Population genetic structure of Indian shad, *Tenualosa ilisha* Inferred from variation in mitochondrial DNA sequences

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Abstract

Indian shad, *Tenualosa ilisha,* is a commercially important anadromous fish representing major catch in Indo-pacific region. The present study evaluated partial Cytochrome *b* (Cyt *b*) gene sequence of mtDNA in *T. ilisha* for determining genetic variation from Bay of Bengal and Arabian Sea origins. The genomic DNA extracted from *T. ilisha* samples representing two distant rivers in the Indian subcontinent, the Bhagirathi (lower stretch of Ganges) and the Tapi was analyzed. Sequencing of 307 bp mtDNA Cytochrome *b* gene fragment revealed the presence of 5 haplotypes, with high haplotype diversity (Hd) of 0.9048 with variance 0.103 and low nucleotide diversity (π) of 0.14301. Three population specific haplotypes were observed in river Ganga and two haplotypes in river Tapi. Neighbour-joining tree based on Cytochrome *b* gene sequences of *T. ilisha* showed that population from Bay of Bengal and Arabian Sea origins belonged to two distinct clusters.

Introduction

Indian shad hilsa, *Tenualosa ilisha* (Ham.) is one of the most important tropical fish of Indo-Pacific region and has great market demand for its taste, flavor and culinary properties. The fish is rich in oils unlike many estuarine and freshwater fish species and provide valuable fatty acids for human health (Mohanty *et al.,* 2012). *Tenualosa ilisha* forms a predominant catch in Bangladesh and India particularly in the Hooghly-Bhagirathi river system accounting 77,912 tons during year 2011-2012 (Bhaumik, 2012). However, the catch has shown a sharp decline due to various factors including hydro-morphological changes in major spawning habitats, overfishing (of juveniles and brood fish) and habitat degradation resulting in disruption of migratory routes, loss of spawning, feeding and nursing grounds (Bhaumik, 2012). Therefore, stock structure information of hilsa is essential for conservation and sustainable management of this species. In particular, analysis of mitochondrial DNA has proved to be useful in determining population stock of fish species due to its simple mode of transmission avoiding recombination, high mutation rate and predominantly maternal inheritance (Hoolihan *et al.,* 2004). mt DNA has fast evolution rate and its maternal mode of inheritance makes it a very potential genetic marker system, alone or in combination with other nuclear markers such as microsatellites, for analyzing population structure and phylogenetic studies (Luhariya *et al.,* 2012). Cytochrome *b* gene of mtDNA is generally variable in vertebrates and these genes are consistently found to have high evolutionary rate (1.3% per million years) in fish (Perdices *et al.,* 2002; Habib *et al.,* 2011). In the present study, mt DNA marker was used to determine the genetic structure of this economically importance species from Bay of Bengal and Arabian Sea by analyzing the inter and intra-specific genetic variability using mitochondrial Cyt *b* gene.

Materials and Methods

Sample collections: Specimens of Indian shad, *Tenualosa ilisha,* were collected from five locations of two rivers, representing both East and West coast of India. The sites of river Ganga were near Lalgola, Hooghly ghat and Farakka from Bay of Bengal origin (East coast) and river Tapi near Ukai and Nuapada from Arabian Sea origin (West coast) (Fig. 1). A 50 mg of fin tissue,
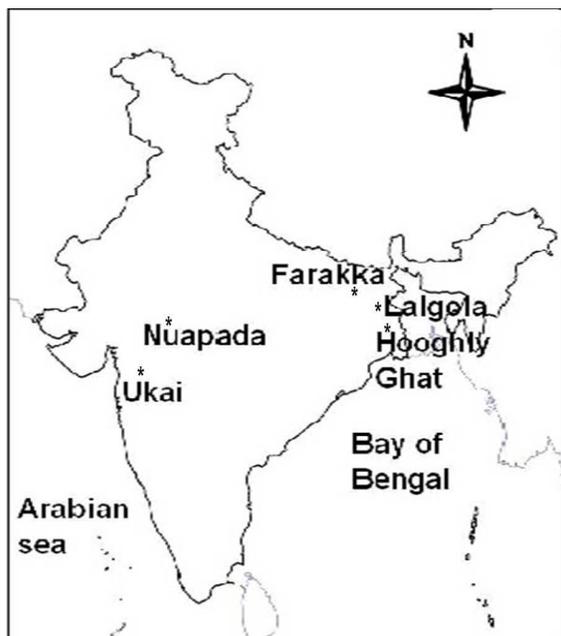
Fig. 1 : Map showing the sampling sites of *T. ilisha* from Nuapada and Ukai (Gujrat) and Farakka, Lalgola and Hooghly Ghat (West Bengal), India

100 mg of muscle and liver tissue samples from each specimen were preserved in 95% ethanol in 1:5 (tissue : ethanol) ratio.

