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Genetic characterisation of wild catla (*Catla catla* Hamilton) populations using microsatellite DNA markers

MD. ABDULLAH AL FAROQUE, MARUF HOSSAIN MINAR, NUR UN NESA[1,*], MD. RAFIQUL ISLAM SARDER AND MD. FAZLUL AWAL MOLLAH

Dept. of Fish. Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

[1]Current address: College of Public Health, Medical and Veterinary Sciences, Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville, QLD 4811, Australia

*Corresponding author'sEmail: nurun.nesa@my.jcu.edu.au

Abstract. The genetic characterisation of catla, *Catla catla* populations from three rivers, the Halda, the Jamuna, and the Padma was performed employing six microsatellite DNA markers *Ccat A12*, *Ccat C3*, *Ccat C6*, *Ccat C8*, *Ccat G1*, and *Ccat G2*. All the studied loci were detected as polymorphic (P_{95}) in nature. The appearance of five rare alleles in Halda, at *Ccat A12* (149 and 153 bp), *Ccat C6* (180 bp), and *Ccat C8* (98 and 104 bp) demonstrated the genetic richness of the Halda population. The average highest (6.33) and lowest (4.67) number of alleles were recorded in the Halda and the Padma populations, respectively. The Halda population exhibited the highest (0.5111) average heterozygosity (H_o) compared to the Jamuna (0.4889) and the Padma (0.4167). The population differentiation (F_{ST}) between the Halda and the Padma populations was the highest (0.0653) and the lowest (0.0366) between the Halda and the Jamuna populations. The highest gene flow (N_m) (6.5747) existed between the Halda, and the Jamuna populations and the lowest (3.5811) between the Halda and the Padma populations. These findings revealed a reduced genetic variability of wild stocks of *C. catla* in relation to allelic diversity and heterozygosity that provided a benchmark for future comprehensive management programme to conserve the genetic resources of wild populations of this species.

Keywords: Genetic variability, *Catla catla*, Heterozygosity, Polymorphism

Introduction

Catla catla (Hamilton, 1822) is the second most economically important species in the aquaculture sector of Bangladesh among the Indian major carps (IMCs) and distributed across India, Myanmar, Pakistan, Nepal, and Laos (Alam and Islam 2005, Hansen *et al.* 2006, Rahman *et al.* 2009). It contributed 3,041,299 Metric Ton (MT) as a major species to the world's total aquaculture production in 2018 valued at approximately 5 billion USD (FAO-FIGIS 2020). In Bangladesh, catla contributed 6.4 % accounting 231,878 MT to the total annual production in inland waterbodies (FRSS 2018). Catla has become an appealing food fish to the Bangladeshi people owing to its elegant appearance, excellent taste, ease of culture, and faster growth rate. It inhabits the major rivers of Bangladesh such that the Padma, Jamuna, Arialkha, Old Brahmaputra, Garai/Madhumati and Halda (Alam and Islam 2005). This species gains reproductive maturity at about 2 years old ($\approx$ 2 kg body weight) and breeds in upstream river throughout the monsoon season (May-August). Depending on the fish length and weight, fertility ranges between 100,000 and 200,000 eggs.kg^{-1} body weight (FAO 2009). Among the riverine sources, Halda is the biggest natural breeding and spawning habitat of IMCs (Patra and Azadi 1985) and produced the highest amount (4.507 of total 9.274 MT) of carp hatchlings in the fiscal year 2017-2018 (FRSS 2018).

Loss in genetic diversity has deleterious effects on fish growth, development, reproduction, and disease tolerance (Liu and Cordes 2004). Increased level of homozygosity causes inbreeding depression that may compromise the population fitness by assembling the lethal alleles or owing to lack of discrepancy in over dominant loci (Hansen *et al.* 2006). For the conservation of gene pool, preventing the deficits of genetic diversity is an imperative management strategy that can

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be achieved by maintaining heterozygosity as well as allelic diversity (Hansen *et al.*, 2006). Allelic diversity is a requirement for preserving evolutionary capacity, in support to allow wild species to respond to changing environmental circumstances and to ensure that selective breeding programme will strengthen the characteristics of captive populations (Hansen *et al.* 2006). Extensive data on the genetic structure of the wild population of catla discerning genetic differentiation over a certain geographic area is fundamental for establishing suitable conservation guidelines of the species.

