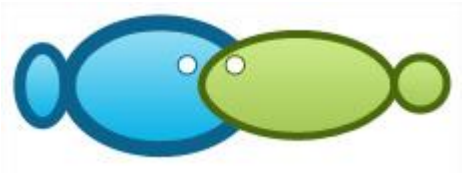

Genetic variation of wild and hatchery populations of the mrigal Indian major carp (*Cirrhinus cirrhosus*) conferred by RAPD markers

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Abstract. In the present study, Random Amplified Polymorphic DNA (RAPD) analysis was performed to compare the genetic variability of five different hatchery populations with the natural population of the Halda River of mrigal (*Cirrhinus cirrhosus*). RAPD analysis of six different populations revealed 128 scorable bands of which 98 bands were polymorphic. Comparatively higher genetic similarity indices were observed between hatchery population pairs and comparatively lower inter-population genetic similarity indices (SIij) values were obtained for hatchery Halda River populations. Unweighted pair group method using arithmetic averages (UPGMA) analysis of the RAPD markers and pairwise genetic similarities of six populations of *C. cirrhosus* were estimated and ranged from 0.000 to 15.166 indicating that the genetic distance among these population is diversified. The hatchery populations were found more homozygous and there is a lacking genetic variability thereby providing reduced growth performance. It was obtained that the Halda River population is genetically more variable than that of other hatchery populations. The outcome of this study indicates carp culture is more profitable with Halda's fry.

Key Words: genetic variability, Halda River, RAPD-PCR.

Introduction. Bangladesh produces 2.77 % of the total world's freshwater aquaculture (Gupta et al 2005). Mrigal, *Cirrhinus cirrhosus* is one of the Indian major carps (IMCs) and is a very tasty (Bloch 1795), popular fish having high market value in Bangladesh as well as in other Asian countries (Dinesh et al 1993). Mrigal (*Cirrhinus cirrhosus*), catla (*Catla catla*) and rohu (*Labeo rohita*) are the major cultured carp species contributing to about 23.82 % of total fish production (DoF 2012) and more than 78% of pond production of Bangladesh (Dey et al 2005). Inland fisheries of Bangladesh are the world's third richest after China and India (Talwar & Jhingran 1991) having a diverse fish fauna of 264 species (Rahman 2005). Carps are the most cultured group of fishes in the world (Billard et al 1995, Hulata 1995, El-Zaeem 1996, Bartfai & Egedi 2003) and about 20 carp species are extensively cultured in Asia and for each species, several traits have been identified as improved (Gupta et al 1997).

Previously, seeds of the species used to be collected from the natural sources and these are believed superior to hatchery produced seeds. Now almost the total demand of seed is being met from hatcheries established under private and public sector in the country. The quality of hatchery produced seeds has been deteriorated due to inbreeding, genetic drift, negative selection, indiscriminate inter-specific hybridization, and improper brood stock management (Alam et al 1996, Hansen et al 2006). Widespread hybridization has occurred among the species of IMCs in hatcheries, but not in the wild (Simonsen et al 2005). There is the loss of genetic variation in the hatchery-reared Indian major carps in Bangladesh (Hansen et al 2006). Inbreeding is responsible for the

reduced growth performance of *C. cirrhosus,* and the increase of genetic variability is the only solution for the increase of production and growth performance (Aflalo et al 2006).

The application of Random Amplified Polymorphic DNA (RAPD) technique in fisheries biotechnology can check and measure the DNA genetic polymorphism of fish genomes, can identify the varieties and breeds for aquaculture, and select the brooders for selective breeding and crossing (Qiu 1996). The RAPD technique is also useful in gene localization and cloning, species classification and molecular evolution studies (Zhu et al 2005). RAPD analysis is based on the polymerase chain reaction (PCR) amplification of discrete regions of genome with short oligonucleotide primers of arbitrary sequence (Williams et al 1990) technique which has been used extensively to detect genetic diversity in plants (Williams et al 1993), animals (Cushwa & Medrano 1996) and microbes (Carretto & Marone 1995). Population subdivision (Kuusipalo 1994), genetic diversity and genetic distance (Naish et al 1995), species identification (Dinesh et al 1993, Bardakci & Skibinski 1994), and the building of genetic linkage maps (Postlethwait et al 1994) have all been studied using this technique. It has been applied in areas such as population genetics, taxonomy, species identification, phylogenetic assessment (Hadrys et al 1992), and management of endangered species.

