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Molecular characterization of hilsa shads in Bangladesh using cytochrome C oxidase subunit I (COI) gene

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Abstract
The national fish of Bangladesh, *Tenualosa ilisha*, is a very commercially important species, and it contributes to about 10% of total fish production of the country. In recent times, the production of hilsa has undergone a drastic decline due to overfishing. Fishermen have overfished the species, and mislabled fish have made their way to the market, thereby hurting sales. An investigation was carried out to properly identify hilsa shads based on morphometric, meristic and molecular characteristics. We have morphologically identified three species of hilsa shads; *Tenualosa ilisha*, *Tenualosa toli*, and *Hilsa kelee*, and further validated our results through DNA barcoding. The evolutionary distances indicated that the mean interspecies distance was 25.52%, while the intraspecies distance was 0.22%. Therefore, the study indicates that there are three sepcies of hilsa, and COI is an ideal genetic marker that can be used to identify and confirm the nature of these species.

Keywords: Hilsa shads, *Tenualosa ilisha*, COI, Bangladesh

Introduction
Bangladesh is a land of rivers filled with fish, hence rice and fish has become the staple food of the citizens. The most prized and national fish of Bangladesh is *Tenualosa ilisha*, more commonly known as the ilish or hilsa shad [1]. The unique taste of hilsa, owing to its abundant omega 3 fatty acids, makes it a highly sought after fish [2]. The sales of this fish contributes to about 1.15% of the total GDP of Bangladesh. According to the Department of Fisheries in Bangladesh, ilish accounted for 10.18% (394951 tons) of the total fish produced in 2015-2016, however, the market of hilsa is in decline compared to total fish production considering the 11.10% produced in 2010-2011 [3]. The improper management of this fish and overfishing can be ascribed to misidentification of hilsa shads [4, 5].

There is an age-old debate as to how many types of hilsa are present in Bangladesh. Some researchers and taxonomists claim there are only two types, while others claim there are three [6-9]. Hilsa is an anadromous species, but a fluvial potamodromous type and a marine type have also been identified. The potamodromous lives and breeds in the middle of the river yearlong. The anadromous hilsa swims upstream for breeding and eventually returns to its estuarian habitat [10]. It is still unclear when the spawning occurs due to large variations. Moreover, it is not known if the migratory species mix with the other species.

It is generally considered that there are three species of hilsa shads in Bangladesh; namely *Tenualosa ilisha*, *Tenualosa toli*, and *Hilsa kelee*. Currently, these fishes are identified based on morphometric and meristic methods, but the confusion remains. Researchers have tried to employ modern molecular biology tools such as RAPD to identify these fishes, but they have not been successful due to the lower resolving power. This confusion among experts has spread among fishermen and has led to improper care and overfishing of hilsa.

DNA barcoding is a molecular technique used in taxonomy to identify and diferentiate various species. In this method, a shortgenetic marker of the mitochondrial cytochrome c oxidase subunit I (COI) is commonly used as the barcode region. This 658 bp region is commonly referred to as the Folmer region. A recent study noted that in 1360 sequences the mean intraspecies divergence was only 0.3% while the mean interspecies divergence was 8.3% [11]. Many other studies have confirmed the high resolution power of DNA barcodes. Hence, the Barcode of Life Data Systems (BOLD) now houses barcodes of around 265,000 species [12].

This study attempts to create a comprehensive guide to identify the hilsa species of Bangladesh. Thus, the primary purpose of this study is to use the combined resolving powers of morphological analysis and molecular identification techniques to identify hilsa shads.

Materials and Method

Sample collection

The target species of hilsa shads were collected from Cox's Bazar, Sunamganj, and Moulvibazar. Immediately after landing the fish on board, a small portion of tissue near the dorsal fin was carefully collected from each sample and preserved in 95% ethanol until used. The samples were then transported to the Advanced Fisheries and DNA Barcoding \Laboratory at the Department of Zoology, University of Dhaka for analysis.

Morphological Analysis

Species identification was done following Whitehead's illustrated catalogue of hilsa shads [13]. Morphometric study was done by measuring the length and width of various parts of the body. Meristic characteristics were analyzed by studying lateral lines, scale count, and fin count.

