A Seminar Paper

on

Population Structure of Hilsa Shad in Bangladesh Using Genetic Marker

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Population Structure of Hilsa Shad in Bangladesh Using Genetic Marker[1]

by

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Abstract

The Hilsa shad (*Tenualosa ilisha*) is locally known as 'Ilish' fish is found in three different ecological environments such as freshwater, brackish water and marine water in Bangladesh. For conservation of Hilsa shad, different stocks are discriminated on the basis of various pattern of genetic variation within different populations of this valuable fish species. Genetic structure investigation was done by different genetic markers like PCR-RFLP, mtDNA sequencing, RAPD etc. By using these population variation markers, sufficient amount of haplotypes diversity, nucleotide diversity, polymorphism were found in case of Hilsa shad of different ecosystems. Distinguishable genetic distance among freshwater, brackish water and marine water hilsa shad is also found by studying with these genetic markers. These indicate that the genetic variability of the present Hilsa shad population falls within the range of good condition and the genetic status of Hilsa shad does not appear to be affected though the catch has decreased due to overexploitation and indiscriminate killing.

Key words: Hilsa shad, brackish, PCR-RFLP, mtDNA sequencing, RAPD, haplotypes, polymorphism, overexploitation.

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Chapter I

INTRODUCTION

Bangladesh is very blessed with diversified aquatic resources. There are about 47 lakh ha of inland water bodies in the form of rivers, canal, haor, baor, beel, estuaries etc. There are also vast amount of sea areas in the Bay of Bengal. The Hilsa shad, *Tenualosa ilisha* belonging to the order Clupeiformes occurs in foreshore areas, estuaries, brackish-water lakes and freshwater rivers of south and south East Asia especially in Bangladesh. Its marine distribution extends from Iran and Iraq in the Persian Gulf to the west coast of India in the Arabian Sea and the Bay of Bengal (Pillay and Rosa Jr, 1963). Hilsa shad is the largest single fishable species in Bangladesh, present in almost all the major river systems, estuaries and marine environments (Bay of Bengal). The Hilsa shad is largely an anadromous species, but two other ecotypes - a fluvial potamodromous type and a marine type - have been recognized. The potamodromous stocks appear to remain in the middle reaches of the rivers throughout the year and breed therein. The anadromous stocks, whose normal habitat is the lower region of the estuaries and the foreshore areas, ascend the rivers during the breeding season and return to the original habitat after spawning (Raja, 1985).

However, fish and fisheries sector is playing an important role for the socio-economic development of Bangladesh. This sector contributes 3.65% to GDP, 22.15% to the agriculture sector, 1.92% in export earnings (DoF, 2016). In 2015-16, the annual production of fish was 3.8 million metric ton where Hilsa production was 0.5 million metric ton. This Hilsa fishery sector contributes 11% of the total fish production and 1% of the national GDP in the country (DoF, 2016).Hilsa is termed as the national fish of Bangladesh due to its popularity and economic importance. Among the fish, the national fish Hilsa has made its place into the Bengali culture and it is highly nutritious and delicious. It is an important food fish, rich in omega-3 polyunsatured fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Mohanty et al. 2012). The Hilsa fishery generates employment and income for millions of people in India, Bangladesh and Myanmar, worth over US$ 2 billion (BOBLME 2012).

Hilsa shad is endemic to the Bay of Bengal, Indian Ocean and Arabian Sea, and is well-distributed in the Ganga-Brahmaputra-Meghna drainage systems of the India and Bangladesh. In India, the Hooghly, lower stretch of the Ganga, the Brahmaputra, the

Godavari and the Narmada rivers yield a rich Hilsa catch (Bhaumik 2012). In Bangladesh, the Padma, the Meghna, the Jamuna, the Rupsa, the Shibsa, the Bishkhali and the Pyra rivers are the major sources of riverine Hilsa catch throughout the year (Haldar et al. 1992). The species has a large market demand, with a global average annual catch of about 0.72 million tons, of which approximately 50% to 60% originates from Bangladesh, 20% to 25% from Myanmar, 15% to 20% from India and 5% to 10% from other countries (e.g., Iraq, Kuwait, Malaysia, Thailand and Pakistan) (Milton 2010; Rahman et al. 2010).

However, at present the total catch has drastically declined in India and Myanmar, while the catch rate (CPUE) has decreased in Bangladesh (BOBLME 2014). These declines are attributed to a number of factors, including: low water discharge from the upstream rivers, loss of spawning, feeding and nursing grounds; blockade of natural migration for breeding due to dams and barrages across rivers (De et al. 1994); high rates of fishing pressure at different life stages in all habitats (i.e., marine, estuary and river stretches); alteration in physico-chemical parameters of rivers and estuaries because of addition of industrial pollutants and domestic effluents; reduction in water flows and depth due to increase in water abstraction and climate change; and higher rates of sedimentation in rivers. All these factors, either individually or combined, are responsible for habitat loss resulting in decline of Hilsa catch (BOBLME 2014).So, for increasing the catch rate as well as for conservation of this Hilsa fish, population structure should be identified.

Genetic variation refers to the differences in the heredity constituents of the individuals of species, which is important in maintaining the developmental stability and biological potential of fish populations. The degree of genetic variation in a population clearly specifies what kind of changes it might have experienced in the past, what the current situation is, and what the probability of sustenance is in future. Low levels of genetic diversity have a negative correlation with the potential for adaptation to changing environmental conditions, and that the survival of endangered species may be threatened. The study of genetic variation is important to conserve this species and increase adaptability based on changed environment.

RFLP (Random Amplified Length Polymorphism) are presently the most preferred molecular markers because of their co-dominant nature and option of performing analysis with the use of polymerase chain reaction. Through the flanking sequence RFLP are usually specific for the particular species. RFLP (Restriction Fragment length polymorphism) is a powerful genetic marker for quantifying genetic variations within and between populations of species, vital tool in genome mapping, genetic fingerprinting, useful in the characterization of genetic diversity or breeding patterns in animal populations. RFLP has proven to be a successful method for studying the population genetic structure and differentiation of many fish.

Mitochondrial DNA (mtDNA) is by far the most widely used population genetic marker in animals (Avise, 1987). In a population at mutation/drift equilibrium, the expected level of genetic diversity of a neutral locus is proportional to the effective population size and to the locus mutation rate (Wright, 1931) mtDNA diversity is therefore typically assumed to reflect demographic effects, i.e. variations in population size between species or populations, which makes it a popular tool for conservation purposes (Harrison, 1989).

