

POLYMORPHIC LOCI ANALYSIS OF 16S RIBOSOMAL RNA GENE OF BOMBAY DUCK (*HARPADON NEHEREUS*)

Sristy Saha, Zannatul Ferdous, Hawa Jahan, Ashfaquul Muid Khandaker, Reza Md. Shahjahan and Rowshan Ara Begum*

Genetics and Molecular Biology Laboratory, Department of Zoology, University of Dhaka, Dhaka-1000, Bangladesh

Abstract: Attempts were made to combine morphological and molecular techniques for identification and analysis of evolutionary relationship of commercially important marine lizardfish (Bombay duck) from the Bay of Bengal. For molecular identification, mitochondrial DNA (mtDNA) was extracted using CTAB extraction protocol and 16S rRNA gene was amplified by PCR method. Morphological identification was supported by molecular data generated by 99% identity of BLAST search result of *Harpadon nehereus*. The nucleotide sequence of *H. nehereus* was 586 bp long and the G+C content was 51% and intraspecies polymorphic sites were 0.5%. According to interspecies polymorphic sites analysis, 4.9% polymorphism was observed when *H. nehereus* and *H. microchir* were compared. Phylogenetic tree was constructed and both the *Harpadon* spp. formed a monophyletic clade. This analysis will be used to study molecular diversity as well as conservation of this fish species.

Key words: 16S rRNA gene, Polymorphic loci, mt-DNA, *Harpadon nehereus*

INTRODUCTION

Harpadon nehereus, commonly known as the Bombay duck, is a lizardfish, belongs to the family Synodontidae (Whitehead 1984). It lives in the tropical waters of the Indo-Pacific and traditionally caught in the waters of the Bay of Bengal. In Bangladesh, the species is found mainly in the estuaries and also ascends tidal rivers (Rahman 1989, Rahman 2005, Gopal and Chauhan 2006). About, 100 g flesh of this fish contains 0.85% carbohydrate, 14.61% protein, 2.75% fat, 1.03% ash and 80.75% moisture and calcium 339.15 mg (Bhuiyan 1992, Zafar *et al.* 1993). Dried Bombay duck has commercial importance in the South and Southeast Asia (Rahman 2005).

The elevated market demand and over exploitation of Bombay duck fish may deplete the wild stocks to an unsustainable level (Sarker *et al.* 2017). Nowadays, the best way to protect a species is not only to preserve its ecosystem but also preserve its genetic resources and diversity (Hedrick 2001). To do so first needs to identify the species. Species identification by using conventional keys only

*Author for correspondence: <rowshanbegumdu@yahoo.com>

is not enough. Molecular techniques should also 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, it 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 (Weisburg *et al.* 1991). 16S rRNA gene along with other mitochondrial genes are used to ensure quality checks on canned fishes and meat and investigate the status of cancerous disease, toxicological status of fish (Sultana 2015). These mitochondrial genes are also used for the purposes of environmental study. 16S rRNA gene sequence is also used in the bar coding of certain vertebrates, for examples amphibian, fishes etc. (Vences *et al.* 2005).

To investigate the genetic characteristics of *H. nehereus* in this study a partial sequence of the 16S rRNA gene of *H. nehereus* were determined. The sequence was aligned with other sequence of lizardfish to study the number of polymorphic sites and the phylogenetic relationships.

MATERIAL AND METHODS

The Bombay duck or locally known as loitta (*Harpadon nehereus*) was collected from Cox's Bazar (Fishery Ghats, fish markets). The fresh specimen was brought to the laboratory of Genetics and Molecular Biology, Department of Zoology, University of Dhaka for further experiment. The sample was then morphologically identified by conventional method. The measurement of different body part of collected fish is shown in Fig. 1. The sample was stored in refrigerator, minimum at 4°C.

DNA extraction, PCR amplification and sequencing: Genomic DNA was extracted from 0.05 gm muscle tissue of the observed fish species following a method (Doyle and Doyle 1990) with modification. 100 µl CTAB extraction buffer and 10 µl of proteinase k were added. The mixture was incubated at 56°C overnight. After digestion equal volume of phenol chloroform was added with the supernatant and centrifuged at 13,000 rpm for 5 min. DNA was precipitated with ethanol and dissolved in distilled water. Standard PCR was performed using universal primers and were analyzed by electrophoresis in a 1.5 % agarose

gel (Faucher *et al.* 2016). Then, PCR amplification was purified using a purification Kit and sequenced using direct sequencing method.

