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IDENTIFICATION OF ORISSA FROG *FEJERVARYA ORISSAENSIS* FROM BANGLADESH BASED ON 16S AND 12S rRNA GENES

Hafisha Khatun Anee, Ashfaqul Muid Khandaker
Marufa Akter and Rowshan Ara Begum*

Department of Zoology, University of Dhaka, Dhaka -1000, Bangladesh

ABSTRACT: In this study, we attempted species-level identification of frog specimen collected from Faridpur district of Bangladesh beyond it' s location outside Orissa, India. Specimen was identified morphologically at genus level as *Fejervarya* sp. belonging to the family Dicroglossidae using finger formula F3>F4>F1=F1 where F denote as toe finger. From the study two nucleotide sequences of 16S and 12S rRNA genes were obtained which contained 508bp and 408bp respectively. The Sequences were submitted to Gene Bank database with the accession number OQ231604 and OQ240197 for 16S and 12S rRNA gene sequences. Furthermore, the 16S rRNA gene sequence was used as molecular bar-code for the identified Orissa frog *F. orissaensis* species from Bangladesh. GC content of partial 12S and 16S rRNA genes have been calculated as 44% and 45% respectively. For 16S rRNA gene sequence there was no intra specific divergence. Whereas the inter specific polymorphic divergence were calculated 4.13% and 6.3% when the collected Orissa frog *F. orissaensis* was compared with that of *F. iskandari* and *F. kupitzi,* respectively. In case of 12S rRNA gene intra specific divergence was found 2.45% where the inter specific divergence were 4.41% and 6.86% when the collected Orissa frog *F. orissaensis* was compared with that of *F. iskandari* and *F. limnocaris,* respectively. Maximum likelihood tree also indicates that our sample Orissa frog formed a monophyletic group with *F. orissaensis* in both the cases of 16S and 12S rRNA genes and thus can be concluded as closely related. Therefore, the collected specimen was identified to be belonging to *F. Fejervarya orissaens is* which would be first report from Bangladesh outside Orissa, India.

Key words: *Fejervarya orissaensis*, 16S and 12S rRNA gene Sequencing, First report, Bangladesh

INTRODUCTION

In Bangladesh, the taxa of amphibian species has increased nearly 30% over the last few decades (IUCN Bangladesh 2000, 2015), many of which have

*Author for corresponding: <rowshanbegum@du.ac.bd>
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previously been misidentified morphologically with other existing species. Bangladesh is situated between the Himalayan mountains and the Bay of Bengal (20°34”N-26°33”N, 88°01”E-92°41”E ´), is the ideal habitat for amphibians because of its moisture, shady and warm environment. In recent years, many new species, genera and families of amphibians have been discovered on the Indian subcontinent (Biju and Bossuyt, 2003). Despite these recent discoveries, the variety of frog species in South and Southeast Asia remains underappreciated, owing to amphibian morphological homoplasy (Stuart *et al.* 2006). Gender, season, habitat and a variety of other factors all have a role in the variances that occur among individuals of the same species. For this reason, molecular analysis is a foolproof method of correctly identifying distinct amphibian species in any of their many forms or life phases, regardless of their size, age, gender, or physical state. In fact morphological allozyme and molecular analyses as well as crossing experiments have discovered many undescribed frog species throughout complexes previously thought to be one species in South to East Asian countries (Hasan *et al.* 2012; Sumida *et al.* 2007).

As mitochondrial DNA is naked, it has high mutations rate. Because animal mtDNA evolves at a faster rate than human mtDNA, substantial amounts of closely related species showed sequence variation, which is a useful trait for species identification (Allio *et al.* 2017). Furthermore, mtDNA is transferred maternally in most species due to sperm dilution (Sato and Sato, 2013). As mitochondrial DNA inheritance is fully maternal, the interpretation of species identification data is substantially simplified. It is also easier to recover from low-quantity or deteriorated hair where nuclear DNA is fully absent. For species identification, mtDNA samples have a clear advantage over nuclear genome-based methods since they are present in numerous copies per cell (Yang *et al.* 2013) 12S and 16S rRNA mt gene sequencing has been successfully applied for taxonomic studies, molecular identifications, polymorphism analyses, comprehensive phylogenetic analyses etc. (Cawthorn *et al.* 2012). The accuracy rate for the identification by using 12S, 16S rRNA, and CO1 mt genes is quite impressive and is practical for further exploration of animal diversity.

