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Existence of panmictic Hilsa shad (*Tenualosa ilisha*) populations from two large river ecosystems in Bangladesh revealed by mitochondrial control region

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Abstract. The present study was undertaken to estimate genetic diversity and reveal population structure of Hilsa shad (*Tenualosa ilisha*) from two large river ecosystems, the Padma and Meghna of Bangladesh using mitochondrial control region (D-loop region). Fin tissue samples of 60 individuals were collected from the Padma (ChapaiNawabgonj) and Meghna River (Chandpur). DNA was extracted and D-loopof mtDNA was amplified, purified and sequenced. A total of 35 Sequences (15 Sequences from the Padma and 20 Sequences from the Meghna population) were analyzed and 14 haplotypes were detected. Total16 polymorphic sites were identified that included11 singleton sites and 5 parsimony informative sites. These populations showed high haplotype diversity (*h*) (0.863 in the Meghna and 0.901in the Padma population). We found low nucleotide diversity (P_i) within each population, 0.0058±0.0039 in the Padma to 0.0063±0.004 in the Meghna population. The shared haplotypes among the two populations were identified as 28.57%, and the rest 71.43% were private haplotypes. Estimate of genetic differentiations (F_{st}) suggest that the Meghna population of Hilsa was closely related to the Padma population (F_{st}= 0.058, P=0.07). The TCS genealogy showed three main haplotypes separated by three mutational steps and hap_10 might be the most ancestral haplotype by its internal position in the network. All results suggest panmixia in *T. ilisha* populations between the Meghna and Padma river populations and thus similar management plan could be useful for these two river Hilsa fishery in Bangladesh.
Keywords: Hilsa Shad, Mitochondrial DNA, Genetic structure, Population

Introduction

The river shad or Hilsa shad (*Tenualosa ilisha* Hamilton, 1822), popularly known as *Ilish*, constitutes the largest single species fishery in Bangladesh (DoF 2019). Since the early 1900s, Hilsa is harvested in huge quantities from the extensivezoneof the Bay of Bengal that suggests a substantial increase in hilsa catch from marine environment (estuaries, coastal areas and sea) in Bangladesh, India and Myanmar (BoBLME 2012, ECOFISH-Bangladesh 2017, FAO 2017, Hossain *et al.* 2019). The Hilsa production in Bangladesh was 5.3 Lakh MT in 2019 from Rivers, Sundarbans and Marine (DoF 2019). Hilsa is well-known for its transboundary nature and spends major part of its life in the sea and migrate to upstream major rivers (the Padma, Meghna, Jamuna in Bangladesh) for breeding purpose (BOBLME 2010). In Bangladesh, the major portion of the Hilsa catch (60–70%) is caught from rivers during theirupstream spawning migration and juveniles are harvested on their wayreturn to the sea (Hossain *et al.* 2019). However, inland catch (the Padma, Meghna etc.) has beendecreased severely over the years due to a number of reasons: overexploitation, resource degradation (heavy siltation, low water discharge from the uprivers), disruption of migratory route, and disappearance of essential spawning, feeding and nursery grounds (Mohammed and Wahab 2013). In such circumstances, it is a prerequisite to study the genetic diversity and differentiation of the riverine Hilsa fish populations for sustainable management and conservation purposes.

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For effective management and conservation purpose information on genetic diversity and gene flow among and within populations is essential which can be obtained through molecular markers (Storfer *et al.* 2007). Among the various molecular markers used, mitochondrial DNA marker has been proved to be very useful in analyzing population structure and phylogenetic studies because mtDNA has maternal inheritance, elevated mutation rate and is easily transmitted without any recombination (Hoolihan *et al.* 2004,Luhariya*et al.* 2012). The control region or D-loop of mitochondrial DNA exhibit elevated levels of variation as it is a rapidly evolving region (Lalitha and Chandavar 2018). D-loop or Control Region (CR) is widely used for genetic studies of fish populations due to its high mutation rate and high copy number (Ahmed *et al.* 2004; Mazumder and Alam 2009, Wang *et al.* 2016).

