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ASSESSING SPECIES-LEVEL IDENTIFICATION OF SOME CRYPTIC FROG SPECIES OF BANGLADESH USING 16S AND 12S rRNA GENES

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Keywords: cryptic species, frog, molecular identification, 12S rRNA, 16S rRNA

Abstract

In recent years, a number of new and undescribed anuran species are being discovered at a very fast rate in South and Southeast Asia. With the revelation of more and more species that were previously considered to be some other species, accurate identification of these morphologically cryptic anurans based on morphology alone have become difficult. In this study, we attempted species-level identification of 15 frog specimens belonging to the families Dicroglossidae and Microhylidae of Bangladesh by analyzing their 16S and 12S rRNA gene sequences. The collected specimens were successfully identified to be belonging to eight different species, namely *Fejervarya dhaka, F. asmati, F. pierri and F. orissaensis; Euphlyctis kalasgramensis* and *Euphlyctis* sp.*; Microhyla berdmorei* and *M. mymensinghensis* using BLAST and maximum likelihood analyses. In some cases the frog samples showed morphological resemblance but after molecular analysis they were found to be different and in another case morphologically distant samples were found to be molecularly similar. For example, two of the specimens (ESP 01, ESP 02) turned out to be *E. kalasgramensis* at molecular analysis, however, did not match with the morphological and morphometric descriptions (specially snout-vent length) described previously. Moreover, a species is suspected to be still undescribed and another species namely *M. berdmorei* was thought to be absent in Bangladesh which has been recorded in our study. Thus, morphological ambiguity strengthens the urge for molecular analysis for proper identification.

Introduction

In Bangladesh, the number of amphibian species has increased nearly 30% in the last few years[1,2], many of which have previously been misidentified as other existing species. For example, *Euphlyctis kalasgramensis* and *Microhyla nilphamariensis* have previously been recognized as *E. cyanophlyctis* and *M. ornata*, respectively[3-5].

A cryptic species is essentially two or more species that are otherwise difficult to

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identify based on external morphology alone. Cryptic species are two or more species that are or have been considered to be a single nominal species at any time because of their morphological similarity[6]. Members of cryptic species complexes are hard to distinguish morphologically because of how closely related they are and how similar they are to each other in terms of external morphology[7,8]. In fact, identification based on morphological traits only are now considered unsuitable for cryptic species[9].

Rapid identification of all the cryptic species populations in Bangladesh is important for gaining better knowledge of their actual number and distribution in the country. Without proper identification of these species, it is not possible to manage their populations across the country scientifically. Dicroglossidae and Microhylidae are two such anuran families in Bangladesh with ever increasing numbers of species and cryptic complexes.

Molecular data analysis is therefore considered to be a valuable tool for identifying cryptic species[5,6]. When it comes to cryptic species, molecular identification is slightly trickier than normal, because since many of the cryptic species have been or still are recognized as other species for so long, their reference sequences are also listed as such on the databases. But now that these new species or species complexes are being discovered and their genetic data recorded, it is now possible to reevaluate those earlier sequences to determine which species they actually were[10].

Amphibians as a highly diverse group, each individual species also shows different morph pattern with seasonal changes, genders, stages of life cycle, etc. Due to this morphological ambiguity, it becomes very difficult to identify them only using the morphological data. Here, in addition to an extensive morphological analysis, we take a molecular approach to identify 15 cryptic frog specimens from Bangladesh up to species level through 16S and 12S rRNA gene sequence analysis. This uncovers the possibility of the presence of a new species in Bangladesh.

Materials And Methods

Sample collection and morphological study: A total of 15 specimens belonging to the families Dicroglossidae and Microhylidae were collected (ethical clearance or permit Reference No. 74/Biol. Scs.) from Curzon Hall, Dhaka (FSP 04, FSP 05, FSP 07 and MSP 05); Mirpur, Dhaka (ESP 01 and ESP 02); SAU Campus, Dhaka (FSP 06 and MSP 03) and Kamalganj, Sylhet (FSP 01, FSP 02, FSP 03, ESP 03, MSP 01, MSP 02 and MSP 04). Collection was done without causing any disturbances in the population or destroying any of their habitats. Only the bare minimum of specimens was collected and euthanized for muscle tissue collection in strict accordance with the protocols by the Faculty of Biological Science, University of Dhaka (Reference No. 74/Biol. Scs.) solely for scientific research. After collection they were washed and kept in 70% alcohol before being photographed.

The morphometric measurements (in mm) were taken using digital slide-calipers, noted to the nearest 0.02 mm. A total of 21 morphometric characters were measured by following the definitions described[3,11] (Fig. 1) such as SVL (distance from tip of snout to

vent); HL (head length; distance from tip of snout to the back of mandible); HW (head width; maximum width of the head at the posterior margin of mandible); MN (distance from back of mandible to nostril); SL (snout length; distance from anterior corner of eye to the tip of snout); MFE (distance from back of mandible to front of the eye); MBE (distance from back of mandible to back of the eye); IN (internarial distance); IOD (interorbital distance); EN (distance from front of eyes to the nostril); NS (nostril—snout length); EL (eye width); UEW (maximum width of upper eyelid); HAL (hand length; distance from proximal end of outer palmar metacarpal tubercle to tip of third finger); FAL (forearm length; distance from corner of elbow to proximal end of outer palmar metacarpal tubercle); THIGHL (thigh length; distance from vent to knee); TL (tibia length; distance from knee to heel); TFOL (length of tarsus and foot; distance from heel to tip of fourth finger); FOL (foot length; distance from proximal end of inner metatarsal tubercle to tip of fourth finger).

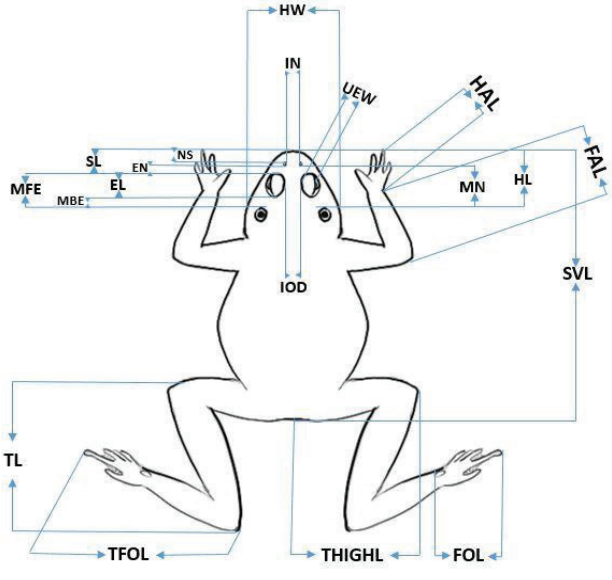


Fig. 1. A schematic illustration of the definitions of morphological traits used in this study.

***	SVL=snout to vent length	SL = snout length	IOD=interorbital distance
	HL =head length	MFE = mandible to front of eye	EN = eyes to nostril
	HW =head width	MBE = back of mandible to back of eye	NS=nostril—snout length
	MN =mandible to nostril distance	IN = internarial distance	THIGHL = thigh length
	EL =eye width	HAL = hand length	TL = tibia length
	UEW =maximum width of upper eyelid	FAL = forearm length	
	TFOL =length of tarsus and foot	FOL = foot length	

DNA extraction and sequencing: Tissue samples were taken from the innermost thigh region of the specimens. For isolation of DNA, a modified CTAB-based DNA extraction method[12] was used. Briefly, collected tissue samples were lysed and homogenized with 600µl CTAB lysis buffer and 10µl Proteinase K solution. The lysate was then incubated

at 55°C for 3 hours and 5µl RNase solution (20mg/µl) was added before proceeding to the next step. Centrifuging at 13000 rpm for 5 minutes, collected supernatant was mixed with equal volume of Phenol:Chloroform (25:24) followed by another bout of centrifugation at the same speed for 5 minutes. The resultant upper DNA layer was again centrifuged after adding isopropanol in it. Then the extracted DNA pellet was washed with 70% alcohol and finally 50-100µl of deionized water was used to elute the DNA. Extraction of DNA using CTAB was successful for both fresh specimens and specimens preserved in 70% alcohol. Muscle tissue from the thigh region gave good quality DNA.

For PCR (Polymerase Chain Reaction) amplification, a total of three pairs of forward and reverse primers were used (listed in Table 1). The PCR was conducted using GoTaq® G2 Hot Start Master Mixes Kit according to the manufacturer›s protocol. Briefly, in a total volume of 50µL PCR mixture, 0.5µL *Taq* DNA polymerase was mixed with 10µL of 5X PCR buffer, 28.17µL nuclease free water, 1µL of 10mM dNTP, 5µL of 25µM MgCl2, 1µL of forward and reverse primer (2.5µM) each, and finally, 3.33µL of template DNA. In thermal cycler, amplification of 16S and 12S rRNA sequences were performed for 30 cycles. Each of these cycles consisted of denaturation for 30 seconds at 94^0C, annealing for 30 seconds at 52^0C for 16S rRNA fragments and 50^0C for 12S rRNA fragments, and elongation at 72^0C for 30 seconds. Besides these cycles, an initial denaturation stage of 2 minutes at 94^0C, and a final elongation stage of 5 minutes at 72^0C were also performed.