RAPD procedures were first developed in 1990 (Williams et al., 1990) using PCR to randomly amplify anonymous segments of nuclear DNA with an identical pair of primers 8–10 bp in length. Because the primers are short and relatively low annealing temperatures are used, the likelihood of amplifying multiple products is great, with each product (presumably) representing a different locus. Because most of the nuclear genome in vertebrates is non-coding, it is presumed that most of the amplified loci will be selectively neutral. Genetic variation and divergence within and between the taxa of interest are assessed by the presence or absence of each product, which is dictated by changes in the DNA sequence at each locus. RAPD polymorphisms can occur due to base substitutions at the primer binding sites or to indels in the regions between the sites.

The present study was conducted with the following objectives:

1. To investigate the pattern of genetic variation within different Hilsa shad population of Bangladesh
2. To discriminate different stocks in order to facilitate development of rational programmes for conservation of Hilsa shad

Chapter II

MATERIALS AND METHODS

This seminar paper is exclusively a review paper. Therefore, all the information were collected from secondary sources with a view to prepare this paper. Various relevant books and journals, which were available in the library of Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), were used for the preparation of this paper. For collecting recent information internet browsing was also be practiced. Good suggestions, valuable information and kind consideration from my honorable Major Professor, course instructors and other resources personnel were taken to enrich this paper. After collecting necessary information, it has compiled and arranged chronologically for better understanding and clarification.

Chapter III

REVIEW OF FINDINGS

3.1 Taxonomy of Hilsa shad

The taxonomy of the species, described below, is based on the studies of Pillay and Rosa (1963), Whitehead (1985), Shad Foundation (1998), FishBase (2012).

Systematic position

Phylum: Vertebrata

Class: Teleostomi

Sub-class: Actinopterygii

Order: Clupeiformes

Family: Clupeidae

Genus: *Hilsa*

Species: *Hilsa kelee*

Genus: *Tenualosa*

Species: *Tenualosa ilisha*

3.2 Geographical Distribution of Hilsa

Country	Names of rivers/lakes
Bangladesh	**Principal rivers** The Meghna, the Padma, the Jamuna and the Brahmaputra **Major rivers** The Sibsa, the Baleswari, the Pasur, the Rupsa, the Madhumati, the Kocha,the Lohalia, the Tetulia, the Biskhali, the Buriswar, the Karnaphuli, the Feni, the Naaf, the Kharkhana, the Arial Khan, the Khairabad, the Muhuri, the Surma, the Halda, the Kushiyara, the Matamuhuri and the Maheskhali Channel. **Minor rivers** The Sangu, the Baral, the Atai-Nabaganga, the Kobadak, the Chitra, the Bhairab, the Betna, the Kumar, the Little Feni, the Selonia, the Mongla, the Ilisha, the Ghuaisakhali, the Bhandra, the Khulpetua and the Kaligang
India (West coast)	The Narbada, the Tapti, the Purna, the Ulhas, the Savitri, the Kali and the Vembanad
India (East coast)	The Hoogly-Bhagirathi, the Godavari, , the Cauvery, the Krishna, the Mahanadi, the ganga and its tributaries, the Padma, the Brahmaputra and the Barak including smaller rivers namely Korayar, Pamaniyar, Vellar, Palar, Pennar, Manneru and Uppeteru and the Chilika lake
Iraq	The Shatt-al-Arab, the Tigris, the Euphrates and the Lake Hammar
Iran	The Shatt-al-Arab
Pakistan	The Sindh (Indus), the Jhelum and the Ravi
Myanmar	The Irrawaddy, the Naaf and the Sittang

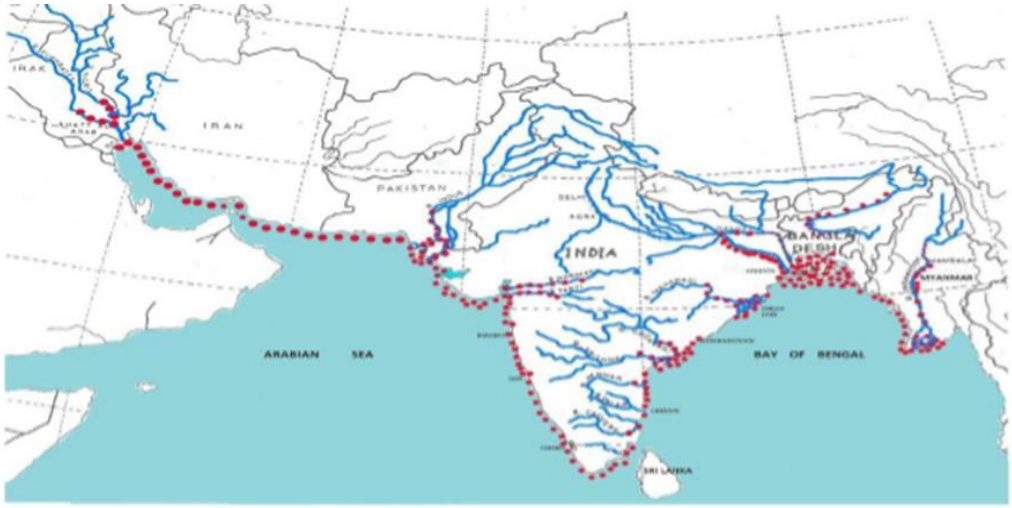

Figure 1. Geographical Distribution of Hilsa Shad (*Tenualosa ilisha*)

3.3 Life cycle of Hilsa

Hilsa shad lives at sea for most of its life but migrates to freshwater rivers for spawning, after which it returns to the sea. However, some stocks/ races of the species do not migrate from the sea to the river or vice versa (Blaber et al. 2003). In freshwater rivers, Hilsa eggs hatch after 23 to 26 hours at an average temperature of 23°C (Jones & Menon 1951). The size of the hatchlings varies between 2.3 and 3.1 mm (Motwani et al. 1957). When the larvae become capable of swimming, they migrate to suitable nursery grounds, normally in the lower regions of rivers and, eventually, to coastal waters. Juvenile hilsa in the nursery grounds are called jatka in Bangladesh (Rahman & Haldar 1998). Juvenile hilsa called Jatka remain around the nursery grounds for about 5 to 6 months and attain a maximum size of 15 to 16 cm (Raja 1985), but with a dominant size of 10 to 12 cm (Rahman & Haldar 1998). Juvenile hilsa slowly acquires the ability to tolerate saline water and move downstream to the estuary.