Here, in addition to an extensive morphological analysis, we take a molecular approach to identify the specimen from Bangladesh up to species level through 16S gene sequence analysis. In addition, 12S rRNA gene sequence was also incorporated.

The objective of the study was to identify *F. Fejervarya orissaensis*, a newly added to the taxa of amphibian found in Bangladesh beyond their claimed endemic geographical areas of India. This is the first record from of the species

F. orissaensis from Bangladesh. Furthermore the data' s may also be helpful in case of conservation and proper management of this species.

MATERIAL AND METHODS

Sample collection and morphological analysis: Frog sample was collected from Faridpur district, Bangladesh. The collected frog was nicely photographed (Figure 2) and further study was conducted at the Laboratory of Genetics and Molecular Biology, Department of Zoology, University of Dhaka. The morphometric features were analyzed and meristic measurements (in mm) were taken using digital slide-calipers (Table 2 and 3). Total 22 morphometric characters were measured by following the description mentioned in Islam *et al.* (2012) and Howlader *et al.* (2015). The sample was preserved in 70% alcohol and stored in at 4°C refrigeration.

DNA extraction, PCR amplification and sequencing: About 25 mg of muscle tissue from the innermost thigh region of the sample was used for DNA extraction.Here, 'Monarch Genomic DNA Extraction Kit by Biolab' was used. PCR amplification of the target regions were performed using GoTaq® G2 Hot Start Master Mixes Kit according to recommended protocol. Universal 16S primer and designed 12S gene primer were used (Table 1) (Faucher *et al.* 2016). Annealing temperature was 55°C for 16S rRNA and 46°C for 12S rRNA, respectively. PCR products were analyzed by 0.8 % agarose gel electrophoresis (Figure 2). Amplified samples were purified at room temperature using GeneJet PCR Purification Kit (ThermoFisher Scientific) according to the manufacturer's protocol. PCR amplicon was sequenced using Sanger dideoxy sequencing method from Macrogen, Korea.

Table 1. Primers used for the study

Primers	Sequences
16S rRNA-Forward	5' - CGCCTGTTTACCAAAAACAT-3'
16S rRNA -Reverse	5'- CCGGTCTGAACTCAGATCACGT-3'
12S rRNA-Forward	5'- CGACAGCTAGGAAACAAACTGG-3'
12S rRNA-Reverse	5'- CCATGTTACGACTTGCCTCTTC-3'

Bioinformatic analysis: 16S and 12S rRNA gene sequences of sample frog were matched with other existing sequence of closely related frogs which were available at NCBI GenBank database to identify the specimen at species level. Mega 11 software was used to perform multiple sequence alignment with our

sequencing data. In addition, a Maximum Likelihood tree based on the Tamura-Nei model was utilized to determine the phylogenetic relationship, and polymorphic sites were also counted and polymorphic divergence were calculated to find out the intra and inter species variation to determine the genetic distance between our sample sequence and the data from GenBank. The species-level identification was attempted to be confirmed by evaluating all of these data.

RESULT AND DISCUSSION

Morphological Analysis: The mormophometric and meristic properties were analyzed. Body length was calculated 42mm. The snout was comperatively more pointed than the other closely related *Fejervarya* species. Hind limbs was moderately long. Finger and toe tips was rounded, slightly swollen at the tip. Fourth toe webbing extends less than half way between the distal and penultimate subarticular tubercles. Limbs contains complete or incomplete dark cross bars (Deuti *et al* 2013). Based on these data, the frog was identified up to genus level as *Fejervarya* sp. (Deuti *et al* 2013, Oliver 2015 and Matsui *et al.* 2005) (Table 2 and 3).

