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Mitochondrial DNA insights into population structure for sustainable management of hilsa fishery

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Abstract

The rapidly declining populations of the Indian hilsa shad *Tenualosa ilisha* (Hamilton, 1822), a commercially important species in India, necessitate detailed understanding of its population structure, which appears to be shaped by a balance between anadromous mixing and philopatric isolation. In this study, *ATPase 8/6* and *cyt b* gene sequences of mitochondrial DNA were generated and analysed from seven populations of *T. ilisha*, sampled from Andhra Pradesh, Gujarat, Odisha, and West Bengal. The results revealed significant genetic differentiation among populations from various geographical regions. A total of 12 and 18 haplotypes were reported, with average haplotype diversities of 0.794 and 0.844, and nucleotide diversities of 0.004 and 0.003 for *ATPase 8/6* and *cyt b*, respectively. High haplotype diversity, along with low nucleotide diversity, suggests that hilsa populations may have experienced recent population expansion or gene flow. Analysis of molecular variance (AMOVA) of the *ATPase 8/6* dataset showed that 77.55% of the genetic variation was within populations, while 22.45% was among populations. For the *cyt b* dataset, 90.07% of the variation was found within populations, with only 9.93% variation among populations, indicating greater genetic homogeneity among populations based on the *cyt b* gene. Given that hilsa ascends rivers annually, there is a strong probability that philopatric migration has facilitated the accumulation of local genetic adaptations and the formation of distinct gene pools across different geographic regions. Therefore, hilsa populations from the northern and western parts of the Bay of Bengal and the Arabian Sea may require distinct restocking programs, fisheries management strategies, and conservation measures to address local genetic differences and to ensure the long-term sustainability of these populations.

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Introduction

The Indian shad, *Tenualosa ilisha* (Hamilton, 1822), is an anadromous fish species belonging to the family Dorosomatidae. Originally described as *Hilsa ilisha*, it was later reclassified as *T. ilisha* (Fisher and Whitehead, 1974). The species has a wide geographical distribution, encompassing the Persian Gulf, Red Sea, Arabian Sea, Bay of Bengal, Vietnam Sea, and China Sea (Bhaumik, 2013). Its broad range reflects adaptability to diverse marine environments, while its anadromous life cycle involves migration from the sea into freshwater rivers for spawning. *T. ilisha* is of considerable commercial and cultural importance, playing a vital role in the fisheries of these regions. *Tenualosa ilisha* contributes 20–25% of the total fish landings in the Hooghly–Bhagirathi river system alone (Ahsan, 2014), highlighting its significance for local fisheries. Renowned for its high nutritional content, soft oily texture, rich flavour, and exceptional mouthfeel, the species is locally known as 'Macher Raja,' or the king of fish. Despite its iconic status, *T. ilisha* populations in the Ganga River system have experienced substantial declines in recent years, primarily due to overfishing.

The decline of *T. ilisha* is further exacerbated by multiple anthropogenic and environmental factors, including reduced water discharge from barrages, heavy siltation, disruption of migratory routes, loss of critical feeding, spawning, and nursery habitats, and the indiscriminate harvesting of juveniles (Jatka) (Bhaumik, 2017; Mandal *et al*., 2018; Amin *et al*., 2004). To ensure the sustainability of hilsa fisheries, the Indian Council of Agriculture

Research (ICAR) has implemented management interventions, including induced spawning, larval rearing, and genetic improvement programs (Mahapatra *et al*., 2014). Population differentiation in migratory species, such as hilsa, may result from schooling behaviour, natal philopatry (Weatherhead and Forbes, 1994), geographical isolation, and adaptive responses to local environmental and climatic conditions (Brown *et al*., 2000). Understanding the population genetic structure and gene flow of a species is critical for developing effective restocking programs and guiding conservation and management strategies (Brown *et al*., 2003). Recent advances in molecular techniques have enabled the development of genetic markers that provide insights into fish stock migration, breeding population dynamics, and overall species conservation (Melamed *et al*., 2002).