In the present study the RAPD assay is used as a source of genetic markers to generate species specific RAPD profiles and to estimate genetic variation among the different hatchery and natural stocks for the *C. cirrhosus*. The DNA samples of the fish of different stocks and species is tested for genetic distinctness employing PCR techniques, using known primers. The objectives of the study were to test the DNA variability of different stocks of *C. cirrhosus* and compare the genetic diversity between riverine and hatchery stocks to measure the genetic distinctness of the different stocks.

Material and Method

Sample collection. Fish samples of *C. cirrhosus* were collected from five different hatcheries namely Kapotakkha (K), MaFatema (MF) and Chowdhury (C) hatcheries in Jessore, Sonali (S) hatchery in Khulna, Niribili (N) hatchery in Faridpur district and from the natural population of Halda River (H) of Chittagong district. A total of 60 samples (10 from each population) were tested for RAPD variability analysis. Sampling locations are presented in the map from Figure 1.

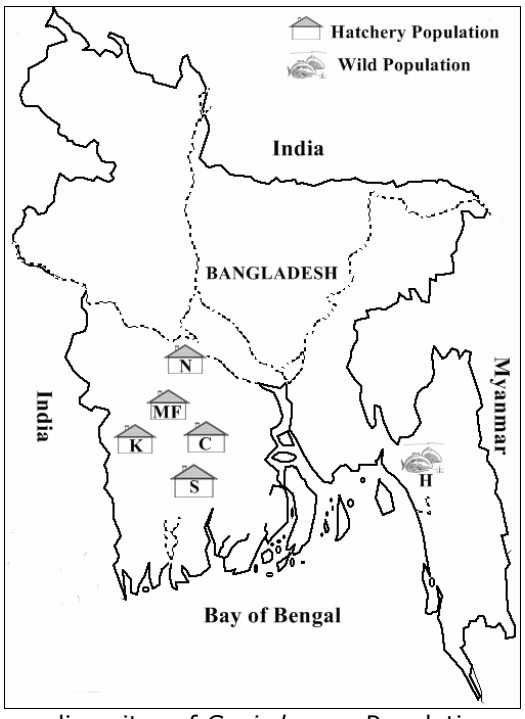

Figure 1. The map showing sampling sites of *C. cirrhosus*. Populations are marked by abbreviations that correspond to Table 2.