Table 1. Primers for 16S rRNA and 12S rRNA genes used for polymerase chain reaction

Primer	Primer sequence 5'-3'	Reference
16S rRNA F-Primer	5'-CGC CTG TTT ACC AAA AAC AT-3'	Knight & Mindell 1993
16S rRNA R-Primer	5'-CCG GTC TGA ACT CAG ATC ACG T-3'	Kocher *et al*. 1989
12S rRNA F-Primer(1)	5'-CGA CAG CTA GGA AAC AAA CTG G-3' CGACAGCTAGGAAACAAACTGG	This study
12S rRNA R-Primer(1)	5'-CCA TGT TAC GAC TTG CCT CTT C-3'	Wilkinson *et al*. 2002
12S rRNA F-Primer(2)	5'-AAC GCT AAG ATG AAC CCT AAA AAG TTC T-3'	Sumida *et al.* 1998
12S rRNA R-Primer(2)	5'-GGG TAT CTA ATC CCA GTT TG-3'	Sumida *et al.* 1998, modified

Amplified samples were purified at room temperature using GeneJet PCR Purification Kit (ThermoFisher Scientific) according to the manufacturer's protocol. The purified PCR products were sequenced by standard sequencing technique using ABI prism 3730XL sequencer (Macrogen Inc., South Korea).

Bioinformatics analysis: For bioinformatics analyses, all the newly generated sequences were analyzed first by aligning the forward and reverse sequences in SeaView v4.7[13] and visually comparing them with the corresponding chromatographs on ChromasPro v2.1.5 (Technelysium Pty Ltd, South Brisbane, Queensland, Australia). Then BLAST analysis[14] was performed using the NCBI BLAST tool for each of the sequences. After that multiple sequence alignments were prepared using the ClustalW[15] method on MEGA7[16] for sequences of the specimens of each genus; homologous sequences of voucher specimens for

all available known species of that genus from NCBI GenBank sequence database[17] were included for comparison (Supplementary Table 2). Using the multiple sequence alignment, maximum likelihood (ML) analysis[18] was done on MEGA7 with 100 bootstrap replicates[19].

Results and Discussion

Morphological analysis: In the current study, the genera of the specimens were easy to determine based on morphology and morphometric analysis [3,4,20-23]. The morphometric measurements of different morphological features of each specimen and their various ratios as calculated are described.

According to the morphological and morphometric analysis the specimens belong to three genus: *Fejervarya* (Table 2), *Euphlyctis* (Table 3) and *Microhyla* (Table 3), (Fig. 2,3).

Table 2. Morphological measurements of *Fejervarya (=Minervarya)* spp

Morphometric Measurements (mm)	*Fejervarya dhaka* (FSP 01)	*F. pierrei* (FSP 02)	*F. asmati* (FSP 03)	*F. pierrei* (FSP 04)	*F. dhaka* (FSP 05)	*F. orissaensis* (FSP 06)	*F. dhaka* (FSP 07)
SVL	23.68	38.28	29.39	27.71	26.96	38.85	22.98
HL	6.64	11.41	89.52	7.22	8.00	11.28	6.02
HW	7.51	12.56	9.67	8.28	8.40	13.72	7.07
MN	4.82	8.16	6.16	5.94	6.22	8.43	4.35
SL	3.78	5.76	1.29	4.03	4.24	5.24	1.31
MFE	2.71	5.81	3.99	3.73	4.08	5.54	2.55
MBE	0.59	1.54	0.52	0.67	1.06	1.23	0.79
IN	1.62	3.25	1.92	0.86	1.92	1.25	1.56
IOD	2.41	2.74	1.75	1.70	1.58	1.59	1.60
EN	1.83	2.81	1.02	1.09	1.42	2.65	1.58
NS	1.38	2.30	1.06	0.70	1.67	1.63	0.94
EL	2.97	4.71	4.21	3.22	3.51	5.36	2.50
UEW	1.64	3.00	2.22	2.06	2.08	3.70	1.55
TD	1.56	2.14	1.56	1.36	1.32	2.31	1.23
HAL	5.32	8.34	6.92	5.97	5.82	8.30	4.59
FAL	4.19	8.18	6.52	5.40	4.98	7.38	3.91
THIGH	11.73	18.96	13.92	12.42	13.61	16.53	9.94
TL	13.83	22.86	15.95	14.81	15.66	19.47	11.93
TFOL	21/02	30.28	22.51	21.00	23.12	26.54	17.06

FOL	14.60	19.13	14.71	14.33	15.30	19.56	11.31
F1	2.70	4.81	3.25	2.80	3.23	4.61	2.29
F2	2.46	3.57	3.03	2.00	2.44	3.00	2.01
F3	3.33	5.00	4.00	4.08	4.29	4.88	3.20
F4	2.22	3.47	2.17	2.02	2.39	2.78	1.73
RFL	3>1>2>4	3>1>2>4	3>1>2>4	3>1>4 ≅2	3>1>2≅4	3>1>2≅4	3>1>2≅4
HL:SVL	0.28	0.30	0.96	0.26	0.30	0.29	0.26
HL:HW	0.88	0.91	1.81	0.87	0.95	0.82	0.85
SL:HL	0.57	0.54	0.50	0.56	0.53	0.46	0.22
EN:NS	1.33	1.22	0.14	1.56	0.85	1.63	1.68
IN:NS	1.17	1.41	0.91	1.23	1.15	0.77	1.66
EL:HL	0.45	0.41	0.06	0.45	0.44	0.48	0.42
EL:SVL	0.13	0.12	0.94	0.12	0.13	0.14	0.11
IOD:IN	1.49	0.84	0.92	1.98	0.82	1.27	1.03
MBE:HL	0.09	0.13	0.06	0.09	0.13	0.11	0.13
FAL:HAL	0.79	0.98	0.94	0.90	0.86	0.89	0.85
FOL:TL	1.06	0.84	0.92	0.97	0.98	1.00	0.95

Table 3. Morphological measurements of *Euphlyctis* spp. and *Microhyla* spp

Morphometric Measurements (mm)	***Euphlyctis kalasgramensis* (ESP 01)**	***E. kalasgramensis* (ESP 02)**	***E. aloysii* (ESP 03)**	**Morphometric Measurements (in mm)**	***Microhyla berdmorei* (MSP 01)**	***M. berdmorei* (MSP 02)**	***M. mymensinghensis* (MSP 03)**	***M. mymensinghensis* (MSP 04)**	***M. mymensinghensis* (MSP 05)**
SVL	63.02	47.31	72.55	SVL	31.34	33.07	15.85	18.42	21.90
HL	16.11	13.38	18.99	HL	7.36	8.45	3.84	5.01	6.67
HW	22.34	16.31	25.21	HW	9.20	10.62	4.37	6.96	6.65
MN	12.41	8.60	14.57	MN	6.47	6.68	2.91	3.29	3.88
SL	8.62	6.02	8.02	SL	3.77	3.51	1.65	2.06	2.22
MFE	9.35	6.02	11.06	MFE	4.56	5.15	1.88	2.85	3.34
MBE	2.74	1.54	1.80	MBE	0.92	1.24	0.25	0.51	0.74
IN	2.26	2.55	2.56	IN	1.38	1.63	0.42	0.60	0.57
IOD	5.83	2.55	2.44	IOD	2.48	2.70	0.67	1.65	1.25
EN	3.08	3.48	5.33	EN	1.70	1.67	0.81	1.23	1.01
NS	1.76	3.65	3.07	NS	1.20	0.98	0.24	0.35	0.30
EL	6.14	6.55	9.78	EL	3.85	3.19	1.43	2.15	2.45

UEW	3.05	3.75	5.48	UEW	1.80	2.11	0.88	0.82	1.20
HAL	16.27	13.11	18.00	HAL	8.16	7.61	2.97	4.08	4.37
FAL	14.58	9.39	13.59	FAL	5.29	5.31	2.37	3.43	3.23
THIGH	35.19	21.84	38.41	THIGH	18.33	17.32	8.03	9.90	10.14
TL	47.06	24.18	33.03	TL	23.84	22.32	8.41	11.17	11.65
TFOL	49.04	37.61	51.67	TFOL	29.05	26.92	12.08	15.43	16.70
FOL	35.63	27.49	33.15	FOL	19.77	18.11	7.28	9.97	11.27
F1	8.72	5.40	9.26	F1	1.13	1.02	0.12	0.06	0.45
F2	8.19	5.40	8.87	F2	2.26	2.28	0.89	0.85	1.33
F3	9.75	7.23	9.41	F3	4.55	4.75	1.65	2.44	2.07
F4	7.58	6.35	7.46	F4	3.09	3.22	0.63	1.11	1.29
RFL	3>1>2>4	3>4>1=2	3>1>2>4	RFL	3>4>2>1	3>4>2>1	3>2>4>1	3>4>2>1	3>2>4>1
HL:SVL	0.26	0.28	0.26	HL:SVL	0.23	0.26	0.24	0.27	0.30
NS:EN	0.57	1.05	0.56	NS:EN	0.87	0.60	0.26	0.28	0.30
NS:SVL	0.03	0.08	0.04	EL:HL	0.47	0.66	0.37	0.43	0.37
EL:SVL	0.10	0.14	0.13	IN:NS	0.52	0.38	1.75	1.71	1.90
TL:SVL	0.75	0.51	0.46	UEW:EL	1.42	1.70	0.62	0.38	0.49
FOL:SVL	0.57	0.58	0.46	IOD:IN	1.80	1.66	1.56	2.75	2.19
TD	5.04	3.18	6.21	HL:HW	0.80	0.80	0.88	0.72	1.00

Seven of the collected specimens were the genus *Fejervarya (=Minervarya)* (FSP 01-07), three were *Euphlyctis* (ESP 01-03), and five *Microhyla* (MSP 01-05). Species determination based on morphological analysis was mostly unsuccessful due to the morphological features and morphometric measurements or ratios overlapping or not matching with the descriptions followed.