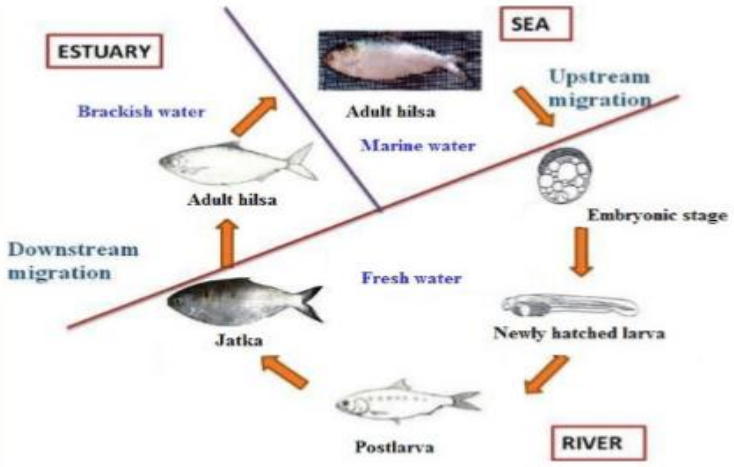

Figure 2. Life cycle of Hilsa Shad (*Tenualosa ilisha*)

3.4 Population stratification of Hilsa shad in Bangladesh by PCR-RFLP

The genetic structure of three populations of Hilsa shad *Tenualosa ilisha* belonging to three different ecological zones such as freshwater (Chadpur), brakish (Kuwakata) and marine (Cox's Bazar) were studied by Polymerase Chain Reaction- Restriction fragment Length Polymorphism of mitochondrial DNA.

3.4.1 Restriction fragment Length Polymorphism analysis and geographic variation of mt DNA haplotype

Table 1(a). Restriction fragment patterns of Hilsa mtDNA obtained by seven endonuclease digestion

Endonucleases types	**XbaI**		**BamHI**	**EcoRI**		**EcoRV**	
Fragment site (bp)	**A**	**N**	**N**	**A**	**B**	**A**	**B**
	1302	2168	2168	-	1440	-	1608
	887	-		1395	-	1489	-
				785	-	705	-
				-	750	-	588
Total length (bp)	2189	2168	2168	2180	2190	2194	2196
No. of fragments	2	1	1	2	2	2	2

Table 1(b). Restriction fragment patterns of Hilsa mtDNA obtained by seven endonuclease digestion

Endonucleases types	**HaeIII**				**Hpa II**	
Fragment site (bp)	**A**	**B**	**C**	**D**	**A**	**B**
	-	-	1596	-	-	1196
	1269	-	-	-	-	999
	-	1214	-	1214	950	-
	-	976	-	-	660	-
	602	-	602	-	546	-
	-	-	-	510		
	-	-	-	390		
	321	-	-	-		
Total length (bp)	2192	2190	2198	2114	2156	2195
No. of fragments	3	2	2	3	3	2

(Source: Ahmed *et.al*, 2004)

The size of each of the amplified mtDNA D-loop regions for all samples of Hilsa was approximately 2.2kb. The number of DNA fragments generated from the restriction enzymes ranging from two to three bands with the size varying from 55 to 2029 bp showed in table 3(a) and 3(b). The estimated molecular weights of all the restriction fragments are also shown here. The number of cleavage patterns produced by site variation was two (A and B) for EcoRI, EcoRV, Hind III and Hpa II and four (A, B, C, D) for Hae III. Xba I yielded a monomorphic pattern 'N' and a polymorphic pattern 'A' and Bam HI yield a monomorphic pattern in all the populations.

Table 2. Distribution of mtDNA D-loop region haplotypes and their frequencies in three populations of Hilsa shad.

		Population		
Haplotype No.	**Haplotypes**	**Chandpur**	**Kuwakata**	**Cox's Bazar**
1	ANAAAAA	1(0.143)		
2	NNABBAB	1(0.143)		
3	ANAAAAB	2(0.286)	1(0.143)	
4	NNABAAB	1(0.143)		
5	NNAAABA	1(0.143)		
6	ANAAACB	1(0.143)		
7	ANAAABA		1(0.143)	
8	ANAABAB		1(0.143)	
9	ANABABB		2(0.286)	1(0.143)
10	NNABBBB		1(0.143)	
11	ANAAABB		1(0.143)	
12	ANBAADB			1(0.143)
13	NNAAADB			3(0.429)
14	NNABBDA			1(0.143)
15	ANAAADA			1(0.143)

(Source: Ahmed *et.al*, 2004)

A total of fifteen mtDNA D-loop haplotypes multiple for each population were identified in the Hilsa samples of the three populations. The haplotypes were mostly population specific except haplotype 3 which was shared by Chandpur and Kuwakata populations and haplotype 9 which was shared by Kuwakata and Cox's Bazar populations. All the three populations have one dominant haplotype each while the haplotype 3 was dominant in Chandpur population, haplotype 9 was dominant in Kuwakata population and haplotype 13 was dominant in Cox's Bazar population.

3.4.2 Genetic Variation within Population

Table 3. Genetic Variability within the three different populations Hilsa shad

	Populations		
	Chandpur	**Kuwakata**	**Cox's Bazar**
Sample size	7	7	7
No. of haplotypes	6	6	5
Rate of haplotype	0.857	0.857	0.714
Haplotype diversity	0.979	0.979	0.918
Nucleotide diversity	36.75%	36.75%	25.56%

(Source: Ahmed *et.al*, 2004)

PCR-RFLP analysis was conducted for 3 populations of Chandpur, Kuwakata and Cox's Bazar. As haplotypes 3 was shared by both Chandpur and Kuwakata population and haplotype 9 was shared by Kuwakata and Cox's Bazar, so Chandpur, Kuwakata and Kuwakata and Cox's Bazar population are close respectively and genetic variation within these population is also very low. Random mating or distribution can be reported also for these Chandpur, Kuwakata as well as Kuwakata and Cox's Bazar populations. Haplotypes were found at a rate of 0.857 within the populations of Chandpur and Kuwakata and 0.714 in Cox's Bazar population. The haplotype diversity was 0.979 in both Chandpur and Kuwakata population and 0.918 in Cox's Bazar population. The intrapopulation nucleotide diversity was 36.75% in each of Chandpur and Kuwakata and 25.56% in Cox's Bazar population. Genetic identity was 0.9789 for Chandpur and Kuwakata population, 0.8333 for Chandpur and Cox's Bazar population and 0.8513 for Kuwakata and Cox's Bazar population.