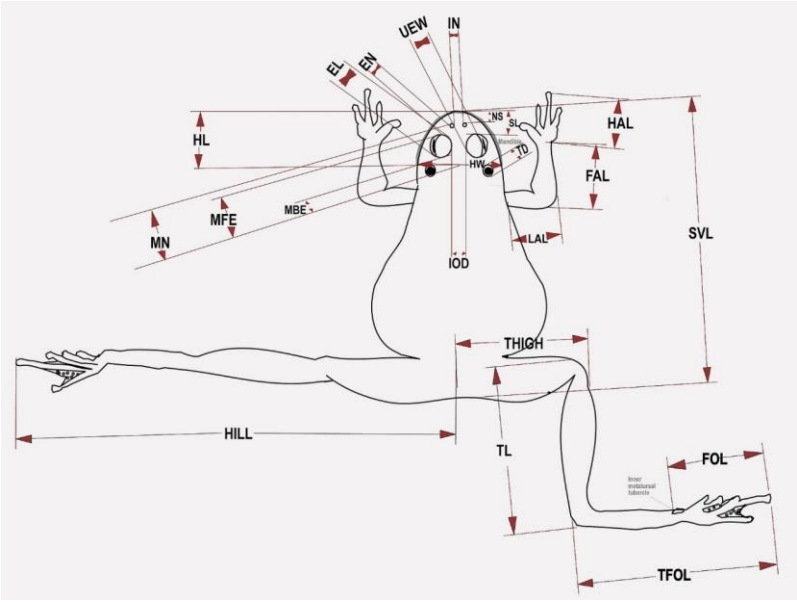

Fig.1**.** Illustration of morphological traits used in this study of a typical frog Oliver *et al.* (2015). Here: SVL (tip of snout to vent distance); HL (head length); HW (head width); NS (nostril snout length); EL (eye width); MN (distance from back of mandible to nostril); MFE (from back of mandible to front of the eye); MBE (distance from back of mandible to back of the eye); SL (snout length; from anterior corner of eye to the tip of snout); IN (internarial distance); IOD (interorbital distance); EN (from front

of eyes to the nostril); UEW (maximum width of upper eyelid); HAL (hand length; distance from proximal end of outer palmar metacarpal tubercle to tip of third finger); THIGHL (thigh length; distance from vent to knee); TFOL (length of tarsus and foot; from the heel to tip of fourth finger distance); TL (tibia length; distance from knee to heel);.

Table 2. Morphometric study of sample frog for the current study

Index	***Fejervarya* sp. *(sample frog)***
Body size	Small (42mm)
Head size	Small (11mm)
Dorsal colour	Brownish grey warty skin with darker blotches
Ventral colour	White

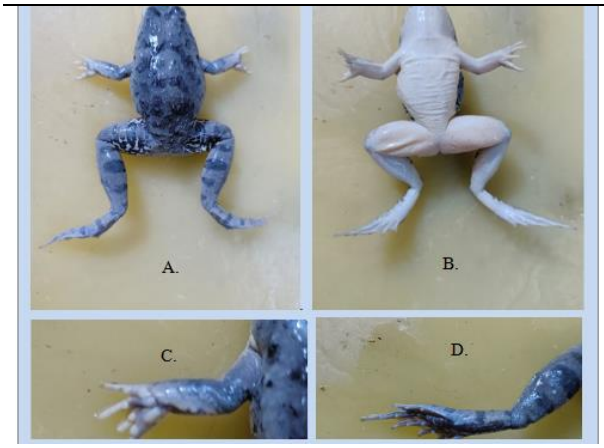

Fig. 2. Morphological presentation of the Orissa frog *Fejervarya* sp. from the current study where figure (A) is showing dorsal view, (B), (C) and (D) showing ventral view, magnified view of ventral side of foot and hand respectively. Toe finger formula 3>4>2=1. Collected from Saltha, Faridpur, Bangladesh.

