World Journal of Fish and Marine Sciences 7 (1): 38-44, 2015
ISSN 2078-4589

DOI: 10.5829/idosi.wjfms.2015.7.1.92119

Population Genetics of Stinging Catfish (*Heteropneustes fossilis*) in Bangladesh Analyzed by Microsatellite DNA Markers

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Abstract: Stinging catfish (*Heteropneustes fossilis*) is an important aquaculture fish species in Bangladesh with high nutritive value. Three microsatellite loci (*Cba02, Cba19* and *Cba20*) were tested to study the genetic variation in this species. Samples were collected from two natural populations namely Mohongonj and Narsingdi and from a hatchery population located in Mymensingh of Bangladesh. All the three loci were found to be polymorphic (P_{95}) in the three studied populations. The average observed heterozygosity (H_o) value was the highest in Narsingdi population (0.80) followed by the Hatchery (0.70) and the Mohongonj population (0.67). Only Mohongonj population was significantly deviated from Hardy-Weinberg Equilibrium in one locus. The population differentiation value (F_{ST}) between all the population pairs was insignificant. The highest genetic distance value (D = 0.47) was found between the Mohongonj and Hatchery populations while the smallest value (D = 0.34) was found between the Mohongonj and Narsingdi as well as Narsingdi and Hatchery populations. The UPGMA dendrogram based on genetic distance resulted in two major clusters: Hatchery stock alone is in one cluster and the remaining two natural stocks are in other cluster. The study also revealed that the *Clarias batrachus* microsatellites could be effectively used in the assessment of genetic structure of the stinging catfish *H. fossilis*

Key words: Stinging Catfish · Microsatellite Loci · Polymorphism · Bangladesh

INTRODUCTION

Catfishes are an extremely large group of diverse teleosts generally regarded as single order, the Siluriformes, or as a suborder, of the order Cypriniformes [1]. There are over 2400 species of catfishes, divided into 344 families containing 412 genera. Members of the family Heteropneustidae are collectively known as 'airsac catfishes'. The stinging catfish (*Heteropneustes fossilis* Bloch), a member of Heteropneustidae, often called "shingi" is commercially as well as aquaculturally an important species in many Asian countries [2]. Stinging catfish is native to Bangladesh, Pakistan and India including the Andaman Islands, Nepal, Srilanka, Burma, Thailand, Indus Basin and Laos [3]. In Bangladesh, the total annual production of catfish in 2007-2008 was estimated to be 85,869 metric ton, which was 3.35 (weight%) of total inland fish production [4]. As a major catfish species the contribution of *H. fossilis* is great in total catfish production. It is also popular from nutritional and medical point of view. The muscles of the fish have been reported to have very high content of iron (226mg/ 100g) and fairly high level of calcium compared to many other freshwater fishes (fat content only 2.57% ±0.24 on fresh weight basis). Due to high nutritive value, the fish is recommended in the diet of sick and convalescents.

Genetic variation within and among populations is essential for an evolutionary interpretation of interactions and for the management of endangered or commercially important taxa. It is the raw material that is exploited in a selective breeding program. Genetic variability is maintained naturally in an infinitely large panmictic population while hatchery stock is finite. As a result, the hatchery populations are always subjected to inbreeding

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and genetic drift that quickly destroy a population's variance. Information on genetic variation within hatchery stocks indicates the level of success in their management and also the status of their broodstock. Maintenance of genetic variation is the most important concern for the management of hatchery stocks of any species [5, 6]. Genetic variation serves as a useful tool for characterization of different species or strains within a species, comparison of farmed strains with wild populations and evaluation of changes in genetic structure of species over time. To manage any biological resource effectively, we must identify the level of genetic variation within and among populations.