3.4.3 Phylogenetic relationships among populations

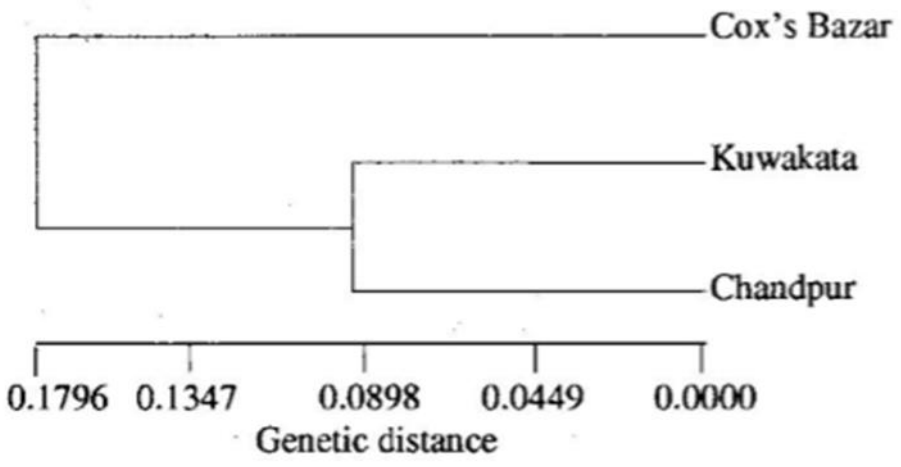

Figure 3. UPGMA dendrogram summarizing genetic distance between Hilsa populations of Cox's Bazar, Kuwakata, Chandpur (Source: Ahmed *et.al*, 2004)

The genetic distances were 0.0941, 0.1796 and 0.1458 between Chandpur and Kuwakata populations, Chandpur and Cox's Bazar populations and Kuwakata and Cox's Bazar populations respectively.

3.5 Population stratification of Hilsa shad in Bangladesh by PCR-RFLP

Genetic variability and divergence in two riverine (the Jamuna and the Meghna), two estuarine (Kuakata and Sundarbans) and one marine (Cox's Bazar) populations of *T. ilisha* were done by Polymerase Chain Reaction- Restriction fragment Length Polymorphism of mitochondrial DNA of D-loop region.

3.5.1 Restriction fragment Length Polymorphism analysis

Table 4. Restriction fragment patterns of Hilsa mtDNA obtained by four endonuclease digestion

		Haplotypes						
Restriction enzyme	**Recognition sequence**	**A**	**B**	**C**	**D**	**E**	**F**	**G**
XbaI	T/CTAGA	1608 568	1241 930	2168				
EcoRI	G/AATTC	1439 566 95	1566 566	1529 566				
EcoRV	GAT/ATC	1432 707	1296 707	1296 675	1375 707	1432 675		
HaeIII	GG/CC	680 660 440 425	680 660 541 200	680 660 409 390	680 660 580 200	1210 580 200	680 660 409 200	849 680 660

(Source: Mazumder *et.al*, 2009)

The size of each of the amplified mtDNA D-loop regions for all samples of Hilsa was approximately 2.2kb. The number of DNA fragments generated from the restriction enzymes ranging from two to four bands with the size varying from 95 to 1608 bp showed in table 6. The estimated molecular weights of all the restriction fragments are also shown here.The cleavage patterns produced due to variations in restriction sites were three for XbaI and EcoRI, five for EcoRV and seven for HaeIII. In some cases, however, the sum of the fragment sizes did not exactly equal the total size of the amplified region, probably due to small fragments being lost or bands of similar size co-migrating.

High levels of haplotype and gene diversity within and significant variation among populations of *T. ilisha* were observed.

3.5.2 Genetic Variation within Population

Table 5. Genetic Variability within the five different populations Hilsa shad

	Populations				
	Balashi	**Chandpur**	**Cox's Bazar**	**Sundarbans**	**Kuakata**
Sample size	18	18	18	18	18
No. of haplotypes	11	9	12	13	11
No. of polymorphic sites	20	20	23	24	19
Rate of haplotype	0.611	0.500	0.677	0.722	0.611
Haplotype diversity	0.948±0.05	0.882±0.05	0.967±0.03	0.960±0.03	0.954±0.02
Expected heterozygosity	0.451±0.29	0.363±0.26	0.531±0.16	0.569±0.24	0.343±0.09
F_{ST} indices	0.099	0.105	0.092	0.085	0.079
Tajima's D test	0.751	0.150	1.280	0.082	0.565
Fu's Fs test	-1.057	0.178	-3.26	-1.899	2.814

(Source: Mazumder *et.al*, 2009)

Polymorphic haplotypes were observed in all the five populations. The rates of haplotypes (number of haplotypes observed divided by the sample size) ranged from 0.500 (Chandpur) to 0.722 (Sundarbans). Haplotype diversity was high in all the five populations, ranging from 0.882 (Chandpur) to 0.960 (Sundarbans). The average gene diversity across the loci was highest in the Sundarbans population (0.569) and lowest in the Kuakata (0.343).The overall Fixation Index (FST) across the populations was 0.092 and the population specific FST indices ranged from 0.085 (Sundarbans) to 0.105 (Chandpur). Tajima's D test (Tajima, 1989) and Fu's F test (Fu, 1997) were not significant ($p > 0.05$) in all the populations.

3.5.3 Phylogenetic relationships among populations

Table 6. Estimates of pairwise FST (Population differentiation) values (below diagonal) and genetic distance (above diagonal) among five *T. ilisha* populations in Bangladesh.

	Ba	Ch	CB	Su	Ku
Ba	0.00000	0.00243	0.04816	0.07628	0.01959
Ch	0.00563	0.00000	0.07252	0.07847	0.03334
CB	0.10497	0.15380	0.00000	0.05637	0.03402
Su	0.16109	0.16531	0.12173	0.00000	0.00145
Ku	0.044120	0.07391	0.07536	0.00334	0.00000

Note: Ba: Balashi, Ch: Chandpur, CB: Cox's Bazar, Su: Sundarbans, Ku: Kuakata,

(Source: Mazumder *et.al*, 2009)

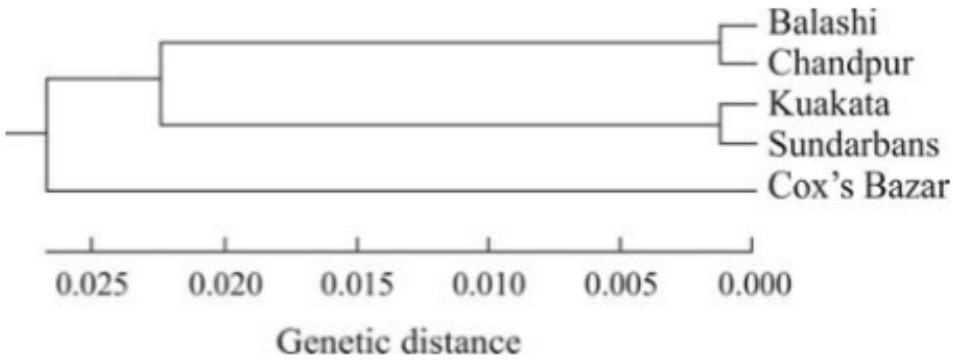

Figure 4. UPGMA dendrogram summarizing genetic distance between Hilsa populations of Balashi, Chandpur, Kuakata, Sundarbans and Cox's Bazar (Source: Mazumder *et.al*, 2009)