Effective conservation and management of hilsa fisheries requires comprehensive information on the genetic diversity within and among populations. Various markers, including morphometric, protein, and DNA markers have been employed to assess stock differentiation (Pillay and Rosa, 1963; Ghosh *et al*., 1968; Rahman *et al*., 1997). Allozyme studies suggest the presence of a single population across rivers flowing into the Bay of Bengal (Salini *et al*., 2004; Lal *et al*., 2004), whereas RAPD (Shifat *et al*., 2003; Brahmane *et al*., 2006) and PCR-RFLP (Mazumder and Alam, 2009) markers have successfully distinguished populations among various rivers in Bangladesh. In India, population studies have often relied on a single gene, such as the Cytochrome b marker (Brahmane *et al*., 2013; Behera *et al*., 2015), or investigated mitochondrial control region and microsatellite markers separately (Verma *et al*., 2016a, b). Mohindra *et al*. (2019) studied hilsa populations from three river systems. Therefore, validation of genetic stock structure using two highly informative mitochondrial genes, *ATPase 8/6* and *cyt b*, which differ in evolutionary rates (Hewitt, 2004), across Indian rivers draining into both the Bay of Bengal and the Arabian Sea, is essential for robust conservation planning.

Mitochondrial markers are highly effective for assessing variability at both inter- and intra-specific levels due to their maternal inheritance, high mutation rates, and lack of recombination. In addition, these markers are cost-effective and technically simple, requiring only small amounts of frozen, fresh, or alcohol-preserved tissue, in contrast to microsatellite or single-nucleotide polymorphism analyses (Zimmerman *et al*., 2020). mtDNA markers have been widely applied in stock structure analyses across diverse vertebrate groups, including fish (Heist and Gold, 1999), birds (Mila *et al*., 2000), mammals (Menotti-Raymond and O'Brien, 1993), and reptiles (Shanker *et al*., 2004). Mitochondrial genes evolve at different rates (Meyer, 1993); for instance, the 16S rRNA gene evolves slowly and is useful for comparing genetic variability among individuals of the same species or closely related taxa, whereas rapidly evolving regions, such as the D-loop (control region) and ATPase 6/8 genes, are commonly used in population structure studies of several fish species (Chow *et al*., 1997; Fang *et al*., 2022). The cytochrome b (*cyt b*) gene encodes a conserved protein but exhibits intraspecific variation, particularly at the third codon position, making it valuable for identifying fish stocks (Habib *et al*., 2010).

Materials and methods

Sample collection

Samples of *T. ilisha* were collected with the help of local fishermen from across the different geographical locations in India (Table 1), including rivers emptying into the Bay of Bengal (*i.e.*, Ganga, Godavari, Hooghly) and Arabian Sea (*i.e.*, Tapti and Narmada) as well as marine landings at Diamond Harbour and Paradip Port. The fish species were identified based on morphological features described by Fisher and Whitehead (1974) and Talwar and Jhingran (1991). The sampled specimens photographed after being placed on graph paper, and meristic counts were recorded and then compared. The vouchers were preserved in 10% (v/v) formaldehyde, and muscle and fin tissues were preserved in 95% (v/v) ethanol. Specific and explicit codes were given to tissue samples and vouchers for each fish specimen (Table 1). All sampled materials are stored at the ICAR-National Bureau of Fish Genetic Resources (ICAR-NBFGR), Lucknow, Uttar Pradesh, India.

DNA isolation, PCR amplification and DNA sequencing

Genomic DNA (gDNA) was isolated from approximately 50 mg of muscle tissue using the standard Phenol: Chloroform:Isoamyl Alcohol (PCI) extraction method in a 25:24:1 (v/v) ratio, following the protocol described by Sambrook and Russell (2001). The precipitated DNA was dissolved in TE buffer, and its concentration was estimated using a Nanodrop 2000 (Thermo Scientific, USA). Primers ATI1L (5'-TAAATTGGGCCTAGCGTTAGCC-3') and ATI3H (5'-GGGTCATTAGGGTGACTGAATGG-3') were designed for amplification of the ATPase8/6 gene. While primers TCBF (5'-TCTGCCCGGACTTTAACCG-3') and TCBR (5'-GCTTTGGGAGTTAGGGGTGGAAG-3') were designed for amplification of the *cyt b* gene. All primers were designed using Primer3 (Rozen and Skaletsky, 2000) and validated using BLASTn (Altschul *et al*., 1990) to ensure specificity. The PCR reaction, with a 50 µl volume, comprised 1X buffer, 2 mM $MgCl_2$, 100 µM dNTPs, 3 U