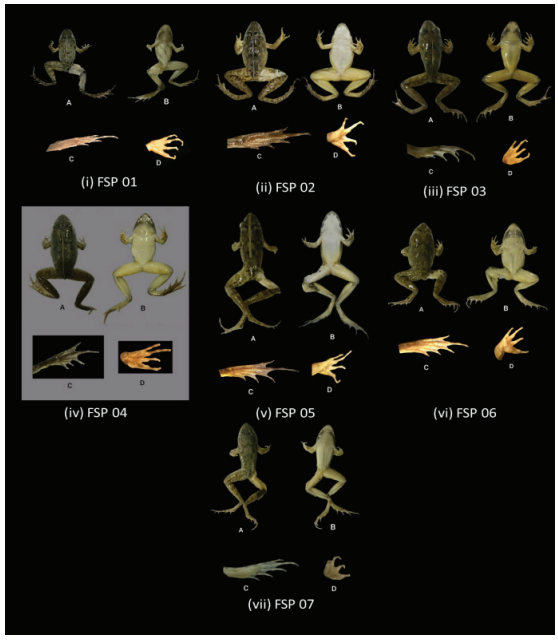
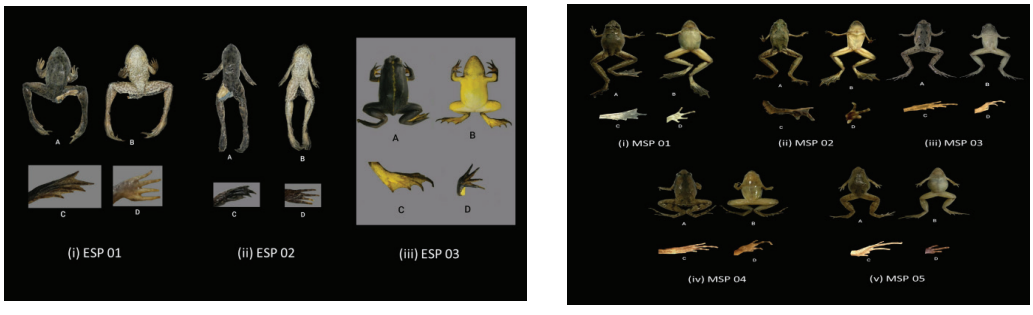


Fig. 2. Morphological presentation of FSP 01-07 from the current study where figure (A), (B), (C) and (D) show dorsal view, ventral view, magnified view of ventral side of foot and hand respectively.



(a) **(b)**

Fig. 3. (a) Morphological presentation of ESP 01-03 from the current study where figure (A), (B), (C) and (D) show dorsal view, ventral view, magnified view of ventral side of foot and hand, respectively. (b) Morphological presentation of MSP 01-05 from the current study where figure (A), (B), (C) and (D) show dorsal view, ventral view, magnified view of ventral side of foot and hand, respectively.

Molecular analysis: A total of 23 partial nucleotide sequences were gained from the specimens; fifteen from 16S rRNA gene (GenBank Accession No.: MK635480-MK635494) and eight from 12S rRNA gene (GenBank Accession No.: MK635471-MK635478). The final partial nucleotide sequences of 16S rRNA gene contained 506-605 base pairs (bp), while for

12S rRNA gene sequences the lengths were 397-486 bp.

BLAST search results utilizing the 16S and 12S rRNA gene sequences of *Fejervarya* specimens showed three *Fejervarya* specimens (FSP 01, FSP 05 and FSP 07) to be closest to *Fejervarya dhaka,* one specimen (FSP 03) to be closest to *F. asmati*, another specimen (FSP 06) to *F.* sp. Large (=*F. orissaensis*), and two other specimens (FSP 02 and FSP 04) were closest to *F. pierrei* (Table 4).

Thus among the seven specimens of *Fejervarya* in this study, four different species were found. One of these, denoted as *Fejervarya* sp. large[11] has been confirmed as *F. orissaensis* in 2019[22], thus further confirming the presence of *F. orissaensis* in Bangladesh. Another species, *F. dhaka,* has just recently been described as a separate species[23]. Before that, this species was described as '*Fejervarya* sp. Medium type'[11]. Among all the *Fejervarya* species of Bangladesh, *F. dhaka* and *F. asmati* bear very close resemblance to each other in terms of morphology[22,23]. Distinguishing between these two species using molecular methods was successful in the present study. The three specimens of *F. dhaka* found in the present study all match 100% with the 16S rRNA gene nucleotide sequence of the specimens described[23]. *F. pierrei* of the present study which were collected from Dhaka showed 99% or more 16S rRNA gene sequence similarity to the *F. pierrei* found in Chitwan, Nepal[23] (Supplementary Table 1).

BLAST searches of the partial 16S rRNA genes of two *Euphlyctis* specimens (ESP 01 and ESP 02) showed them to be closest to *Euphlyctis kalasgramensis*, while *E. hexadactylus* was the closest match for specimen ESP 03. BLAST searches using the partial 12S rRNA sequences of ESP 01 and ESP 02 were inconclusive. Specimens from the *Euphlyctis* genus also produced quite interesting results. Two of the specimens (ESP 01, ESP 02) turned out to be *E. kalasgramensis* even though they did not quite match with the morphological and morphometric descriptions provided[3]. Even the snout-vent lengths of the two specimens were quite longer than the snout-vent length described[3]. This brings forward the question whether the description of *E. kalasgramensis* is in need of subsequent revisions in future studies. Another specimen of *Euphlyctis* (ESP 03) from the present study which showed similarity to the descriptions of *E. hexadactylus* of Bangladesh, turned out to be genetically different than the *E. hexadactylus* found in Karnataka, India[3,5,24].

Table 4. List of collected specimens with their molecular identification

Genus name	Specimen No.	Species name (based on molecular identification)	GenBank Accession No. (16S)	GenBank Accession No. (12S)
Fejervarya (=*Minervarya*)	FSP 01	*Fejervarya dhaka*	MK635480	Not done yet
	FSP 02	*Fejervarya pierrei*	MK635481	MK635471
	FSP 03	*Fejervarya asmati*	MK635482	MK635472
	FSP 04	*Fejervarya pierrei*	MK635483	Not done yet
	FSP 05	*Fejervarya dhaka*	MK635484	MK635473
	FSP 06	*Fejervarya orissaensis*	MK635485	MK635474
	FSP 07	*Fejervarya dhaka*	MK635486	Not done yet
Euphlyctis	ESP 01	*E. kalasgramensis*	MK635487	MK635475
	ESP 02	*E. kalasgramensis*	MK635488	MK635476
	ESP 03	*Euphlyctis sp.*	MK635489	Not done yet
Microhyla	MSP 01	*Microhyla berdmorei*	MK635490	MK635477
	MSP 02	*Microhyla berdmorei*	MK635491	Not done yet
	MSP 03	*M. mymensinghensis*	MK635492	Not done yet
	MSP 04	*M. mymensinghensis*	MK635493	MK635478
	MSP 05	*M. mymensinghensis*	MK635494	Not done yet

BLAST searches for the 16S and 12S rRNA gene sequences of two *Microhyla* specimens (MSP 01 and MSP 02) showed them to be closest to *M. berdmorei*. Partial 16S rRNA gene sequence of three *Microhyla* specimens (MSP 03, MSP 04 and MSP 05) matched closest to that of *M. mymensinghensis.* Partial 12S rRNA gene sequence of the specimen MSP 04 had no available match on GenBank. The three specimens of *Microhyla mymensinghensis* species found in the present study show different percentages of 16S rRNA gene nucleotide similarity to the sequence of the voucher specimen from Golapganj, Sylhet gained from GenBank[5]. The specimen (MSP 04) that was collected from Kamalganj, Sylhet for the present study matches 100% with the aforementioned voucher specimen from Golapganj, Sylhet. On the other hand, the two *M. mymensinghensis* specimens (MSP 03, MSP 05) that were collected from Dhaka show 0.38-0.57% divergence in the 16S rRNA gene nucleotide sequences from the voucher specimen. These results may allude to the geographic divergence of this species in different populations. The other two specimens of *Microhyla* from the present study show a high percentage of similarity to the *M. berdmorei* population found in its type locality in Myanmar[25]. This negates the previous speculation about the *M. berdmorei* present in Bangladesh being different from that of the original *M. berdmorei*[5,31].