DNA extraction and amplification

DNA extraction was carried out using organic method. A 20 mg size of tissue was cut, chopped, homogenized, and suspended in 500 µl TNES buffer (10 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0, 120 mM NaCl, and 1% SDS). The tissue was then digested using 10 µl of 20 mg/ml Proteinase K and incubation at 56°C overnight. After digestion, 500 µl Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and centrifuged for 15 minutes at 13000 rpm. The aqueous phase was transferred to a new tube and added to an equal volume of Chloroform: Isoamyl alcohol (24:1) and centrifuged at 13000 rpm for 15 minutes. The upper layer was transferred to a fresh, sterilized micro-centrifuge tube; a double volume of chilled absolute Ethanol was added and centrifuged at 13,000 rpm for 15 minutes. The supernatant was discarded, and the pellet was retained. This was followed by another wash with absolute Ethanol and subsequent centrifugation at 13,000 rpm for 15 minutes. The pellet was air-dried and resuspended in 50µl nuclease free water.

The COI gene was amplified in a 25 µL volume with 12.5 µl of GoTaq® G2 Hot Start Colorless Master mix (Ref. M743A, Promega, Madison, WI USA), 1 µl (0.01 mM) of forward primer, 1 µl (0.01 mM) of reverse primer, 8.5 µl of nuclease free water, and 2 µl of genomic DNA (Ref. P119A, Promega, Madison, WI USA). For the amplification of the COI gene the following primers were used [14]:

FishF1: 5′-TCAACCAACCACAAAGACATTGGCAC-3′

FishR1: 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′

The PCR conditions consisted of an initial step of 5 min at 95 ˚C followed by 40 cycles of 30 s at 94 ˚C, 30 s at 54 ˚C and 1 min at 72 ˚C, followed in turn by a final extension of 7 min at 72 ˚C. The PCR products were visualized in a 1.2% agarose gels, and the most intense products were selected for sequencing.

Sequencing, Phylogenetic and Statistical analyses

The purified PCR products were sent to the First BASE Laboratories SdnBhd, Malaysia for sequencing. The sequencing data from the chromatogram was curated and converted into FASTA format using Sequencer v5.4.1.Each sequence was identified using Nucleotide Basic Local Alignment Search Tool (Blastn). The barcodes determined in this study were deposited in the GenBank database under the Accession No KX657721, KY124381, and KX657720. A total of 18 sequences from the Clupeidae family were also included in the study as a reference. Evolutionary analyses of the aligned sequences were conducted in the program MEGA7 [15]. The evolutionary history was inferred using the Neighbor-Joining method after the sequences were aligned with MUSCLE [16]. Pairwise genetic distance, intraspecies and interspecies distances were calculated using the Kimura 2-parameter distance model [17].

Results and Discussion

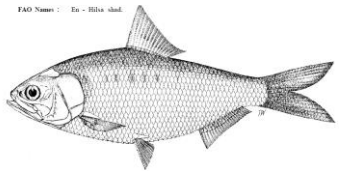
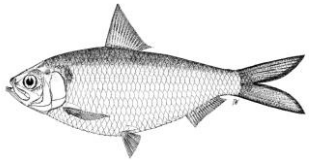
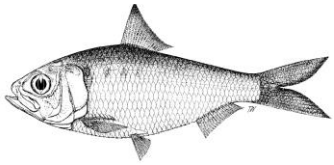
Morphological Analysis

The species were identified by shape, size, markings, scales, and fins. A comprehensive table to identify the different species of hilsa was then created (Table1).

Analysis of PCR product

The extracted DNA from *T. ilisha*, *T. toil* and *Hilsa kelee* were amplified by PCR using COI gene-specific primer to obtain a 658 bp amplicon. Amplified PCR products were resolved and detected in 1% (w/v) agarose gel with Ethidium Bromide.

Table 1: Morphometric and meristic charactersitics of the species

Feature	*T. ilisha*	*T. toli*	*Hilsa kelee*
Picture			
Shape	Body moderately deep, compressed, belly with 30 to 33 scutes. Head length 28 to 32% of standard length.	Body moderately deep, compressed, belly with 28 to 30 scutes. Head length 25 to 27% of standard length.	Body fairlydeep and compressed, belly with distinct keel of scutes.
Size	60 cm standard length, commonly 35 to 40 cm.	50 cm standard length.	24.4 cm standard length, usually about15 to 18 cm.
Spots	A dark blotch behind gill opening, followed by a series of small spots along the flank.	At most, a dark diffuse mark behind gill opening, but no other spots on the flank.	A black spot behind gill.