Table 1. Detailed information on fish (*T. ilisha*) samplings and GenBank accession numbers

Collection site	Sample size		Latitude/ Longitude	GenBank Accession No.	
	cyt b	*ATPase 8/6*		*ATPase 8/6*	*cyt b*
Hooghly Feeder Canal, Farraka, West Bengal	10	9	24.48N/ 87.55E	KF475255-63	KC816486- 95
Ganga River, below Farraka Barrage, Malda, West Bengal	7	7	24.47N/ 87.55E	KF475281-87	KC816514- 20
Diamond Harbour, West Bengal	11	8	22.10N/ 88.10E	KF475247-54	KC816475- 84, KC816521
Paradip Port, Odisha	10	9	20.19N/ 86.36E	KF475264-72	KC816496- 505
Godavari River, Rajahmundry, Andhra Pradesh.	8	8	16.56N/ 81.44E	KF475273-80	KC816506- 13
Narmada River, Barkal, Gujarat	12	13	21.55N/ 73.25E	KF475288- KF475300	KC816522- 33
Tapti River, Ukai Dam, Surat, Gujarat	10	8	21.15N/ 73.35E	KF475238-45	KC816465- KC816474

Taq DNA polymerase, 10 picomoles of each primer, and 200 ng of template DNA. PCR was performed in Veriti®96-Wells Fast Thermal Cycler (Applied Biosystems, Inc., USA). The thermal cycle consisted of initial denaturation of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C (ATPase8/6) and 50°C (*cyt b*) for 45 s, extension at 72°C for 80 s with final extension at 72°C for 10 min. The quality of PCR amplicons was checked on a 1% agarose gel stained with ethidium bromide and documented using a gel documentation system (UVP BioImaging Systems, USA). Sequencing of the amplicons was performed using the dideoxynucleotide chain termination method (Sanger *et al*., 1977).

DNA sequence analysis

DNA sequence analysis was performed using MEGA version 11 (Tamura *et al*., 2021). Forward and reverse sequence reads were aligned using the Clustal W tool to generate full-length consensus sequences. The lengths of the *ATPase 8/6* and *cyt b* genes used for analysis were 842 bp and 1,152 bp, respectively. The resulting full-length sequences were submitted to GenBank, and their accession numbers are provided in Table 1. The evolutionary analysis was calculated using the Maximum Likelihood method (Felsenstein, 1981) with the bootstrap value from 1000 replicates in MEGA version 11. Branches consistent with less than 50% of bootstrap replicates were merged. The percentage of replicated trees in which the related taxa grouped together was displayed close to the branches (Felsenstein, 1985). The Kimura 2-parameter model was used to calculate evolutionary distances as the number of base substitutions per site (Kimura, 1980).

Haplotype analysis

Haplotype analysis was conducted using DnaSP v5 (Librado and Rozas, 2009) to determine the number of polymorphic sites (S), nucleotide composition, nucleotide diversity, and haplotype diversity (Tajima, 1983; Nei, 1987).

Population genetic analysis

Arlequin v3.5.1.3 (Excoffier and Lischer, 2010) was used to estimate pairwise FST statistics for testing isolation by distance and to perform analysis of molecular variance (AMOVA) to assess the hierarchical distribution of genetic diversity. The significance of pairwise population comparisons was evaluated using 20,000 permutations (Guo and Thompson, 1992). Minimum spanning networks were constructed using NETWORK v4.6.1.3 to infer relationships among haplotypes.

