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MOLECULAR CHARACTERIZATION OF COPY'S FROG *HYDROPHYLAX LEPTOGLOSSA* BASED ON 16S rRNA GENE

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Abstract: Copy's frog, commonly observed in Bangladesh, is ecologically important for evergreen forest. Sample was collected from Satchari National park, Hobigonj, Sylhet. Attempts were made to combine morphological and molecular techniques for identification and analysis of evolutionary relation of Copy's frog with anuran species. Morphological identification of *Hydrophylax* sp. was conducted based on distinguishing characteristics of the body and different body measurement parameter. The species was identified morphologically as *Hydrophylax leptoglossa* based on the taxonomic key of finger toes and formula was 3>1>4>2. Simultaneously genomic DNA was isolated using CTAB extraction method. The 16S rRNA gene was amplified from the genomic DNA by polymerase chain reaction method using the pair of universal primers. Amplified product was a 308 bp fragment that generated AT/GC ratio of 1.08% once sequenced. In the present study comparative analysis of intraspecific and interspecific variation was conducted based on nucleotide sequence of 16S rRNA gene collected from GenBank. The intraspecific variation compares between Southeast Asia and Bangladesh was 1.29% for 16S rRNA gene. The interspecific genetic divergence was studied between *H. leptoglossa* and *H. tytleri* was observed 14.29%. The nucleotide sequence of the present study will be used as DNA barcode for that particular individual collected from Sylhet, Bangladesh. Furthermore, a molecular phylogenetic tree was constructed, where all the anuran species from a monophyletic group. Four species of *Hydrophylax* form monophyletic group and is consistent with relation based on morphology.

Key words: 16S rRNA gene, Polymorphic loci, *Hydophylax leptoglossa*

INTRODUCTION

International Union for Conservation of Nature has categorized the amphibians as an endangered group of animals (IUCN 2015, Berger *et al.*, 1998). The number of amphibian species is still increasing and there is

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least a third of known amphibian species (Bickford *et al.*, 2007, Fouquet *et al.*, 2007). While some amphibian species are disappearing every year, a number of new species are being discovered (Welsh *et al.*, 1998). They have different life stages, high phenotypic plasticity and high level of cryptic diversity between species. These traits often make it most difficult to correctly identify species, especially at early life-stages (Larson *et al.*, 1993). Moreover, most amphibian have complex life cycles which make the identification complicated (Bickford *et al.*, 2007). Hence, recently they have been in the center of research and public attention.

Morphometrics, refers to the quantitative analysis of form, a concept that encompasses size and shape is commonly used for morphological identification. Morphometric analyses are commonly performed on organisms, and are useful in analyzing their fossil record, the impact of mutations on shape, developmental changes in form, covariances between ecological factors and shape, as well for estimating quantitative-genetic parameters of shape (Hasan *et al.*, 2014).

Nowadays species identification by using conventional keys only is not enough. Molecular techniques should be implemented for correct identification. In that case, mitochondrial DNA (mtDNA) plays an important role to species identification and evolutionary relation study. Among mtDNA genes 16S ribosomal RNA (16S rRNA) gene is a special region of mitochondrial genome and has been considered as one of the most informative regions used in phylogenetic studies (Bej *et al.*, 2012; Patwardhan *et al.*, 2004). It is a component of the 30S small subunit of prokaryotic ribosome and is 1.5 kb in length (Devereux and Wilkinson 2004). 16S rRNA gene variation between species and within species is stable (Yang *et al.*, 2014). Although it was originally used to identify an organism, 16S rRNA sequencing was subsequently found to be capable of reclassifying the organism into completely new species, or even genera and is also used for bar coding of certain amphibians (Vences *et al.*, 2005; Weisburg *et al.*, 1991).

To investigate the genetic characteristics of *H. leptoglossa* in this study a partial sequence of the 16S rRNA of *H. leptoglossa* was determined. The sequence was aligned with other sequence of *H. leptoglossa* and *H. tytleri* to study the number of polymorphic sites. Phylogenetic relationship was observed by construct a phylogenetic tree.

MATERIAL AND METHODS

Morphological analysis: The Cope's frog or locally known as long-tongued frog, *Hydrophylax leptoglossa* was collected from Satchari National Park, Hobigonj, Sylhet. The fresh specimen was brought to the laboratory of Genetics

and Molecular Biology, Department of Zoology, University of Dhaka for further study. The sample was morphologically identified by conventional method. The measurement of different body part of collected frog is shown in Fig. 1. The sample was stored in refrigerator, minimum temperature at 4° C as voucher sample, labeled as H1.