The ML tree based on the partial 16S rRNA genes showed three of the *Fejervarya* specimens (FSP 01, FSP 05 and FSP 07) forming a monophyletic clade with *F. dhaka*, FSP 03 showed proximity to *F. asmati*; FSP 06 to *F.* sp. Large (=*F. orissaensis*)[11,24] while two other specimens (FSP 02 and FSP 04) showed proximity to *F. pierrei* (Fig. 4).

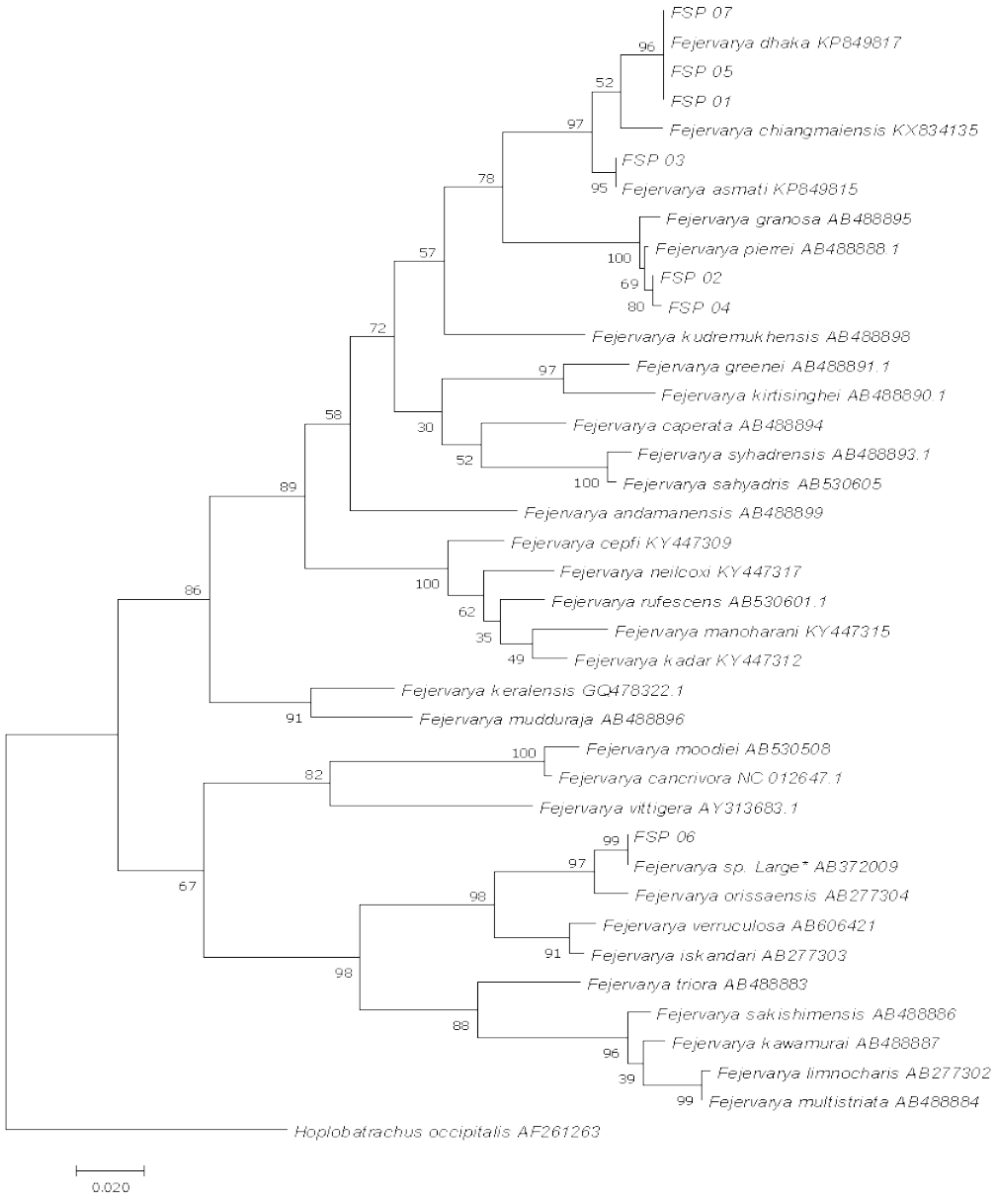


Fig. 4. Phylogenetic relationships among different species of Fejervarya by Maximum Likelihood method using 16S rRNA gene sequences. FSP 01 to 07 indicate the specimens used in the present study. *Hoplobatrachus occipitalis* was used as an outgroup. Numbers on branches represent bootstrap support values.

However, the 12S rRNA gene-based ML tree showed results conflicting with the abovementioned results (Fig. 5).

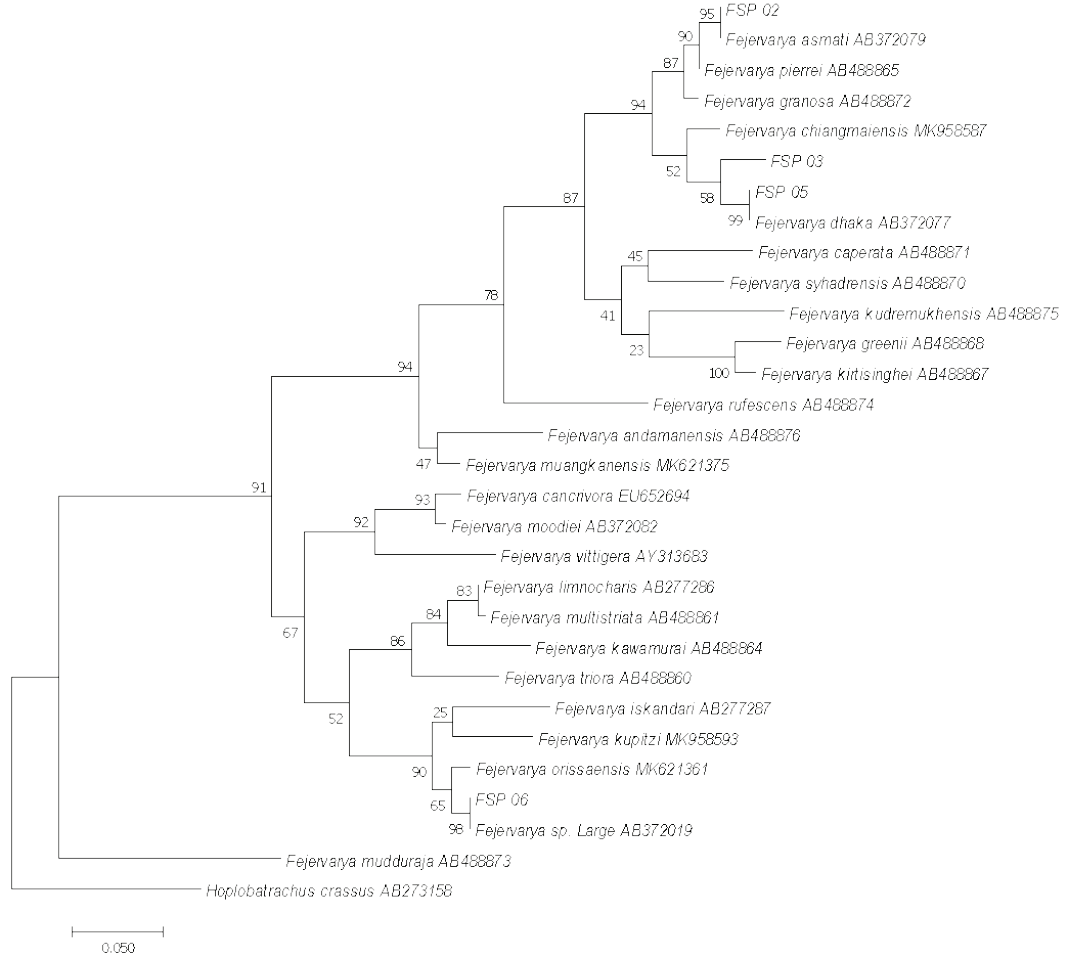


Fig. 5. Phylogenetic relationships among different species of Fejervarya by Maximum Likelihood method using 12S rRNA gene sequences. FSP 02, FSP 03, FSP 05 and FSP 06 are the specimens used in the present study. Hoplobatrachus crassus was used as an outgroup. Numbers on branches represent bootstrap support values.

On the 16S rRNA gene-based tree for *Euphlyctis*, two specimens (ESP 01 and ESP 02) formed a monophyletic clade with *E. kalasgramensis*, while ESP 03 formed a monophyletic clade with the species *E. aloysii* (Fig. 6).