Genomic DNA extraction from fish tissue. Genomic DNA of fish samples collected from different populations was isolated by using Genomic DNA Extraction Kit, (AccuPrep®, Bioneer, Korea) following the manufacturer's standard procedure. The samples (25-50 mg) were disrupted (or homogenized) with a mortar and pestle, they were placed in a clean 1.5 ml tube and 200 µl of tissue lysis buffer (TL) was added. The muscles were pestled carefully as incomplete disruption will lead to significantly reduced yield and can cause clogging of binding column tube. Then 20 µl of proteinase K was added, and mixed well by vortex mixer, and incubated at 60°C until the tissue was completely lysed. After change in clarity from turbid to clear, the tube was then briefly spun down to remove drops from the inside of the lid and 200 µl of binding buffer (GC) was added and immediately mixed well by vortex mixture. It was then incubated at 60°C for 10 minutes. After incubation, 100 µl of isopropanol was added in the tube and mixed well by pipetting. Then the tube was briefly spun down to get the drop cleaning under the lid. Without wetting the rim, the lysate was gently placed into the upper reservoir of the binding column tube (fit in a 2 ml tube). The tube was closed and centrifuged at 8,000 rpm for 1 minute and again at 10,000 rpm. After that it was opened, and binding column tube was transferred to a new 2 ml tube (supplied) for filtration. Then, 500 µl of washing buffer 1 (W1) was added without wetting the rim, and the tube was covered and centrifuged at 8,000 rpm for 1 minute. The tube was opened, and the solution was poured from the 2ml tube into a disposal bottle. Carefully, 500 µl of washing buffer 2 (W2) was added without wetting the rim, and the tube was then closed and centrifuged at 8,000 rpm for 1 minute. It was centrifuged once more at 12,000 rpm for 1 minute to completely remove ethanol. The binding column tube was transferred to a new 1.5 ml tube for elusion, and 200 µl of elusion buffer (EL) was added onto binding column tube and left at room temperature (15-25°C) until EL is completely absorbed into the glass fiber of binding column tube. The tube was then centrifuged at 8,000 rpm for 1 minute to elute and about 180 µl to 200 µl of eluent was obtained.

Primer selection. Five commercially available decamer primers of random sequence (Operon Technologies Inc., Alameda, CA, USA) were selected for the RAPD analysis of *C. cirrhosus*. Primers had been identified on the idea of intensity or resolution of bands, repeatability of markers and consistency within individuals and it makes possible to differentiate the populations (polymorphism). Table 1 represents the name of the selected primers, G-C content sequences and size ranges corresponding to their annealing temperatures.

DNA purity and concentration determination. The concentration and purity of extracted DNA samples were determined from the ratio of absorbance at A260 and A280 (absorbance at 260 nm and 280 nm) using a spectrophotometer. Before loading the next sample, the DNA sample with the cuvette was thoroughly cleaned. The DNA concentration was determined as, Double-stranded DNA concentration (C), µg/ml = Absorbance at A260 × 50 × 500 the formulae, and purity as, Purity = Absorbance at A260/Absorbance at A280.

PCR amplification. The concentrations of the extracted DNA samples were adjusted between 20-25 ng/µl by diluting the DNA samples with the aid of de-ionized nuclease free sterile distilled water. The PCR reactions were carried out in a 20µl reaction mixture containing 1µl DNA sample (containing 2025 ng of template DNA), 2µl (10 pico-mole/µl) oligonucleotide primers (Bioneer, Korea), 2µl 10X reaction buffer (Bioneer, Korea), 2µl 10mM dNTPs mixture (Bioneer, Korea), 2µl Taq DNA polymerase (1 unit) and 11µl de-ionized distilled water. The reaction mixtures were then placed in a DNA thermal cycler (C1000^{TM}, BIO-RAD, USA) for polymerase chain reaction. For target DNA amplification, the PCR conditions were extended denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 32-34°C for 1 minute and elongation at 72°C for 1 minute.

Agarose gel electrophoresis. After the completion of thermal cycling, 8 µl of each PCR product was analyzed electrophoretically by running through a 2% agarose gel and the amplified product size was determined by comparing with a 100 bp DNA size marker which is known as DNA Ladder (Bioneer, Korea).The 2% agarose gel constituted ethidium bromide and 1X TAE buffer. The electrophoresis apparatus (Bioneer, A-7020, Korea) 1X TAE buffer was poured to soak the agarose gel and the electrophoresis process was maintained at 120 V electric power for 45 minutes. The DNA ladder provided 13 different bands of 100 to 2000 base pairs (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1600 and 2000 base pairs). The bands were observed on UV-trans illuminator and photograph was taken by a Gel Cam Polaroid camera.