DNA extraction, PCR amplification and sequencing: Genomic DNA was extracted from 0.05 gm muscle tissue of the observed frog species following a method (Doyle and Doyle 1987) with few modification (Saha *et al.*, 2019). 100µl CTAB (Cetyltrimethyl ammonium bromide) extraction buffer and 10µl of proteinase k were added. This mixture was incubated at 56° C for overnight. After digestion, equal volume of phenol chloroform was added with the supernatant and centrifuged at 13000 rpm for 5 min. DNA was precipitated with ethanol and dissolved in distilled water. Partial region of 16S rRNA gene was amplified using universal primers and was visualized in 1.5% agarose gel (Faucher *et al.*, 2016). Then, the amplified PCR product was purified using a purification kit (Saha *et al.*, 2019). Finally, nucleotide sequence was determined using direct sequencing method.

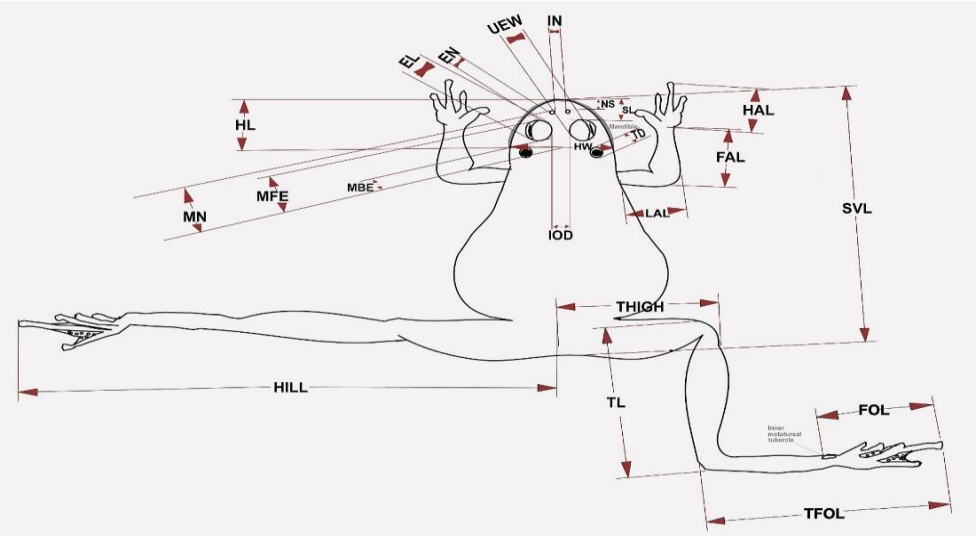

Fig. 1: Measurement of different part of Cope's frog.

Bioinformatics analysis: 16S rRNA gene sequence of experimental frog species and other existing sequence of Cope's frog were collected from GenBank database (NCBI). Alignment was done with the help of serial cloner software. MEGA 7 software was also used for multiple sequence alignment which was

followed by polymorphic site analyses. Phylogenetic tree was constructed using neighbor joining method (Saha *et al.,* 2019).

RESULTS AND DISCUSSION

Morphological analysis: The dorsal part of the copy's frog *H. leptoglossa* was dark brown with scattered black spots (Figure-2, Table-1). Head and back skin is distinctly granulated. Tympanum is distinct and about three-fourth of the eye diameter. Fingers are free and toes are two-third webbed. Tips of all digits are slightly dilated. The length of fingers was compared and 3rd one was found to be largest and 2nd one was smallest. The rest two are as follows and formula was 3>1>4>2 (Table-2). Tibio-tarsal articulation reaches beyond the snout. Collected sample was under the Class Amphibia, Order Anura. That species is under family Ranidae. The genus was *Hylarana* and species was *leptoglossa* (Hasan *et al.*, 2014)

Collected sample was morphologically similar to that of *H. leptoglossa*. It was initially identified by body color, special mark and other measurement as describe by Hasan *et al.*, 2014. Present study found the SVL length of *H. leptoglossa* was 51.816 mm and similar length was observed by Hasan *et al.*, 2014 mentioned the SVL length of *H. leptoglossa* was between 45-70 mm. They also mentioned that head was granulated and tymphanum was distinct which was similar to my data. They mentioned relative finger length was 3>1>4>2 and was fully matched with present investigation. So, it was clear that the species was *H. leptoglossa* according to Hasan *et al.*, 2014. Recently *Hylarana leptoglossa* is renamed *Hydophylax leptoglossa* (https://www.uniprot.org/taxonomy/1659774).