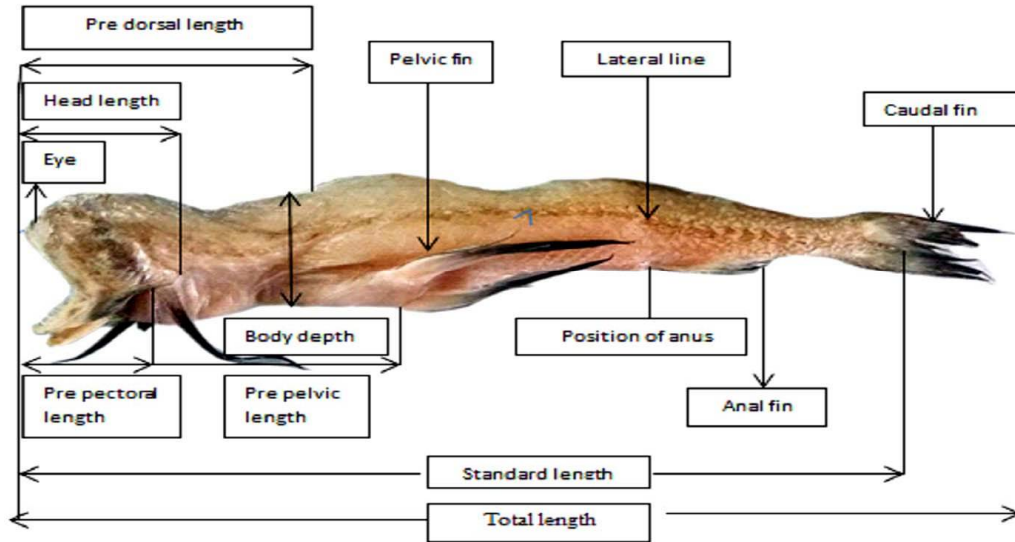


Fig. 1. Measurement of different part of Bombay duck fish.

Bioinformatics analysis: 16S rRNA gene sequence of experimental fish species and other existing sequence of lizard fish were collected from GenBank database (NCBI). Local alignment was done with the help of serial cloner software. MEGA 7 software was used for multiple sequence alignment which was followed by polymorphic sites analysis and for construction of phylogenetic tree. Sequence alignment and pairwise distance calculation is used to conduct two or more biological sequences of similar length.

RESULTS AND DISCUSSION

After analyses the morphology of *Harpadon nehereus*, it was observed that body shape lateral elongated, dorsal head profile clearly convex, eyes more or less normal, mouth/snout more or less normal, position of mouth terminal, scales restricted to posterior half of the body, posterior tip of pectoral fin reaching origin of pelvic. Meristic analysis of different body part of the observed fish species are shown in Table 1.

After analyzing the morphological characteristics, the fin formula for the current supplement fish species was found D 12, Pc 11; Pe 9, A 15. Similar type of fin formula finding had been shown in previously conducted research by Whitehead 1984.

Table 1. Meristic analysis of different body parts (parameters) of observed fish species

Body measurement (cm)		Number of spines and soft rays		Fin base length	
TL	31	DFN	1	DFB	0.37
SL	28	DFS	0	PcFB	0.4
BD	0.9	DFSR	12	PeFB	0.8
PDL	11	PcFN	1	AFB	0.6
HL	8.0	PcFS	0		
ED	0.3	PeFN	1		
PrOL	0.6	PeFSR	9		
PoOL	4.6	AFN	1		
IOL	0.5	AFS	0		
		AFSR	15		

TL = Total length, SL = Standard length, BD = Body depth, PDL = Pre dorsal length, HL = Head length, ED = Eye diameter, PrOL = Pre orbital length, PoOL = Post orbital length, IOL = Inter orbital length, DFN = Dorsal fin number, DFS = Dorsal fin spine, DFSR = Dorsal fin soft ray, PcFN = Pectoral fin number, PcFS = Pectoral fin spine, PcFSR = Pectoral fin soft ray, PeFN = Pelvic fin number, PeFSR = Pelvic fin soft ray, AFN = Anal fin number, AFS = Anal fin number, AFSR = Anal fin soft ray, DFB = Dorsal fin base, PcFB = Pectoral fin base, PeFB = Pelvic fin base, AFB = Anal fin base.