Data analysis. DNA banding patterns produced by RAPD were scored as 1 for bright bands (presence of bands) 0 for absence of bands; each amplified and allelic frequencies were supported presence or absence of the band of every locus. In the present study, similarity coefficient was calculated across all possible pair wise comparison of individuals both within and between the populations using the method of Lynch (Lynch & Milligan 1994) with the formula, SI = 2NAB / (NA + NB). Where, NAB = number of fragments shared by individual A and B, NA and NB = number of fragments scored for each individual. Dendrogram was constructed based on the un-weighted paired group method with arithmetic averaging (UPGMA) employing the sequential, agglomerative, hierarchal, and nested clustering (SAHN) program by using RAP Distance version 1.04 (Armstrong et al 1994). Genetic distance was calculated by using the formula: D = 1 – C, where C is the correlation between spot clusters.

Results. RAPD analysis of six different populations of *Cirrhinus cirrhosus* in the present study revealed 128 scorable bands of which 98 bands were polymorphic. Number of bands produced varied with different primers and samples. The amplified PCR fragment sizes ranged from 100 bp to 2.0 kb with the scorable region being from 150 bp to 2.0 kb. The number of bands generated by the primers ranged from 10 to 40 with an average of 23 (Table 1).

Table 1

List of primers used for RAPD amplification, GC content, annealing temperature (Tm.), total number of loci, the level of polymorphism and size range of fragments

Primer	*Sequence (5′ to 3′)*	*Fragments size range (bp)*	*No. of bands scored*	*Polymorphic bands*	*Proportion of polymorphism (%)*	*GC content (%)*	*MW (g/mole)*	*Tm (°C)*
OPA1	CAGGCCCTTC	300-1600	21	15	71.43	70	2963.9	34
OPA18	AGGTGACCGT	100-1600	31	13	41.94	60	3068.0	32
OPB2	TGATCCCTGG	200-2000	27	21	77.78	60	3018.9	32
OPB12	CCTTGACGCA	200-2000	32	32	100	60	2987.9	32
OPC3	GGGGGTCTTT	200-1600	17	17	100	60	3090.0	32

The 5 different decamer oligonucleotide primers produced 128 bands, ranging in size between 100 to 2000 bp (Figure 2). Out of these 128 bands scored, 98 bands were polymorphic (76.56%) and rest of the 30 (23.44%) bands were monomorphic (Table 1). Total numbers of bands for different primers were scored at 21 (15 polymorphic), 31 (13 polymorphic), 27 (21 polymorphic), 32 (all polymorphic) and 17 (all polymorphic) for the primers OPA1, OPA18, OPB2, OPB12 and OPC3 respectively, presented in Table 2.

Table 2

Proportions of polymorphism produced by different populations and intra population genetic similarity indices (SI). "K" indicates Kapotakkha hatchery; "MF" indicates MaFatema hatchery; "C" indicates Chowdhury; "S" indicates Sonali hatchery; "N" indicates Niribili hatchery; "H" indicates Halda River

Population	*No. of bands scored*	*Polymorphic band*	*Proportion of polymorphism (%)*	*Size range (bp)*	*SI (%)*
K	16	16	100	200-1600	43.61
MF	22	17	77.27	200-1600	54.71
H	40	25	62.5	200-2000	66.90
S	16	16	100	300-1600	65.80
S	10	10	100	300-1600	49.20
N	24	24	100	100-1600	46.50

The primers OPB12 and OPC3 revealed 100% polymorphism. On the other hand, Sonali, Chowdhuri and Niribili hatcheries also showed 100% polymorphism. Again, total numbers of bands scored for different populations were scored at 16, 22, 40, 16, 10 and 24 for Kapotakkha, Ma-Fatema, Halda, Sonali, Chowdhuri and Niribili respectively. The highest similarity (66.90%) was observed within the individuals of the Halda River population, and the lowest genetic similarity (43.61%) was observed within the individuals of Kapotakkha hatchery population. The RAPD profiles (banding patterns) of different populations for five different primers are represented in Figure 2.