Marks	No	No	Top of head with numerous fronto-parietal striae
Notch	Distinct median notch in upper jaw.	A distinct median notch in upper jaw.	Upper jaw with median notch.
Gillrakers	Gillrakers fine and numerous, about 100 to 250 on the lower part of the arch.	Gillrakers fine but not numerous, 60to 100 on the lower part of the arch (barely increasing after 10 cm standard length).	Gillrakers about 100 to 175, those on innerarches distinctly curled; outer row of gill filaments on the first arch not more than half length of gillrakers.
Special scales	No	No	A series of small triangular scales above the axis of pectoral fin; hind part of body scales perforated.
Caudal fin	Caudal fin moderate, 25 to 31% of standard length.	Caudal fin short, 31 to 34% standard length.	

BLAST result analysis

The BLAST analysis revealed that the partial COI gene sequences of the three species have similarity (99%) with the respective sequences of the mitochondrial region in the GenBank database (Table 2).

Table 2: The BLAST similarities of sample sequences from NCBI GenBank

Query sequence IDs	Accession no.	Percent similarity	E-value	Accession no. of the best match
Tenualosa ilisha	KX657721.1	99%	0.0	MF588658.1
Tenualosa toli	KY124381.1	99%	0.0	JX983317.1
Hilsa kelee	KX657720.1	100%	0.0	JF493643.1

Phylogenetic and genetic divergence analysis

The sequence analysis revealed that the overall GC content was 47.71% (S.E. = 0.52), with *Hilsa kelee* having the highest (52.04%) GC content. All the species exhibited unique barcodes that could be distinguished from one another, and all individuals within species and genus could be differentiated. The mean interspecies genetic divergence was 25.52%, while the mean intraspecies divergence was only 0.22% indicating a high DNA barcoding gap (Table 3).

Table 3: The genetic divergence (K2P Distance %) within the species and between the species

		K2P Distance (%)				
Comparison	**No. of sequences**	**Mean**	**Min**	**Max**	**S.E.[a]**	**Overall mean**
Intraspecies	21	0.22	0.00	0.80	0.0008	24.49
Interspecies		25.52	0.50	33.84	0.011	
[a] Standard Error						