Results

DNA sequence analysis

In the complete nucleotide sequence of 842 bp of ATPase8/6 genes, upstream 168 bp constitute *ATPase8* gene and downstream 684 bp constitute *ATPase 6*, with an overlapping region of 10 bp from 159-168 positions. Out of the 842 bp, 29 positions (3.4%) were found to be mutable with 11 parsimony informative sites. Average frequencies of the bases in *T. ilisha* were: A=25.9%; T= 27.9%; C=30.3% and G=15.9%, which displayed bias towards the over-representation of C over G. Out of 1,152 nucleotide positions in *cyt b* gene, 23 (~2%) were found to be variable with 16 parsimony informative sites. The average frequencies were A: 23.9%; T: 27.8%; C: 30.4%; G: 17.9%, which also showed a bias towards the over-representation of C over G**.**

Haplotype analysis

In the *ATPase8/6* dataset, we observed 12 haplotypes from seven populations of *T. Ilisha* and the phylogenetic tree generated from these haplotypes formed two clades (one clade of Arabian sea populations and another of Bay of Bengal populations), both originating from haplotype Hap_2 (Fig. 1) . Out of 12 haplotypes, five were (~41.6%) shared among individuals of different populations (Hap_1, Hap_2, Hap_3, Hap_6, Hap_7) and remaining seven were private haplotypes, each confined to a particular population (Fig. 2). The Diamond Harbour population displayed the highest haplotype diversity (0.929), while the same was lowest in the Narmada population (0.410). In *cyt b* dataset, 18 haplotypes were observed from seven subject populations (Table 2) and the phylogenetic tree of these haplotype sequences formed a single clade (Fig.3) . Out of 18 haplotypes, 7 (~39%) were shared among different populations (Hap_1, Hap_2, Hap_5, Hap_6, Hap_7, Hap_8, Hap_12) and remaining 11 were private haplotypes, each confined to a particular population. The haplotype Hap_2 was common in all populations, and all haplotypes originated from Hap_2, directly or through successive mutations (Fig. 4) . The Pradip Port population displayed the highest haplotype diversity with the values of 0.911, while the same was lowest in the Tapti population (0.64444).

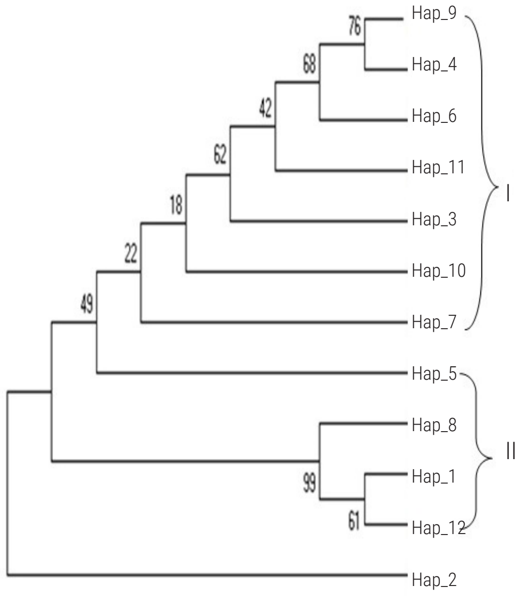

Fig.1. Molecular phylogenetic analysis of 12 haplotypes of *T. ilisha* constructed by Maximum Likelihood method (*ATPase8/6*).

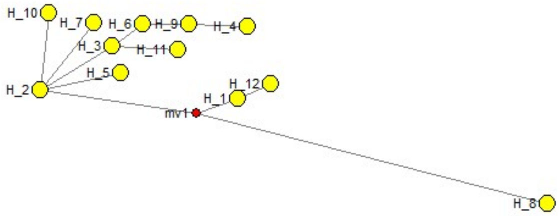

Fig. 2. Minimum spanning network of *T. ilisha* based on *ATPase8/6* haplotypes. Haplotypes separated by single lines are one mutation apart, and small circles along lines represent missing haplotypes (not sampled or extinct).