Table-1. Morphometric characteristics of *H. leptoglossa*

Objective	Charateristics
Body size	Medium
Dorsal color	Dark brown
Ventral color	Gray with white spot
Head	Granulated
Tympanum	Distinct
Teeth	Vomerine
Web (finger)	Present and free
Web (toe)	Two third webbed

DNA extraction: DNA extraction of the samples used in the present study was performed successfully. Band of the extracted genomic DNA was visualized under the ultraviolet transilluminator and shown in Figure 3.

Table-2. Morphometric measurement of different body parts (parameters) of observed in *H. leptoglossa*

Body measurement (mm) Criteria Size		**Toe length** Criteria Size	
* SVL	51.816	F1	7.493
EN	3.606	F2	5.359
HL	14.222	F3	7.950
EL	2.59	F4	6.629
HW	13.766		
UEW	4.648		
MN	12.87		
TD	3.454		
SL	7.772		
HAL	11.637		
MFE	9.982		
FAL	8.02		
MBE	4.013		
THIGH	20.75		
IN	4.318		
TL	23.59		
IOD	5.538		
TFOL	30.60		

{*Abbreviations are explained as follows: SVL (snout-vent length), HL (head length), HW (head width), MN (distance from back of mandible to nostril), SL (snout length), MFE (distance from back of mandible to front of 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 nostril), NS (nostril–snout length), EL (eye length), UEW (maximum width of upper eyelid), TD (tympanum diameter), TEL (tympanum–eye length), HAL (hand length), FAL (forearm length), THIGHL (thigh length), TL (tibia length), TFOL (length of tarsus and foot), FOL (foot length), and IMTL (inner metatarsal tubercle length).}

Fig.2. Dorsal view of Cope's frog observed in present study.

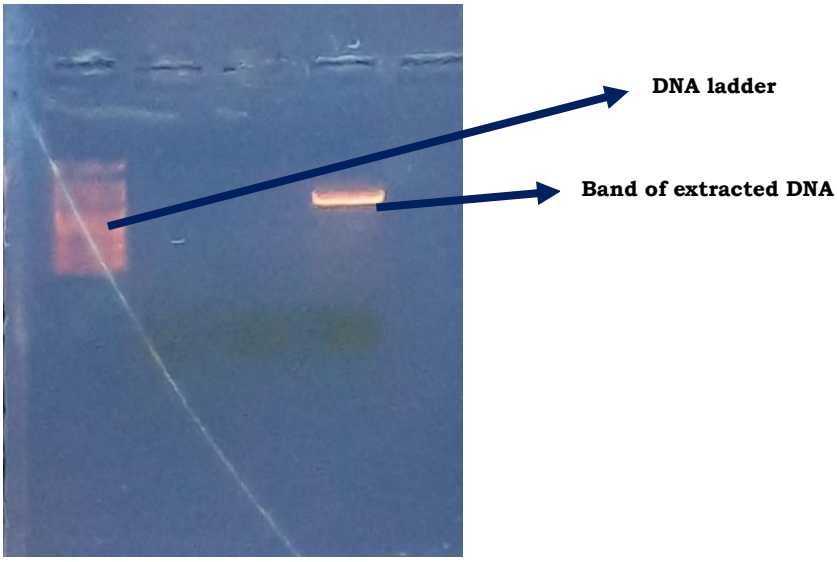

Fig. 3. Extracted genomic DNA band of *H. leptoglossa* showing in 1.0% agarose gel.

Amplification of 16S rRNA gene by polymerase chain reaction: Extracted genomic DNA of observed species was prepared for polymerase chain reaction. The amplified PCR product was then visualized by running them through agarose gel of concentration 1.5% and shown in Figure 4. Compared with DNA marker, it was revealed that amplified gene was around 600 base pair long.

