Use of RAPD Fingerprinting for Discriminating Two Populations of Hilsa shad (*Tenualosa ilisha* Ham.) from Inland Rivers of Bangladesh

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The Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) was applied to analyze the genetic variation of the Hilsa shad, *Tenualosa ilisha* Ham., from the two major inland rivers (Padma and Meghna) in Bangladesh. Twenty-eight random 10-mer primers were primarily scored in 8 individuals from each of the two locations. Fifteen primers, which gave polymorphism, were selected and used in the final analysis of 34 individuals from the two sites. Using these primers, 480 scorable DNA fragments were found, of which 98 (20.41%) were polymorphic. By comparing the RAPD banding patterns, variations were found between and within the populations. A dendrogram was constructed with the polymorphic fragments to analyze the genetic distances between the Hilsa shad populations. The results show two major clusters of Padma and Meghna, assuming different spawning populations with different stocks or races of Hilsa shad in the major Bangladesh rivers.

Keywords: Genetic Diversity, RAPD, *Tenualosa ilisha*

Introduction

Hilsa shad (*Tenualosa ilisha* Hamilton), one of the most important tropical fish of the Clupeidae family, is the national fish of Bangladesh. It is primarily an anadromous species and occurs in the coastal shelf, brackish, estuarine, and fresh water rivers of the western division of the Indo-Pacific faunistic region. Its geographical distribution extends from the Persian Gulf eastward to Myanmar, including the western and eastern coasts of India, the upper Bay of Bengal, and the South China Sea. However, 75% of the catch is reported from Bangladesh water (Anon, 1987; Mazid and Islam, 1991). Moreover, in

Bangladesh, Hilsa fishery contributes a vital role, approximately 30% of the total fish production (Hossain *et al*., 1987; Mazid and Islam, 1991). Either directly or indirectly, about 40% of the fishermen or 2% of the total population of the country earn their livelihood from this fishery. The fish is recognized as a delicious food and one of the major sources of protein for the huge, poor, and unnourished population of Bangladesh. Therefore, the economic contribution from this single species of fish is very high in an agriculture-based country like Bangladesh.

The fish lives in the sea or lower region of estuaries for most of its life. During the commencement of the southwest monsoon, they start the spawning migration towards the estuaries and rivers (from July till October, also from January to February/March). During this migratory period, the catch percentage of hilsa from the inland rivers is very high, and 80% of the females are found in the egg-releasing condition to support the spawning status of the fish (*source*: Hilsa Shad Profile, http://www.pathcom.com/~mizan.html). After breeding, the juveniles of Hilsa shad, from 4-15 cm (locally known as Jatka), are abundant from February to May in the foreshore and riverine waters of Bangladesh's deltaic rivers, including the Padma and Meghna (Rahman, 1996). After growing 1-2 years in the sea, the fish matures and reaches a size of 32-35 cm prior to their spawning migration towards the inland rivers, and the cycle continues (*source*: Virtual Bangladesh: The Story of the Hilsa shad, http://www.virtualbangladesh.com/ilish.html).

However, the exact spawning season for the species is still controversial. Reportedly the spawning varies from a few months to year round; this duration depends on the river or different parts of the regions where the species is distributed. Therefore, the exact stocks are still in dispute. For a solution, many authors investigated their biology and migratory strategy from the 19th century until the present. Jenkins (1938) pointed out that there may be two or more races or varieties of hilsa with different spawning grounds. Moreover, Mojumdar (1939) and Dutt (1966) claimed that the fish persist in three ecotypes: (1) Saline water of the sea. (2) Muddy fresh water of

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rivers like Padma and Hoogly. (3) Clear fresh water, like Meghna. Raja (1985) also investigated and discovered that hilsa stocks are different in each of the major river systems in the Indo-Bangladesh regions. Furthermore, Mazid and Islam (1991) examined and described three breeding grounds of hilsa; one in Padma and two in Meghna.

Some morphological differences were revealed among the hilsa stocks by morphometric and meristic studies that were sampled from different rivers and localities in Bangladesh. Quddus *et al.* (1984a) found significant differences in the body height; consequently they divided the hilsa stocks into Broad and Slender types. Also, there were observed differences in age and growth (Quddus *et al.*, 1984b) and spawning season and fecundity (Quddus *et al.*, 1984c) between these two groups. Currently there is no published data available for discriminating the stocks into subspecies.