Table 3. Meristic measurements of sample frog for the current study

***Fejervarya* sp. (sample frog)**	
Morphological Traits	**Measurement(mm)**
SVL	23.75
HL	7.5
HW	9.25
MN	7.5
SL	4
MFE	5.5
MBE	2.0
IN	1.5
IOD	3.5
EN	1.5
NS	1.0

EL	2.75
UEW	1.75
TD	2
HAL	5
THIGH	10.5
TL	12
TFOL	15.5
F1	2
F2	2
F3	4
F4	2.5
Finger Formula:	F3>F4>F2=F1

Molecular Analysis: In *Fejervarya* sp. (sample frog) 16S and 12S rRNA genes were amplified (Figure 3). Both forward and reverse sequences were aligned properly and no ambiguities observed, for that reason only one sample was used for each gene analysis (data not shown). The total length of these genes were 508 bp and 408bp respectively. 16S and 12S rRNA gene sequences were submitted to NCBI GenBank database with the accession number OQ231604 and OQ240197 respectively which are first reported data for both 12S and 16S rRNA genes from Bangladesh for the Orissa frog *F. orissaensis*. GC content of partial 12S and 16S rRNA genes have been calculated as 44% and 45% respectively. Where AT/GC ratio is 1.27 and 1.22 for 12S and 16S rRNA respectively.

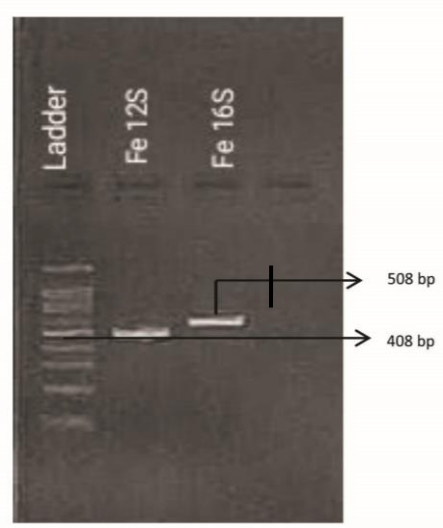

Fig. 3. PCR gel run bands of sample frog *Fejervarya* sp. for 12S and 16S rRNA gene at 55° annealing temperature.

In *Fejervarya* sp. (sample frog) 16S rRNA gene sequence 100% matches with *F. orissaensis* from Myanmer regions (MK621431) (Table 5) and in case of

12S rRNA gene sequence, it matches about 96% with *F. orissaensis* from Thailand (AB277288) and Indian (AB277289) regions (Table 6). From this analysis it can be initially confirmed as *F. orissaensis.* Yet extensive studies based on CO1, Cyt b, ND1 gene of mitochondrial genome as well as some other genes from nuclear genome are needed for further confirmation.

Table 5. Comparison of Partial 16S rRNA nucleotide sequence of sample *Fejervarya* sp. with the other gene sequences of *Fejervarya* sp. collected from NCBI gene bank database

Scientific Name	Percentage Identity	Accession Number	Location
Fejervarya orissaensis	100.00%	MK621431	Myanmar, 2019
Fejervarya sp.	100.00%	AB372009	Mymensingh, BAU Campus
Fejervarya sp.	100.00%	AB530504	Bangladesh:Sylhet, Golapganj
Fejervarya iskandari	95.28%	AJ292016	Indonesia
Fejervarya Kupitzi	93.7%	MK958580	Myanmer

Table 6. Comparisn of Partial 12S rRNA nucleotide sequence of sample *Fejervarya* sp. with the other gene sequences of *Fejervarya* sp. collected from NCBI gene bank database

Scientific Name	Percentage Identity	Accession Number	Location
Fejervarya orissaensis	95.90%	AB277288	Thailand, 2011
Fejervarya orissaensis	95.90%	AB277289	India, 2011
Fejervarya sp.	100.00%	AB372073	Bangladesh: Mymensingh, BAU Campus
Fejervarya iskandari	94.41%	AB277287	Indonesia
Fejervarya limnocharis	93.2%	KU840479	Brazil