DNA extraction and observed banding pattern of isolated DNA: DNA extraction of the samples used in the present study was performed successfully. Agarose gel electrophoresis of extracted genomic DNA was done for being confirmed of successful DNA extraction. Band of the extracted genomic DNA was visualized under the ultraviolet transilluminator was clear and concise as shown in Fig. 2.

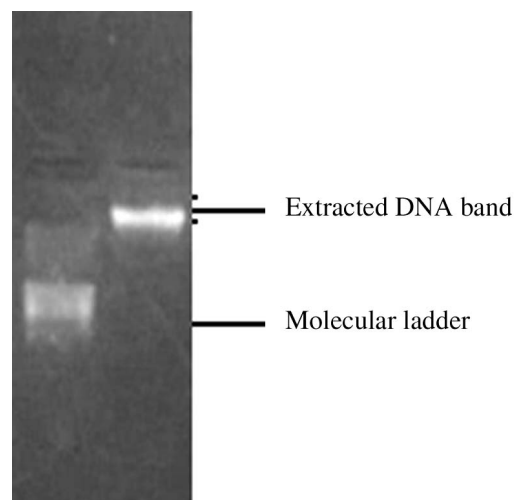


Fig. 2. Extracted genomic DNA band of sample from the collected specimen *H. nehereus* showing in 1.0% agarose gel.

Amplification of 16S rRNA gene by polymerase chain reaction: Extracted genomic DNA from observed species was prepared for polymerase chain reaction. The amplified PCR product was then observed by running them through agarose gel of concentration 1.5% (Fig. 3). Compared with DNA marker, it was revealed that the amplified gene was around 600 base pair long.

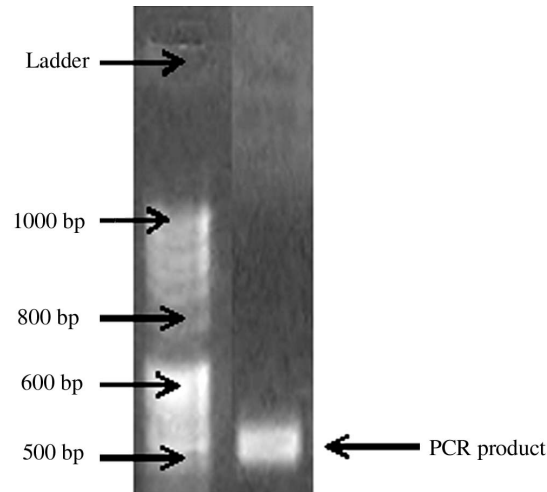


Fig. 3. Amplified DNA band of 16S rRNA gene of *H. nehereus* showing in 1.5% agarose gel.

Nucleotide sequences of 16S rRNA gene: Nucleotide sequence was obtained by direct sequencing method and 586 bp of nucleotide sequence was determined. The obtained sequence is submitted to the GenBank and the accession number is (MK641100). Nitrogen base was analyzed by percentages are shown in Table 2.

Table 2. Percentages of nucleotide base pair of observed fish species *H. nehereus*

Nucleotide base name	Percentage
Adenine (A)	27.8
Cytosine (C)	27.0
Guanine (G)	23.2
Thymine (T)	21.5
G-C content	51
A-T content	49
AT/GC ratio	0.9

According to this analysis, the region amplified was GC rich, whereas the whole 16S rRNA gene shows AT richness, compared to GC (Bo *et al.* 2013).

Intraspecific variation of 16S rRNA gene: For further analysis multiple sequence alignment was done to investigate the intraspecific comparison between *H. nehereus* from Bangladesh used in the present study and China (JX534239). The alignment of 16S rRNA gene of *H. nehereus* from two different region indicated nucleotide polymorphism varies at 3 positions with 0.5% polymorphism and location are shown in Table 3.

Table 3. Polymorphic sites analysis of 16S rRNA gene of *Harpadon nehereus* from Bangladesh and China

Serial No.	Polymorphic sites	BH	CH
1	204	T	A
2	242	C	T
3	357	T	C

Sequences labelled "BH" stands for *H. nehereus* from Bangladesh used in the present study and "CH" stands for *H. nehereus* from China.

Interspecific variation of sequences: In this study the interspecific variations of partial regions of 16S rRNA gene between *H. nehereus* and *H. microchir* (AP002919.1) from Bangladesh and China are shown in Table 4.