DNA isolation and PCR amplification : Genomic DNA was isolated following Asahida *et al.* (1996) with slight modifications. The quantity of isolated DNA was estimated using a UV spectrophotometer and diluted to a final concentration of 100 ng μl^{-1} for PCR analysis. Mitochondrial Cytochrome *b* gene was amplified in 50 µl reaction volume with 5µl of Taq polymerase buffer, 0.2 mM of each dNTP, 0.4 µM of each primer, 1.0 U of Taq DNA polymerase and 50 ng of genomic DNA using thermal cycler Gene Amp PCR System 9700 (Applied Biosystems, USA). The universal Cytochrome *b* gene primers, L14841 (5´-AAAAGCTTCCATCCAACATCTCAGCATGATGAA A-3´) and H15149 (5´- AAACTGCAGCCCCTCAGAATGATATTT

GTCCTCA- 3´) were used for amplification of partial region of Cytochrome *b* gene. The thermal profile used was: 35 repetitions of a three step cycle consisting of denaturation at 94 °C for 1 min, annealing at 56 °C for 1.5 min and extension at 72 °C for 2 min including 4 min for initial denaturation at 94 °C and 7 min for final extension at 72 °C. The PCR products were visualized on 1.8% agarose gels and the most intense products were selected for sequencing.

DNA sequencing and analysis: The PCR amplified Cyt *b* gene products were sequenced in both directions using an ABI 3730xl capillary sequencer to check validity of the sequenced data. The contigs were prepared using DNA Baser software version 4.13. All the DNA sequences of mtDNA Cyt *b* gene of *T. ilisha* were submitted to NCBI GenBank. All DNA sequences were aligned using ClustalW (Thompson *et al.,* 1994). Pair-wise evolutionary distance among haplotypes was determined by the Kimura 2-Parameter method (Kimura, 1980) using software program MEGA 5 (Molecular Evolutionary Genetics Analysis) (Tamura *et al.,* 2011) UPGMA tree was constructed to verify the robustness of tree, bootstrap analysis was carried out using 1000 pseudo-replications. The number of polymorphic sites and nucleotide diversity (π), nucleotide composition and number of transition and transversion between species were determined using software DnaSP version 5.10 (Librado and Rozas, 2009). Analysis of molecular variance (AMOVA), as implemented in Arlequin version 3.5 (Excoffier and Lischer, 2010), was used to assess the population genetic structure of *T. ilisha*.

Results and Discussion

Partial Cytochrome *b* gene (307 bp) was sequenced in seven individual samples collected from two rivers of India to determine the genetic variability. The GenBank accession numbers of mitochondrial DNA Cyt *b* sequences are JX213627 to JX213633**.** A total of 89 positions were found to be variable with 5 haplotypes and 66 parsimony informative sites. The average frequencies of four nucleotides for all the samples of *T. ilisha* were A: 24.38%; T: 28.85%; C: 29.73%; G: 17.03%. The nucleotide sequences of Cytochrome *b* were A+T rich (53.23%) with transition to transversion ratio (Ts:Tv) 1.279. Transition substitutions were detected more commonly than transversional

Table 1 : Result of analysis of molecular varience (AMOVA) testing genetic structure of *T. ilisha*

Structure tested (Sampling sites)	Variance	Variation (%)	Φ Statistics	P-values
One group (NUP,UKI,FAR,LAL,HOG)*				
Among populations	0.76928	97.93	0.97	P = 0.08
Within populations	1.78868	2.07		
Two groups (NUP,UKI), (FAR,LAL,HOG)				
Among groups	32.02	78.07	Φ_{CT} = 0.78	P = 0.09
Among population within groups	8.49	20.70	Φ_{SC} = 0.94	P = 0.32
Within populations	0.50	1.23	Φ_{ST} = 0.98	P = 0.08

*NUP-Nuapada, UKI-Ukai, FAR-Farakka, LAL-Lalgola, HOG-Hooghlyghat

Table 2 : Matrix of population pairwise Fst (below diagonal), Population specific Fst (at diagonal) and associated P values (above diagonal) among five different populations of *T. ilisha*