Hilsa is an anadromous fish, hence Quddus *et al.* (1984) and Rahman (1997) made few assumptions regarding the Hilsa stock in Bangladesh: a) A purely marine stock that does not migrate to river b) A purely riverine stock that inhabit it entire life in freshwater and c) A migratory stock that migrates between the seashores of the Bay of Bengal and riverine environment for spawning purpose. Information on genetic diversity and genetic differentiation of Hilsa population can help understand these assumptions. Considering the fact that inland Hilsa fishery has become vulnerable over the years, it is essential to focuson the population genetic studies ofthis speciesin the riverine ecosystems. Asaduzzaman *et al.* (2019) reported that Hilsa shad population of Bangladesh has been divided into two genetic clusters according to local adaptation: 1) marine and estuarine cluster and 2) riverine cluster. Again, among the riverine cluster, population were divided into north-western and north-eastern ecotypes. Population study on hilsa shad using allozyme marker revealed more than one stock (freshwater and estuarine) in Bangladesh (Rahman 1997, Rahman and Naevdal 2000). PCR based technique RAPD fingerprinting have also inferred more than one gene pool in inland rivers in Bangladesh (Shifat *et al.* 2003). However, another study on shad populations revealed no significant differentiations (populations from different geographical locations within and beyond Bangladesh) using allozyme electrophoresis (Hussain *et al.* 1998). Restriction Fragment Length Polymorphism (RFLP) study of the mitochondrial DNA of *T. ilisha* revealed significant differentiation among riverine, coastal and marine populations in Bangladesh (Ahmed *et al.* 2004,MazumderandAlam 2009). Several studies using mitochondrial DNA in Hilsa shad were conducted in India also (Lal *et al.* 2004, Brahmane *et al.* 2013). Lal *et al.* (2004) reported panmixia in Hilsa populations from the Ganga, Padma, Brahmaputra, Hoogly and feeder canal and Brahmane *et al.* (2013) did not identify any population structure between the Ganga and Hoogly populations. So, genetic studies on Hilsa populations conducted both in India and Bangladesh could not provide a specific indication of riverine Hilsa shad population structure. Despite the commercial value of the *T. ilisha* in Bangladesh, available research on genetic diversity and population structureof Hilsa fish using control region of mitochondrial DNA is absent. So, the present study was focused on determiningpopulation structure and genetic diversity of *T. ilisha* from riverine ecosystems by using mitochondrial control region (D-loop).

Materials and methods

Fish sampling: Fish sampling was done from two large riverine ecosystem of Bangladesh namely: the Padma River (Godagari, ChapaiNawabgonj-24.444436°N, 88.301148°E) and the Meghna River (Horinaghat, Chandpur-23.1650734°N, 90.6012752°E) (Fig 1). Thirty (30) individuals were caught from each site from July 2018 to June 2019. Fin tissues collected from the caudal region were preserved in 100% ethanoland stored at -20^{0}C in the Fish Genetics Laboratory atFisheries Department, University of Rajshahi.

DNA extraction: Following the Wizard Genomic DNA Purification Kit (Promega, USA Corporation) instructions, genomic DNA of Hilsa fish was extracted. In total, DNA was extracted from 60 individuals. DNA was eluted to 100 µL final volume and quality was checked on 1% agarose gel. According to the Qubit 4.0 fluorometer (Thermo Fisher Scientific) Kit instruction DNA concentration was estimated.