H. fossilis is popular for its high growth rate, high fecundity, efficient feed utilization capacity and ability to survive in poorly oxygenated waters. Habitat degradation caused by natural and human interventions, injudicious application of pesticides in agricultural fields and release of industrial effluent have recently become great constraints for fish biodiversity in most aquatic ecosystems in Bangladesh. As a result remarkable changes have already been observed in natural *H. fossilis* in different closed water bodies. In order to understand those variations, intensive study on existing genetic diversity of natural populations of *H. fossilis* in Bangladesh is urgently needed. Microsatellite loci are highly polymorphic in natural populations with average expected heterozygosity well above 50% in general, peaking virtually at 100% [7]. Mcrosatellite have been isolated from the genome of only a few catfish species [8, 9]. Despite of the economic importance of this species, genetic data on *H. fossilis* stocks is unavailable not only in Bangladesh but also in other countries. The objectives of the present study, however, were to study the genetic structure and compare the genetic variability between three stocks of *H. fossilis* and to identify the genetically superior fish stocks among the populations.

MATERIALS AND METHODS

Fish Sample Collection and DNA Extraction: *H. fossilis* samples were collected from two natural and one hatchery sources namely Mohongonj *haor* (Netrakona), Belaboo *beel* (Narsingdi) and Al-Amin Hatchery (Mymensingh) (Fig. 1). Thirty fish samples from each stock were analyzed by microsatellite DNA markers. A small piece of

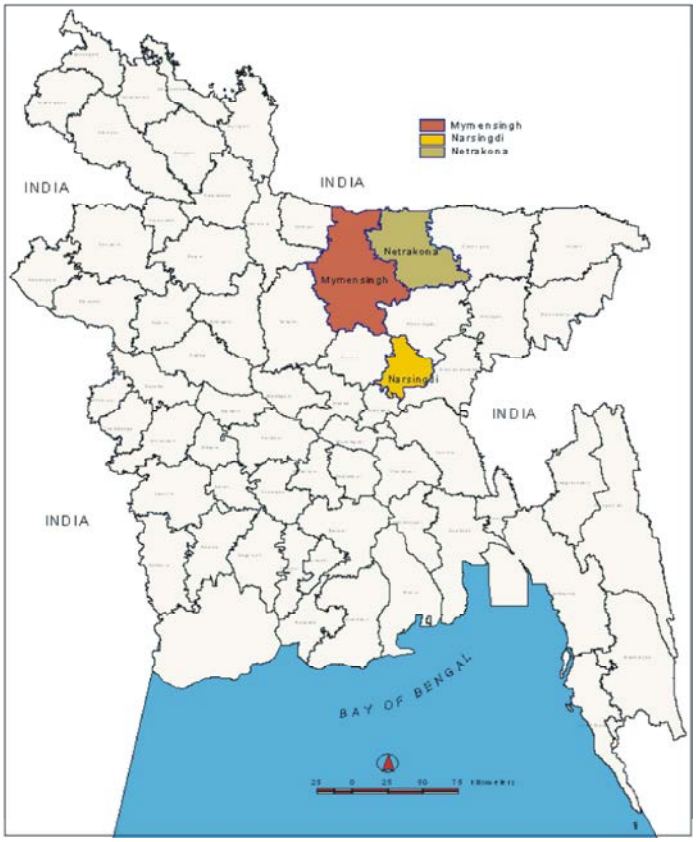

Fig. 1: Map of Bangladesh showing sampling sites (□=Mymensingh, Δ=Narshingdi and ◇=Netrokona) for populations of *H. fossilis*

the caudal fin was cut with scissors and preserved in 95% ethanol until used for genomic DNA extraction. Genomic DNA was extracted from fin tissues according to the method described by Islam and Alam [10] with some modifications. In brief, approximately 30 mg of fin tissues was cut into small pieces and taken into 1.5 ml microfuge tube. The fin tissue was digested with proteinase-K in extraction buffer (100 mM Tris, 10 mM EDTA and 250 mM NaCl, pH= 8.0 and 1% Sodium Dodecyl Sulfate) overnight at 37°C. DNA was purified once with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) and precipitated using 0.6 volumes of isopropanol. The DNA samples were tested qualitatively on 1% agarose gel and quantified by using a spectrophotometer (Biophotometer plus, Eppendorf, Germany).