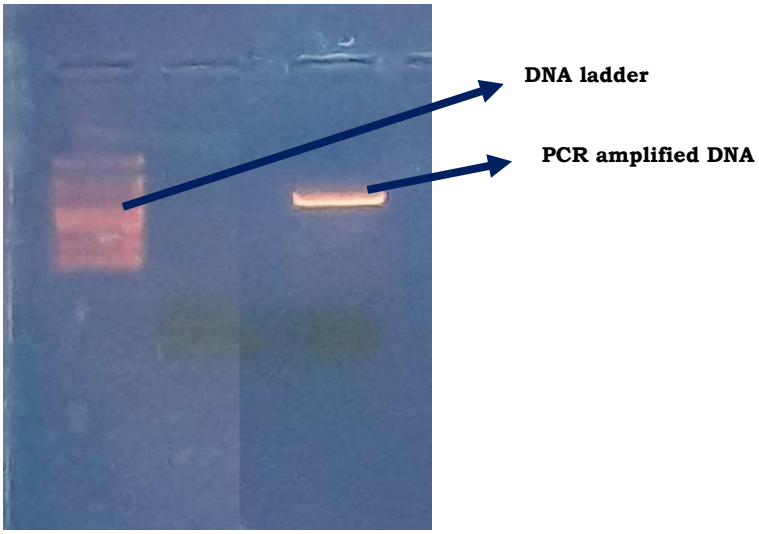

Fig.4. Amplified DNA band of 16S rRNA gene of *H. leptoglossa* showing in 1.5% Agarose gel

Nucleotide sequences of 16S rRNA gene: Nucleotide sequence was obtained by direct sequencing method and 308 bp of nucleotide sequence was determined. The obtained sequence was submitted to the GenBank database and the accession number is MN477195. Of the nucleotide sequences four nitrogen bases were analyzed and percentages are shown in table 3.

Table 3. Percentages of nucleotide base pair of observed anuran species *H. leptoglossa*

Nucleotide base name	Percentage%
Adenine (A)	28.57
Cytosine (C)	24.35
Guanine (G)	23.70
Thymine (T)	23.38
G-C content	8.05
A-T content	51.95
AT/GC ratio	1.08

Within the determined sequence the content of adenine, cytosine, thymine and guanine were 88, 75, 73 and 72, respectively. The AT/GC ratio was 1.08% as shown in table 3 and is matches with that of *H. leptoglossa* found in GenBank database with the accession number AB530528.

Intraspecific variation of 16S rRNA gene: Multiple sequence alignment was done to investigate the intraspecific comparison of the 16S rRNA gene among three individuals of *H. leptoglossa*. The sequence that was determined in the present study was compared with the sequences of other two individuals retrieved from the GenBank database of National Center for Biotechnology Information (NCBI). The sequences obtained from NCBI were done on individuals collected from Bandarban, Bangladesh (accession number AB530528) and Southeast Asia (accession number AB530526). Four polymorphic sites were revealed after comparing the intraspecies sequences among *H. leptoglossa* (Present study, Sylhet), *H. leptoglossa* (Bandarban, Bangladesh) and *H. leptoglossa* (Southeast Asia) and are shown in table 4. All of the sequences were

Table 4. Polymorphic sites analysis of 16S rRNA gene of *H. leptoglossa* from Bangladesh, Southeast Asia and present study

Serial	Polymorphic site	*HS	HB	HSA
01	213	A	T	-
02	214	G	G	-
03	215	C	C	-
04	307	T	T	C

*Sequence labeled "HS" stands for *H. leptoglossa* from present study (Sylhet), HB stands for *H. leptoglossa* from Bandarban, Bangladesh and "HSA" stands for South-east Asia.

Table 5. Polymorphic sites analysis of nucleotide sequences of 16S rRNA gene between *H. tytleri*, and *H. leptoglossa* (present study)

Serial No	Polymorphic sites	HT	HL
1	15	C	G
2	18	T	C
3	32	C	T
4	36	A	G
5	106	T	C
6	122	T	C
7	163	A	T
8	164	A	T
9	165	C	A
10	203	T	A
11	207	C	T
12	211	C	T
13	212	C	T
14	214	G	A
15	215	C	-
16	217	C	T
17	218	C	T
18	219	T	G
19	220	T	A
20	221	T	A
21	224	A	T
22	227	A	T
23	230	C	T
24	233	A	T
25	234	C	T
26	235	A	T
27	236	T	C
28	239	G	A
29	242	A	C
30	244	C	T
31	245	T	-
32	246	G	-
33	250	A	T
34	259	A	T
35	273	A	G
36	284	T	C
37	285	A	T
38	286	T	A
39	294	A	G
40	295	T	C
41	296	G	A
42	299	A	T
43	302	A	T
44	308	C	C

Sequence labeled “HT” Stands for *H. tytleri* and “HL” stand for *H. leptoglossa*.

found to be approximately 308bp in length. The intraspecific genetic diversion was 1.29% where the species were compared with different countries.