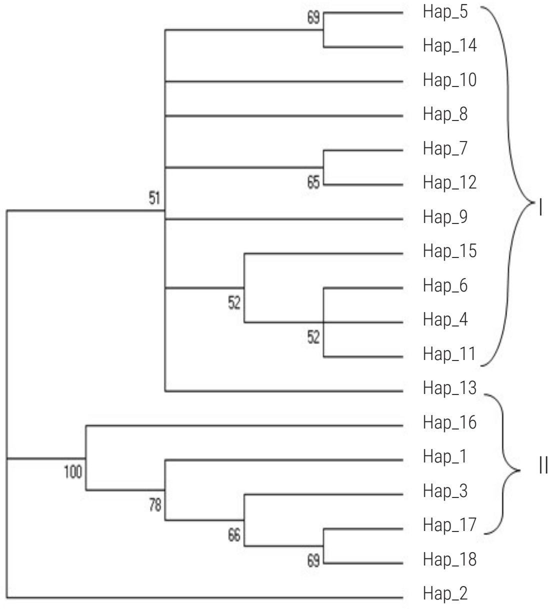

Fig. 3. Molecular phylogenetic analysis of 18 haplotypes of *T. ilisha* constructed by Maximum Likelihood method (*cyt b*).

Population genetic analysis

The AMOVA of the ATPase8/6 sequence dataset reveals that the majority of variation (77.55%) is attributed to within-population variation, while only 22.45% is attributed to among-population variation (Table 3) . Here, four gene pools, *i.e.,* (Tapti, Narmada), (Diamond Harbour, Hooghly, Hooghly Feeder Canal, Ganga), (Godavari), and (Paradip Port), were found to be significantly different. Additionally, 0.224 was reported as a significant FST value at a significance level of p<0.001. In the case of the *cyt b* sequence dataset, the hierarchical analysis of molecular variance (AMOVA) considering all populations as independent groups resulted in non-significant variation. By using one gene pool, two gene pools (Tapti, Narmada) and (Diamond Harbour, Hooghly, Hooghly Feeder Canal, Paradip Port, Godavari, Ganga) and three gene pools (Tapti, Narmada), (Diamond Harbour, Hooghly, Hooghly Feeder Canal, Paradip Port, Ganga) and (Godavari) significant variations were observed. AMOVA revealed 9.93% variation among populations and 90.07% variation within populations (Table 3) , indicating that there is significant geographical structuring within the population, which was further supported by a significant (p<0.01) FST value (*i.e.*, 0.0993).

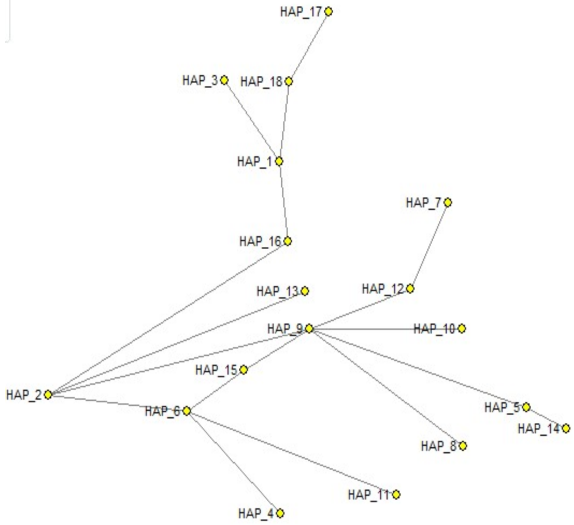

Fig. 4. Minimum spanning network of *T. ilisha* based on *cyt b* haplotypes. Haplotypes separated by single lines are one mutation apart, and small circles along lines represent missing haplotypes (not sampled or extinct).

Table 2. Haplotype and nucleotide diversities in different populations of *T. ilisha.*

Populations	*ATPase 8/6*			*cyt b*		
	No. of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)	No. of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)
Diamond Harbour	6	0.929±0.084	0.0025±0.0005	5	0.709±0.137	0.00129±0.00040
Hooghly Feeder Canal	4	0.694±0.147	0.0066±0.0030	5	0.844±0.080	0.00154±0.00029
Paradip Port	4	0.583±0.183	0.0010±0.0003	7	0.911± 0.077	0.00243± 0.00032
Godavari	4	0.750±0.139	0.0016±0.0004	4	0.786±0.113	0.00158±0.00034
Ganga	3	0.667±0.160	0.0012±0.0003	5	0.905±0.103	0.00190±0.00036
Narmada	3	0.410±0.154	0.0020±0.0010	5	0.66667± 0.141	0.00208±0.00072
Tapti	2	0.571± 0.094	0.0040±0.0006	3	0.64444±0.101	0.00307±0.00041

Table 3. Hierarchical analysis of molecular variance (AMOVA) for *T. ilisha*.