Sampling site	Farakka	Lalgola	Hooghly ghat	Ukai	Nuapada
Farakka	**0.94130**	0.99099	0.99099	0.28829	0.35135
Lalgola	-1.0050	**0.99457**	0.99099	0.99099	0.99099
Hooghlyghat	-1.0050	0.00000	**0.99457**	0.99099	0.99099
Ukai	0.97333	1.00000	1.00000	**0.99457**	0.99099
Nuapada	0.98485	1.00000	1.00000	1.00000	**0.99457**
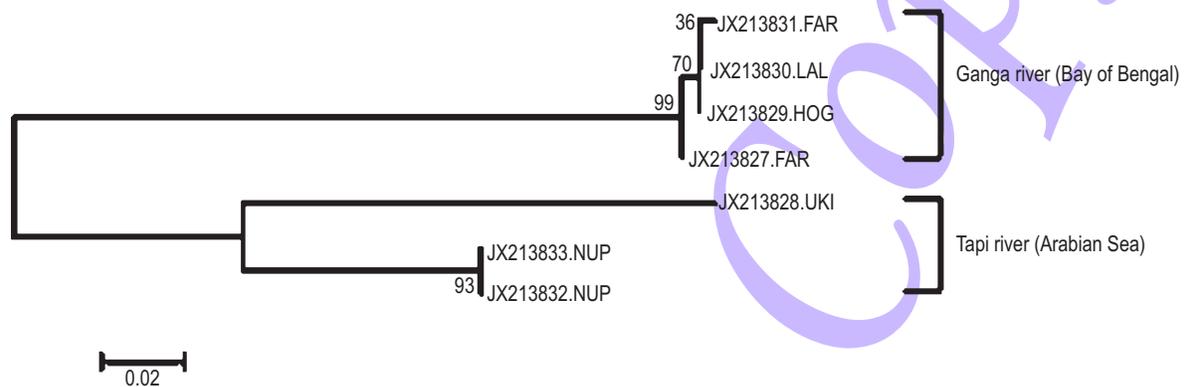
Fig. 2 : Neighbour-joining tree of *T. ilisha* collected from Ganga and Tapi river systems using mitochondrial Cyt *b* gene partial sequences. Bootstrap values after 1000 replications are shown above the nodes

one. The nucleotide diversity (π) for the all the five populations was found to be 0.14301 and haplotype diversity (Hd) was 0.9048 with variance 0.103. Out of the total variation, 97.93% was attributed to 'among populations' differences and only 1.03% was due to 'within populations'. In addition, hierarchical analysis of molecular variance showed significant difference among the two groups (F_{CT} = 0.78) (Table 1). The Fst value (0.97) was found to be non-significant (p = 0.08). Population pairwise Fst values ranged from -0.0050 to 1.000 and Population specific Fst value ranged from 0.94130 to 0.99457 (Table 2). A total of 5 distinct Cyt *b* mitochondrial DNA haplotypes were identified in five populations of *Tenualosa ilisha*; out of this Hap-1 was observed only in Farakka sample. Hap-2 was observed in two different sampling sites within the Ganga (Farakka and Lalgola). Hap-3 was present only in Hooghly ghat, whereas Hap-4 and HAP-5 were only present in Ukai and Nuapada, respectively (Table 3). The Neighbour-joining tree, based on Cyt *b* gene partial sequences, delineated *T. ilisha* into two distinct clusters with the first cluster formed by hilsa samples collected from river Ganga having Bay of Bengal origin and the second cluster was formed by hilsa samples collected from the river Tapi having Arabian Sea origin. The clusters were supported by high bootstrap values (Fig. 2).

The present study revealed genetic differentiation in *T. ilisha* populations collected from five different sites viz., Farakka, Lalgola and Hoogly ghat of river Ganga belonging to the Bay of Bengal origin, and Ukai and Nuapada of river Tapi belonging to

the Arabian Sea origin, indicating two different stocks. Lal *et al.* (2004) reported hilsa population from the Brahmaputra, Padma, Ganga and Hooghly river representing Bay of Bengal origin is of single stock using allozyme markers. Salini *et al.* (2004) reported a significant difference in allele frequencies in hilsa samples of Bangladesh, eastern India, Indonesia, Myanmar and Kuwait. No significant difference in population within Bangladesh or within the Bay of Bengal (Bangladesh, eastern India, Indonesia and Myanmar) was by using allozyme markers and morphometric analysis. Brahmane et al. (2006) studied that genetic structure of hilsa population sampled from the Ganga, Yamuna, Hooghly and Narmada rivers using RAPD and found that the hilsa from Ganga and Yamuna rivers formed one cluster while those of Hooghly and Narmada rivers constituted another cluster.