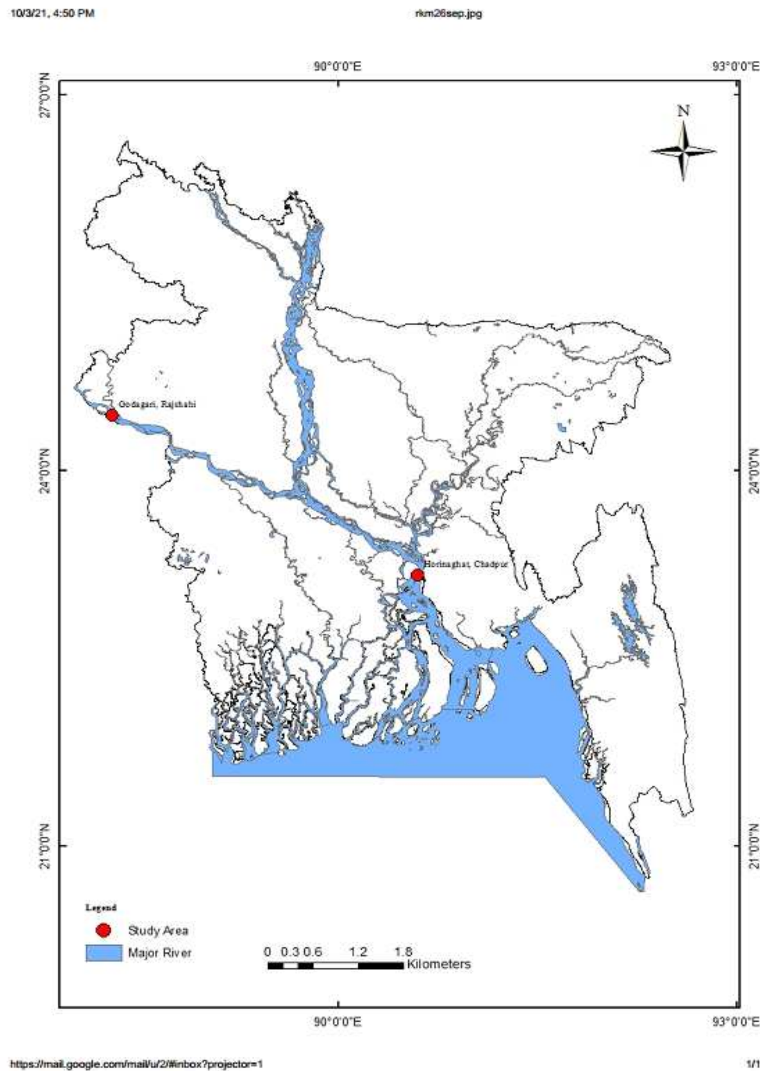

Fig 1. Map showing sampling locations in the Padma and Meghna River indicated with red circle.

Amplification of mtDNAcontrol region: Amplification of 421 base pair (bp) of the control region (CR) was done using Pro-L (5´-CTACCTCCAACTCCCAAAGC-3´) and MT-H (5´-CCTGAAGTAGGAACCAGCTG-3´) primers (Palumbi *et al.* 1991). A reaction volume of 10 µl containing 0.5 µl template DNA, 5 µl of GoTaq® Green Master Mix (Promega Corporation, USA), 0.2 µl of each primer and 4.3 µl of deionized water was used.PCR amplification was performed following this program on the thermocycler (Gene Atlas, Astec Co. Ltd., Japan): 5min

at95°C for denaturation, 35 cycles of 30s at 94°C 30s, at 50°C and 30s at 72°C and a last extension cycle at 72°C for 5min. For confirmation, 2µl of each amplified PCR products were visualized on 1% agarose gels through electrophoresis. The PCR products were cleaned by FavorPrep PCR Clean-up kit (Favorgen Biotech Corporation, Taiwan) following thekit instructions.

DNA sequencing and data analysis: Purified DNA (60 samples) was sent to Macrogen, South Korea for sequencing. Sequences were aligned and edited using Bioedit 7.2 (Hall 1999). Total 35 sequences (15 Sequences from the Padma population and20 from the Magna population) were selected after quality checkfor bioinformaticsanalysis.Control region sequence was derived from forward and reverse sequence reads using MEGA version 7.6.Nucleotide diversity indices such as nucleotide composition, number of polymorphic sites (*S*), haplotype diversity (*h*), and nucleotide diversity were analysed using Arlequin version 3.5 (Excoffier and Lischer 2010), DnaSP version 5.1(Librado and Rozas 2009), and MEGA 7.0 (Tamura *et al.* 2013). Haplotype Network was prepared by statistical parsimony using TCS version 2.1 (Clement *et al.* 2000). Arlequin 3.5 was used to calculate F_{ST} statistics, Tajima's D and Fu's F_S values (Excoffier and Lischer 2010).

Results and Discussion

The overall objective of the present study was to detect genetic diversity and evidence of genetic differentiation between Hilsa (*T. ilisha*) populations of the Padma and Meghna river using mitochondrial control region (D-loop).

Molecular characterization and genetic diversity: Sequence data of 322 bpfragments of CR were aligned, edited and trimmed to the same length. From the 35 Sequences analyzed a total of 14 haplotypes (10 and 8 in the Meghna and the Padma population, respectively) and 16 polymorphic sites were identified. The polymorphic sites included 11 singleton sites and 5 parsimony informative sites. We observed sharing of haplotypes (4 haplotypes) between the two populations (Table I). Average nucleotide compositionof the mitochondrial control region were A: 14.85%; T: 35.47%; C: 35.58%; and G: 14.11% for Padma and Meghna River populations (Table II). Significant difference was not found in nucleotide composition between the two riverine populations.