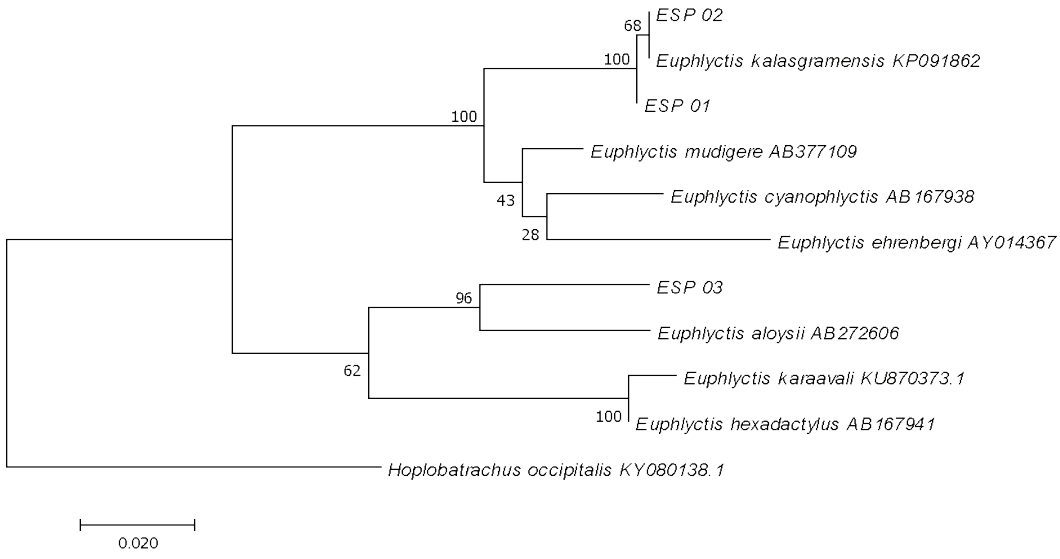


Fig. 6. Phylogenetic relationships among different species of Euphlyctis by Maximum Likelihood method using 16S rRNA gene sequences. ESP 01, 02 and 03 indicate the specimens used in the present study. Hoplobatrachus occipitalis was used as an outgroup. Numbers on branches represent bootstrap support values.

ML analysis using the partial 12S rRNA sequences of ESP 01 and ESP 02 were inconclusive, owing to lack of sufficient reference sequences that were homologous to ours (Fig. 7).

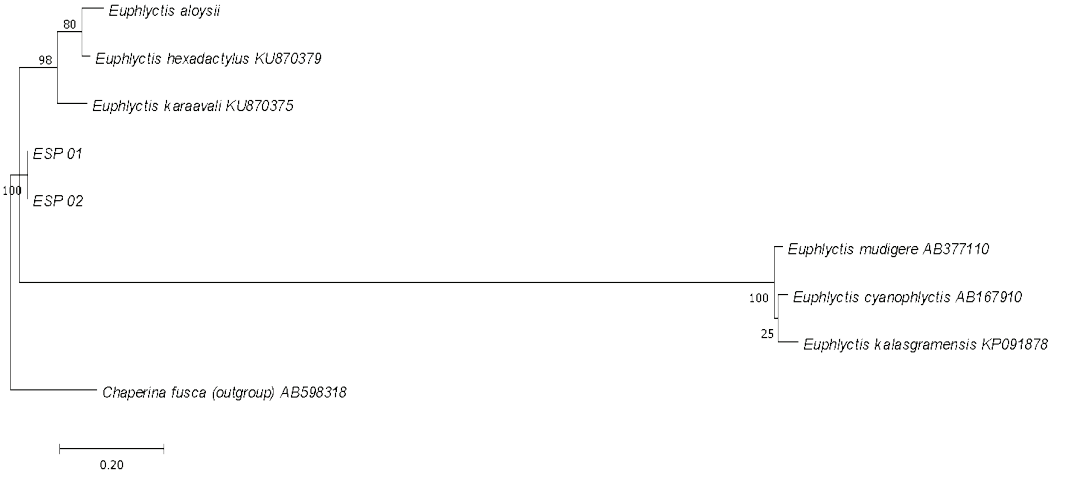


Fig. 7. Phylogenetic relationships among different species of Euphlyctis by Maximum Likelihood method using 12S rRNA gene sequences. ESP 01 and 02 indicate the specimens used in the present study. Chaperina fusca was used as an outgroup. Numbers on branches represent bootstrap support values.

Two *Microhyla* specimens (MSP 01 and MSP 02) showed monophyly with *M. berdmorei* from Myanmar on the 16S rRNA gene-based tree, while three other specimens (MSP 03, MSP 04, and MSP 05) showed proximity to *M. mymensinghensis* (Fig. 8).

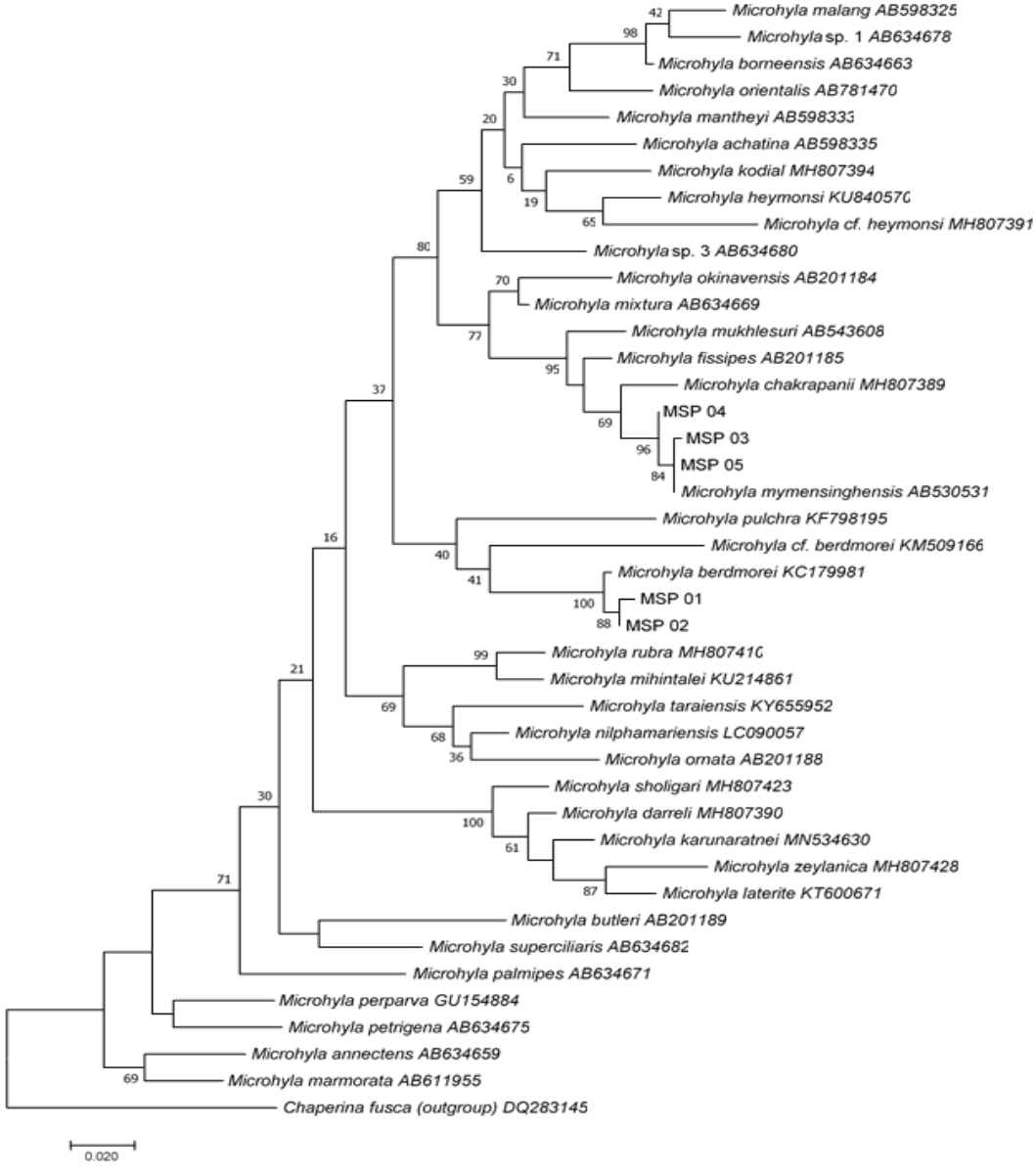


Fig. 8. Phylogenetic relationships among different species of *Microhyla* by Maximum Likelihood method using 16S rRNA gene sequences. MSP 01 to 05 indicate the specimens used in the present study. *Chaperina fusca* was used as an outgroup. Numbers on branches represent bootstrap support values.

Construction of a single ML tree containing all the partial 12S rRNA genes was unsuccessful in the case of the genus *Microhyla* because not all the available sequences collected from GenBank were homologous to ours. When separate trees were built by eliminating the non-homologous sequences, the trees showed multiple anomalies including low bootstrap values, and thus were deemed as unreliable for identification (Fig. 9-10).

