Genetic variation and differentiation in the Stinging catfish, *Heteropneustes fossilis* (Bloch), populations assessed by heterologous microsatellite DNA markers

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Microsatellite DNA markers have been increasingly used in genetic diversity studies. The present study reports on the characterization of genetic variation and differentiation in four different natural populations of the stinging catfish, *Heteropneustes fossilis* (Bloch), in Bangladesh, viz., Mymensingh, Netrakona, Narsingdi and Rangpur, using cross-species microsatellite DNA markers developed from the walking catfish, *Clarias batrachus*. Eighteen polymorphic alleles were found in the 128 diploid individuals (32 from each population), with nine alleles at each of the two loci analyzed. The Netrakona and Rangpur population deviated from the Hardy-Weinberg proportion at one locus. The population differentiation ($F_{ST}$) value between the Narsingdi and Netrakona population was found to be insignificant, while the values between all the other population pairs were found to be significant. The genetic distance values ranged between 0.165 and 0.626. The UPGMA dendrogram based on genetic distance resulted in two clusters: the Mymensingh population alone was in one cluster and the three other populations in the second cluster. This study revealed a fairly high level of genetic variation in the microsatellite loci within and between the four populations, and identified existence of distinct population groups of *H. fossilis*.

**Keywords**: Genetic variation, *Heteropneustes fossilis*, microsatellite, polymorphism

**Introduction**

The stinging catfish, *Heteropneustes fossilis* (Bloch), belongs to the Family Heteropneustidae of the Order Siluriformes. It is native to Bangladesh, India, Pakistan, Myanmar, Nepal, Sri Lanka and Thailand, and has been introduced in Iraq and Iran¹. This species of freshwater catfish is found mainly in ponds, ditches, swamps and marshes, but sometimes occurs in muddy rivers. They breed in confined waters during the monsoon months, but can also breed in ponds and ditches when sufficient rain-water accumulates. It is commercially as well as aquaculturally an important species in many Asian countries including Bangladesh. It is popular not only for its good taste but also from nutritional and medical points of view. The muscles of the fish have been reported to contain high amount of iron (226 mg/100 g) and calcium compared to many other freshwater fishes². Being a lean fish (fat content only 2.57±0.24% on fresh wt basis), it is suitable to those people for whom animal fats are undesirable³.

Popularity of this species for cultivation is high due to extreme hardiness, good growth, high market price, efficient feed utilization and high market demand.

Until the seventh decade of the 20th century, the stinging catfish was abundant in natural water bodies, like beels (low-lying seasonal water bodies), haors (large natural depressions), ditches and ponds in Bangladesh. Its abundance has been greatly reduced by overexploitation and serious destruction and fragmentation of most aquatic ecosystems, particularly from injudicious application of insecticides in the paddy fields⁴. The loss of abundance was aggravated by an outbreak of epizootic ulcerative syndrome (EUS) during the late eighties and early nineties of the last century. The species has already been categorized as vulnerable based on IUCN red list of 1994 in natural water bodies of India⁵. Thus, it is a great concern whether the reduction in population size as reflected by poor catch and less availability in the market has had any impact on genetic variability of this species. Reduction in effective breeding number increases the chance of inbreeding, thereby reduces genetic variability. Genetic variation refers to the differences in the hereditary constituents of the individuals of...
species, which is important in maintaining the developmental stability and biological potential of fish populations. The genetic characterization of populations allows evaluation of genetic variability, which is a fundamental element in working out stock improvement and genetic conservation plans. Therefore, it is imperative that the current level of genetic diversity and differentiation within and between populations of this particular species be investigated as a basis for conservation and sustainable management recommendations. Molecular markers have provided the opportunity to access this variability as they contribute information on every region of the genome, regardless of the level of gene expression. Microsatellites 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 (PCR). These markers have added new dimensions in the field of fisheries and aquaculture as many populations are subjected to bottleneck, inbreeding, genetic drift and as a consequence exhibit low variation that cannot be detected by other markers.

Though the flanking sequence of the microsatellites (highly polymorphic simple sequence repeat) are usually specific (conserved) for the particular species, markers developed from one species can some time amplify markers in closely related species. Microsatellite markers have been developed from the genome of several catfish species and used for the study of population genetic structure. Since microsatellite markers have not yet been isolated from the stinging catfish \( (H. \text{ fossilis}) \), the markers developed by Yue et al. from another catfish species, \( Clarias \text{ batrachus} \) (Linn.) have been used. The authors report here the cross-species amplification of the microsatellite markers and the genetic variation and differentiation in four different populations of \( H. \text{ fossilis} \) in Bangladesh.