Amplification of Microsatellite Loci: Three primer pairs (*Cba02*, *Cba19* and *Cba20*) developed by Yue *et al*. [11] from *C. batrachus*, were used for PCR amplification in this study. Annealing temperatures of the three primer pairs were adjusted to 62°C to obtain better resolution of the amplified products. PCR was performed in a 10 µl reaction volume containing 50 ng of template DNA, 0.25 mM of each primer, 0.25 mM of each dNTP, 1 U Taq DNA polymerase (GENEI Pvt. Ltd., Bangalore, India) and 1 µl 10X reaction buffer containing 15 mM $MgCl_2$. Thermal profile consisted of 3 min initial denaturation at 94°C, followed by 35 cycles, each of 30 s at 94°C, 30 s at the respective annealing temperature and 1 min at 72°C, ending with an additional 5 min at 72°C for final elongation. An oil free thermal cycler (Master Cycler Gradient, Eppendorf, Germany) was used for conducting the polymerase chain reaction.

Electrophoretic Separation and Visualization of PCR Products: Three microliters from each of the PCR products was electrophoresed on a 6% denaturing polyacrylamide gel ontaining 19:1 acrylamide:bis-acrylamide and 8 M urea. Electrophoresis was conducted using the SequiGen GT sequencing gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA). A prerun of the gel for 30 min at 120 W was followed by a final run at 60 W and 50°C upon loading of denatured PCR products for a specified period of time depending on the size of amplified DNA fragment (usually 1 h for 100 bp). A molecular weight marker DNA (100 bp DNA ladder, New England Biolabs Inc., Beverly, MA) was loaded on either side of the gel. After completion of electrophoresis, the DNA fragments were visualized, essentially following the Promega (Madison, WI) silver-staining protocol.

Scoring and Analysis of Microsatellite Data: The bands representing particular alleles at the microsatellite loci were scored manually and designated as a, b, c, etc from the bottom to the top of the gel. Scoring of genotype was done for a specific locus as aa (140/140), bb (150/150) etc. for homozygous or as ab (140/150) etc. for heterozygous. A single genotypic data matrix was constructed for all loci. The software DNAfrag version 3.03 was used to estimate marker length and allelic length [12]. The GenAlEx program version 6 [13] was used for estimating the number of alleles (N) and frequency of alleles. G-stat program [14] was used to calculate expected and observed heterozygosities (H_e, H_o), to test pairwise homogeneity, to estimate fixation index (f) and to calculate Nei's [15] genetic distance (D) in different population pairs. Fit to Hardy-Weinberg proportions was estimated using the PopGene program version 1.31 by a chi-square test [16] with 1,000 simulated samples. The same program was also used to estimate gene flow (Nm) between populations. Fstat program version 2.9.3 [17] was used to test for differences (F_{ST}) between populations (1,000 permutations). The unweighted pair group method with averages (UPGMA) dendrogram was drawn based on Nei's [15] genetic distances using the Treeview program [18].

Table 1: Allele frequencies at three microsatellite loci in three stocks of *H. fossilis*

Locus name	Allele size (bp)	Mohongonj	Narsingdi	Hatchery
Cba02	126	0.000	0.000	0.100
	130	0.000	0.050	0.200
	132	0.550	0.100	0.200
	134	0.050	0.200	0.150
	138	0.100	0.250	0.150
	140	0.100	0.250	0.150
	144	0.000	0.100	0.050
	150	0.100	0.050	0.000
	154	0.100	0.000	0.000
Cba19	192	0.250	0.200	0.100
	206	0.300	0.400	0.450
	211	0.150	0.000	0.100
	221	0.300	0.400	0.350
Cba20	115	0.000	0.050	0.000
	116	0.100	0.000	0.050
	119	0.100	0.200	0.000
	120	0.000	0.100	0.050
	123	0.250	0.150	0.150
	126	0.250	0.150	0.000
	129	0.050	0.000	0.500
	131	0.100	0.150	0.000
	133	0.000	0.050	0.100
	134	0.050	0.000	0.000
	138	0.000	0.100	0.100
	139	0.100	0.000	0.000
	142	0.000	0.050	0.050

RESULTS

Allele Frequency and Size Variation Within Stocks: In the present study three microsatellite loci *Cba02, Cba19* and *Cba20* in three populations of *H. fossilis* were analyzed. The allele size and frequencies of three microsatellite loci in three populations are shown in Table 1. The size of the alleles at all loci in the three stocks ranged from 115 to 221 bp (126 bp to 154 bp, 192 bp to 221 bp and 115 bp to 142 bp for the locus *Cba02, Cba19* and *Cba20* respectively).