However, the production of Hilsa fishery has been significantly decreased in Bangladesh waters during the past few years. This is due to several natural and man-made barriers as well as the overexploitation of juveniles and egg-carrying females from the inland rivers. It is important to re-establish the Hilsa fishery in the inland rivers and more extensively in northern rivers (e.g. Padma, Jamuna, Bramhaputra, etc.) where it was once abundant. For better management, it is quite necessary to reveal the genetic characterization of all possible hilsa stocks. For this purpose, Rahman *et al.* (2000) studied the hilsa shad population that was sampled from marine, estuaries, and fresh water (River Meghna) locations. Genetic variation among the individuals and groups with allozyme electrophoresis were found. They found that hilsa shad of Bangladesh belong to more than one gene pool, rather than just a single panmictic population. This discrimination between the same sampled populations was confirmed with a RAPD-PCR analysis (Dahle *et al.*, 1998).

It was supposed that a RAPD analysis (Williams *et al.*, 1990) could more quickly and less expensively yield valuable information on the population structure of hilsa (Dahle *et al.*, 1998) than, for example, a tagging experiment (Pillay *et al.*, 1963; Blower *et al.*, 1981; Ward and Grewe, 1995). It is also more reliable than morphomertic studies. Quddus *et al.* (1984a) and Shafi *et al.* (1977) found the morphological differences among the riverine hilsa populations. Moreover, it is noted that hilsa caught in the Padma and Meghna rivers can be distinguished by their distinctive taste. It is well known that hilsa of the Padma are tastier and fetch a higher price than hilsa from the Meghna. The taste difference that is also accompanied by some morphological differences may suggest the presence of two separate races or varieties within the different spawning grounds. Therefore, in the present study, the RAPD markers were analyzed to investigate whether the Hilsa shad populations that were sampled from the two major inland rivers, Padma and Meghna, are genetically different.

Fig. 1. Map showing the two locations: Aricha Ghat, Daulatdia, Goalundo (Padma), and Chandpur (Meghna).

Materials and Methods

Sampling During May 1999 till April 2002, two different sampling stations, based on different freshwater locations (Fig. 1), were chosen to collect samples of Hilsa. These included Aricha ghat, Daulatdia, and Goalunda of the Padma and Chandpur of the Meghna. The muscle, liver, and brain tissue were isolated from the freshly caught fish and preserved at −20ºC for future use.

DNA extraction For the isolation of the total genomic DNA, a short procedure was applied according to the protocol modified from Dahle *et al.* (1998). Tissues (150-200 mg) were placed in a 1.5 ml tube that contained a 0.5 ml lysis buffer (4 mM NaCl, 0.5 mM EDTA, 0.1% SDS, 0.01% Proteinase K), then chloroform (250 µl) and phenol (250 µl) were added, mixed gently, and centrifuged at 14,000 rpm for 5 min. The supernatant was then transformed to a fresh eppendorf tube. Next, 250 µl of chloroform was added, mixed, and centrifuged at 14,000 rpm for 5 min. The DNA was precipitated with 250 µl 7.5 M ammonium acetate and two volumes of 99% ethanol (ice-cold). The DNA pellet was washed with 70% ethanol, dried, dissolved in a TE buffer (10 mM Tris-HCl, 1 mM EDTA), and stored at −20ºC.

PCR conditions The reaction mixture (25 µl) for PCR was

Table 1. 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

composed of 10 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM MgCl$_2$, 200 µM dNTPs, 60 ng primer, 1.0 unit of *Taq* DNA polymerase, and 30 ng template DNA. After preheating for 5 min at 94ºC, PCR was run for 45 cycles. It consisted of a 94ºC denaturation step (0.45 min), 37ºC annealing step (1 min), and 72ºC elongation step (1.5 min) in a Thermal Cycler (Eppendorf Mastercycler Gradient, Hamburg, Germany). At the end of the run, a final extension period was appended (72ºC, 7 min), then stored at 4ºC until the PCR products were analyzed. After amplification, the PCR products were resolved by electrophoresis on 2% agarose gel and stained with ethidium bromide (0.5 µg/ml), followed by destaining and visualization under UV light. The gels were then photographed.