The microsatellite markers consisting of tandem repeated nucleotide sequences (one-to-eight), arbitrarily dispersed in the genome, have rapidly become the preferred molecular marker for population genetic studies that is suitable to identify the genetic difference of marginally distinct natural and cultured fish species (Hansen *et al.* 2006, Zolgharnein *et al.* 2011). Few studies have revealed the genetic structure of catla using RAPD for wild (Islam *et al.* 2005, Rahman *et al.* 2009), allozyme for hatchery reared population (Alam *et al.* 2004, Hansen *et al.* 2006) and only one study by microsatellite DNA markers (Alam and Islam 2005) from the identical three major rivers though from varied distant locations. Moreover, studies demonstrated that genetic diversity of species is unevenly distributed even across the short stretches of river basin, a characteristic that should be taken into consideration for future management and conservation to avoid irreversible biological damage (Ferreira *et al.* 2015). Thus, this study focused on the genetic characterisation of wild populations of *C. catla* in three rivers using microsatellite markers.

Materials and Methods

***Ethical statement*:** The care and use of experimental animals complied with the standards of Animal Welfare and Experimental Ethics Committee as approved by Bangladesh Agricultural University, Mymensingh.

Fish sample collection: *C. catla* fry samples were collected from three rivers, i.e., the Halda, the Jamuna and the Padma (Fig. 1) speculating that these sampling locations will reflect the genetic diversity across a wide variety of the geographical region. The fry was held in rectangular earthen nursery ponds in the proximity of Fisheries Faculty, Bangladesh Agricultural University. The fish fry was nurtured for 60 days feeding with the nursery and traditional supplementary feed.

DNA extraction and genotyping*:* The DNA from fin tissues of 30 fishes from each sampling site was extracted following the method developed by Alam and Islam (2005). For genotyping. six pairs of microsatellite markers namely *Ccat A12*, *Ccat C3*, *Ccat C6*, *Ccat C8*, *Ccat G1*, and *Ccat G2* (Naish and Skibinski, 1998; McConnell *et al.,* 2001) were used (Table I). PCR was carried out at a reaction volume of 10 µL containing 50 ng genomic DNA template, 2.0 µM each primer, 0.25 mM each dNTPs, 1.5 mM $MgCl_2$, 1-unit *Taq* DNA polymerase, and 1 µL 10× PCR buffer. The thermal profile for PCR was set at 94 °C, 3 min for denaturation followed by 35 cycles of 30 sec at 94 °C, 30 sec at the corresponding annealing temperature, 1 min at 72 °C for extension, and final phase with 5 min extension at 72 °C. After PCR completion, the DNA amplicons were separated on a 6 % denatured polyacrylamide- bis-acrylamide (19:1) gel containing 5M urea. Electrophoresis was performed employing a Sequi-Gen GT sequencing gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). An

initial run was conducted for 30 min at 120 W following a final run at 60 W until the loading dye hit the end of the electrophoresis plate. The fragments of DNA were visualised on the gel according to the Promega silver-staining (Madison, WI, USA) protocol.

