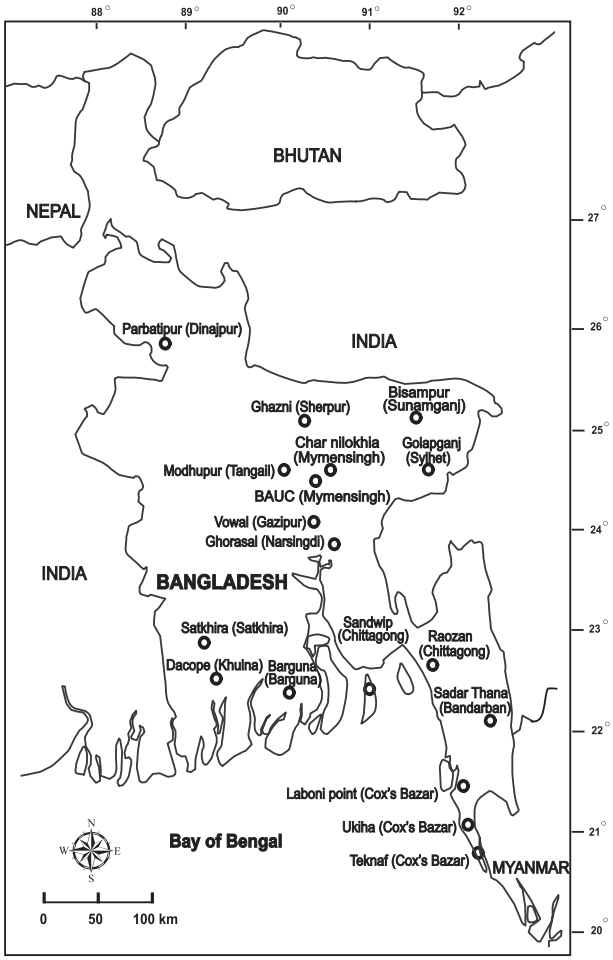

Fig. 1. Map showing the collecting sites of Bangladeshi frogs used for this study. Each black circle represents a sampling site with locality and district name in parenthesis. Bangladesh neighboring countries are also shown in this map.

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 (–In*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.