Table I. Haplotypes identified among the populations

Haplotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Padma (Godagari-15)	2	2	-	-	-	-	-	2	-	4	2	1	1	1
Meghna (Chandpur-20)	3	7	1	1	1	1	1	2	1	2	-	-	-	-

Table II. Nucleotide composition (%) of mitochondrial control region of *T. ilisha*

Nucleotide (%) / Rivers	A	T	C	G
Padma	14.82	35.51	35.59	14.08
Meghna	14.88	35.43	35.56	14.13
Average	14.85	35.47	35.58	14.11

The control region haplotype network displayed a complex pattern of haplotype and nucleotide diversity and was not star-shaped. The TCS genealogy showed four main haplotypes (Hap-1, Hap-8, Hap-2 and Hap-10) separated by three mutational steps (Fig. 2). This network suggests that Hap-10 might be the most ancestral haplotype by its internal position in the network. Other shared haplotypes were originated from this haplotype. From the genealogy, four missing mutations were also observed which could be due to small number of individuals sampled from only distant located populations. A star shaped haplotype network with one or two very common internal haplotypes surrounded by several low frequency newly derived haplotypes can be indicative of recent population expansions whichis visible with longer internal branches in the haplotype network (Slatkinand Hudson 1991,Ramírez-Soriano *et al.* 2008). If populations experience a bottleneck event, these low frequency haplotypes have a greater chance of being lost from the population resulting in a pattern with several common haplotypes connected by extinct haplotypes and displaying longer internal branches (Aris-Brosou and Excoffier 1996)**.** However, we did not observe a star shaped haplotype network, so there are no recent population expansion.

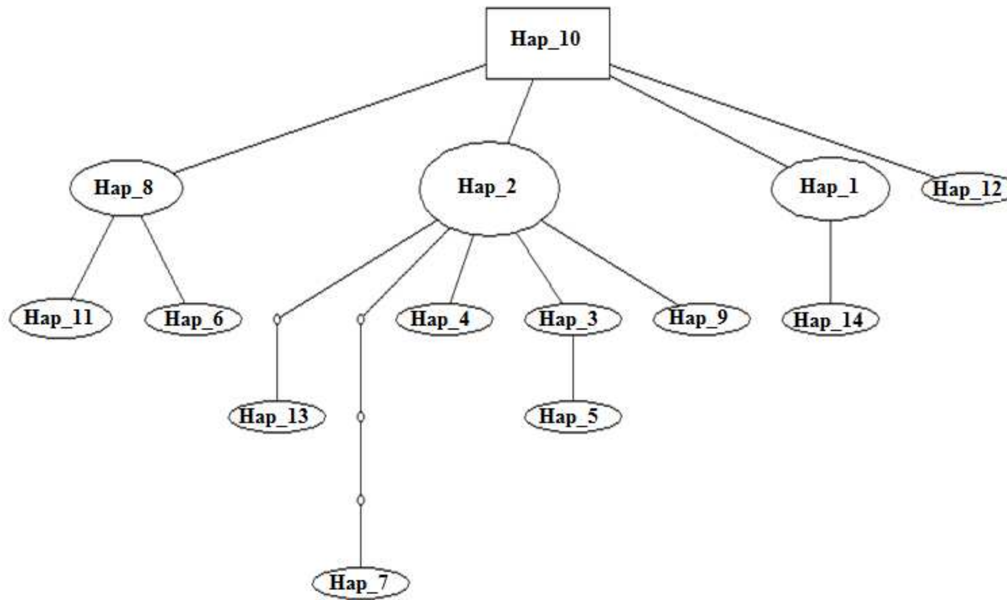

Fig. 2. Haplotype network of mitochondrial DNA in *T.ilisha*.

In the Padma population, number of segregating sites (S) was 8 whereas 16 segregating sites were observed in the Meghna population which was twice as much (Table III). Similarly almost double number of haplotypes was found in the Meghna population (14) than the Padma population (8). These results suggest that the Meghna population is more diversified than the Padma population. These could be resulted from the movement of individuals due to downstream waterflow from the Padma River to the Meghna River.The present study indicates low nucleotide diversity and high haplotype diversity between the Padma and Meghna populations. Haplotype diversity (H_d) was higher in the Padma population (0.905) whereas nucleotide diversity was higher in the Meghna population (0.0063±0.004).These findings suggest that *T. ilisha* has a high level of genetic diversity.Similarly findings were also observed by Verma *et al.* (2016) and Behera *et al.* (2015) who reported high genetic diversity and low nuclear diversity among riverine populations

using mtDNA marker. In general, large panmictic populations show high levels of genetic diversity due to their migratory nature (Santos *et al.* 2007), so the effect of genetic drift is minimized in such circumstances.