The UPGMA dendrogram based on genetic distance resulted in two clusters. The first cluster contained four populations and the second contained only the Cox's Bazar population. The first cluster was subsequently divided into two sub-groups. The two river populations, Balashi and Chandpur, formed one group and the two estuarine populations, Kuakata and Sundarbans, the second Significant differentiation between the riverine and marine (Cox's Bazar) populations, but not between the marine and one of the estuarine. There is no evidence of spawning in the sea, but there is evidence of *T. ilisha* estuarine spawning in Bangladesh (BOBP, 1987). Thus, fishes from the Cox's Bazar region must migrate to any of the rivers or at least up to the estuaries.

Fishes from the Cox's Bazar region may go up to the Kuakata region, but not to the Meghna and Jamuna rivers, as significant differentiations between the Cox's Bazar and the two river populations have been observed, but not between the Cox's Bazar and Kuakata.

3.6 Population stratification of Hilsa shad in Bangladesh by Mitochondrial DNA sequence

Indian shad, *Tenualosa ilisha*, is a commercially important anadromous fish representing major catch in Indo-pacific region. Partial Cytochrome b (Cyt b) gene sequence of mtDNA in *T. ilisha* was used for determining genetic variation from Bay of Bengal and Arabian Sea origins. Sequencing of 307 bp mtDNA Cytochrome b gene fragment revealed the presence of 5 haplotypes. Haplotype was considered when single base differs from others. Variable sites named haplotype h1, h2, h3, h4, h5 are given below.

Table 7. Relative frequency of five haplotype of *T. ilisha* from Bay of Bengal and Arabian Sea origins

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

(Source: Behera *et.al*, 2015)

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 8. Result of analysis of molecular varience (AMOVA) testing genetic structure of *T. ilisha*

Structure tested (Sampling sites)	Variance	Variation (%)
One group (NUP,UKI,FAR,LAL,HOG)*		
Among populations	0.76928	97.93
Within populations	1.78868	2.07
Two groups (NUP,UK), (FAR,LAL,HOG)		
Among groups	32.02	78.07
Among population within groups	8.49	20.70
Within populations	050	1.23

(Source: Behera *et.al*, 2015)

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

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.

Table 9. Matrix of population pairwise Fst (below diagonal), Population specific Fst (at 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
Hooghly ghat	-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**

(Source: Behera *et.al*, 2015)

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.

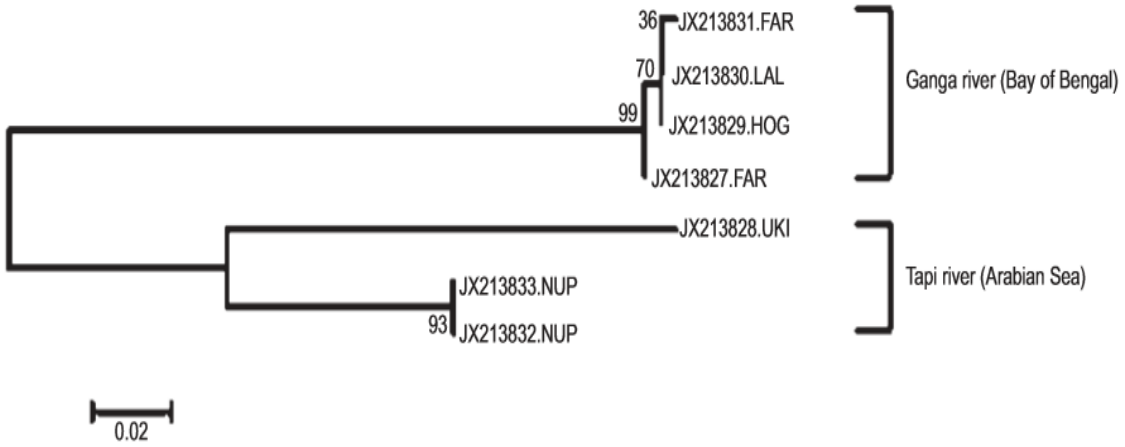

Figure 5. Neighbour-joining tree of *T. ilisha* collected from Ganga and Tapi river systems using mitochondrial Cyt b gene partial sequences. (Source: Behera *et.al*, 2015)

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.

3.7 Use of RAPD fingerprinting for delineating Hilsa shad population

RAPD was used to delineate the Hilsa populations collected from the Ganga, Yamuna, Hooghly and Narmada rivers at six different locations.

Table10. Number and size of fragments amplified by different random amplified polymorphic DNA primers

Primer	Sequence (5' to 3')	G + C content (%)	Total number of fragments	Size range of fragments (bp)
OPA-10	GTGATCGCAG	60	16	255-1000
OPA-11	CAATCGCCGT	60	16	340-1200
OPA-19	CAAACGTCGG	60	15	300-1300
OPC-01	TTCGAGCCAG	60	16	265-1200
OPD-11	AGCGCCATTG	60	18	285-1200
OPD-19	GGGGTGACGA	70	17	300-990

(Source: Brahmane *et al.*, 2006)

Six degenerate primers were used to generate the fragment patterns. All the primers were highly polymorphic and generated high numbers of amplification products. The six primers OPA-01, OPA-11, OPA-19, OPC-01, OPD-11 and OPD-19 were employed to perform the amplification reactions. All primers generated a high number of bands. The six primers generated 98 bands of which 98% were polymorphic.