**Materials and Methods**

**Collection of Sample and Isolation of Genomic DNA**

Live specimens of \( H. \text{ fossilis} \) (Bloch) were collected from four different populations in Bangladesh, namely, Kella Beel (Mymensingh), Rajdhala Beel (Netrakona), Bellabo Beel (Narsingdi) and Pargang Beel (Rangpur) during March, 2006 to August, 2006 (Fig. 1). Thirty two fish from each of the four sources were anaesthetized with MS 222 and ~40 mg of caudal fin tissue was clipped from each individual. The fin clips were cut into small pieces and ground with a tissue grinder in a 1.5 mL microcentrifuge tube. The genomic DNA was isolated following SDS (sodium dodecyl sulphate)-proteinase-K digestion, phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) extraction, and ethanol precipitation method as described by Islam and Alam. The quality of DNA was tested by electrophoresis on 1% agarose gel and the quantity was determined by using a spectrophotometer (Spectronic® Genesis™).

**PCR Amplification of Microsatellite Markers and Electrophoresis**

Five pairs of primers developed from the Walking catfish, \( C. \text{ batrachus} \) by Yue et al. were used in this study. Two pairs of primers namely \( Cba02 \) (Forward: GCCCTGCCAACATCTCCA, Reverse: TGGCTCCAGCACAACAA) and \( Cba19 \) (Forward: CAGGCCTAAATTACCCATAATCA, Reverse: GCCATGTGTTATAACATGTGAGG), which gave clear scorable bands, were finally used for analysis of the samples of the four populations of \( H. \text{ fossilis} \). The annealing temperature was adjusted to 56°C for \( Cba02 \) and 58°C for \( Cba19 \). PCR was performed in a 10 µL reaction volume containing...
50 ng template DNA, 0.25 µM of each primer, 0.25 mM of each of the dNTPs, 1 unit of Taq DNA polymerase (Genei, Bangalore, India)) and 1 µL 10× reaction buffer containing 1.5 mM MgCl₂. PCR amplification was performed in a gradient thermal cycler (Master Cycler Gradient, Eppendorf, Germany) with a temperature profile comprising 3 min initial denaturation at 94°C, followed by 35 cycles, each of 30 sec at 95°C, 30 sec at 56°C/58°C and 1 min at 72°C. Finally, an additional one cycle of 5 min at 72°C was added to allow elongation of the amplified products. The PCR product (10 µL) was mixed with 2.5 µL loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol) and half of the mixture was run on 2% standard agarose gel to check the quality of amplification products. The PCR products showing good resolution on agarose gel were separated on 6% denaturing polyacrylamide gel containing 19:1 acrylamide:bis-acrylamide and 7 M urea. Electrophoresis was conducted using a Sequi Gen GT sequencing electrophoresis system (BIORAD Laboratories, Hercules, CA). The DNA fragments were visualized essentially following the Promega (Madison, WI) silver staining protocol.

Scoring and Analysis of Microsatellite Data

Genotype of each individual fish was determined and recorded from the silver-stained gels for each microsatellite locus. Bands representing particular alleles at the microsatellite loci were scored from the plate manually and the size of the bands were estimated using the DNAfrag program version 3.03. Scoring of genotypes for a specific locus was performed on the basis of the number of alleles of a particular size present in that locus. For example, a locus carrying two alleles of 193 bp represented by a single band was considered as homozygous (193/193), while a locus carrying two alleles of different sizes (193 bp and 197 bp, for example) represented by two separate bands was considered as heterozygous (193/197). A single genotypic data matrix was constructed for all loci. Estimation of allelic variations, heterozygosity values (observed and expected), deviation from Hardy-Weinberg equilibrium, population differentiation (FST) values and Analysis of Molecular Variance (AMOVA) were performed using the software Arlequin v 3.0. The Nei’s genetic distance values and gene flow between populations were estimated using the software GenAlex v 6.1. A dendrogram was drawn based on the genetic distance between the populations following Unweighted Pair Group method of Averages (UPGMA) using the software MEGA 4.