Table III. Genetic diversity among the Padma and Meghna River populations of *T. ilisha*

Parameters	Total	Padma	Meghna
Number of sequences	35	15	20
Number of haplotypes (h)	14	8	10
Haplotype diversity (H_d)	0.886	0.905	0.863
Average no. of nucleotide differences (K_t)	2.03	1.89	2.03
Nucleotide diversity (P_i)	0.006±0.004	0.0059±0.004	0.0063±0.004
Number of segregation sites/ polymorphic sites	16	8	12

Genetic differentiations (F_{st})

The present study revealed insignificant genetic differentiation in *T. ilisha* populations sampled from the Padma and Meghna River. Estimate of genetic differentiations (F_{st}) suggests that the Meghna population of Hilsa was closely related to the Padma population (F_{st}=0.058, P=0.07). However, the existence of private haplotypes in these populations suggests that they are not completely separated from each other.Several direct investigations have been performed using molecular approaches to infer levels of genetic variation and to detect the population structure of hilsa shad across the riverine, coastal and marine environments in Bangladesh and India. Both genetic and otolith chemistry data put support for presence of single stock of hilsa shad across riverine network and coastal areas in Bangladesh (Milton and Chenery 2001, BOBLME 2010). Ahmed et al. (2004) suggested more than one population of hilsa shad across the riverine and coastal environments in Bangladesh.Similarly, the control region study showedpopulation structuring on broad scale between the Bay of Bengal and Arabian Sea (F_{st}=0.044, p<0.001), it was failed to determine structure among riverine populations (Verma *et al.* 2016). RAPD markers could differentiate genetically the Hooghly and Narmada Riverhilsastock from the Ganga/Yamuna rivers populations (Brahmane *et al.* 2006) whereas mitochondrial cytochrome b region analysis did not identify any population differentiation and the genetic diversity was low in the Hooghly and Ganga Rivers (Brahmane *et al.* 2013). Rahman (1997) conducted a population study on Hilsa shad using allozyme electrophoresis and PCR based technique RAPD fingerprinting of mtDNA and have inferred more than one gene pool in Bangladesh. On another occasion, RAPD markers detected three separate populations from the foreshore waters of the Bay of Bengal (Dahle *et al.* 1997). Again, Rahman and Naevdal (2000) found several hilsa stock in the inland and marine waters of Bangladesh using allozyme marker. So, in most cases it was observed that there wasexistenceonly one population in the riverine system but genetically distinct population in the estuarine and marine environment. Asaduzzaman *et al.* (2019) suggested that local adaptation could be the reason behind genetic differentiation of riverine, coastal and marine Hilsa populations in Bangladesh waters.

Neutrality test

The demographic pattern in *T. ilisha* populations in the Padma and Meghna Rivers was examined using five neutrality test statistics namely: Tajima's D, Fu's F_S, Fu and Li's F^* and D^* and R^2 to test whether the diversity was a product of selection or demographic change of populations (Tajima 1989, Fu 1997). All tests of neutrality were not significantly different from neutral

expectation (Table IV). Non-significant neutrality tests suggest that genetic variation in populations is as expected under a neutral model of evolution and that most of the genetic variation could be explained by genetic drift and mutation (Tajima 1989, Fu 1997).The non-significant values ($p>0.05$) of Tajima's *D* and Fu's Fs statistical tests suggestthat there is no selection pressure influencing these riverine populations and a genetically equilibrium condition prevails among them. From the neutrality tests and pairwise-differences, it can be concluded that *T. ilisha* populations were panmictic i.e. there are no genetic differentiations among the Padma and Meghna riverine populations. Besides, the population of *T. ilisha* was stable and did not have any demographic change recent times. So, single management program for Hilsa fishery in the riverine populations could be effective for the sustainable capture in Bangladesh.

Table IV. Neutrality test results for the Padma and Meghna River populations

Tests	Test Statistics	Significance
Tajima’s D	-1.69	NS
Fu’s FS	-7.401	NS
Fu and Li’s D*	-2.91	NS
Fu and Li’s F*	-2.96	NS
Ramos (R^2) statistics	0.06	NS
Reggedness index ®	0.056	NS
Tau	2.032	NS

Conclusions

There was not any population structure among the Padma and Meghna Hilsa populations, rather high genetic diversity prevailed. It can be concluded from the above results that both populations could be managed with single management plan. This study could providebeneficial information for effective management and conservation projects of artisanal Hilsa fishery in Bangladesh. However, we recommend the application of several moderately evolving markers such as mitochondrial ATPase8/6, microsatellites, Single Nucleotide Polymorphisms (SNPs) for elucidating Hilsapopulation structure in the riverine ecosystems.

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