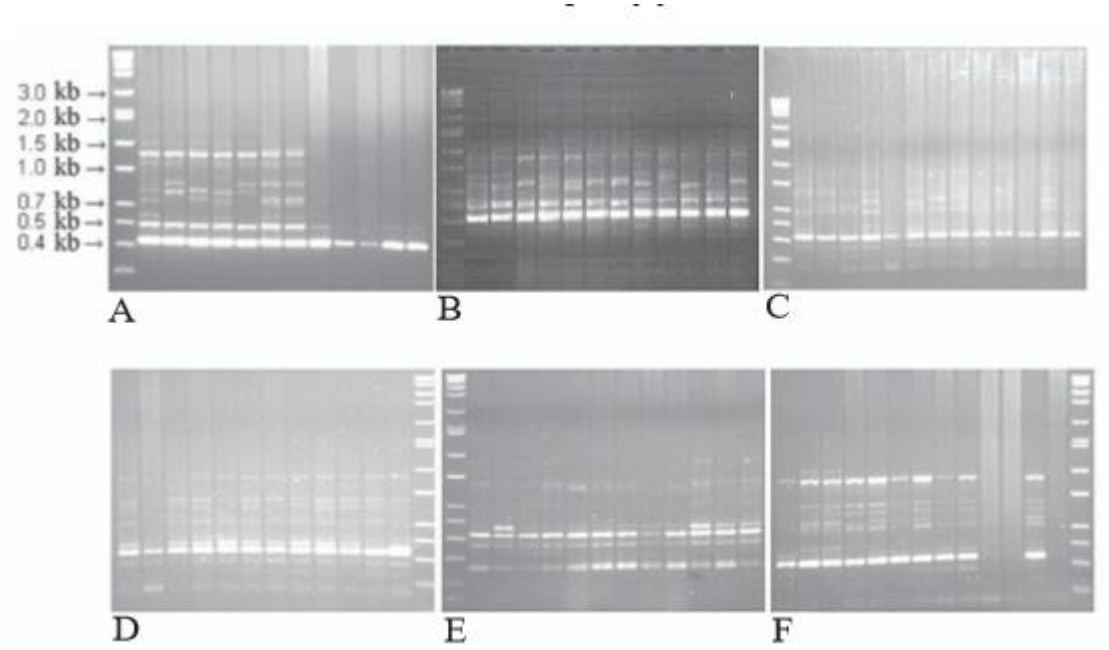

Figure 6. Random amplified polymorphic DNA fragment patterns generated using primer OPA-10. A. Yamuna-Allahabad; B. Ganga-Beniagram; C. Ganga-Lalgola; D. Feeder Canal-Farakka; E. Hooghly-Nawabganj; F. Narmada-Bhadbhud. M is the molecular marker Directload wide range DNA marker (Sigma). (Source: Brahmane *et al.*, 2006)

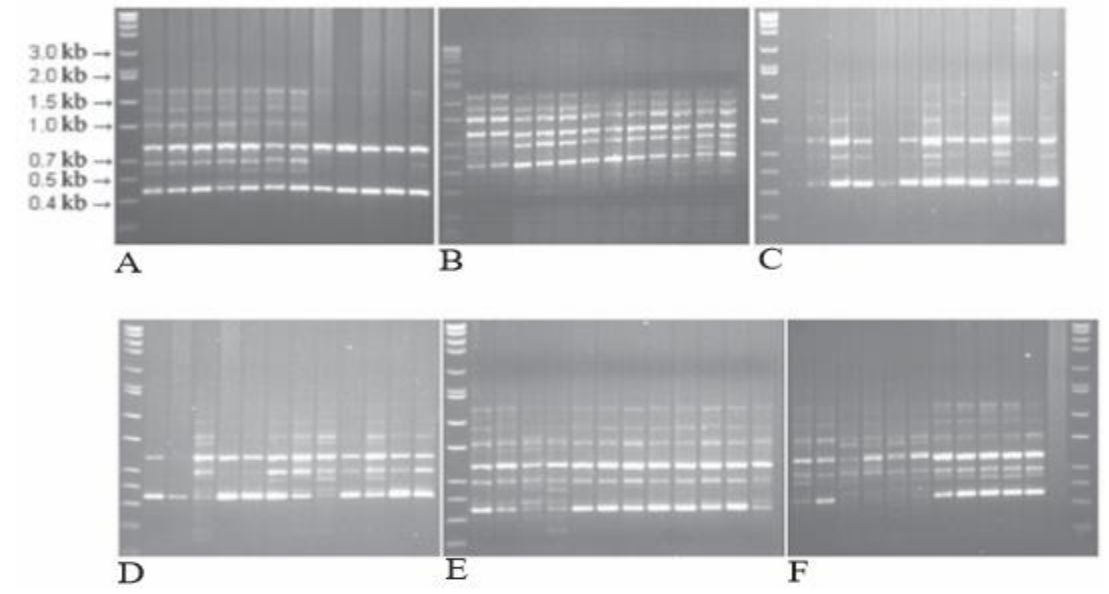

Figure 7. Random amplified polymorphic DNA fragment patterns generated using primer OPA-11 A. Yamuna-Allahabad; B. Ganga-Beniagram; C. Ganga-Lalgola; D. Feeder Canal-Farakka; E. Hooghly-Nawabganj; F. Narmada-Bhadbhud. M is the molecular marker Directload wide range DNA marker (Sigma) (Source: Brahmane *et al.*, 2006)

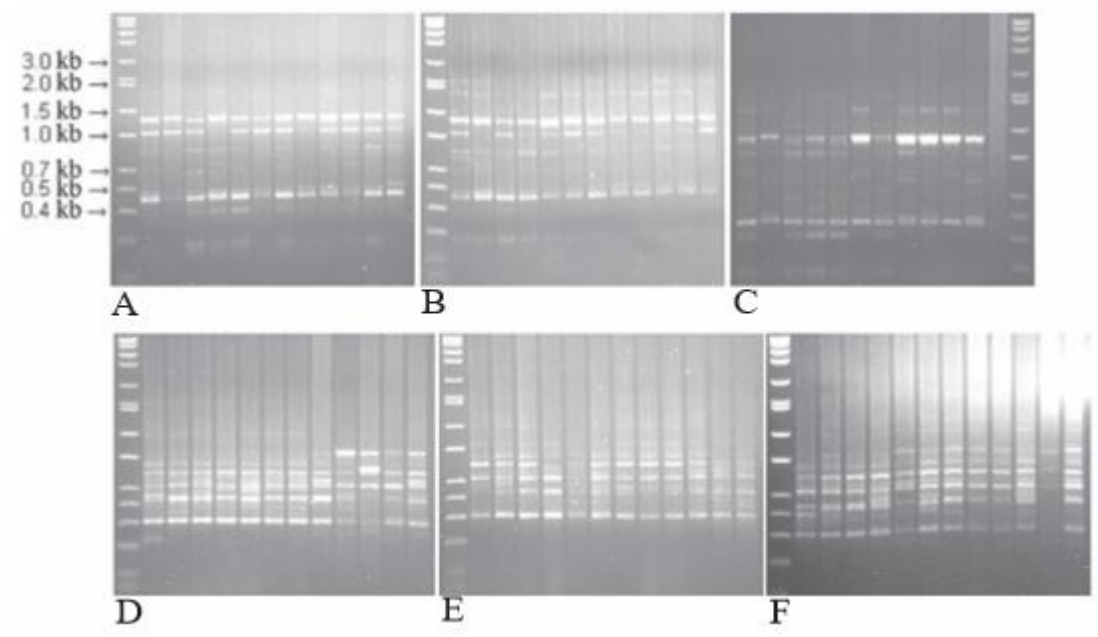

Figure 8. Random amplified polymorphic DNA fragment patterns generated using primers OPA-19: A. Yamuna-Allahabad; B. Ganga-Beniagram; C. Ganga-Lalgola, and OPD-13: D. Feeder Canal-Farakka; E. Hooghly-Nawabganj; F. NarmadaBhadbhud. M is the molecular marker Directload wide range DNA marker (Sigma). (Source: Brahmane *et al.,* 2006)

The RAPD profiles of different primers are shown in the figure 2, 3, 4.