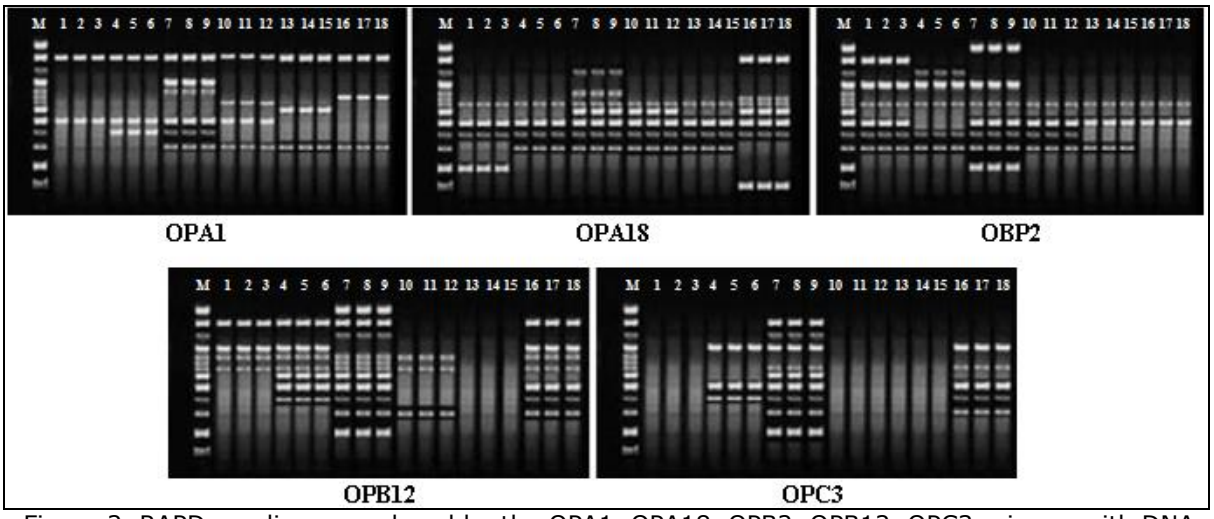

Figure 2. RAPD amplicons produced by the OPA1, OPA18, OPB2, OPB12, OPC3 primers with DNA from 6 different populations of *Cirrhinus cirrhosus*. Gels are representative of ten independent experiments of *Cirrhinus cirrhosus*. Lane M, molecular marker; lane 1 – 3, Kapotakha hatchery population; lane 4 – 6, Ma-Fatema hatchery population; lane 7 – 9, Halda river population; lane 10 – 12, Sonali hatchery population; lane 13 – 15 Chowdhuri hatchery population; lane 16 – 18, Niribili hatchery population.

Inter-population genetic similarity indices and genetic distances between different population pairs have been presented in Table 3. Comparatively higher genetic similarity indices were observed between hatchery population pairs and comparatively lower inter-population genetic similarity indices (SIij) values were obtained for hatchery-Halda River populations. The highest genetic distance (0.835) was obtained for the Halda River-Sonali hatchery pair and the lowest value was obtained at 0.175 for the Kapotakkha-Chowdhuri pair.

Table 3

Inter-population genetic similarity indices (SIij) and genetic distances (D). Population codes as in Table 2.

Paired population	*SIij(%)*	*Genetic distance(D)*
H – N	56.82	0.373
H – K	55.36	0.560
H – S	60.56	0.835
H – C	59.05	0.637
H – MF	64.55	0.703
N – K	54.32	0.298
N – S	58.45	0.736
N – C	67.07	0.421
N – MF	55.83	0.526
K – S	63.45	0.625
K – C	60.56	0.175
K – MF	75.11	0.325
S – C	70.90	0.545
S – MF	63.17	0.444
C – MF	61.00	0.181

The dendrogram provides two main clusters for the six different populations: hatchery population in one side and the Halda river population in the other side in Figure 3. Within the hatchery population Niribili hatchery falls in a side and Kapotakha, Ma-Fatema, Chowdhuri, Sonali hatcheries fall in another side. Thus, the genetic status-diversity of the Halda River population is far higher over the hatchery populations; providing better genetic status of this population. Out of the hatchery populations, the genetic status of Niribili hatchery was found higher compared to the others. Unweighted pair group method using arithmetic averages (UPGMA) analysis of the RAPD markers and pairwise genetic similarities of six populations of *C. cirrhosus* were estimated and ranged from 0.000 to 15.166 indicating that the genetic distance among these population is diversified.