Genetic Variation: All of the three microsatellite loci *Cba02, Cba19* and *Cba 20* were found to be polymorphic (P_{95}) in all the studied populations. The number of alleles over the loci ranged from 4 to 13. The locus *Cba20* in the Narsingdi population had the highest number of alleles (9) while the locus *Cba19* in the Narsingdi had the least number of alleles (3) (Table 2). The average number of allele in the Narsingdi population was 6.30 and that in the Mohongonj and the Hatchery population was 6.00 (Table 2).

Table 2: Allelic and genotypic variation at three microsatellite loci in three stocks of *H. fossilis* (N=Number of alleles, N_e=Effective number of alleles, H_o=Heterozygosity observed, H_e= Heterozygosity expected, I= Information index)

Microsatellite loci	Parameters	Mohongonj	Narsingdi	Hatchery
Cba02	N(9)	6	7	7
	N_e	2.90	5.26	6.25
	H_o	0.20	0.70	0.60
	H_e	0.65	0.81	0.84
	1- H_o/H_e	0.69	0.14	0.29
	I	1.40	1.76	1.88
Cba19	N(4)	4	3	4
	N_e	3.77	2.77	2.89
	H_o	1.00	1.00	1.00
	H_e	0.73	0.64	0.65
	1- H_o/H_e	-0.37	-0.56	-0.38
	I	1.35	1.05	1.118
Cba20	N(13)	8	9	7
	N_e	5.88	7.40	3.33
	H_o	0.80	0.70	0.60
	H_e	0.83	0.86	0.70
	1- H_o/H_e	0.036	0.19	0.14
	I	1.91	2.08	1.54
Average H_o over loci		0.67	0.80	0.70
Average H_e over loci		0.74	0.77	0.73
Average number of alleles		6.0	6.3	6.0
Polymorphism		1.00	1.00	1.00

Table 3: Hardy- Weinberg (H-W) Expectations (x^2 values) at three microsatellite loci in three populations of *H. fossilis* (x^2 values in parentheses indicate degree of freedom in parentheses)

Parameter	Microsatellite loci	Mohongonj	Narsingdi	Hatchery
H-W test	*Cba02*	$39.17(15)^{***}$	$13.20(21)^{NS}$	$21.53(21)^{NS}$
	Cba19	$8.67(6)^{NS}$	$6.25(3)^{NS}$	$3.51(6)^{NS}$
	Cba20	$26.40(28)^{NS}$	$36.39(36)^{NS}$	$22.73(21)^{NS}$

Statistically significant values are marked with asterisks
NS=not significant, *** P<0.001

Table 4: Multilocus F_{ST} (above diagonal) and Nm (below diagonal) values between pairs of three stocks of *H. fossilis* across all loci

Stocks	Mohongonj	Narsingdi	Hatchery
Mohongonj	--	0.044	0.061
Narshingdi	5.43	--	0.043
Hatchery	3.87	5.54	--

Table 5: Genetic distance between pairs of three stocks of *H. fossilis* across all loci

Stocks	Mohongonj	Narshingdi	Hatchery
Mohongonj	--	0.34	0.47
Narshingdi		--	0.34
Hatchery			--

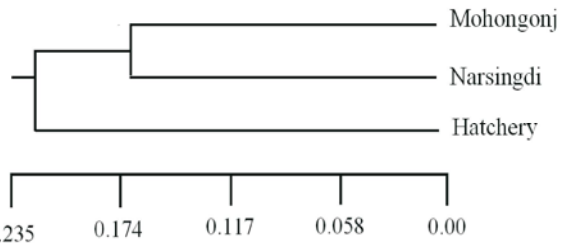

Fig. 2: UPGMA dendrogram based on genetic distance, summarizing the data on differentiation between three stocks of *H. fossilis*, according to the microsatellite DNA analysis.