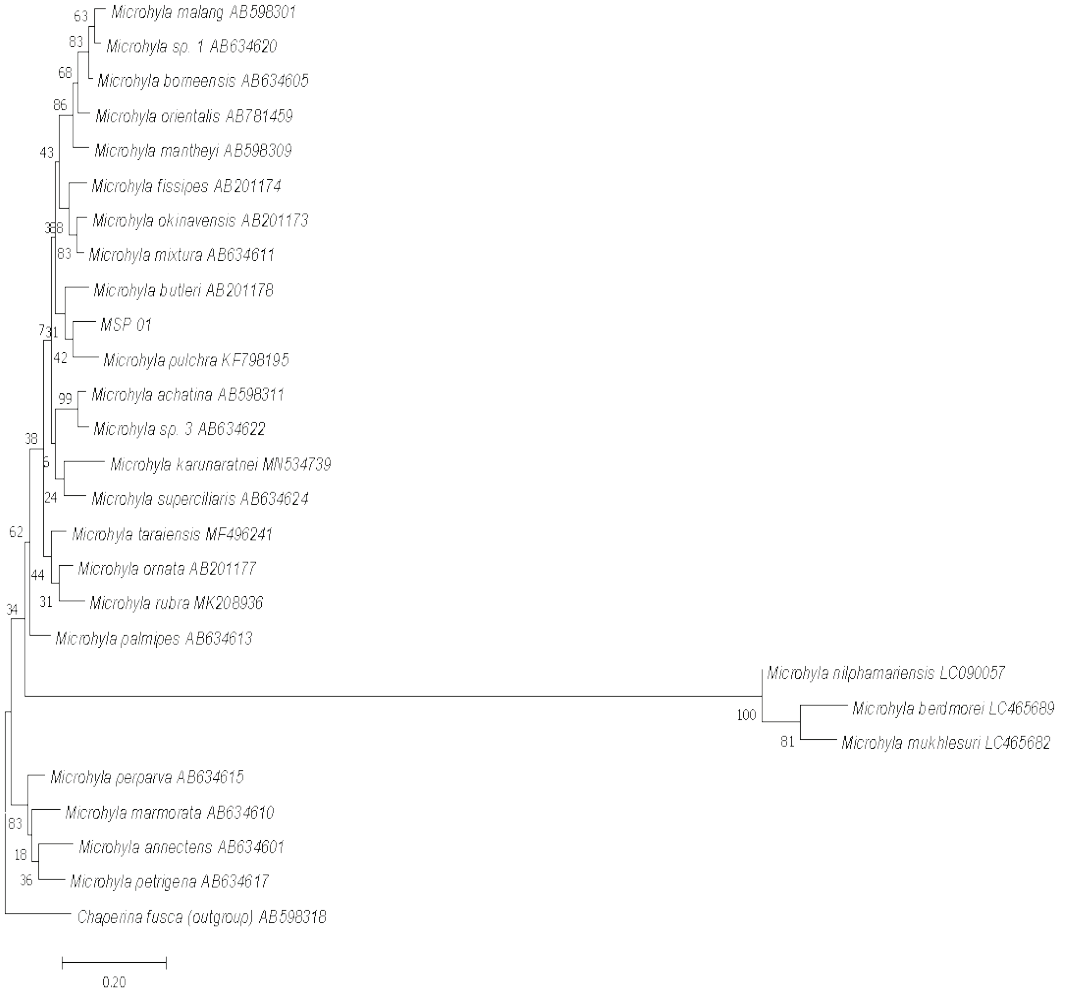


Fig. 9. Phylogenetic relationships among different species of *Microhyla* by Maximum Likelihood method using 12S rRNA gene sequences. MSP 01 indicates a specimen used in the present study. *Chaperina fusca* was used as an outgroup. Numbers on branches represent bootstrap support values.

Fig.10. Phylogenetic relationships among different species of *Microhyla* by Maximum Likelihood method using 12S rRNA gene sequences. MSP 04 indicates a specimen used in the present study. *Chaperina fusca* was used as an outgroup. Numbers on branches represent bootstrap support values.

Construction of ML trees using partial 16S rRNA gene sequences were more successful than those using partial 12S rRNA gene sequences. Multiple sequence alignment using partial 12S rRNA gene sequences were faced with difficulties in some cases where homologous sequences were not available on GenBank. Also, few of the anuran species did not have any 16S or 12S rRNA gene nucleotide sequences available in the GenBank database. Furthermore, ML trees constructed from 12S rRNA gene sequences showed erroneous results contradicting the known phylogeny of the taxa, however the reason is not understood. This suggests that 16S rRNA gene is potentially a better option for molecular identification of frogs. Otherwise, whole mitogenome analysis, where possible, could be more effective.

This study alludes that individuals of the same species of the same locality are now diversifying rapidly. In the case of *E. kalasgramensis*, individuals with very different

morphologies turned out to be the same species. On the other hand, in the case of *Fejervarya,* despite being quite similar in appearance, the individuals turned out to be of different species.

Recently in 2017 it was hypothesized that different species that contain groups with a high rate of diversification are at greater risk of extinction compared to slowly diversifying lineages[26]. Specialized amphibian species generated by these groups are also at a greater risk of being lost forever[26]. This is alarming for the various anuran populations of Bangladesh considering the many reports of cryptic unidentified species being present among them[11,27,28]. Extensive study using molecular markers for various anuran populations may reveal even more genetic diversity than what was previously perceived[29-32].

Implementation of molecular techniques can facilitate continuous observation of the divergence of these animal groups over time. Further and extensive studies are needed involving all the available groups of cryptic anurans of this country. It is high time these amphibians, especially the rapidly diversifying cryptic groups were given the much-needed attention. The cryptic groups of anurans need to be taken under an extensive plan of conservation. For that to be effective and sustainable, a rich database containing detailed information on their morphological as well as genetic information has become a dire necessity. Otherwise, a vast amount of genetic diversity may be lost before they are even discovered.

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Author Contributions

Conceived and designed the project: HJ. Performed the laboratory work: HJ, DN and MSA. Analyzed the data: HJ, DN and MSA. Contributed reagents & materials: HJ, DN and RAB. Wrote the paper: HJ, DN, HKA and RAB.

Competing Interests Statement

We have no conflict of interest to disclose.

Data Accessibility Statement

The data that support the findings of this study are openly available at NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The accession numbers have been included within the article. Sampling locations and morphological data are also included within the article.

References

1. IUCN BANGLADESH 2000. Red Book of Threatened Amphibians and Reptiles of Bangladesh. IUCN Bangladesh.
2. IUCN BANGLADESH 2015. Red List of Bangladesh: A Brief on Assessment Result 2015. IUCN Bangladesh.
3. Howlader MSA, A Nair, SV Gopalan J Merilä 2015a. A new species of *Euphlyctis* (Anura: Dicroglossidae) from Barisal, Bangladesh. PLoS ONE. **10**(2): e0116666.
4. Howlader MSA, A Nair, SV Gopalan and J Merilä 2015b. A new species of *Microhyla* (Anura: Microhylidae) from Nilphamari, Bangladesh. PLoS ONE. **10**(3).
5. Hasan M, MM Islam, MMR Khan, MS Alam, A Kurabayashi, T Igawa, *et al*. 2012. Cryptic anuran biodiversity in Bangladesh revealed by mitochondrial 16S rRNA gene sequences. Zool. Sci. **29**(9): 162–172.
6. Bickford D, DJ Lohman, NS Sodhi, PKL Ng, R Meier, K Winker, KK Ingram and I Das 2007. Cryptic species as a window on diversity and conservation. Trends Ecol.and Evol. **22**(3): 148– 155.
7. Fišer C and M Zagmajster 2009. Cryptic species from cryptic space: The case of *Niphargus fongi* sp. n. (Amphipoda, Niphargidae). Crustaceana. **82:** 593–614.
8. Schlick-Steiner BC, FM Steiner, B Seifert, C Stauffer, E Christian abd RH Crozier 2010. Integrative taxonomy: A multisource approach to exploring biodiversity. Annual Review Ento. **55:** 421–438.
9. Fišer C, CT Robinson and F Malard 2018. Cryptic species as a window into the paradigm shift of the species concept. Mol. Ecol. **27**(3): 613–635.
10. Garg S, A Das, RG Kamei and SD Biju 2018. Delineating *Microhyla ornata* (Anura, Microhylidae): mitochondrial DNA barcodes resolve century-old taxonomic misidentification. Mitochond. DNA Part B. **3**(2): 856-861.
11. Islam MM, N Kurose MMR Khan, T Nishizawa, M Kuramoto and MS Alam 2008. Genetic divergence and reproductive isolation in the genus *Fejervarya* (Amphibia: Anura) from Bangladesh inferred from morphological observation, crossing experiments, and molecular analyses. Zool. Sci. **25***:* 1084-1105.
12. Alam MS, I Jahan, S Rahman, H Jahan and K Fatema 2021. Regulatory elements in the upstream region of metallothionein gene in tilapia species. Dhaka Univ. J. Biol. Sci. **30**(1): 95–103.
13. Gouy M, S Guindon and O Gascuel 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Bio. and Evol. **27**(2): 221-224.
14. Altschul SF, W Gish, W Miller, EW Myers and DJ Lipman 1990. Basic local alignment search tool. J. Mol. Biol. **215**(3): 403–410.
15. Higgins D, J Thompson, T Gibson, JD Thompson, DG Higgins and TJ Gibson 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research. **22:** 4673-4680.