Data recording and analysis The DNA profile or fingerprint of each fish population was recorded. The data was also scored for the presence or absence of the amplified fragments for all individuals. The data matrix was generated and each individual profile constructed using the following criterion: if a given amplified fragment was present in an individual, then it was assigned as ' 1 ' and when the fragment was absent, it was assigned as ' 0 '. Data were analyzed for genetic relationship using STATISTICA: Cluster Analysis (StatSoft, North Lincolnshire, UK, 1994) software.

Results

Twenty-eight primers (Operon 10-mer primers) were screened on a group of 8 randomly-chosen individuals from each of the two populations, Padma and Meghna. Of the 28 primers, five (OPAB-9, OPE-3, OPH-14, OPQ-1, and OPS-14) produced no fragments, and eight (OPAB-1, OPAB-12, OPG-7, OPH-5, OPH-19, OPQ-5, OPQ-14, and OPQ-17) showed no polymorphic fragments among the hilsa shad that were sampled under the PCR conditions that were chosen for this study. Fifteen of the 28 primers showed polymorphic banding patterns, and the number of bands that were generated per primer varied. For the final analysis, primers were selected if they produced clearly resolved DNA banding patterns within

and between populations. Twelve of 15 primers met these requirements (Table 1) and were studied on all 34 individuals from two locations because they gave several reproducible polymorphic bands.

For the different hilsa samples, Table 2 shows the differences in the number of bands that were obtained by the RAPD primers that were used in the construction of the dendrogram and for determining their genetic distances. This table also shows two triangular sections, the upper portion of triangle shows Squared Euclidean, the number of band differences between pairs of hilsa individuals and the lower triangle shows the distances between hilsa individuals that constitute two populations. From these data, we easily distinguished between the tested populations. Distances between 2 & 4, 3 & 7 of Padma individuals were closer than the distances between the rest of the Padma individuals that were studied. The distances between 23 & 24 of Meghna individuals were also closer than the other Meghna individuals.

A dendrogram was constructed with the two Hilsa shad populations using Squared Euclidean distances (Table 2) by STATISTICA software to analyze the genetic distances (Fig. 2). The resulting dendrogram clustered into two major groups; one for the Padma population and the other for the Meghna population. However, two smaller clusters formed part of the one for major Meghna. Also, Padma 9 clustered with the Meghna population and showed a closer relationship to them.

Discussion

Using a RAPD analysis, the intrapopulation variation was detected with different primers in tilapia (Bardakci and Skibinski, 1994). This technique was more sensitive than the mtDNA analysis which failed to reveal the variation within the tilapia populations (Capili, 1990; Seyoum and Kornfield, 1992). The RAPD method was successfully used to detect the
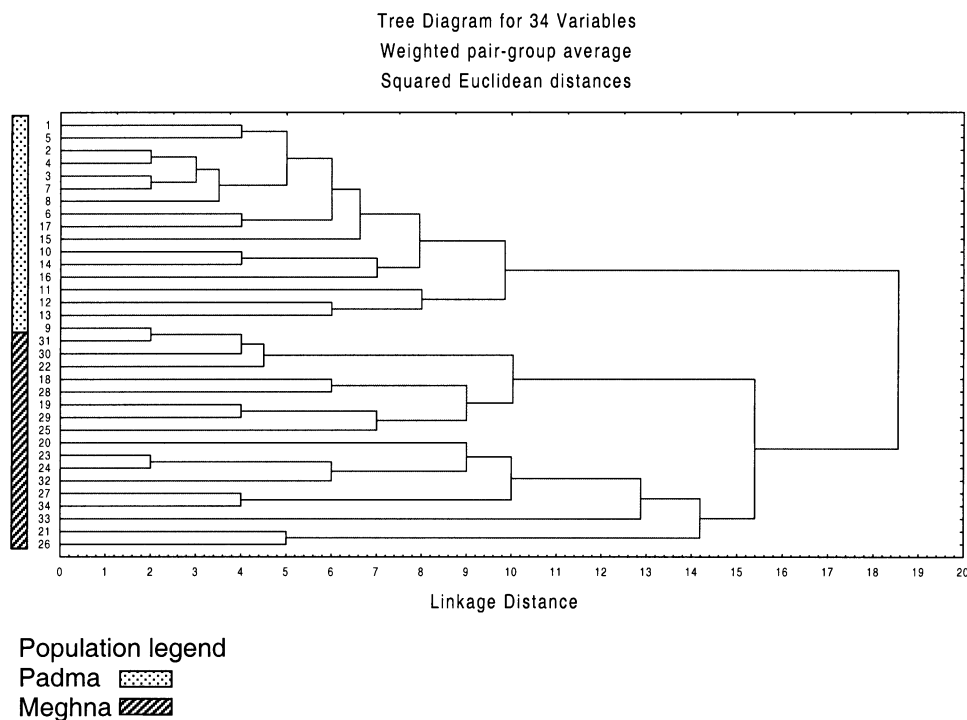
Table 2. Number of band differences and Squared Euclidean distances between Hilsa shad populations