The observed average heterozygosity (H_o) value in the Narsingdi population was the highest (0.80) followed by the Hatchery population (0.70) and the Mohongonj population (0.67) (Table 2). In the three stocks, at locus *Cba19* the 1-H_o/H_e values were negative indicating excess of heterozygosity. The 1-H_o/H_e values were positive for the three stocks at the other two loci (*Cba02* and *Cba20*), which means that the stocks were deficient in heterozygosity (Table 2).

Deviation from Hardy-Weinberg Expectation: Significant deviation from Hardy-Weinberg Equilibrium (HWE) was detected in only one out of the nine tests (Table 3). Of the three microsatellite loci tested in this study, Mohongonj stock was found to be deviated from Hardy Weinberg proportion at locus *Cba02* (P<0.001).

Inter Population Genetic Structure: The F_{ST} value between the population pairs have been compared and found that the population differentiation values between all the population pairs were insignificant. The population differentiation (F_{ST}) value between the Mohongonj and the Narsingdi population was the highest (0.061) and that value was the lowest (0.043) between the Narsingdi and the Hatchery populations (Table 4). The gene flow value (N_m) between Hatchery and Mohongonj populations was lowest (3.87) while that of the Narsingdi and Hatchery populations was highest (5.54) (Table 4).

Genetic Distance: A matrix of genetic distance was built based on allelic frequencies of all loci (Table 5). The largest genetic distance value (D = 0.47) was measured between the Mohongonj and the Hatchery population while the smallest value (D =0.34) was found between the Mohongonj and the Narsingdi populations and between the Narsingdi and the Hatchery population.

Dendrogram: The UPGMA dendrogram based on genetic distance resulted in two major clusters. The Hatchery population alone was in one cluster and remaining two populations (the Mohongonj and the Narsingdi) in other cluster (Fig. 2).

DISCUSSION

For conducting genetic studies with a view to stock improvement, particularly when such works are pioneering for a species, it is important to have clear- cut rationale on which the strategy of the experiment can be based. For *H. fossilis* such a rationale is the fact that very little molecular work has been performed on the species. The microsatellite technique has been found to be suitable for characterizing the three stocks of *H. fossilis* in this study. Based on microsatellite DNA marker the abundance and high heterozygosity have been used as tool for assessing the genetic variability among intra-species and inter species fish populations.

Five primers developed from *Clarias batrachus* (*Cba02, Cba07, Cba12, Cba19* and *Cba20*) were tested for PCR amplification using shing DNA as template. Two primers (*Cba07* and *Cba12*) have been failed to amplify probably due to lack of priming sites in *H. fossilis* genome, unresponsiveness of these primer pairs or inaccuracy of set PCR profiles.

Primers developed for one species sometimes cross- amplify microsatellite loci in closely related species [19]. Yue *et al*. [11] tested cross-amplification of microsatellite loci isolated from *C. batrachus* in other catfish such as *C. fuscus, C. gariepinus, C. macrocephalus, Heteropneustes fossilis, H. longfilis, Phractrocephalus hemiolipterus* and an unclassified catfish. For genetically close relationship between *C. batrachus* and *H. fossilis*, the loci *Cba02, Cba19* and *Cba20* of *C. batrachus* were found to be more or less homologous in *H. fossilis* which was not very unusual.

In the locus *Cba02*, the alleles 126bp, 130bp and 144bp of were absent in Mohongonj stock, the alleles 126bp and 154 bp were absent in Narsingdi stock and the alleles 150 bp and 154 bp of locus *Cba02* were absent in Hatchery stock. On the other hand, in the locus *Cba20*, the alleles 115 bp, 120 bp, 133 bp, 138 bp and 142 bp absent in Mohongonj population, the alleles 116 bp, 129 bp, 134 bp and 139 bp were absent in Narsingdi population and the alleles 115 bp, 119 bp, 126 bp, 131 bp, 134 bp and 139 bp of locus *Cba20* were absent in Hatchery population. In the locus *Cba19*, only the allele 211 bp was absent in Narsingdi population.