However, results of the present study contradict the findings of Brahmane *et al.* (2006). This contradiction might be due to the limitation of RAPD technique which did not differentiate between homozygosity and heterozygosity of the samples analyzed. In the present study, two different stocks of hilsa were observed belonging to the Arabian Sea and the Bay of Bengal origin which is supported by the findings of Salini *et al.* (2004). Cytochrome *b* gene amplified in the present study has been reported to be useful in detecting intra-specific variation in several species. This region has been found to be polymorphic and used successfully for intra-specific genetic diversity analysis in various fish species like *Salmo trutta* and *Sardina pilchardus* (Tinti *et al.,*

Table 3 : Relative frequency of five haplotype of *T. ilisha*

Haplotype	Farakka (2)	Lalgola (1)	Hoogly ghat (1)	Ukai (1)	Nuapada (2)
Hap-1	1	0	0	0	0
Hap-2	1	1	0	0	0
Hap-3	0	0	1	0	0
Hap-4	0	0	0	1	0
Hap-5	0	0	0	0	2
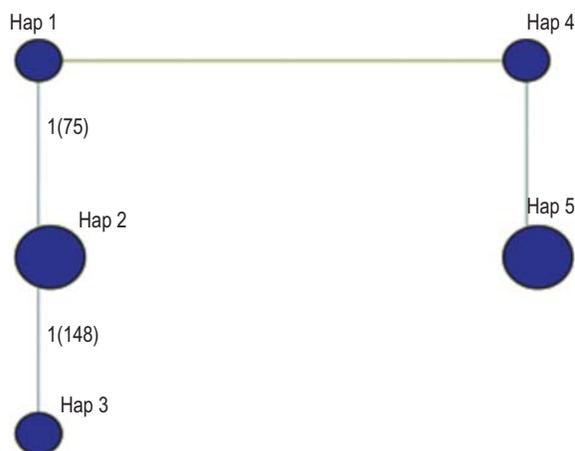
Fig. 3 : Median-joining network of haplotypes of *T. illisha*. Each circle represents a haplotype and circle size is proportional to haplotype frequency. Numeral indicates number of mutations and mutated positions are given in bracket

2002). The AMOVA analysis revealed a very low variation within population in *T. ilisha* (1.03%) and high variation among population. It was earlier reported that a migratory fish specie has 85 and 15% of each diversity within and between local populations and 67.6 and 32.4% for a non-migratory fish. The level of genetic divergence among populations of *T. ilisha* observed in this study was higher than that reported for a migratory fish, which also reflect the migratory nature of *T. ilisha.* Fst value (0.97) also supported the presence of significant genetic difference among population of the Bay of Bengal and the Arabian Sea origin. Such high intra-specific diversity could be expected as the sampling sites belonged to distant locations and it was possible that population investigated could have evolved in isolation after fragmentation from common ancestors. The results demonstrated that, partial mt DNA Cytochrome *b* gene was observed to be a potential marker for studying variation both within and among population in *T. ilisha*. This result clearly indicated that differences were occurring within the same population as in case of river Ganga (3 haplotypes) and river Tapi (2 haplotypes) were observed. Such studies have provided useful information in case of many other fish species. The success of conservation programme and effective management of fisheries

biodiversity depend on the level of genetic divergence within and among species, and developing strategies to maintain natural genetic diversity (Lakra *et al.,* 2009). Cytochrome *b* fragment is a promising marker to determine distribution and pattern of genetic variation across the native distribution of *Tenualosa ilisha.*

Presently, the abundance of species has drastically dwindled in almost all the river systems due to extensive fishing pressure and habitat degradation. The loss in habitat is directly related to the recruitment potential of hilsa fishery. Therefore, the need of the hour is conservation of this species through mass scale production. This may be considered as one of the possible approach so that a pond reared strain may be developed for the potential aquaculture. Information on genetic stock generated from this study will be useful to plan for stock specific strategies for breeding, conservation and management of wild population of *Tenualosa ilisha.*

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