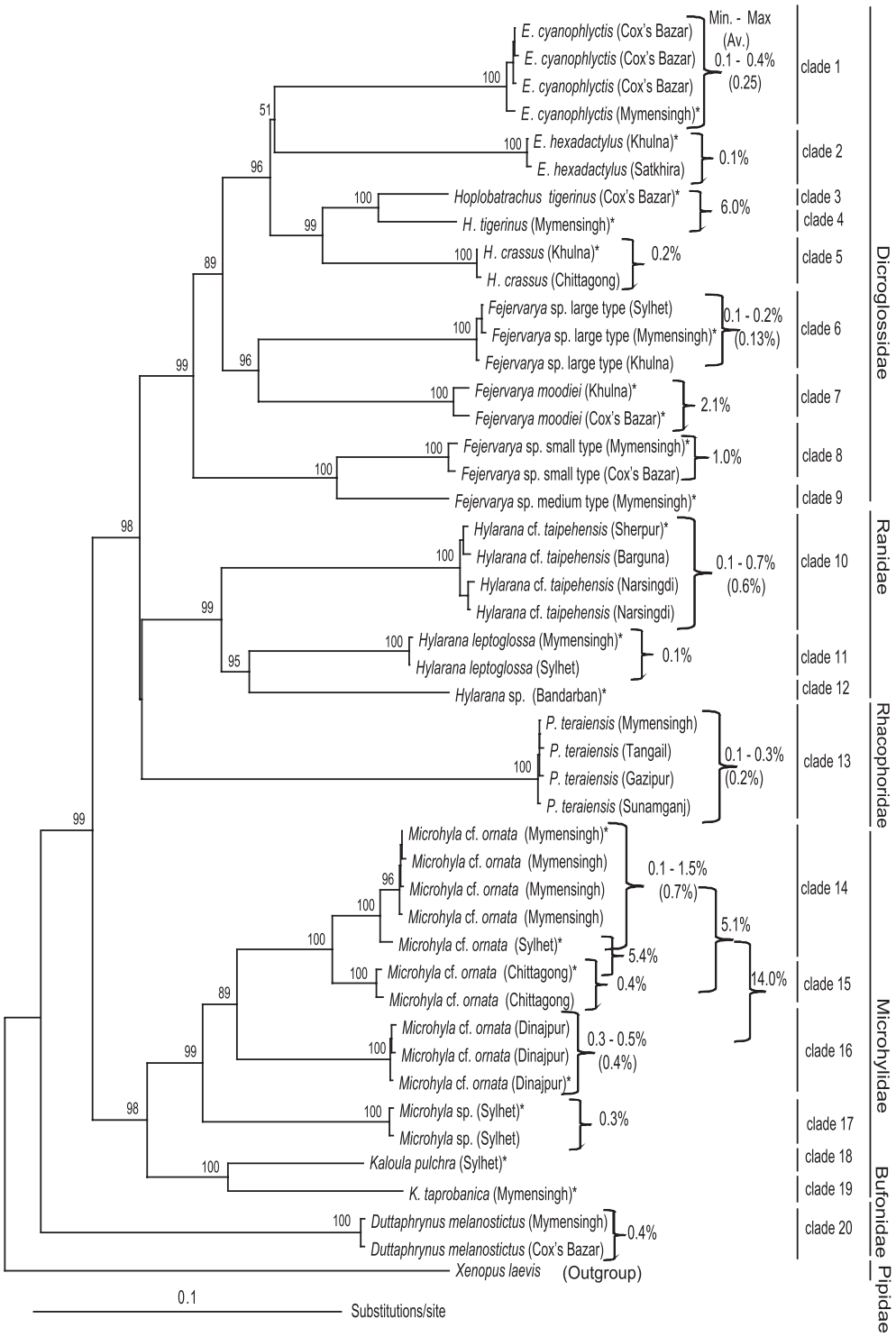

Fig. 2. Neighbor Joining (NJ) tree based on nucleotide sequences of mitochondrial *16S* rRNA gene using the GTR + I + G substitution model from 45 haplotypes with *Xenopus laevis* as an outgroup. The bootstrap support (> 50%) is given above the branches and is based on 1000 replicates. The scale bar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