Results

Allelic and Genotypic Variation within Population and Deviation from Hardy-Weinberg Expectation

Initially five heterologous microsatellite markers developed from C. batrachus were tested for analyzing the population genetic structure of H. fossilis. Of the five, two loci, Cba02 and Cba19, were amplified successfully and resulted in clearly scorable bands on the standard polyacrylamide sequencing gel visualized by silver nitrate staining. Both of the loci were found to be polymorphic (P93) revealing a total of 18 alleles in 128 diploid individuals. The size of the alleles ranged from 188 to 219 bp at the locus Cba02 and 208 to 237 bp at the locus Cba19 (Table 1). The average number of allele ranged from 6.5 (Netrakona) to 9.0 (Mymensingh). However, the effective number of alleles ranged from 3.68 to 6.63. Two private alleles were detected in Mymensingh population, whereas no private alleles were observed in rest of the populations (Table 2). The total number of lost alleles (alleles not amplified in any of the individuals of a population) was the highest in the Netrakona population (5), followed by the Rangpur (4) and the Narsingdi (3) population (Table 1). The observed heterozygosity (Ho) values ranged from 0.500 to 0.773, while the expected heterozygosity (He) ranged from 0.746 to 0.869 (Table 1). Except in Netrakona population, the Fixation Index (F) values were close to zero indicating random mating in these populations. A reasonably high level of Fixation value in Netrakona population might have resulted from the incident of inbreeding in this population or due to undetected null alleles. Significant deviations from Hardy-Weinberg proportion were detected in two out of a total of eight tests. The Netrakona and Rangpur populations both deviated from Hardy-Weinberg proportion at the locus Cba02.

Inter-population Genetic Structures and Genetic Distance

The Nei’s genetic distance (D), population differentiation (FST) and gene flow (Nm) values between the population-pairs are shown in Table 2. The D values ranged from 0.165 to 0.626 in the population-pairs. The mean D value between the Narsingdi and Mymensingh population-pair was the highest (0.626) and that between the Narsingdi and Netrakona population was the lowest (0.165). The Nm value between the Narsingdi and Netrakona
population was the highest, while that between the Narsingdi and Mymensingh population was the lowest. The AMOVA revealed that 95% of the variation contained within the population and only 5% distributed among the populations (AMOVA Table not shown). The \( F_{ST} \) values between the population pairs ranged from 0.022 to 0.083 (Table 2). The value between the Narsingdi and Netrakona population was insignificant (\( P>0.10 \)), while the values between the other populations pairs were found to be significant (\( P<0.05 \) or \( 0.0001 \)).

### Table 1—Allele size range, number of alleles observed (Na), effective number alleles (Ne), heterozygosity observed (Ho), heterozygosity expected (He), Fixation Index (F), deviation from Hardy-Weinberg equilibrium (H-W), percent of polymorphic loci (\( P_{95} \)), number of private alleles (Pa) and number of alleles lost (La) in four different populations of the stinging catfish (\( H. \text{fossilis} \))