Interspecific variations of sequences: Inter specific sequences of 16S rRNA gene of *H. leptoglossa* and *H. tytleri* were compared. All the sequences were found to be approximately 308 bp in length. 44 polymorphic sites were observed (Table 5). Inter species variation among these species was 14.29%.

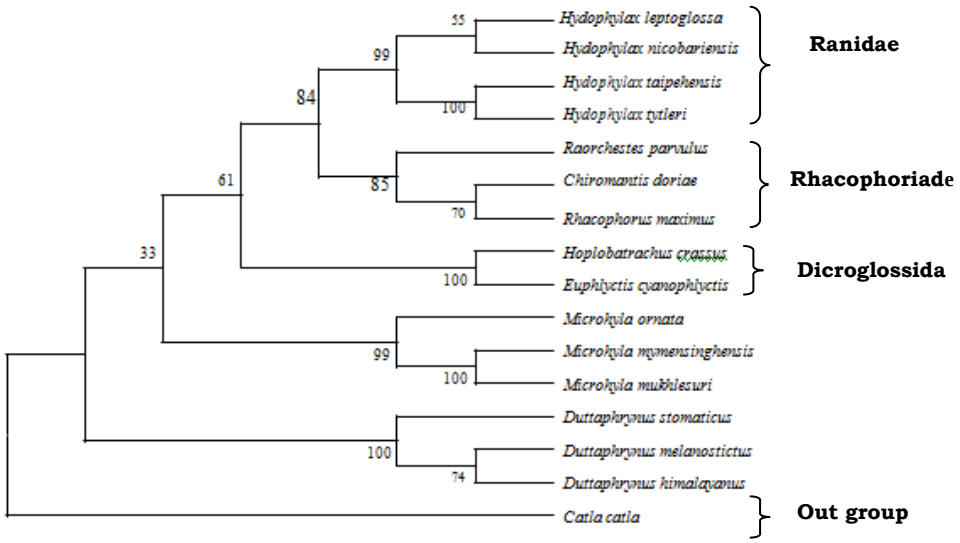

Fig.5. Molecular phylogenetic tree for Cope's frog according to the neighbor joining method based on the partial nucleotide sequences for the 16S rRNA gene where *Catla catla* used as outgroup.

Phylogenetic analysis: To identify the phylogenetic position of *H. leptoglossa* of the present study a phylogenetic tree was constructed. As Cope's frog was an anuran species, a comparative study was performed among anuran from Ranidae, Rhacophoridae, Dicroglossidae, Microhylidae and Bufonidae family using the neighbor joining method, where *Catla catla* was used as an out group based on 16S rRNA. In the present study, four species of *Hydophylax* namely *H. leptoglossa*, *H. nicoberebsis*, *H. taipenensis* and *H. tytleri*, belong to same family Ranidae, form a monophyletic clade with bootstrap value 99 (Figure-5). Similar monophyletic clade was observed by Hasan (2012). Among 15 Anuran species *H. leptoglossa* and *H. tapiensis* formed a monophyletic group with bootstrap value was 99. Furthermore, *Rhacophorus parvulus*, *Chiromantis doriae* and *Rhachophorus maximus* belong to Rhacophoriade family form a monophyletic clade which bootstrap value was 84 and this relationship was not acceptable. So far, the species belong to family Ranidae was closely related to those of the family Rhacophoridae. So, the phylogenetic relationship of present study was similar to that of Hasan (2012) study and the phylogeny based on morphology.

CONCLUSION

Partial nucleotide sequence of 16S rRNA gene of copy's frog *Hydrophylax leptoglossa* was determined and will be used as DNA barcode for that particular

individual collected from Sylhet, Bangladesh. The sequence was initially used to analyze a molecular phylogenetic tree. To confirm the present observation of the molecular taxonomic position of the present anuran species, further study is required based on nucleotide sequences of 12S rRNA, COI, cytochrome *b*, ND4 and ND1 genes from the mitochondrial genome.

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