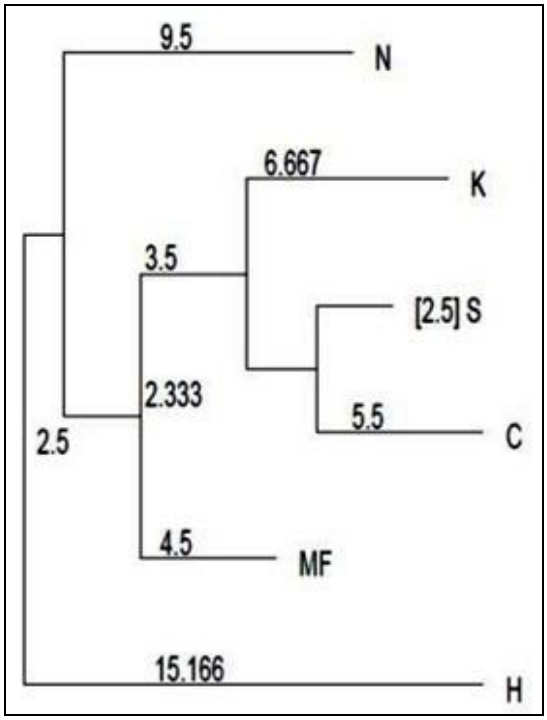

Figure 3. This figure shows unweighted pair group method of arithmetic mean dendrogram based on Nei's (1978) genetic distance. Population codes as in Table 2.

Discussion. In the present study, RAPD analysis was performed to compare the genetic variability of five different hatchery populations of *C. cirrhosus* with the natural population of the Halda River in Chittagong. It was found that the Halda River population is genetically more variable than that of other hatchery populations used in the present study. The seeds produced from hatcheries have lower genetic variation due to the use of skewed sex ratios and negative selection and the aquaculture sector has been suffering a serious problem of genetic quality deterioration. The results of this study indicate culture of *C. cirrhosus* will be more profitable with the use of Halda's fry.

Overall proportions of polymorphism obtained for different populations involved 100%, 77.27%, 62.5%, 100%, 100% and 100% for Kapotakkha, Ma-Fatema, Halda, Sonali, Chowdhuri and Niribili, respectively. The level of genetic variation determines the genetic status for selective improvement of a stock (Islam et al 2015). The presence of genetic variability among different populations of the species and between individuals within a population provides fitness to survive well and successfully respond to various environmental changes (Ryman 1983). Islam and Alam (2004) studied the genetic variability of *Labeo rohita* from four different stocks and found 46.5 % polymorphic bands. Das et al (2005) found 25.4 % polymorphic bands from four species of IMCs.

In the present study, a higher rate of polymorphism was observed for all the populations. Allozyme and microsatellite analysis of six different populations revealed almost similar patterns of polymorphism in hatchery stocks of *C. cirrhosus*, although the overall genetic variation was comparatively higher in the riverine populations (Alam et al 1996). Simonsen et al (2005) observed a high incidence of hybridization in the hatchery stocks of the three IMCs by isozyme electrophoresis. Liu et al (1999) observed that very low levels of intra-specific polymorphisms for both *Ictalurus punctatus* and blue catfish *Ictalurus furcatus* strains although distinct phenotypes were observed and inherited in each of these strains. In the present study, 80% of the bands were found to be polymorphic, much higher than that obtained by Liu et al (1999) in catfish and indicating that the RAPD system may be more useful to generate molecular markers for genetic characterization in *C. cirrhosus.* In the present study, a higher number and percentage of polymorphic bands were recorded. This is because of lack of genetic improvement programs in Bangladesh that leads to genetic drift, inbreeding, hybrid introgression and thereby ensuring genetic quality loss in the hatchery seeds of IMCs.