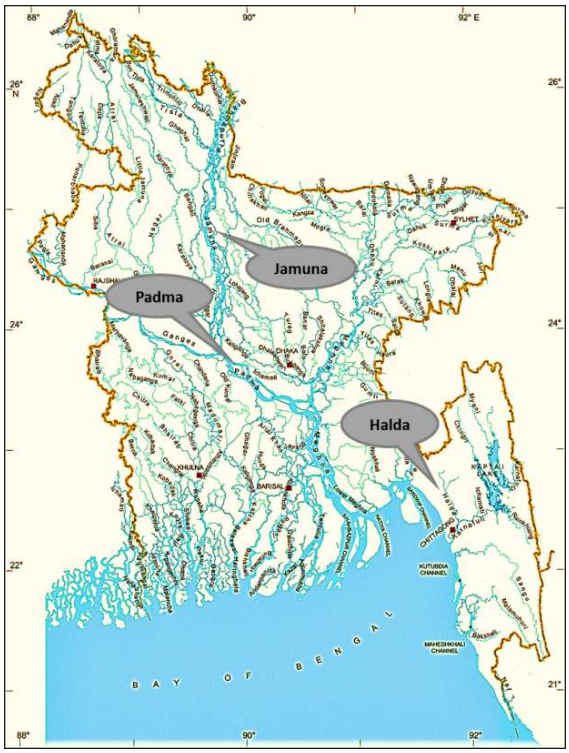

Fig. 1. A map of Bangladesh presenting the three sampling sites of *G. catla*.

Table I. The size and sequence of microsatellite markers for genetic variation analysis of *C. catla*

Primers	Allele size (bp)	No. of alleles		Primer sequence (5´-3´)	Annealing Temperature (°C)
Ccat C3	135	5	For.	AGGCAATTCAGTCTGTTAGAG	56 °C
			Rev.	TAACAACATGCTAATACCTFGC	
Ccat G1	140	3	For.	AGCAGGTTGATCATTTCTCC	60 °C
			Rev.	TGCTGTGTTTCAAATGTTCC	
Ccat G2	419	6	For.	GTCCGCTGTAAAACGGAGATTCCTG	60 °C
			Rev.	ACCCCCATGTCTCTGTGACATTC	
Ccat A12	144	4	For.	GCACAATATATTGTCTCCATATCGG	61 °C
			Rev.	AATGCTGGATATATGAAATGGACAG	
Ccat C6	180	6	For.	ATTTGAGGTTAAAGGTTAAAAG	50 °C
			Rev.	AAGAACTCTAATGATGCCAG	
Ccat C8	138	6	For.	GAGTGACATTTTCATTTATT	50 °C
			Rev.	ACACTCAGGAATGAGCAG	

***Microsatellite data scoring for statistical analysis*:** The allelic length was measured with the DNAfrag software version 3.03 (Nash 1991). Allele frequencies were estimated from observed

genotypes and a single genotypic matrix data was assembled for all the loci. The GenAlEx program version 6.1 (Peakall and Smouse 2006) was used for assessing the frequency and number of alleles (N). For allelic variation and fitting of genotypic data to Hardy-Weinberg proportions, the POPGENE software version 1.31 (Yeh *et al.* 1999) was applied using a chi-square (X^2) test with 1000 simulated samples. POPGENE progam was also used to determine the expected heterozygosity (H_e) and observed heterozygosity (H_o), pair-wise homogeneity test, genetic differentiation (F_{ST}), gene flow (Nm) and the genetic distance (D) between the population pairs.

Results

***Allelic and genetic variation*:** In total, 38 alleles were identified across six microsatellite loci in three populations of *C. catla*. The allelic sizes ranged from 98 to 434 bp (98-112 bp, 130-143 bp, 130-146 bp, 137-153 bp, 180-210 bp, 404-434 bp) for the loci *Ccat C8, Ccat C3, Ccat G1, Ccat A12, Ccat C6,* and *Ccat G2,* respectively (Table II).

Table II. Allele frequencies at six microsatellite loci in three populations of *C. catla*

Locus	Allele size	Halda	Jamuna	Padma
Ccat A12	137	0.083	0.150	0.333
	141	0.033	0.267	0.250
	142	0.050	0.017	0.217
	143	0.050	0.033	0.000
	144	0.133	0.033	0.000
	146	0.117	0.150	0.000
	147	0.100	0.100	0.167
	149	0.217	0.000	0.000
	150	0.150	0.250	0.133
	153	0.067	0.000	0.000
Ccat C3	130	0.033	0.125	0.250
	131	0.083	0.017	0.017
	134	0.250	0.217	0.233
	137	0.383	0.467	0.300
	142	0.150	0.200	0.200
	143	0.017	0.017	0.000
Ccat C6	180	0.017	0.000	0.000
	183	0.183	0.050	0.000
	196	0.367	0.267	0.400
	200	0.183	0.200	0.083
	206	0.150	0.150	0.183
	210	0.100	0.350	0.333
Ccat C8	98	0.017	0.000	0.000
	104	0.083	0.000	0.000
	106	0.333	0.275	0.450
	108	0.333	0.483	0.317
	110	0.150	0.233	0.167
	112	0.083	0.067	0.067