Variable	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1	0	2	3	0	2	1	1	2	1	3	2	2	4	3	2	0	1	6	3	8	4	1	8	6	1	9	6	2	1	2	3	1	1	4
2	6.0	0	1	2	4	3	1	0	1	1	0	0	2	1	0	2	1	8	5	10	2	1	10	8	3	11	8	4	3	0	1	3	3	6
3	5.0	3.0	0	3	5	4	2	1	2	0	1	1	1	0	1	3	2	9	6	11	1	2	11	9	4	12	9	5	4	1	0	4	4	7
4	6.0	2.0	3.0	0	2	1	1	2	1	3	2	2	4	3	2	0	1	6	3	8	4	1	8	6	1	9	6	2	1	2	3	1	1	4
5	4.0	4.0	5.0	4.0	0	1	3	4	3	5	4	4	6	5	4	2	3	4	1	6	6	3	6	4	1	7	4	0	1	4	5	1	1	2
6	7.0	5.0	4.0	3.0	7.0	0	2	3	2	4	3	3	5	4	3	1	2	5	2	7	5	2	7	5	0	8	5	1	0	3	4	0	0	3
7	5.0	3.0	2.0	3.0	5.0	4.0	0	1	0	2	1	1	3	2	1	1	0	7	4	9	3	0	9	7	2	10	7	3	2	1	2	2	2	5
8	4.0	4.0	3.0	4.0	6.0	5.0	3.0	0	1	1	0	0	2	1	0	2	1	8	5	10	2	1	10	8	3	11	8	4	3	0	1	3	3	6
9	21.0	21.0	20.0	21.0	23.0	18.0	22.0	19.0	0	2	1	1	3	2	1	1	0	7	4	9	3	0	9	7	2	10	7	3	2	1	2	2	2	5
10	9.0	9.0	10.0	9.0	11.0	10.0	10.0	9.0	16.0	0	1	1	1	0	1	3	2	9	6	11	1	2	11	9	4	12	9	5	4	1	0	4	4	7
11	10.0	8.0	9.0	10.0	10.0	13.0	11.0	10.0	21.0	7.0	0	0	2	1	0	2	1	8	5	10	2	1	10	8	3	11	8	4	3	0	1	3	3	6
12	8.0	10.0	9.0	10.0	12.0	11.0	9.0	8.0	21.0	7.0	8.0	0	2	1	0	2	1	8	5	10	2	1	10	8	3	11	8	4	3	0	1	3	3	6
13	8.0	8.0	7.0	10.0	12.0	9.0	9.0	8.0	19.0	9.0	8.0	6.0	0	1	2	4	3	10	7	12	0	3	12	10	5	13	10	6	5	2	1	5	5	8
14	7.0	9.0	8.0	7.0	9.0	8.0	8.0	7.0	18.0	4.0	9.0	7.0	9.0	0	1	3	2	9	6	11	1	2	11	9	4	12	9	5	4	1	0	4	4	7
15	8.0	8.0	7.0	8.0	10.0	7.0	5.0	4.0	19.0	9.0	12.0	8.0	10.0	7.0	0	2	1	8	5	10	2	1	10	8	3	11	8	4	3	0	1	3	3	6
16	6.0	8.0	7.0	8.0	6.0	7.0	7.0	8.0	19.0	7.0	12.0	10.0	10.0	7.0	8.0	0	1	6	3	8	4	1	8	6	1	9	6	2	1	2	3	1	1	4
17	5.0	7.0	6.0	5.0	9.0	4.0	6.0	5.0	20.0	8.0	11.0	7.0	7.0	6.0	5.0	9.0	0	7	4	9	3	0	9	7	2	10	7	3	2	1	2	2	2	5
18	20.0	20.0	19.0	20.0	22.0	17.0	21.0	18.0	11.0	17.0	18.0	18.0	16.0	19.0	20.0	20.0	19.0	0	3	2	10	7	2	0	5	3	0	4	5	8	9	5	5	2
19	19.0	19.0	18.0	21.0	21.0	18.0	20.0	17.0	8.0	16.0	17.0	15.0	15.0	18.0	17.0	17.0	20.0	9.0	0	5	7	4	5	3	2	6	3	1	2	5	6	2	2	1
20	16.0	14.0	15.0	12.0	14.0	13.0	15.0	16.0	19.0	17.0	18.0	18.0	20.0	17.0	18.0	16.0	15.0	20.0	19.0	0	12	9	0	2	7	1	2	6	7	10	11	8	8	2