For interspecies polymorphic sites analysis of Bombay duck fish, the sequence of *H. nehereus* and *H. microchir* were aligned. Alignment of the sequence indicated single nucleotide polymorphism varies at 29 positions with 4.9% polymorphism and positions are shown in Table 4. Similar type of interspecies polymorphism showed in *Barilius* spp. (Singh et al. 2015) when 16S rRNA gene was compared among *Barilius bakeri*, *B. gatensis*, *B. vagra*, *B. bendelisis* and *B. tile*.

Phylogenetic analysis: As Bombay duck is a lizardfish, a comparative study was performed among lizardfish from Harpadontidae and synodontidae family using the neighbor joining method, with bootstrap value 1000 where *Labeo rohita* was used as an out group. In the present study, *Harpadon nehereus* and *H. microchir*, belong to the family Harpadontidae, form a monophyletic clade with bootstrap value 96 (Fig. 4). Monophyly of these two species of *Harpadon* in the present study agreed with the result based on cytochrome c oxidase sub unit I (COI) gene (Nugroho et al. 2017). Furthermore, *Saurida wanieso* and *S. umeyoshii* along with *S. undosquamis* belong to the family Synodontidae form a monophyletic clade with bootstrap value 99 and similar result was also observed based on COI gene (Nugroho et al. 2017).

Table 4. Polymorphic sites analysis of nucleotide sequence of 16S rRNA gene between *Harpadon nehereus* and *H. microchir* (AP002919.1)

Serial No.	Polymorphic sites	HM	HN
1	51	C	T
2	52	G	A
3	108	T	C
4	116	A	G
5	159	A	C
6	180	C	T
7	182	T	C
8	199	A	G
9	202	A	T
10	203	A	T
11	204	C	T
12	254	A	G
13	255	G	A
14	262	A	G
15	265	C	G
16	266	C	T
17	267	A	G
18	269	-	A
19	274	G	A
20	294	G	C
21	309	C	T
22	358	A	T
23	362	A	C
24	368	A	G
25	372	C	T
26	379	T	C
27	382	C	T
28	411	C	A
29	422	T	C

Sequences labelled "HM" stands for *H. microchir* and "HN" stands for *H. nehereus*.

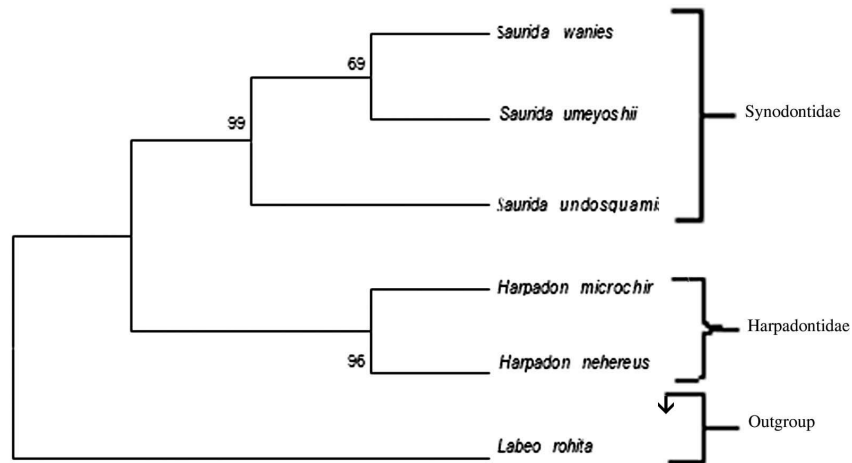


Fig. 4. Molecular phylogenetic tree for lizardfish according to the neighbor joining method based on the partial nucleotide sequences for the 16S rRNA gene.

Where *Harpadon nehereus* was used in the present study and *H. microchir* (AP002919.1), *S. wanieso* (NC 025940.1), *S. undosquamis* (NC 003162.1), *S. umeyoshii* (KM189365.1) were collected from GenBank database. *Labeo rohita* (KR185963.1) was used as out group.

Partial nucleotide sequence of 16S rRNA gene of *Harpadon* spp. of the present study was initially used to analyze the polymorphic loci and to construct a molecular phylogenetic tree. To confirm the present observation, further study is required based on nucleotide sequences of 12S rRNA, COI, cytochrome *b*, ND4 and ND1 genes.

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