Ccat G2	404	0.167	0.033	0.033
	408	0.267	0.250	0.333
	417	0.450	0.300	0.117
	425	0.083	0.283	0.300
	429	0.017	0.050	0.167
	434	0.017	0.083	0.050
Ccat G1	130	0.267	0.333	0.283
	133	0.433	0.450	0.517
	140	0.250	0.167	0.133
	146	0.050	0.050	0.067
No. of missed allele across loci		0	5	10

A total of 5 private alleles (*Ccat A12*$_{149}$, *Ccat A12*$_{153}$, *Ccat C6*$_{180}$, *Ccat C8*$_{98}$ and *Ccat C8*$_{104}$) were found in Halda population. The allelic richness ranged from 4 to 10 and highest average number of alleles was (6.33) detected in Halda population and lowest (4.67) in Padma populations (Table II). The average H_o was highest (0.5111) in the Halda population followed by the Jamuna (0.4889) and the Padma populations (0.4167). However, the average H_e was highest in the Padma (0.7554) followed by the Jamuna (0.7310) and the Halda populations (0.7200) (Table III). In all cases, the 1-H_o/H_e values were positive except for loci *Ccat C3* for Halda and Padma populations, indicating that all populations were lacking in heterozygosity (Table III).

Table III. Allelic and genetic variation of three river populations of *C. catla* at six microsatellite loci

Microsatellite loci	Parameters	Halda	Jamuna	Padma
Ccat A12	*N(4)*	10	8	5
	Ho	0.9667	0.8667	0.5333
	He	0.8864	0.8226	0.7701
	1-*Ho / He*	-0.0906	-0.0536	0.3074
	Nei	0.8717	0.8089	0.7572
	ne	7.7922	5.2326	4.1190
Ccat C3	*N(4)*	6	6	5
	Ho	0.6667	0.4000	0.5000
	He	0.7232	0.6994	0.7655
	1-*Ho / He*	0.0781	0.4281	0.3468
	Nei	0.7111	0.6878	0.7528
	ne	3.4615	3.2028	4.0449
Ccat C6	*N(4)*	6	5	4
	Ho	0.7000	0.4000	0.4000
	He	0.7785	0.7554	0.7000
	1-*Ho / He*	0.1008	0.4705	0.4286
	Nei	0.7656	0.7428	0.6883
	ne	4.2654	3.8877	3.2086
Ccat C8	*N(4)*	6	4	4
	Ho	0.4000	0.5667	0.3237
	He	0.7537	0.6718	0.6650
	1-*Ho / He*	0.4692	0.1564	0. 5143
	Nei	0.7411	0.6606	0.6889
	ne	3.8627	2.9460	2.9851

Ccat G2	*N(4)*	6	6	6
	Ho	0.3667	0.3333	0.3588
	He	0.6876	0.6672	0.6412
	1-*Ho / He* Nei ne	0.4667 0.6761 3.2374	0.5004 0.6561 4.1096	0.4404 0.6543 4.0632
Ccat G1	*N(4)*	4	4	4
	Ho	0.4000	0.5000	0.2333
	He	0.7028	0.7695	0.7667
	1-*Ho / He* Nei ne	0.4308 0.6911 3.0875	0.3502 0.7567 2.9079	0.6957 0.7339 2.7068
Average H_o over loci		0.5111	0.4889	0.4167
Average H_e over loci		0.7200	0.7310	0.7554
Average number of alleles		6.33	5.5	4.67
Polymorphism (P_{95})		1.00	1.00	1.00

N = No. of alleles, H_o = heterozygosity observed, H_e = heterozygosity expected

***Departures from Hardy-Weinberg Equilibrium*:** Out of 18, 8 tests showed significant departures from Hardy-Weinberg Equilibrium (HWE) for two populations (Table IV). The test for fit to HWE exposed that the Halda and Padma populations departed at 5 and 3 loci, respectively. Among all the populations, the Halda showed the maximum departure from HWE was at locus *Ccat C3* ($p < 0.001$). Jamuna population did not depart from HWE at any locus tested (Table IV).