21	18.0	20.0	17.0	20.0	20.0	21.0	17.0	16.0	15.0	21.0	20.0	18.0	20.0	21.0	18.0	20.0	21.0	16.0	13.0	14.0	0	3	12	10	5	13	10	6	5	2	1	5	5	8
22	20.0	18.0	17.0	20.0	20.0	17.0	19.0	18.0	5.0	17.0	20.0	20.0	18.0	19.0	18.0	16.0	21.0	14.0	7.0	16.0	16.0	0	9	7	2	10	7	3	2	1	2	2	2	5
23	16.0	14.0	15.0	14.0	14.0	13.0	15.0	16.0	17.0	19.0	18.0	20.0	20.0	19.0	18.0	14.0	17.0	20.0	19.0	6.0	14.0	14.0	0	2	7	1	2	6	7	10	11	8	8	2
24	16.0	14.0	15.0	14.0	14.0	13.0	15.0	16.0	17.0	19.0	18.0	20.0	20.0	19.0	18.0	14.0	17.0	20.0	19.0	8.0	16.0	16.0	2.0	0	5	3	0	4	5	8	9	5	5	2
25	21.0	17.0	18.0	19.0	19.0	20.0	20.0	19.0	12.0	16.0	15.0	15.0	15.0	18.0	19.0	15.0	22.0	13.0	6.0	19.0	15.0	9.0	19.0	19.0	0	8	5	1	0	3	4	0	0	3
26	21.0	19.0	18.0	19.0	19.0	22.0	18.0	19.0	18.0	24.0	19.0	21.0	23.0	24.0	21.0	21.0	24.0	21.0	14.0	11.0	5.0	15.0	11.0	13.0	12.0	0	3	7	8	11	12	8	8	5
27	14.0	12.0	11.0	12.0	12.0	13.0	13.0	12.0	17.0	17.0	14.0	16.0	16.0	17.0	14.0	14.0	15.0	16.0	13.0	8.0	14.0	14.0	10.0	10.0	15.0	11.0	0	4	5	8	9	5	5	2
28	22.0	20.0	19.0	20.0	22.0	17.0	21.0	20.0	7.0	17.0	20.0	18.0	18.0	19.0	20.0	18.0	21.0	6.0	7.0	18.0	16.0	8.0	18.0	18.0	7.0	17.0	16.0	0	1	4	5	1	1	2
29	21.0	19.0	18.0	19.0	23.0	18.0	20.0	17.0	8.0	14.0	17.0	15.0	15.0	16.0	19.0	19.0	20.0	9.0	4.0	21.0	13.0	11.0	23.0	23.0	8.0	16.0	17.0	7.0	0	3	4	0	0	5
30	20.0	20.0	19.0	22.0	22.0	21.0	21.0	18.0	5.0	17.0	20.0	20.0	18.0	20.0	18.0	23.0	14.0	9.0	20.0	14.0	4.0	18.0	20.0	11.0	17.0	18.0	10.0	9.0	9.0	0	1	3	3	6
31	19.0	19.0	18.0	19.0	21.0	18.0	20.0	17.0	2.0	14.0	19.0	19.0	17.0	16.0	19.0	17.0	20.0	11.0	8.0	19.0	15.0	5.0	19.0	19.0	10.0	18.0	17.0	7.0	6.0	3.0	0	4	4	7
32	13.0	11.0	10.0	11.0	11.0	12.0	12.0	13.0	18.0	16.0	15.0	17.0	15.0	16.0	17.0	11.0	16.0	17.0	18.0	11.0	15.0	15.0	7.0	5.0	14.0	14.0	11.0	15.0	20.0	17.0	16.0	0	0	3
33	19.0	15.0	16.0	17.0	17.0	20.0	18.0	17.0	12.0	18.0	17.0	21.0	21.0	20.0	19.0	17.0	22.0	17.0	16.0	17.0	15.0	13.0	15.0	15.0	12.0	14.0	11.0	13.0	16.0	13.0	12.0	14.0	0	3
34	14.0	12.0	11.0	10.0	12.0	11.0	13.0	12.0	15.0	15.0	14.0	18.0	18.0	15.0	14.0	14.0	13.0	14.0	15.0	10.0	18.0	14.0	12.0	12.0	15.0	15.0	4.0	14.0	17.0	18.0	15.0	11.0	9.0	0