Source of variation	Variance	% Total	Fixation indices	p value
One gene pool (Tapti, Narmada, Diamond Harbour, Hooghly, Hooghly Feeder Canal, Paradip Port, Godavari, Ganga)				
ATPase 8/6				
Among populations	0.0918	22.45	0.2245	p<0.001
Within population	0.3172	77.55	-	-
cyt b				
Among population	0.04244	9.93	0.09932	p<0.01
Within population	0.38486	90.07	-	-
Two gene pools (Tapti, Narmada) and (Diamond Harbour, Hooghly, Hooghly Feeder Canal, Paradip Port, Godavari, Ganga)				
ATPase 8/6				
Among groups	0.1430	29.94	0.299	p<0.05
Among populations within group	0.0175	3.67	0.052	NS
Within population	0.3172	66.39	0.336	p<0.001
cyt b				
Among groups	0.06546	14.24	0.14243	p<0.05
Among population within groups	0.00928	2.02	0.02354	NS
Within population	0.38486	83.74	0.16261	p<0.01
Three gene pools (Tapti, Narmada), (Diamond Harbour, Hooghly, Hooghly Feeder Canal, Paradip Port, Ganga) and (Godavari)				
ATPase 8/6				
Among groups	0.1309	29.03	0.290	p<0.05
Among populations within group	0.0028	0.63	0.008	NS
Within population	0.3172	70.34	0.296	p<0.001
cyt b				
Among groups	0.06012	13.43	0.13427	p<0.01
Among population within groups	0.00279	0.62	0.00720	NS
Within population	0.38486	85.95	0.14050	p<0.01
Four gene pools (Tapti, Narmada), (Diamond Harbour, Hooghly, Hooghly Feeder Canal, Ganga), (Godavari) and (Paradip Port)				
ATPase 8/6				
Among groups	0.1097	25.54	0.255	p<0.05
Among populations within group	0.0027	0.64	0.008	NS
Within population	0.3172	73.83	0.261	p<0.001

Discussion

Local genetic adaptation and philopatry in *T. ilisha* may have contributed to the formation of a local gene pool across their geographic distribution. Other factors, such as geographic isolation, habitat variation, and migration, play substantial roles in the genetic structuring of fish populations (Roy *et al*., 2024). The mitochondrial genes exhibit a high rate of evolution, characterised by high genetic variability between haplotypes, even in small sample sizes (Habib *et al*., 2012; Das *et al*., 2024). In the present study, we observed 12 and 18 haplotypes from seven populations of *T. ilisha* in the *ATPase 8/6* and *cyt b* dataset respectively. Haplotype network and Maximum Likelihood tree analysis of both *ATPase 8/6* and *cyt b* support the monophyletic origin of the hilsa population and all haplotypes created from haplotype Hap_2 directly or through consecutive mutations, which is in accordance with the coalescent theory (Posada and Crandall, 2002). This theory suggests that the oldest mitochondrial lineage is represented by the most common haplotypes, *i.e.* haplotype Hap_2, and is likely to be the ancestral source of origin for all haplotypes. Except for common (Hap_2) as well as shared haplotypes (Hap_1, Hap_3, Hap_6, Hap_7 in ATPase8/6 and Hap_1, Hap_2, Hap_5, Hap_6, Hap_7, Hap_8, Hap_12 in *cyt b*), the remaining private haplotypes were likely to have originated independently through mutations. The occurrence of shared haplotypes indicated gene flow among stocks, while returning to the natal river, some individuals might have taken the wrong route, whereas the presence of private haplotypes suggests that some individuals isolated and evolved as localised populations in new habitats, so it may be concluded that populations have been fragmented and consequently expanded as an evolutionarily significant unit. The phylogenetic tree from *cyt b* and *ATPase 8/6* did not form distinct clusters, indicating the phylogenetic tree was unable to show a clear relationship between geographical location and haplotypes.