Table IV. Departures from Hardy–Weinberg genotype frequency expectations in three different populations of *C. catla* (χ^2 values, followed by degrees of freedom in parentheses)

Parameters	Loci	Halda	Jamuna	Padma
H-W test	*CcatA12*	56.212^{ns} (45)	18.82^{ns} (21)	9.06^{ns} (6)
	Ccat C3	68.18*** (15)	22.94^{ns} (15)	31.45***(10)
	Ccat C6	37.230** (15)	11.32^{ns} (10)	19.07*** (6)
	Ccat C8	54.83** (10)	8.12^{ns} (6)	4.22* (6)
	Ccat G1	24.809*** (3)	0.17^{ns} (3)	1.47^{ns} (3)
	Ccat G2	40.102*** (15)	19.23^{ns} (15)	20.51^{ns} (15)

Statistically significant values are marked with asterisks. ns= not significant, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

***Population differentiation (F_{ST}) and gene flow (N_m)*:** Pair wise population comparisons of allele frequency indicated that there were significant genetic differentiations between populations. The F_{ST} value was the highest (0.0653) between the Halda and Padma populations, while the lowest (0.0366) F_{ST} was between the Halda and Jamuna populations. In contrast, the highest N_m was between the Halda and Jamuna populations (6.5747) and lowest between the Halda and Padma populations (3.5811) (Table V).

Table V. Multilocus F_{ST} and N_m values between pairs of three populations of *C. catla* across all loci

Population	Pair-wise F_{ST}	Mean F_{ST}	Pair-wise N_m	Mean N_m
Halda-Jamuna	0.0366		6.5747	
Halda-Padma	0.0653	0.04727	3.5811	5.3883
Jamuna-Padma	0.0399		6.0091	

Discussion

A primary obligation for prospective fisheries management strategies is understanding the population genetic structure that delineates whether contemporary management techniques are adequate for a sustainable harvest (Reiss *et al.* 2009). For conservation-oriented carp breeding programme, appropriate genetic data from population structure analyses are critical that incorporates diverse and evolving population systems into novel management procedures. Thus, we characterised the genetic structure of *C. catla* based on six microsatellite loci to generate genetic data for their conservation. The high heterozygosity found in the current study at microsatellite loci, makes it possible to identify and manipulate a large amount of genetic differentiation for stock recognition.

Alam and Islam (2005) studied *C. catla* collected from same three rivers using eight microsatellite loci (*Ccat A12*, *Ccat C3*, *Cc6*, *Cc7*, *Cc8*, *Cc9*, *Ccat G1*, and *Ccat G2*) where 6 (*Ccat A12*, *Ccat C3*, *Cc6*, *Cc8*, *Ccat G1*, and *Ccat G2*) are common to the present study. They found a total of 38 alleles ranging from 99 to 418 bp and their reported average number of alleles ranged from 3.50-4.38. The results of the present study coincide with the findings of Alam and Islam (2005) in majority cases such as the total number of alleles (38), and detection of polymorphism in all the common loci tested, apart from higher average number of alleles identified (4.67-6.33). The Halda population indicated its genetic richness in allelic variation with the presence of largest average (6.33) number of alleles. Moreover, the existence of five private or rare alleles at some microsatellite loci of Halda population revealed genetic abundance and a propensity of gene flow restriction among the populations.