In the present study, all the three populations of *H. fossilis* were found to be polymorphic in the three loci. The result is consistent with the findings of Islam *et al*. [20] in case of *C. batrachus* of different populations in Bangladesh while working with microsatellite markers. Similar result was also reported in case of four natural populations of *C. macrocephalus* [21]. The average observed heterozygosity of the three stocks ranged from 0.67 to 0.80. The average expected heterozygosity was highest in Narsingdi stock (0.77) and lowest in Hatchery stock (0.73). The average observed heterozygosities of all populations in all loci were lower than corresponding expected heterozygosities. Islam *et al*. [20] found that the loci *Cma-3, Cba02* and *Cba19* were polymorphic having 8, 5 and 9 alleles per locus and average heterozygosity 0.67 to 0.83 in *C. batrachus*. Yue *et al.* [11] identified 16 polymorphic microsatellite loci (allele number: 2-10/locus and expected heterozygosity (0.30-0.87) in the walking catfish (*C. batrachus*). Na-Nakorn *et al*. [21] studied polymorphic loci in four natural populations of *C. macrocephalus* collected from different locations in Thailand and found mean number of alleles per locus 8.0, 8.7, 6.0 and 10.0 with mean heterozygosities 0.744, 0.765, 0.718 and 0.810, respectively.

The 1-H_o/H_e values were negative at locus *Cba19* referring that the populations did not lose heterozygosity and individuals could be considered as naturally outbred only at this locus rather than as bottlenecked. Negative values of 1-H_o/H_e were also reported in almost all populations across all microsatellite loci analyzed by Islam *et al*. [20] in *C. batrachus* experiencing recent bottleneck in those populations. The positive values of 1-H_o/H_e at loci *Cba02* and *Cba20*, meaning that studied populations were deficient in heterozygosities. However, heterozygote excess in the populations is not as common as heterozygote deficiency.

In the present study, Hardy-Weinberg proportion revealed that only Mohongonj stock was found to be deviated significantly ($P<0.001$) from Hardy-Weinberg Equilibrium at locus *Cba02*. The remaining stocks were in equilibrium across all loci. Almost similar result was reported by Na-Nakorn *et al*. [22] in which they observed that six out of twenty-six populations of *C. macrocephalus* differed significantly from Hardy-Weinberg Equilibrium by RAPD marker analysis. The population differentiation (F_{ST}) values were low (0.043 to 0.061) and insignificant. This result indicates that the populations are homogeneous, i.e., the populations are genetically almost similar to each other and the results are consistent with findings in Indo-Pacific king mackerel (*Scomberomorus guttatus*) [23] and silver pomfret (*Pampus argenteus*) [24], both inferred from microsatellite analysis. As documented by Abedi *et al*. [23], low F_{ST} values (0.004- 0.038) were observed among the population pairs of Indo-Pacific king mackerel. Pairwise F_{ST} values in silver pomfret also revealed low (<0.040) genetic differentiation between 5 sampling sites across Persian Gulf and Oman [24]. The gene flow values (3.87-5.54) indicated that sufficient gene flow was prevailing among three populations allowing no occurrence of regional genetic differentiation.

The genetic distance between the population pairs ranged from 0.34 to 0.47. The highest genetic distance was found between the Hatchery and the Mohongonj population (0.47) which might be due to relatively distant origin of broods. Although no significant genetic differentiation was evident and gene flow was sufficient among the populations, there might have certain phylogenetic relationship and genetic distance based on geographical sources of individuals. Almost similar result was also found by Na-Nakorn *et al.* [21] in *C. macrocephalus* from Chiangrai and Pattani or Pattalung population in Thailand when analyzed with microsatellite marker.

CONCLUSION

Population genetics and genetic variation is the baseline information for conservation management of any fish species. The present study would be helpful in identifying the brood catfish bearing good genetic data to aid in selective and induced breeding programs. Side by side, utilization of good quality broods and avoidance of indiscriminate inbreeding and/or hybridization by the hatchery owners and breeders is essential to maintain proper genetic diversity of aquaculture fish species. However, molecular diversity analysis and genetic characterization may play a pivotal role in the breeding, conservation and sustainable aquaculture of the catfish species.

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