High haplotype diversity (h = 0.929 ± 0.084) and moderate nucleotide diversity (π = 0.00660 ± 0.003) were found in all populations analysed for the *ATPase 8/6* marker (Table 2), suggesting a recent population expansion followed by a small effective population size, triggered by founder events or bottlenecks (Grant and Bowen, 1998). These populations face a high risk of extinction due to poor adaptation to environmental changes and the persistence of lethal alleles resulting from genetic drift in a small effective population. Therefore, the present results underscore the need to halt the overexploitation of hilsa populations and inform conservation policies based on current genetic diversity data.

AMOVA revealed high within-population variation (77.55% in *ATPase 8/6* and 90.07% in *cyt b*) and low among-population variation (22.45% in *ATPase 8/6* and 9.93% in *cyt b*). AMOVA and significant F_{ST} values indicated significant geographical structuring within the population. The diversity within and between populations of migratory fish species was reported to be 85% and 15%, while that of non-migratory fish was 67.6% and 32.4% (Vrijenhoek, 1998), respectively. Our result, showing intermediate within-population variation, corroborates the migratory nature of *T. ilisha*, but at the same time indicates a mechanism of population isolation that is more effective than random migration in fish. Marine migratory fishes exhibit high levels of gene flow and a low degree of differentiation, largely due to their high dispersal abilities and large effective population size. However, in this study, *T. ilisha* shows restricted gene flow as revealed by pairwise FST and hierarchical AMOVA, suggesting the absence of a single panmictic hilsa population; thus, the null hypothesis of panmixia can be rejected for the studied hilsa populations.

Mohindra *et al*. (2019) studied the hilsa population from three river systems of Hooghly, Brahmaputra, and Padma (Farraka) and marine samples from Kakdwip and Digha. Their study suggested the role of temporal stability in population structuring of hilsa and considered the presence of distinct stocks within the Hooghly River in winter and monsoon, thus demanding differential stock-specific management strategies. Brahmane *et al*. (2013) reported the Ganga and Hooghly Rivers as single populations due to low genetic diversity. Behera *et al.* (2015) reported that the *T. ilisha* population from the Arabian Sea and the Bay of Bengal belonged to two distinct clades, as determined by Cytochrome b marker studies. Verma *et al*. (2016a; b) concluded that population structure defined by AMOVA and location-explicit haplotypes refined populations up to sea level (Bay of Bengal and Arabian Sea) but failed to refine up to basin level by D-loop, but microsatellite studies declared five distinct genetic groupings of *T. ilisha*, Godavari, Paradip Port, Tapti, Narmada, and locations around Bay of Bengal (Diamond Harbour, Hooghly Feeder Canal and Ganga River). As a marine migratory fish, hilsa shad populations from Diamond Harbour, the Hooghly feeder canal, and the Ganga River do not demonstrate genetic differentiation due to high gene flow and a large effective population size (Carreras *et al.*, 2017). Our study corroborates previous studies, suggesting that populations from the northern part of the Bay of Bengal (Hooghly, Ganga, and Diamond Harbour) share a common gene pool. It also suggests four distinct hilsa populations, namely the northern part of the Bay of Bengal (Hooghly, Ganga, and Diamond Harbour), Paradip Port, Godavari, and the Arabian Sea (Narmada and Tapti), thus requiring differential fisheries management and conservation policies. Additionally, the Narmada population exhibited a significantly divergent haplotype frequency distribution, accompanied by higher nucleotide diversity, pairwise nucleotide divergences, and significant pairwise FST values across all populations. These results suggest that an unknown mechanism resulted in the isolation of this population and prevented its mixing with other populations.

This study highlights the importance of using a combination of genetic markers in population genetics research, as their sensitivity and resolution depend on their mutation rates. While *cyt b* failed to differentiate populations at the river-basin level, *ATPase 8/6* revealed four distinct groupings, providing finer resolution for population differentiation. Therefore, we recommend using *ATPase 8/6* in combination with a nuclear DNA marker, as this approach offers a greater potential for detecting genetic variation and population structure than mitochondrial DNA alone.

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