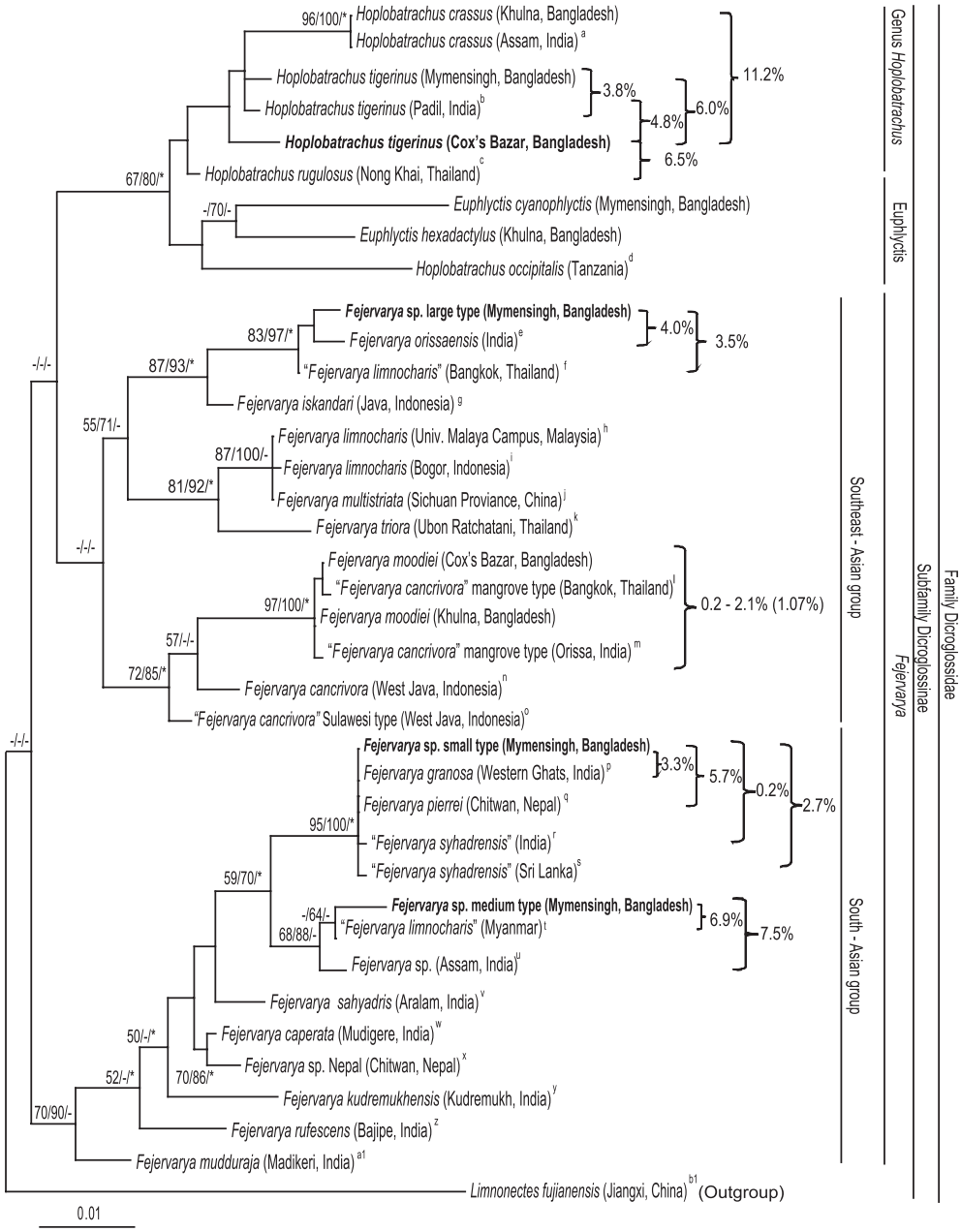

Fig. 3. Maximum Likelihood (ML) tree of dicroglossid frogs based on nucleotide sequences of the mitochondrial *16S* rRNA gene using the SYM + I + G substitution model with *Limnonectes fujianensis* as an outgroup. The bootstrap support (> 50%) is given in order for ML (500) and NJ (1000) replicates. 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