Intra specific variation of 16S and 12S rRNA genes: Multiple sequence alignment was operated to investigate the intra specific variation of 16S rRNA and 12S rRNA genes among the individuals of *F. orissaensis* with our sample Orissa frog (*F. orissaensis*). The sequence that was found in the present study was compared with other sequences retrieved from NCBI the GenBank database. The 16S rRNA sequence of our sample Orissa frog matches 100% with *F. orissaensis* (MK621431) and *Fejervarya* sp. (AB372009 and AB530504) (Table 5). Thus showing no significant intra species variation (Table 7). In case of 12S rRNA, 100% match was found with *Fejervarya* sp. collected from Bangladesh: Mymensingh, BAU Campus with accession number AB372073. But this was not identified up to species level. 10 polymorphic sites were revealed after comparing the sequences of *F. orissaensis* (Present study) and *F. orissaensis* (AB277289) and are shown in Table 6. The intraspecific genetic diversion was 2.45% for 12S rRNA gene (Table 8). According to Chowdhury *et al.* (2021) when *Hydrophylax tytleri* and *H. leptoglossa* from different countries were compared the intraspecific variation was found to be 1.29%.

In case of *Microhyla ornata* about similar type of intra species variation of 1.52% was observed between the sequences with accession number AB201177 and MN534723. From this it can be said that the previously unidentified *Fejervarya* sp. from Bangladesh could be actually *F. orissaensis.*

Table 7: Intra and inter specific polymorphic sites analysis of 16S rRNA gene sequence of *F. orissaensis* (present study)

Polymorphic sites	*F. Orissaensis (Presnt study)* (OQ231604)	Intra Specific variation	Inter Specific Variation	
		F. orissaensis (MK621431)	*F. Iskandari* (AJ292016)	*F.Kupitzi* (MK958580)
108	A			G
130	T		C	
170	A			T
173	A			T
211	C			T
218	T		C	C
225	T		C	C
226	T		A	A
234	C		T	
237	T			C
245	A		C	C
246	T			C
248	C		T	T
260	C	No divergence found	T	
261	C		T	
283	G		A	A
296	C		T	T
305	T		C	
314	A		G	G
317	T			C
334	A		G	
335	C			T
336	T		C	
340	C		G	T
341	C			T
354	T		C	C
359	C		T	T
367	C		T	T
368	A		T	
378	A			G
380	A			T
429	A		T	-
430	A			-
433	G			A
434	C			G
438	T			C
439	A			T
440	T			A
441	C			T
442	G			C
443	C			A
444	G			C
457	G			-

Inter specific variation of 16S and 12S rRNA gene: Inter specific variation of 16S rRNA gene sequence of *F. orissaensis* (present study) was calculated after comparing it with *F. iskandari (*AJ292016) and *F. Kupitzi* (MK958580) which were 4.13% and 6.3% respectively (Table7). In case of 12S rRNA gene sequence, it was compared with *F. iskandari (*AB277287) and *F. limnocharis* (KU840479) and the polymorphic divergence calculated were 4.41% and 6.86% respectively (Table 8). Another research showed around 14% polymorphic divergence between *H. leptoglossa* and *H. tytleri* in case of 16S rRNA gene sequence*(*Chowdhury *et al.* 2021). The sequence length was much shorter, which may have caused the higher polymorphic divergence. The high polymorphic divergence may also caused by the fact that the frogs have become evolutionary too distant from each other. The nucleotide sequence divergences of the 12S rRNA gene were 0.25–4.83% within the Far Eastern frogs, 0.25-6.22% within the European frogs which correspond to our study (Sumida *et al.* 2000).

Table 8. Intra and inter specific polymorphic sites analysis of 12S rRNA gene of *F. Orissaensis (Present study)*

Polymorphic sites	*F. Orissaensis G. (*present study) (OQ240197)	Intra Specific variation	Inter Specific Variation	
		F. Orissaensis (AB277289)	*F. iskandari* (AB277287)	*F. limnocharis* (KU840479)
3	A		T	
7	T	C	C	
8	T			A
12	C			T
15	A			G
17	C			A
20	C			A
28	A	C		C
42	C			T
43	T			C
44	T	C		
97	A			C
120	A		T	T
125	T	A	A	A
145	C			T
162	T			C
184	C		A	
186	C			T
189	C	T	T	T
191	C		T	
199	T	A		
203	T	C	C	C
204	T	C	C	A
205	C	A		
206	C		T	T
207	C	T		T
208	C			-
234	G			A
237	C			T