<table>
<thead>
<tr>
<th>Population</th>
<th>Locus</th>
<th>Size Range (bp)</th>
<th>Na</th>
<th>Ne</th>
<th>Ho</th>
<th>He</th>
<th>F</th>
<th>H-W</th>
<th>( P_{95} )</th>
<th>Pa</th>
<th>La</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narsingdi</td>
<td>Cba02</td>
<td>190-219</td>
<td>8.00</td>
<td>6.05</td>
<td>0.773</td>
<td>0.854</td>
<td>0.074</td>
<td>NS</td>
<td>100</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Cba19</td>
<td>213-237</td>
<td>7.00</td>
<td>3.68</td>
<td>0.727</td>
<td>0.745</td>
<td>0.001</td>
<td>NS</td>
<td>100</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>-</td>
<td>7.50</td>
<td>4.87</td>
<td>0.750</td>
<td>0.799</td>
<td>0.038</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Netrakona</td>
<td>Cba02</td>
<td>193-219</td>
<td>7.00</td>
<td>3.92</td>
<td>0.500</td>
<td>0.762</td>
<td>0.329</td>
<td>***</td>
<td>100</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cba19</td>
<td>211-232</td>
<td>6.00</td>
<td>4.32</td>
<td>0.727</td>
<td>0.786</td>
<td>0.054</td>
<td>NS</td>
<td>100</td>
<td>0</td>
<td>3</td>
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<tr>
<td></td>
<td>Mean</td>
<td>-</td>
<td>6.50</td>
<td>4.12</td>
<td>0.614</td>
<td>0.774</td>
<td>0.192</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rangpur</td>
<td>Cba02</td>
<td>190-214</td>
<td>7.00</td>
<td>5.83</td>
<td>0.773</td>
<td>0.848</td>
<td>0.069</td>
<td>*</td>
<td>100</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cba19</td>
<td>211-237</td>
<td>7.00</td>
<td>3.94</td>
<td>0.727</td>
<td>0.746</td>
<td>0.025</td>
<td>NS</td>
<td>100</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>-</td>
<td>7.00</td>
<td>4.89</td>
<td>0.750</td>
<td>0.797</td>
<td>0.047</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mymensingh</td>
<td>Cba02</td>
<td>188-219</td>
<td>9.00</td>
<td>6.63</td>
<td>0.773</td>
<td>0.869</td>
<td>0.090</td>
<td>NS</td>
<td>100</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cba19</td>
<td>208-237</td>
<td>9.00</td>
<td>4.77</td>
<td>0.773</td>
<td>0.809</td>
<td>0.022</td>
<td>NS</td>
<td>100</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>-</td>
<td>9.00</td>
<td>5.70</td>
<td>0.773</td>
<td>0.839</td>
<td>0.056</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*=Statistically significant at \( P<0.05 \), ***=Statistically significant at \( P<0.001 \), NS=Not significant

### Table 2—Nei’s genetic distance (D), population differentiation (\( F_{ST} \)) and gene flow (Nm) between the population-pairs of the stinging catfish (\( H. \text{fossilis} \)) across the loci analyzed

<table>
<thead>
<tr>
<th>Population pair</th>
<th>D</th>
<th>( F_{ST} )</th>
<th>Nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narsingdi-Netrakona</td>
<td>0.165</td>
<td>0.022NS</td>
<td>11.245</td>
</tr>
<tr>
<td>Narsingdi-Rangpur</td>
<td>0.198</td>
<td>0.025*</td>
<td>9.788</td>
</tr>
<tr>
<td>Netrakona-Rangpur</td>
<td>0.274</td>
<td>0.045***</td>
<td>5.329</td>
</tr>
<tr>
<td>Narsingdi-Mymensingh</td>
<td>0.626</td>
<td>0.083***</td>
<td>2.745</td>
</tr>
<tr>
<td>Netrakona-Mymensingh</td>
<td>0.541</td>
<td>0.081***</td>
<td>2.818</td>
</tr>
<tr>
<td>Rangpur-Mymensingh</td>
<td>0.307</td>
<td>0.039***</td>
<td>6.091</td>
</tr>
</tbody>
</table>

NS=Not significant, *=Significant at \( P<0.05 \), ***=Significant at \( P<0.0001 \)

The UPGMA dendrogram based on Nei’s genetic distance, summarizing the data on differentiation between four populations of \( H. \text{fossilis} \), according to microsatellite DNA marker analysis

> Fig. 2—UPGMA dendrogram based on Nei’s genetic distance, summarizing the data on differentiation between four populations of \( H. \text{fossilis} \), according to microsatellite DNA marker analysis

The UPGMA dendrogram based on the genetic distances resulted in two major clusters: the Mymensingh population alone placed in one cluster and the remaining three populations were placed in the other cluster (Fig. 2). The second cluster was subsequently separated into two sub-clusters: the Rangpur population being alone in one sub-cluster and the Narsingdi and Netrakona populations together were in the other sub-cluster.