Table11. Nei's (1978) measure of genetic distances between Hilsa populations from six different locations.

River/Sampling	**Location**					
	Yamuna Allahabad	**Ganga Beniagram**	**Ganga Lalgola**	**Feeder canal Baghirathi**	**Hooghly Nawabganj**	**Narmada Bhadbhud**
Yamuna/Allahabad	0					
Ganga/Beniagram	0.277					
Ganga/Lalgola	0.213	0.231				
FeederCanal/Baghirathi	0.292	0.259	0.231			
Hooghly/Nawabganj	0.372	0.360	0.268	0.288		
Narmada/Bhadbhud	0.394	0.325	0.374	0.258	0.284	0

(Source: Brahmane *et al.,* 2006)

The genetic distances were calculated according to Nei (1978). The highest genetic distance value 0.394 which was found between Allahabad and Lalgola.

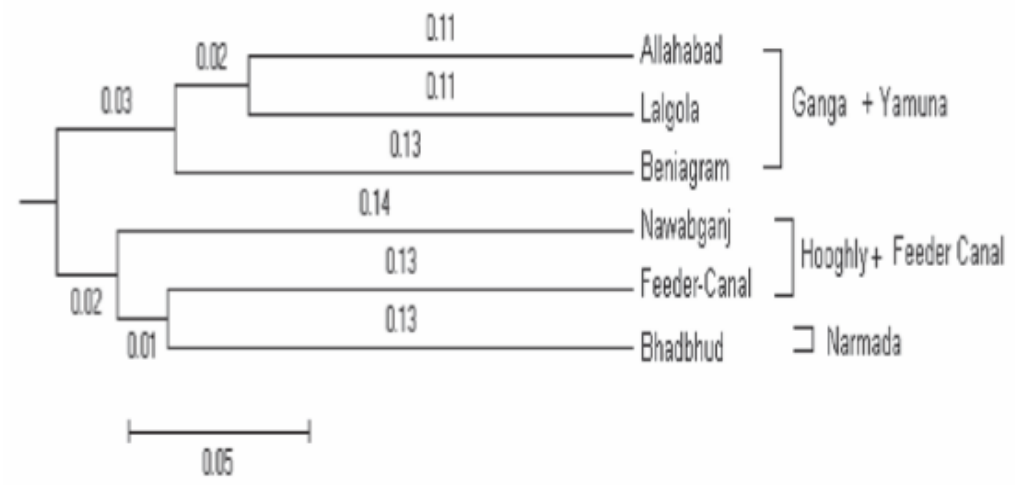

Figure 9. UPGMA dendrogram summarizing genetic distance between populations of Hilsa collected from India. (Source: Brahmane *et al.*, 2006)

The UPGMA dendrogram based on Nei's (1978) genetic distance indicated the segregation of the *Tenualosa ilisha* population collected from different sites into two clusters. The populations from Allahabad (Yamuna), Beniagram (Ganga) and Lalgola (Ganga) belonged to one cluster and those from the feeder canal, Nawabganj (Hooghly) and Bhadbhud (Narmada) belonged to other.

3.8 Use of RAPD fingerprinting for discriminating two populations of Hilsa Shad *Tenualosa ilisha* from inland rivers of Bangladesh

RAPD was applied to analyze the genetic variation of the Hilsa shad *Tenualosa ilisha* from the two major inland rivers (Padma and Meghna) in Bangladesh.

Table12. The 12 DNA primers that produced the 39 polymorphic bands useful for characterizing each of the tested fish population

DNA Primer		Number of bands	
Name	**Sequence (from 5' to 3')**	**Total**	**Polymorphic**
OPAB-3	TGGCGCACAC	36	9
OPAB-8	GTTACGGACC	41	11
OPE-1	CCCAAGGTCC	38	7
OPE-13	CCCGATTGCG	29	5
OPE-17	CTACTGCCGT	35	8
OPG-3	GAGCCCTCCA	52	10
OPG-6	GTGCCTAACC	43	6
OPG-9	CTGACGTCAC	37	8
OPG-15	ACTGGGACTC	39	6
OPH-4	GGAAGTCGCC	26	7
OPH-13	GACGCCACAC	48	9
OPN-13	AGCGTCACTC	56	12

(Source: Shifat *et al.*, 2003)

Twelve degenerate primers were used to generate the fragment patterns. All the primers were highly polymorphic and generated high numbers of amplification products. The twelve primers OPAB-3, OPAB-8, OPE-1, OPE-13, OPE-17, OPG-6, OPG-9, OPG-15, OPH-4, OPH-13, OPN-13 were employed to perform the amplification reactions. All primers generated a high number of bands.