In the present study, the H_o were significantly lower than the H_e in all the riverine populations which is similar to the findings of Alam and Islam (2005), that shows over the years, the river originated wild populations of *C. catla* have reduced genetic diversity, for which precise explanations are difficult to clarify. The decline in river population is believed because of uncontrolled fishing practices such that indiscriminate harvesting of river spawn, insufficient and irregular river flow due to construction of dam and water pollution from anthropogenic activities (Alam *et al.* 2009). Moreover, ongoing climatic changes and global warming are predicted to cause frequent rise of salinity level and habitat water temperature that may exhaust fish population adversely influencing their natural breeding and spawning activities (Nesa *et al.* 2018). However, the highest H_o in the Halda population may be caused by genetic intermixing of individuals, inflow of alleles after mating is confirmed between local and migrating individuals that eventually contribute to overall genetic variation. Additionally, Halda is the richest spawning ground for carp species (Islam and Alam 2004, Patra and Azadi 1985) and presumed to have pure hydrology compared to other river systems, acting as a buffer against environmental change that allows large populations for random mating with unlimited number of effective individuals (N_e) that perhaps produced higher genetic diversity (Rahman *et al.* 2009).

Heterozygote deficiencies were detected in all the river populations of *C. catla* with the positive values of 1-H_o/H_e by all loci except the locus *Ccat A12* that were parallel to the detections of Alam and Islam (2005). Reduction in gene pool caused by population bottlenecking, higher degree of genetic drift experienced by the remaining small population and non-random mating in the form of inbreeding could explain an elevated level of observed heterozygote deficiency (Caballero and Hill 1992, Rana *et al.* 2004, Tonny *et al.* 2014).

Eight tests revealed significant departures from HWE where Halda population departed at loci *Ccat C3*, *Ccat G1*, *Ccat G2* ($p<0.001$); *Cc6*, *Cc8* ($p<0.01$) and Padma population at loci *Ccat C3*, *Cc6*, and *Cc8* ($p<0.001$). The departures are largely due to deficit of heterozygotes and an excess of homozygotes. The general abundance of homozygotes may arise from mixture of populations with different allele frequencies that is known as Wahlund effect, assortative mating among phenotypic similar individuals, and the existence of null alleles for most allele size groups (Karlsson and Mork 2005).

F_{ST} estimates derived from the observation of different river populations was highest (0.0653) between the populations of the Halda and Padma with the lowest N_m (6.0091). The variability between F_{ST} and N_m values possibly due to geographical distance and physical barriers between the populations. Halda is remotely located from Padma by geographical separation, which is most isolated tidal river with freshwater flow originating at the hilly terrains of eastern region of Bangladesh (Alam and Islam 2005, Islam *et al.* 2005). Thus, the probability of mixing this Halda stock with others is much smaller. A close association of increasing genetic distance with increasing geographical distance was observed between populations of European eel, *Anguilla anguilla (L.)* where distantly located eel samples have a small but substantial amount of F_{ST}, indicating a restricted amount of gene transfer between spawning populations (Maes and Volckaert 2002). Furthermore, higher F_{ST} and limited N_m may also derive from reproductive barriers or limited population size producing unfit individuals with low dispersal for reproduction to transfer novel genes, genetic drift, habitat fragmentation, mutation (Hartl and Clark 1997), and changes in environmental conditions i.e., temperature and salinity (Bekkevold *et al.* 2005). Although Halda is also geographically and hydrologically furthest from Jamuna, the highest N_m (6.5747) was observed between the Halda and Jamuna populations where geographical distance did not represent a barricade that may impede the gene flow indicating an intensification of natural transmission of genes between the Halda and Jamuna by translocation through stocking and aquaculture activities (Hamilton *et al.* 2019).

C. catla is an important aquaculture species that has gained much popularity among the aquaculturist. However, the aquaculture producers of Bangladesh face challenges to produce maximum benefit from the cultivation of *C. catla* due to insufficient supply of quality fingerlings that demands the improvements of genetic quality of this species by selective breeding. Our study demonstrated application of microsatellite markers in stock identification can differ among populations and found suitable in identifying the most effective loci in genetic structure analysis of *C. catla* population. Overall, this study illustrates the genetic assembly of *C. catla* population that will help in detecting the superior broodstock from the wild populations for conservation of genetic resources through important breeding strategies such as cryopreservation of gametes.

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