251	T	C	
252	G	T	
270	A	G	
271	T		C
272	C	T	
292	T	C	C
305	C		A
308	A		-
336	A	G	
342	A		T
351	A		T
368	A	G	
369	C		T
403	C	T	G

Maximum Likelihood Tree: Phylogenetic tree were constructed in order to identify the phylogenetic position of *F. orissaensis* of the present study. A comparative study based on 16S and 12S rRNA gene sewuences was performed among other *Fejervarya* sp. where *Panthera tigris* was used as an out group using the maximum likelihood method (Figure 4 and 5). Maximum likelihood tree indicates that our sample Orissa frog formed a monophyletic group with *F. orissaensis* in both the cases of 16S and 12S rRNA gene sequences and thus can be concluded as closely related.

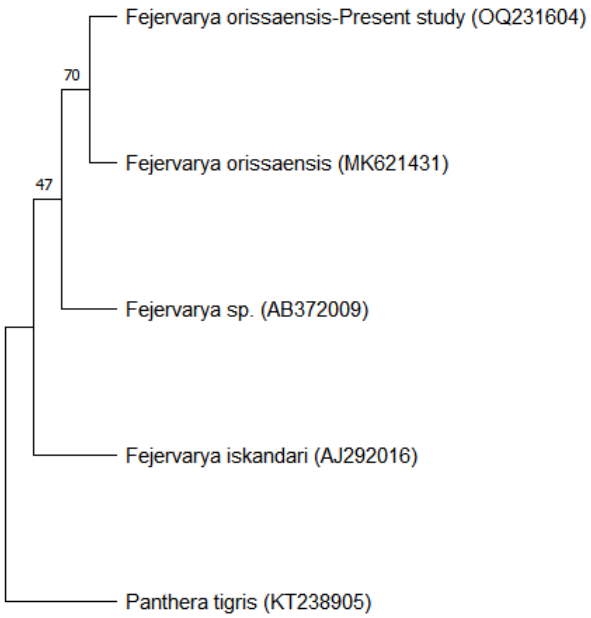

Fig. 4. Evolutionary tree based on maximum likelihood method indicating the evolutionary relation of our sample Orissa frog *F. orissaensis* with other species based on 16S rRNA gene sequence.

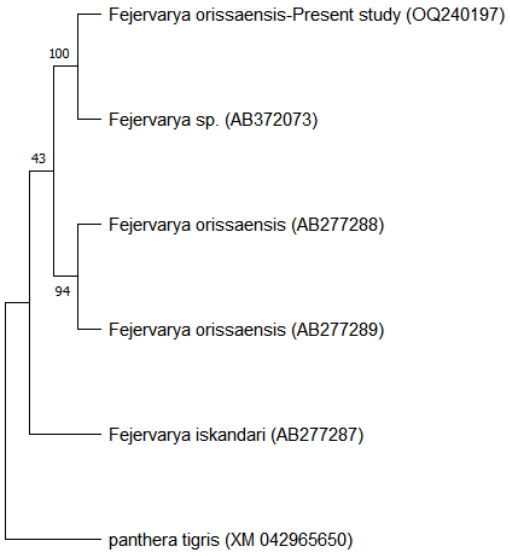

Fig. 5. Evolutionary tree based on maximum likelihood method indicating the evolutionary relation of our sample Orissa frog *F. orissaensis* with other species based on 12S rRNA gene sequence.

CONCLUSION

Using molecular techniques can be helpful when species identification is hard using only morphological characteristics. Molecular data is also helpful for understanding phylogenetic relationship of this species with other species. Proper identification of each species is also very important for their proper management and conservation. Present study attempted to identify *F. orissaensis* at molecular level based on 16S rRNA and 12S rRNA gene sequences. More studies based on CO1, Cyt b, ND1 genes of mitochondrial genome and certain nuclear genes needed to be studied for further clarity.

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