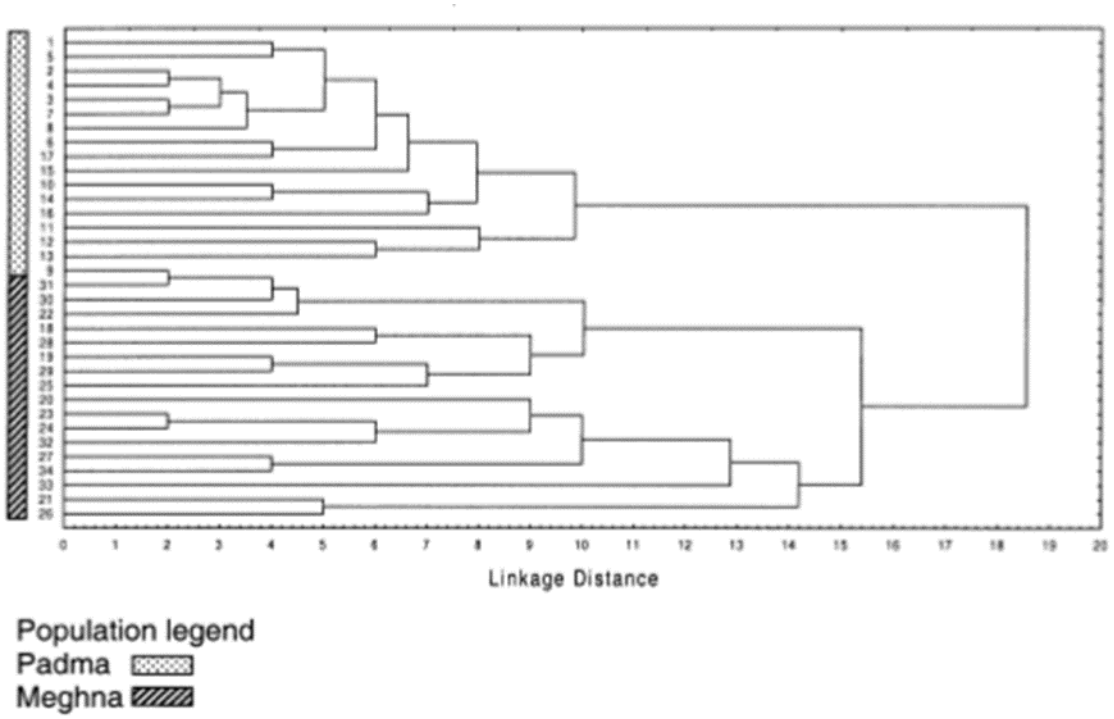

Figure 10. UPGMA dendrogram summarizing linkage distance between populations of Padma and Meghna (Source: Shifat *et al.*, 2003)

A dendrogram was constructed with the two Hilsa shad populations to analyze the genetic distances. The resulting dendrogram clustered into two major groups. One for the Padma population and another from Meghna population.

3.9 Use of RAPD fingerprinting for discriminating two populations of Hilsa Shad *Tenualosa ilisha* from inland rivers of Bangladesh

RAPD was applied to analyze the genetic variation of the Hilsa shad *Tenualosa ilisha* from Chandpur (freshwater), Barguna (brackish water) and Cox's Bazar (salt water) in Bangladesh.

Table13. The maximum number of scored fragments for the seven Operon primers selected for this study

Primer	Maximum number of fragments
OPA-10	8
OPA-11	18
OPA-19	7
OPC-01	12
OPC-12	7
OPD-11	9
OPD-13	11

(Source: Dahle *et al.*, 1997)

Seven primers were used to generate the fragment patterns. All the primers were highly polymorphic and generated high numbers of amplification products. The seven primers OPA-10, OPA-11, OPA-19, OPC-01, OPC-12, OPD-11, and OPD-13 were employed to perform the amplification reactions. All primers generated a high number of bands.

Table14. Estimated similarity (Sij) for each primer between the different samples of Hilsa shad

Primer	Chandpur/ Cox's Bazar	Chandpur/ Barguna	Cox's Bazar/ Barguna
OPA-10	0.469	0.489	0.465
OPA-11	0.524	0.510	0.476
OPA-19	0.344	0.446	0.487
OPC-01	0.358	0.336	0.344
OPC-12	0.343	0.324	0.316
OPD-11	0.413	0.393	0.391
OPD-13	0.370	0.434	0.387
Mean	0.403	0.419	0.409
SD	0.070	0.072	0.067

(Source: Dahle *et al.*, 1997)

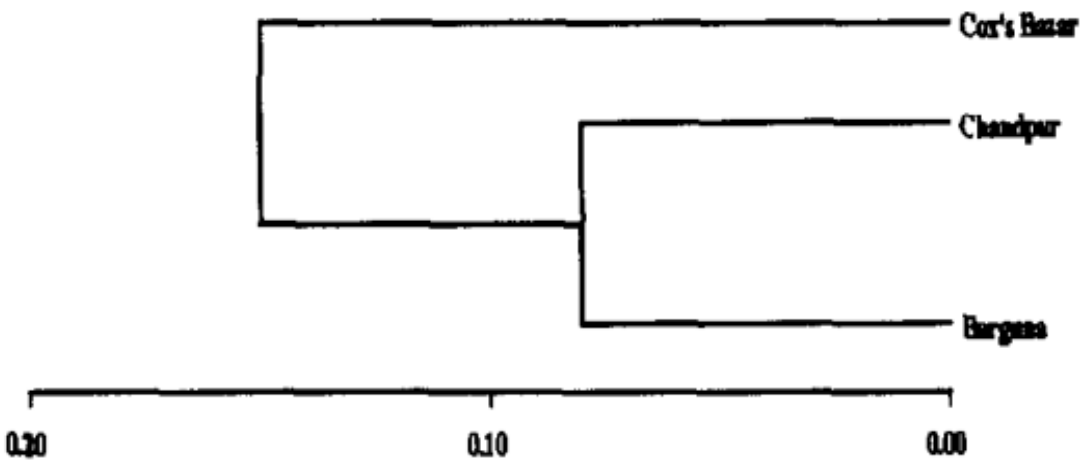

Figure11. UPGMA dendrogram summarizing linkage distance between populations of Cox's Bazar, Chandpur and Barguna

(Source: Dahle *et al.*, 1997)

A dendrogram was constructed with the three Hilsa shad populations to analyze the genetic distances. The resulting dendrogram clustered into two major groups. One for the Cox's Bazar and another two for Chandpur and Barguna population.

Chapter IV

CONCLUSIONS

Hilsa shad (*T. ilisha*) is the national fish of Bangladesh. It is found in freshwater river systems, estuaries and marine environment. Population structure analysis with the samples of Bangladesh and surrounding areas of Bangladesh by different genetic markers like PCR-RFLP, mtDNA sequence analysis and RAPD revealed three population genetic variation patterns and finally divided into three groups corresponding to the three different environments riverine, estuarine and marine. However, certain degree of gene flow between estuaries and marine environment is also reported.

The delicious taste and contribution of this fish has led to its position as one of the most economically important fish in this country. Furthermore, Hilsa fishery constitutes the largest single species fishery in the riverine, estuarine and marine ecosystems of the country. It is essential to recognize that geographically and genetically different populations as different stocks should be managed separately (Carvalho and Hauser, 1994). MtDNA PCR-RFLP analysis, mtDNA sequence analysis and RAPD analysis in *T. ilisha* indicate that population sub-division does indeed exist in this species. On the basis of the Analysis of Molecular Variance, it is concluded that there are three stocks of Hilsa with a substantial level of inter-population genetic divergence among river, estuarine and marine populations. However, these experiments with genetical marker to reveal the population stratification of Hilsa shad were done with limited resources. Therefore, to reach a more definite conclusion, larger samples from throughout the distribution range of the species in the country and surrounding areas should be analyzed by sequencing of mtDNA with faster evolving molecular markers, such as micro-satellite loci.

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