16. Kumar S, G Stecher and K Tamura 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. **33**(7): 1870-1874.

17. Sayers EW, M Cavanaugh, K Clark, J Ostell, KD Pruitt and I Karsch-Mizrachi 2019. GenBank. Nucleic Acids Research. **47:** D94–D99.

18. Fisher RA 1921. On the "probable error" of a coefficient of correlation deduced from a small sample. Metron. **1:** 3-32.

19. Nei M and S Kumar 2000. Mol. Evol. Phylogen. Oxford University Press.Asmat GSM 2007. Update list of the amphibians of Bangladesh (last updated 7 December, 2007). Bonnoprani **4**(3): 1-2.

20. Hasan MK, MMH Khan and MM Feeroz 2014. *Amphibians and Reptiles of Bangladesh – A Field Guide*. Arannyak Foundation.

21. Howlader MSA 2011. A new species of *Fejervarya* (Anura: Dicroglossidae) from Bangladesh. Zootaxa. **2761**: 41-50.

22. Howlader, M. S. A., Nair, A., & Merilä, J. (2016) A new species of frog (Anura:Dicroglossidae) discovered from the mega city of Dhaka. PloS ONE 11(3), e0149597.

23. Köhler G, L Mogk, KPP Khaing and NL Than 2019. The genera *Fejervarya* and *Minervarya* in Myanmar: Description of a new species, new country records, and taxonomic notes (Amphibia, Anura, Dicroglossidae). Verteb. Zool. **69**(2): 183–226.

24. Kurabayashi A, M Kuramoto, H Joshy and M Sumida 2005. Molecular phylogeny of the ranid frogs from the Southwest India based on the mitochondrial ribosomal RNA gene sequences. Zool. Sci.**22***:* 525-534.

25. Matsui M, A Hamidy, DM Belabut, N Ahmad, S Panha and S Sudin 2011. Systematic relationships of oriental tiny frogs of the family Microhylidae (Amphibia, Anura) as revealed by mtDNA genealogy. Mol. Phylogen. Evol. **61***:* 167–176.

26. Greenberg D and A Mooers 2017. Linking speciation to extinction: Diversification raisescontemporary extinction risk in amphibians. Evol. Letters. **1:** 40-48.

27. Mahony S and AHMA Reza 2008. A herpetofaunal collection from the Chittagong hill tracts, Bangladesh, with two new species records for the country. Zool. Sci. **27**(5): 386-395.

28. Funk WC, M Caminer and SR Ron 2011. High levels of cryptic species diversity uncovered in Amazonian frogs. Proceedings Royal Society B: Bio. Sci. **279**(1734): 1806–1814.

29. Ochoa-Vázquez D, R Rosas-Valdez, EA Martínez-Salazar and O Flores-Villela 2019. Identification of leopard frogs (Anura: Ranidae: Lithobates) distributed in some localities of the southern mexican plateau using mitochondrial DNA sequences. Mitochondrial DNA Part A. 1–10.

30. Kotaki M, A Kurabayashi, M Matsui, M Kuramoto, HT Djong and M Sumida 2010. Molecular phylogeny of the diversified frogs of genus *Fejervarya* (Anura: Dicroglossidae). Zool. Sci. **27**(5): 386-395.

31. Sanchez E, SD Biju, MM Islam, M Hasan, A Ohler, M Vences and A Kurabayashi 2018. Phylogeny and classification of fejervaryan frogs (Anura: Dicroglossidae). Salamandra. **54**(2): 109–116.

32. Fouquet A, A Gilles, M Vences, C Marty, M Blanc and NJ Gemmell 2007. Underestimation of species richness in neotropical frogs revealed by mtDNA analyses. PLoS ONE. 2: e1109.

APPENDICES

Supplementary Table 1. Specimens from the present study and their closest match; percentage within brackets denotes the similarity percentage between 16S rRNA gene sequence of the present study specimen and 16S rRNA gene sequence of the voucher specimen from GenBank.

Specimen No.	Closest Match (with 16S similarity percentage in brackets)
FSP 01	*Fejervarya dhaka* (100%)
FSP 02	*Fejervarya pierrei* (99.14%)
FSP 03	*Fejervarya asmati* (99.80%)
FSP 04	*Fejervarya pierrei* (99.00%)
FSP 05	*Fejervarya dhaka* (100%)
FSP 06	*Fejervarya orissaensis* (100%)
FSP 07	*Fejervarya dhaka* (100%)
ESP 01	*Euphlyctis kalasgramensis* (99.80%)
ESP 02	*Euphlyctis kalasgramensis* (100%)
ESP 03	*Euphlyctis aloysii* (93.32%)
MSP 01	*Microhyla berdmorei* (98.77%)
MSP 02	*Microhyla berdmorei* (99.12 %)
MSP 03	*Microhyla mymensinghensis* (99.43%)
MSP 04	*Microhyla mymensinghensis* (100%)
MSP 05	*Microhyla mymensinghensis* (99.62%)

Supplementary Table 2. GenBank Accession Numbers of sequences used for comparison in the present study

Species	Locality	GenBank Accession No.	Gene name
Fejervarya greenei	Nuwara Eliya, Sri Lanka	AB488891.1	16S rRNA
Fejervarya kirtisinghei	Laggalla, Sri Lanka	AB488890.1	16S rRNA
Fejervarya pierrei	Chitwan, Nepal	AB488888.1	16S rRNA
Fejervarya syhadrensis	Kamool, India	AB488893.1	16S rRNA
Fejervarya rufescens	Bajipe, India	AB530601.1	16S rRNA
Fejervarya keralensis	Western Ghats, India	GQ478322.1	16S rRNA
Fejervarya mudduraja	Madikeri, India	AB488896	16S rRNA
Fejervarya granosa	Mudigere, India	AB488895	16S rRNA
Fejervarya caperata	Mudigere, India	AB488894	16S rRNA

Species	Locality	GenBank Accession No.	Gene name
Fejervarya asmati	Bangladesh	KP849815	16S rRNA
Fejervarya sp. Large* (Islam *et al.* 2008)	Mymensingh, Bangladesh	AB372009	16S rRNA
Fejervarya kawamurai	Hiroshima, Japan	AB488887	16S rRNA
Fejervarya limnocharis	Bogor, Indonesia	AB277302	16S rRNA
Fejervarya moodiei	Khulna, Bangladesh	AB530508	16S rRNA
Fejervarya multistriata	Husa, China	AB488884	16S rRNA
Fejervarya orissaensis	Orissa, India	AB277304	16S rRNA
Fejervarya cancrivora	--	NC_012647.1	16S rRNA
Fejervarya dhaka	Dhaka, Bangladesh	KP849817	16S rRNA
Fejervarya sahyadris	Aralam, India	AB530605	16S rRNA
Fejervarya neilcoxi	Kerala, India	KY447317	16S rRNA
Fejervarya manoharani	Kerala, India	KY447315	16S rRNA
Fejervarya kadar	Kerala, India	KY447312	16S rRNA
Fejervarya chiangmaiensis	Omkoi, Thailand	KX834135	16S rRNA
Fejervarya cepfi	Maharashtra, India	KY447309	16S rRNA
Fejervarya andamanensis	Andaman, India	AB488899	16S rRNA
Fejervarya kudremukhensis	Kudermukh, India	AB488898	16S rRNA
Fejervarya vittigera	Quezon Province, Philippines	AY313683.1	16S rRNA
Fejervarya verruculosa	Ende, Indonesia	AB606421	16S rRNA
Fejervarya triora	Ubon Ratchani, Thailand	AB488883	16S rRNA
Fejervarya sakishimensis	Iromote, Japan	AB488886	16S rRNA
Fejervarya iskandari	Cianjur, Indonesia	AB277303	16S rRNA
Hoplobatrachus occipitalis (outgroup)	--	AF261263	16S rRNA
Fejervarya asmati	Bangladesh: Cox's Bazar	AB372079	12S rRNA
Fejervarya pierrei	Nepal: Chitwan	AB488865	12S rRNA
Fejervarya granosa	India: Mudigere	AB488872	12S rRNA
Fejervarya chiangmaiensis	Myanmar:Chin	MK958587	12S rRNA
Fejervarya dhaka	Bangladesh: Mymensingh	AB372077	12S rRNA