Squared Euclidean distances

variation between the different species of fish (Bardakci and Skibinski, 1994) and separation of mollusc species (Crossland *et al.*, 1993). Moreover, Dahle *et al.* (1998) found the genetic variation between the three different populations of Hilsa shad that were sampled from Bangladesh waters using the RAPD technique. In the present study, a RAPD analysis discriminated between the two different freshwater fish populations of Bangladesh. RAPD fragments, observed in the 34 individuals, showed a high degree of polymorphism both within and between the populations.

By analyzing the band patterns that were generated by the primers (OPAB-3, OPAB-8, OPE-1, OPE-13, OPE-17, OPG-3, OPG-6, OPG-9, OPG-15, OPH-4, OPH-13, and OPN-13), the population of Padma are found to be genetically distant from that of Meghna and also genetic variability is present within the Padma as well as Meghna populations.

The constructed dendrogram showed a differentiation into two major clusters, assuming the different spawning populations. The larger cluster was comprised of 18 individuals (17 of Meghna and 1 of Padma). It is interesting to note that one individual of Padma (Padma 9) clustered with the individuals of Meghna. It might have been caught from the Meghna during its downstream migration from the Padma to the Bay of Bengal. However, two clusters were also found within the Meghna population. These might represent individuals that originated from the two separate breeding grounds of Meghna.

These results are similar to the findings of Jenkins (1938), Raja (1985), and Mazid and Islam (1991), that there are two or more races or varieties of hilsa with different spawning grounds. Moreover, Mojumdar (1939) and Dutt (1966) stated that the hilsa persist in three ecotypes; seawater, muddy fresh water of rivers like Padma, and clear fresh water like Meghna. The present analysis confirms this divergence between the

Tree Diagram for 34 Variables
Weighted pair-group average
Squared Euclidean distances

Population legend
Padma
Meghna

Fig. 2. STATISTICA dendrogram of two populations of Hilsa shad, based on values of genetic distance calculated from data for 12 primers. The numbers to the right indicate specimen number.

populations that were sampled from the latter two ecotypes. Furthermore, the morphological differences that were found by Quddus *et al.* (1984a) can also be correlated by this study. Dahle *et al.* (1998) found variations among the three populations of hilsa shad. Using the same methods, this study shows the genetic variation between and within the two populations from the two major inland rivers of Bangladesh. This assumes the presence of separate stocks or races of hilsa, depending on the spawning grounds, nursery grounds of the juveniles, and their seasonal migration in the different river systems.

This method of fingerprinting is important since it is relatively easy to obtain valuable data. It allows for a more introspective interpretation of diversity within a population. The present study may serve as a reference point for future examinations of the genetic variation within populations of *Tenualosa ilisha*. Furthermore, it can be used as a model for other studies relating to genetic diversity. As the production of Hilsa fishery for various reasons are daily declining in the inland waters, then stock assessments of their juveniles and young fish from their respective nursery grounds are of utmost importance. Once their population structure is understood well, the management of this valuable resource could be used to optimize the harvest, as well as to protect the populations.

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