### Discussion

Population genetic structures of a fish species is subject to change over time under some biological and physical factors including overexploitation, contamination of native gene pools through introgression, inbreeding, bottleneck effect,
environmental pollution, habitat degradation, hydrological manipulations, and mutation at various levels. Microsatellite markers have been proved to be effective and efficient in detecting very low levels of genetic change induced by the above-mentioned factors. Microsatellite markers developed from one species some time cross-amplify microsatellite loci in closely related species. Islam et al., for example, reported amplification of the South-east Asian freshwater catfish, *C. macrocephalus* (not available in Bangladesh), microsatellite markers in *C. batrachus*, a freshwater catfish native to Bangladesh. In the present study, we have used the microsatellite markers developed from *C. batrachus* by Yue et al. to analyze population genetic structure of the stinging catfish (*H. fossilis*). Among the five markers tested, two markers gave good resolution and have been finally used for analysis of the samples collected from the four different populations of this species. Conservation of priming sites has been reported previously among closely related species of fish, indicating a promising trans-specific utility of microsatellite primers. The sizes of the amplified fragments (alleles), however, may not necessarily be the same as those of the homologous species (the species from which the markers have been originally developed). For example, the size of the alleles at locus Cba02 and Cba19 ranged from 188-219 bp and 208-237 bp, respectively in *H. fossilis*, while Islam et al. observed allele sizes in *C. batrachus* ranging from 150-194 bp and 224-276 bp at the loci Cba02 and Cba19, respectively.

In the present study, both of the loci Cba02 and Cba19 were found to be polymorphic in all the four natural populations of *H. fossilis* analyzed. In our previous study, we found these two loci polymorphic in three natural and one hatchery populations of *C. batrachus*. The average observed heterozygosity of the four populations ranged from 0.614 to 0.773. The average expected heterozygosity was highest in the Mymensingh population (0.839) and lowest in the Netrakona population (0.774). The average observed heterozygosity values of all the populations were lower than the corresponding expected heterozygosity values. The result of observed heterozygosity was not consistent with the average number of alleles per locus; however, the expected heterozygosity values were consistent with the average number of alleles per locus in a population. For example, the average number of alleles per locus was highest in the Mymensingh population and the average expected heterozygosity value was also highest in this population. On the other hand, the average number of alleles per locus was the lowest in the Netrakona population and the corresponding average expected heterozygosity value was also lowest in this population (Table 1).

The test for fit to Hardy-Weinberg proportion revealed that the Rangpur and the Netrakona population deviated at locus Cba02. All the populations were found to be in equilibrium at locus Cba19 and the Mymensingh and Narsingdi population were found to be in Hardy-Weinberg equilibrium in both the loci. Violations of the Hardy–Weinberg assumptions can cause deviations from expectation. Reduction in size of a population is considered to be one of the few factors that might be responsible for deviations from Hardy-Weinberg equilibrium. Small population size causes a random change in genotypic frequencies, particularly if the population is very small due to genetic drift. From the present study, it can be postulated that the Netrakona and Rangpur populations deviated from the Hardy-Weinberg equilibrium.

This study revealed that the population pairs were not homogeneous groups, rather they are differentiated. Except between the Netrakona and Narsingdi populations, the FST values between all other population-pairs were significant reflecting a high level of genetic diversity among individuals of the populations. Similar to the present study, Islam et al., using microsatellite markers, found high levels of genetic divergence in the *C. batrachus* populations in Bangladesh. The Mymensingh population has come out as a more differentiated population among the four. Two private alleles (Cba02-188 and Cba19-208) have also been found in Mymensingh population and none of the rest three populations possessed any private alleles. Though both the private alleles were not consistently found, only some of the individuals were carrying the alleles. The phylogenetic dendrogram (Fig. 2) drawn from Nei’s genetic distance also suggests the separation of Mymensingh population from the other three populations. Our previous studies involving *C. batrachus* suggested early separation of a hatchery population, while all the three natural population including the Mymensingh (Kella beel) population formed a single cluster.
why the Mymensingh population, rather than the Rangpur, has been separated since, among the four, Rangpur is the most distantly located populations (Fig. 1).

Analysis of population genetic structure of threatened or commercially important fish species is essential to develop management plan or a stock improvement programme. Genetic monitoring is necessary for an effective management strategy because a population can suffer severe genetic erosion (bottleneck, genetic drift, inbreeding, founder effect, etc) without being detected by the traditional demographic monitoring approach. The microsatellite technique has been found to be suitable for characterizing the genetic structure of four populations of stinging catfish (H. fossilis). The allele numbers and heterozygosity levels observed across the studied loci indicate the presence of a reasonably high level of genetic variability in the stinging catfish populations in Bangladesh. This information should be taken into consideration for any genetic conservation and stock improvement plan. However, further study involving large numbers of populations covering all parts of the country with additional microsatellite loci is recommended to reveal detailed genetic structure of this important fish species in Bangladesh.

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