Species	Locality	GenBank Accession No.	Gene name
Fejervarya caperata	India: Mudigere	AB488871	12S rRNA
Fejervarya syhadrensis	India: Karnool	AB488870	12S rRNA
Fejervarya kudremukhensis	India: Kudermukh	AB488875	12S rRNA
Fejervarya greenii	Sri Lanka: Nuwara Eliya	AB488868	12S rRNA
Fejervarya kirtisinghei	Sri Lanka: Laggalla	AB488867	12S rRNA
Fejervarya rufescens	India: Padil: Mangalore	AB488874	12S rRNA
Fejervarya andamanensis	India: Andaman	AB488876	12S rRNA
Fejervarya muangkanensis	Myanmar: Ayeyarwady	MK621375	12S rRNA
Fejervarya cancrivora	China: Guangxi: Beihai	EU652694	12S rRNA
Fejervarya moodiei	Bangladesh: Khulna	AB372082	12S rRNA
Fejervarya vittigera	Philippines: Quezon Province	AY313683	12S rRNA
Fejervarya limnocharis	Indonesia: Bogor	AB277286	12S rRNA
Fejervarya multistriata	China: Husa	AB488861	12S rRNA
Fejervarya kawamurai	Japan: Hiroshima	AB488864	12S rRNA
Fejervarya triora	Thailand: Ubon Ratchani	AB488860	12S rRNA
Fejervarya iskandari	Indonesia: Cianjur	AB277287	12S rRNA
Fejervarya kupitzi	Myanmar:Sagaing	MK958593	12S rRNA
Fejervarya orissaensis	Yangon: Myanmar	MK621361	12S rRNA
Fejervarya sp. Large* (Islam *et al.* 2008)	Bangladesh: Mymensingh	AB372019	12S rRNA
Fejervarya mudduraja	India: Madikeri	AB488873	12S rRNA
Hoplobatrachus crassus (outgroup)	Bangladesh: Khulna	AB273158	12S rRNA
Euphlyctis aloysii	Bajpe, Mangalore, Karnataka, India	AB272606	16S rRNA
Euphlyctis mudigere	Mudigere, Western Ghats, India	AB377109	16S rRNA
Euphlyctis cyanophlyctis	Mudikari, Karnataka, India	AB167938	16S rRNA
Euphlyctis hexadactylus	Mangalore, Karnataka, India	AB167941	16S rRNA

Species	Locality	GenBank Accession No.	Gene name
Euphlyctis ehrenbergii	Yemen	AY014367	16S rRNA
Euphlyctis kalasgramensis	Kalasgram, Barisal, Bangladesh	KP091862	16S rRNA
Euphlyctis karaavali	Sanikatta, Kumta, India	KU870373.1	16S rRNA
Hoplobatrachus occipitalis	Nyanga, Gabon	KY080138	16S rRNA
Euphlyctis aloysii	Bajpe, Mangalore, Karnataka, India	AB273171	12S rRNA
Euphlyctis hexadactylus	Punducherry, India	KU870379.1	12S rRNA
Euphlyctis karaavali	India	KU870375.1	12S rRNA
Euphlyctis mudigere	Mudigere, Western Ghats, India	AB377110	12S rRNA
Euphlyctis cyanophlyctis	Mudikari, Karnataka, India	AB167910	12S rRNA
Euphlyctis kalasgramensis	Kalasgram, Barisal, Bangladesh	KP091878.1	12S rRNA
Chaperina fusca (outgroup)	Crocker, Sabah, Malaysia	AB598318.1	12S rRNA
Microhyla ornata	Dhawad, Karnataka, India	AB201188	16S rRNA
Microhyla butleri	Bangkok, Thailand	AB201189	16S rRNA
Microhyla perparva	Kubah National Park, Malaysia	GU154884	16S rRNA
Microhyla mixtura	China, Sichuan	AB634669	16S rRNA
Microhyla malang	Malaysia: Sabah, Tawau Hills	AB598325	16S rRNA
Microhyla achatina	Indonesia: Java, Ungaran	AB598335	16S rRNA
Microhyla superciliaris	Pahang, Temerloh, Malaysia	AB634682	16S rRNA
Microhyla annectens	Pahang, Cameron, Malaysia	AB634659	16S rRNA
Microhyla palmipes	Sumatra,Bengkulu, Indonesia	AB634671	16S rRNA
Microhyla marmorata	Houapan, Xamneua, Laos	AB611955	16S rRNA
Microhyla petrigena	Sarawak, Bukit Kana, Malaysia	AB634675	16S rRNA
Microhyla nilphamariensis	Bangladesh:Nilphamari, Barua, Berakhuti	LC090057	16S rRNA

Species	Locality	GenBank Accession No.	Gene name
Microhyla borneensis	Malaysia: Sarawak, Serapi	AB634663	16S rRNA
Microhyla "sp. 1"	Malaysia: Sabah, Crocker	AB634678	16S rRNA
Microhyla orientalis	Indonesia: Bali, Wongaya Gede	AB781470	16S rRNA
Microhyla mantheyi	Malaysia: Selangor, Templer Park	AB598333	16S rRNA
Microhyla kodial	India: Karnataka, Mangalore	MH807394	16S rRNA
Microhyla heymonsi	China: Sichuan, Zihuai	KU840570	16S rRNA
Microhyla cf. heymonsi	India: Andaman Islands	MH807391	16S rRNA
Microhyla berdmorei	Myanmar: Sagaing	KC179981	16S rRNA
Microhyla cf. berdmorei	Myanmar: Magway, Pakoku	KM509166	16S rRNA
Microhyla "sp. 3"	Indonesia: Sumatra, Lampung	AB634680	16S rRNA
Microhyla okinavensis	Japan:Ryukyu, Amami, Amamioshima	AB201184	16S rRNA
Microhyla mukhlesuri	Bangladesh: Chittagong, Raozan	AB543608	16S rRNA
Microhyla fissipes	China: Anhui, Huangshan	AB201185	16S rRNA
Microhyla chakrapanii	India: Andaman Islands	MH807389	16S rRNA
Microhyla mymensinghensis	Bangladesh: Mymensingh	AB530531	16S rRNA
Microhyla pulchra	China: Guangdong	KF798195	16S rRNA
Microhyla rubra	India: Karnataka, Shimoga	MH807410	16S rRNA
Microhyla mihintalei	Sri Lanka: Anuradhapura	KU214861	16S rRNA
Microhyla taraiensis	Nepal: Jamun Khadi, Jhapa district	KY655952	16S rRNA
Microhyla ornata	India:Karnatak, Dharwad	AB201188	16S rRNA
Microhyla sholigari	India: Karnataka, BR Hills	MH807423	16S rRNA
Microhyla darreli	India: Thiruvananthapuram, Karamana	MH807390	16S rRNA
Microhyla karunaratnei	Sri Lanka: Sinharaja FR	MN534630	16S rRNA

Species	Locality	GenBank Accession No.	Gene name
Microhyla zeylanica	Sri Lanka: Horton plains	MH807428	16S rRNA
Microhyla laterite	India: Karnataka, Manipal	KT600671	16S rRNA
Chaperina fusca (outgroup)	Sabah, Danum Valley, Malaysia	DQ283145	16S rRNA
Microhyla malang	Malaysia: Sabah, Tawau Hills	AB598301	12S rRNA
Microhyla "sp. 1"	Malaysia: Sabah, Crocker	AB634620	12S rRNA
Microhyla borneensis	Malaysia: Sarawak, Serapi	AB634605	12S rRNA
Microhyla orientalis	Indonesia: Bali, Wongaya Gede	AB781459	12S rRNA
Microhyla mantheyi	Malaysia: Selangor, Templer Park	AB598309	12S rRNA
Microhyla fissipes	China: Anhui, Huangshan	AB201174	12S rRNA
Microhyla okinavensis	Japan:Ryukyu, Amami, Amamioshima	AB201173	12S rRNA
Microhyla mixtura	China: Sichuan	AB634611	12S rRNA
Microhyla butleri	Bangkok, Thailand	AB201178.1	12S rRNA
Microhyla pulchra	China: Guangdong	KF798195	12S rRNA
Microhyla achatina	Indonesia: Java, Ungaran	AB598311	12S rRNA
Microhyla "sp. 3"	Indonesia: Sumatra, Lampung	AB634622	12S rRNA
Microhyla karunaratnei	Sri Lanka: Sinharaja FR	MN534739	12S rRNA
Microhyla superciliaris	Pahang, Temerloh, Malaysia	AB634624.1	12S rRNA
Microhyla taraiensis	--	MF496241	12S rRNA
Microhyla ornata	--	AB201177.1	12S rRNA
Microhyla rubra	India	MK208936	12S rRNA
Microhyla palmipes	Sumatra, Bengkulu, Indonesia	AB634613.1	12S rRNA
Microhyla nilphamariensis	Bangladesh:Nilphamari, Barua, Berakhuti	LC090057	12S rRNA
Microhyla berdmorei	Thailand: Phrae, Mae Yom	LC465689	12S rRNA
Microhyla mukhlesuri	Thailand: Bangkok	LC465682	12S rRNA

Species	Locality	GenBank Accession No.	Gene name
Microhyla perparva	Malaysia:Sarawak, Mulu	AB634615.1	12S rRNA
Microhyla marmorata	Houapan, Xamneua, Laos	AB634610.1	12S rRNA
Microhyla annectens	Pahang, Cameron, Malaysia	AB634601.1	12S rRNA
Microhyla petrigena	Sarawak, Bukit Kana, Malaysia	AB634617.1	12S rRNA
Microhyla karunaratnei	Sri Lanka: Sinharaja FR	MN534739	12S rRNA
Microhyla sholigari	India: Karnataka, BRTTR	KT600667	12S rRNA
Microhyla laterite	India: Karnataka, Manipal	KT600663	12S rRNA
Microhyla heymonsi	China: Sichuan, Zihuai	KU840495	12S rRNA
Chaperina fusca (outgroup)	--	AB598318.1	12S rRNA

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