The Intra-population SI values obtained in the present study for different populations indicated more genetic similarity within the individuals of the Halda River population. Nesa (2014) reported the average number of alleles was highest in the Halda population. Islam and Alam (2004) observed that the intra-population SI of the hatchery population was the highest (96.9%) followed by that of the Padma, the Jamuna and the Halda River populations, respectively and were higher than that of the present study. Rahman (2005) stated that the SI value was found to be highest in the Jamuna population followed by the hatchery, the Padma and the Halda populations. Barman et al (2003) also observed low levels of within-species genetic variation in farmed stocks of major carps in India. The inter-population similarity index value was the highest between the Kapotakkha hatchery-MaFatema hatchery than the Halda River vs hatchery or other hatchery vs hatchery combinations. The higher genetic similarity between these two hatchery populations indicated the genetic relatedness between the populations; and this may be due to the broods of the hatcheries might have the common source. The lowest genetic similarity index value was obtained for the Niribili hatchery vs. Kapotakha hatchery combination. The SIij value for the Jamuna-Padma population pair was the highest compared with all the other between population comparisons (Islam & Alam 2004). On the other hand, Rahman (2005) has stated that inter-population SI for the Padma River vs hatchery populations was higher than those for all other between population comparisons (Halda–Jamuna; Halda–Padma; Padma–Jamuna; Halda–Hatchery; Jamuna–Hatchery).

The dendogram indicated that Halda River population is genetically more variable than that of other hatchery populations. Despite presenting some differences in genetic distances, the three methods for estimating genetic distances produced UPGMA dendrograms with similar clustering patterns. The clustering of the populations reflects their genetic relationship. The geological events of freshwater fish species are the important factors for their genetic differentiation and dispersal (Borowsky et al 1995).

The genetic variations of Sonali, Ma-Fatema, Chowdhuri and Kapotakha hatcheries are not significant; showed higher rate of similarity and are in the same cluster in the dendogram. This is because of these hatcheries keep only a limited number of broods, do not maintain pedigree records, practice negative selection, and use same broods repeatedly. Inbreeding inevitably occurs in the hatchery and that is the main reason for reduced genetic variation in the hatchery population, as was also reported by Eknath and Doyle (1990). The Niribili hatchery seeds showed a comparatively higher value of genetic distance over the other hatcheries that reflects the better brood stock management in the hatchery. In all cases of pair wise genetic distance values, Halda River-hatchery combinations showed higher genetic distance values that clearly indicate higher genetic variability of Halda seeds than the hatchery populations. The Halda is a geographically isolated freshwater tidal river in eastern Bangladesh that originates in the mountainous region. It is regarded as a pure stock of *Cirrhinus cirrhosus* in Bangladesh. There were no major carp spawning grounds recorded in the region's freshwater tidal zone, except in the Halda (Azadi et al 2003), therefore there was no opportunity of mixing with other stocks.

Conclusions. Genetic variability assay provides the status of relatedness and diversity, genetic distance between different populations of different stocks. Thereby, it is an imperative to check the fitness of different hatchery stocks of the species. As a result, intraspecific or strain crossing can be performed to produce high yielding seeds to enhance the production of fish as well as to find out the solutions of the prevailing problems. In the present study it was found that hatchery seeds of mrigal (*Cirrhinus cirrhosus*) have lower genetic variation and the aquaculture sector have been suffering a serious problem of genetic quality deterioration using these seeds. The outcome of this study indicates culture of *Cirrhinus cirrhosus* will be more profitable with the use of Halda River fry because of its higher genetic variability.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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