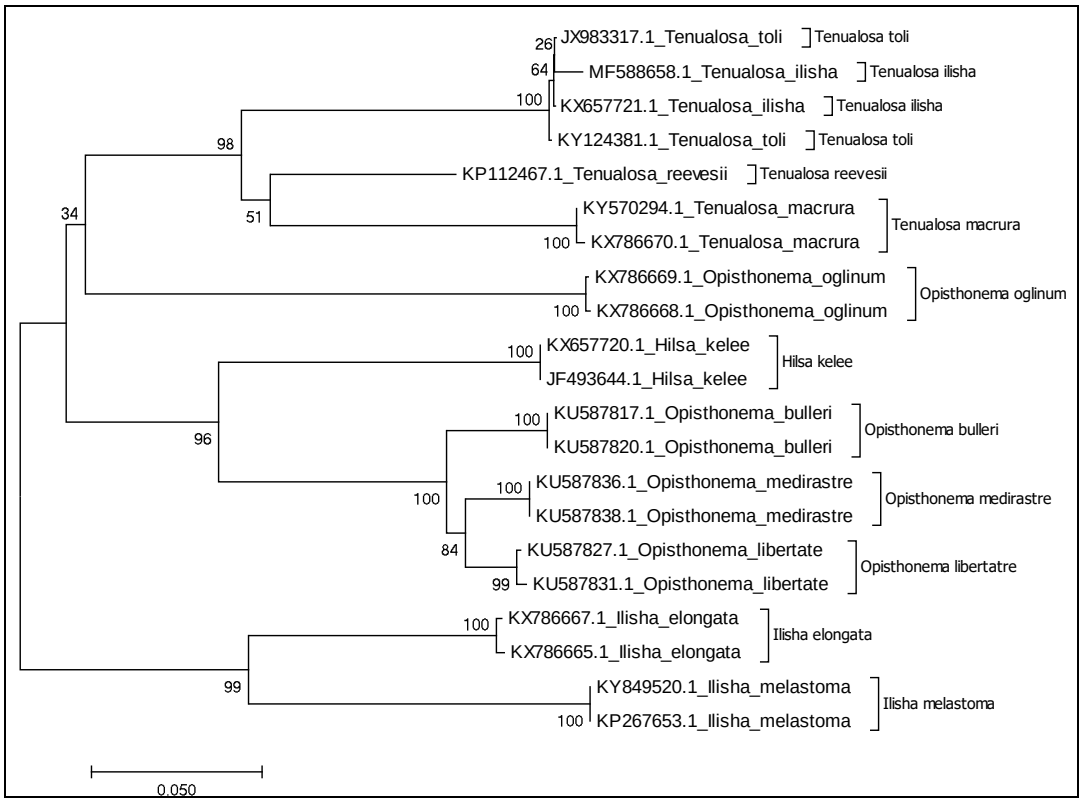


Fig 1: Phylogenetic tree of COI gene sequence of *T. ilisha*, *T. toli* and *Hilsa kelee* with outgroups from the same family. The neighbor-joining (NJ) tree was constructed using K2P model with 1000 replications for bootstrap analysis.

The sequences aligned with MUSCLE were subjected to the K2P-distance model to delineate a phylogenetic tree to trace the origin of the species and infer their evolutionary relationship (Figure 1). The neighbor joining tree seemed to indicate that *T. ilisha* and *T. toli* are the most closely related species, designated by the 100% bootstrap value. *Hilsa kelee* on the other hand seems to be a distantly related species, but still closer to the *Tenualosa* genus compared to the *Opisthonema* and *Ilisha* genus. The phylogenetic tree thus suggests that they should be very close in appearance and overall structure, hence the confusion in morphological identification of the species.

Hilsa is undoubtedly one of the richest resources of the fisheries sector in Bangladesh. This delicacy has been abused for years due to misidentification. Firstly, fishermen have trouble identifying the three species of hilsa. Therefore, this leads to increased fishing pressure, the indiscriminate exploitation of juveniles (Jatka), disruption of migration routes, and loss of spawning, feeding and nursing grounds. This has led to an overall decline in hilsa production in the past years. Secondly, mislabeling of *T. ilisha* as *T. toli* and *Hilsa kelee*, have severely jeopardized the market value of *T. ilisha*.

Several researchers have contributed controversial evidences about the number of species of hilsa in Bangladesh. Mojumdar *et al*. have reported three ecotypes of hilsa based on the living waters of the species [18]. Pillay *et al*. also suggested three types of hilsa based on biometric measurements, while Dahle *et al*. used RAPD techniques to differentiate three forms as well [19 20]. On the other hand, Rahman and Naevdal have suggested just two stocks based on a single polymorphic locus [21]. Shifat *et al*. corroborated their claims by identifying two species based on two habitable rivers [22].

Our results suggest that there are three distinct species of hilsa in Bangladesh. The comprehensive morphological study indicates distinct features that can only be attributable to three different species. Moreover, the DNA barcoding and evolutionary distance points to three separate species of hilsa. The phylogenetic tree was used to trace back to two separate species in a single genus, and another species in a distantly related genus. Although the study suffers from a small sample size, the bootstrap values are quite convincing and indicate proper relationships. Therefore, a larger sample size with other samples from closely related genus of the same family are required for further studies based on populations.

Conclusions

We posit that there are three species of hilsa in the rivers and seas of Bangladesh. We have managed to create an easily discernible comprehensive guide that would allow people to correctly identify the hilsa of Bangladesh. Additionally, the DNA barcodes corroborate our morphological evidence and project information that can be used for both management and conservation purposes. The delimitation and recognition of hilsa species is a requirement in management of fisheries, authentication of food products, to identify species, and uncover biological diversity. Proper management is required to counteract reduction in population sizes and to maintain the genetic diversity of hilsa in Bangladesh.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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