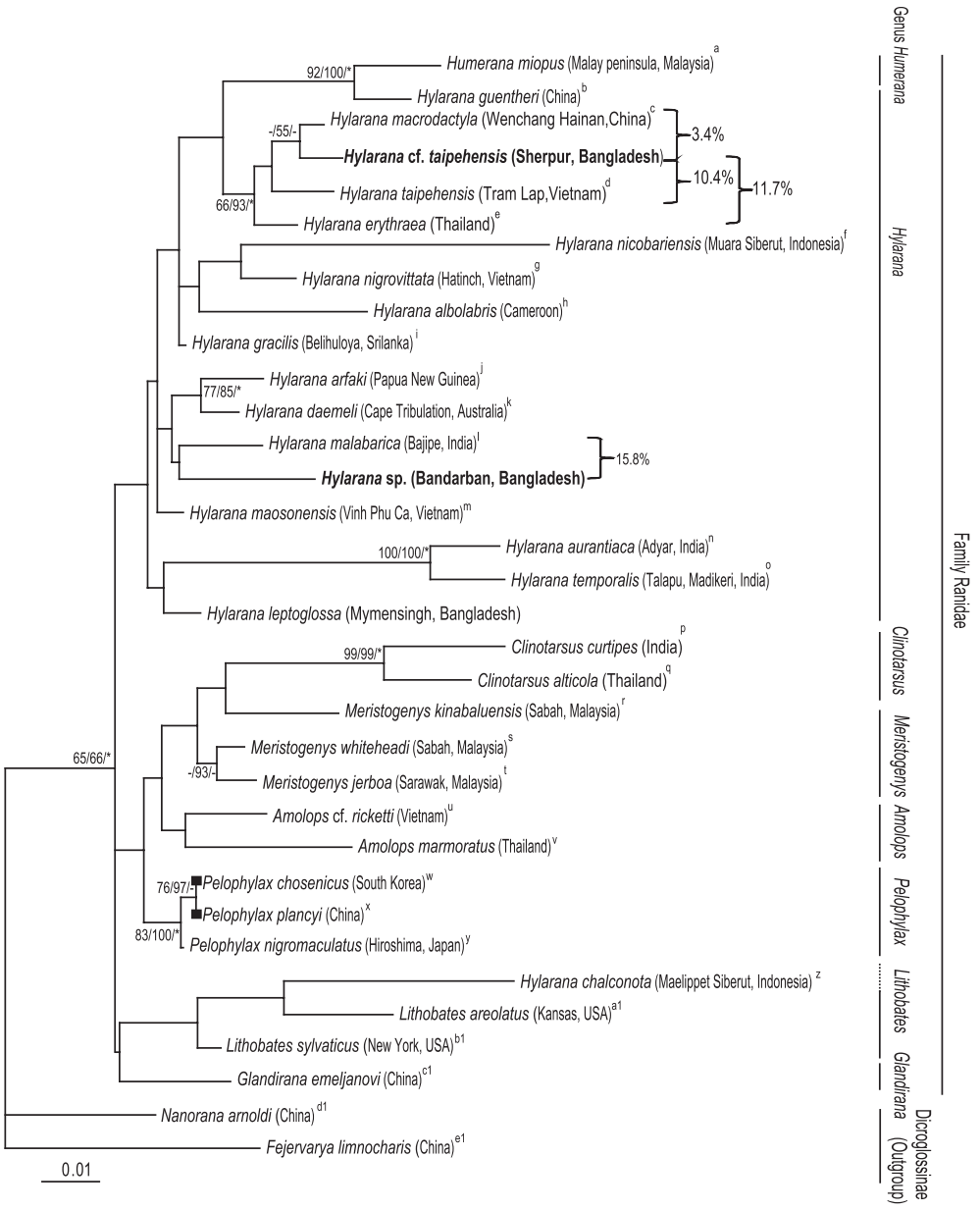

Fig. 4. Maximum Likelihood (ML) tree of ranid frogs based on nucleotide sequences of the mitochondrial *16S* rRNA gene using the GTR + I + G substitutions model with *Nanorana arnoldi* and *Fejervarya limnocharis* as outgroups. The bootstrap support (> 50%) is given in order for ML (500) and NJ (1000) 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.

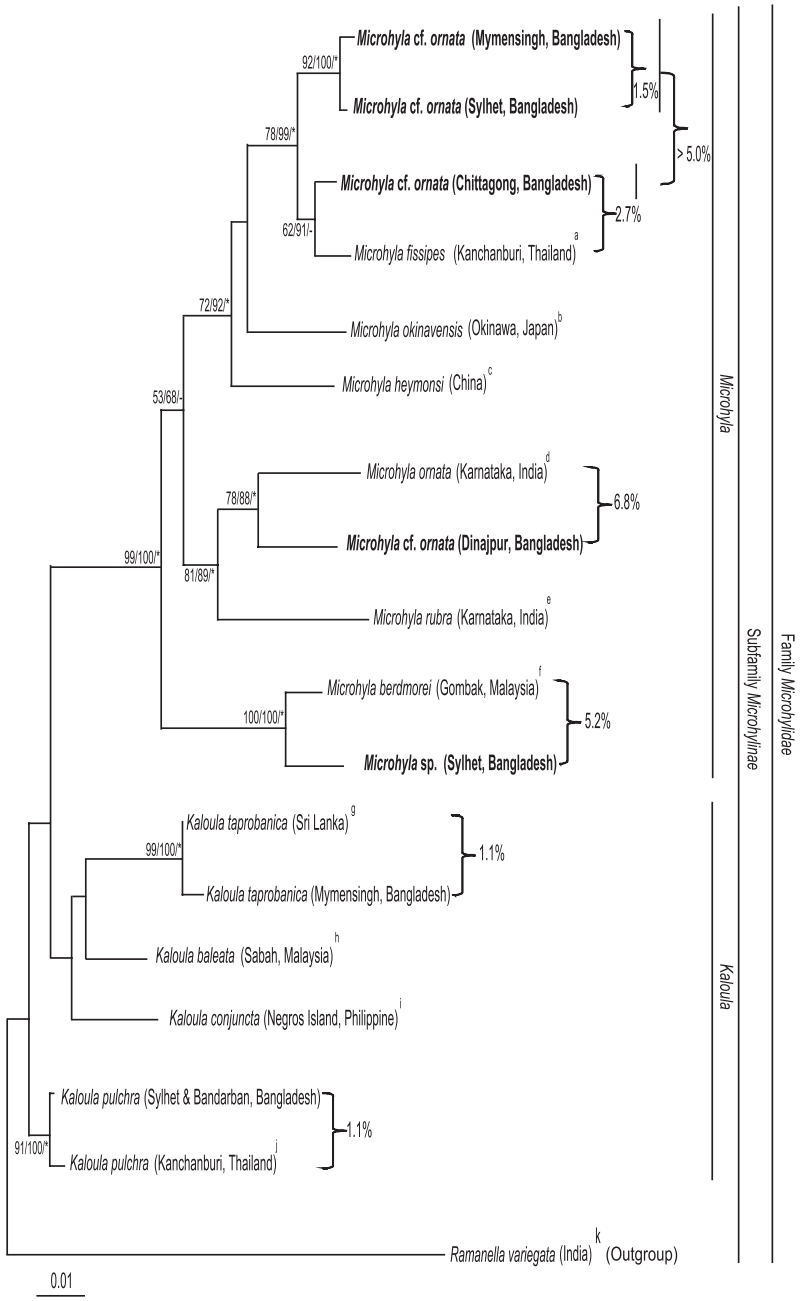

Fig. 5. Maximum Likelihood (ML) tree of microhylid frogs based on nucleotide sequences of the mitochondrial *16S* rRNA gene using the GTR + I + G substitutions model with *Ramanella variegata* as an outgroup. The bootstrap support (> 50%) is given in order for ML (500) and NJ (1000) replicates. Asterisks 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).

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 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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