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Microsatellite DNA Markers Revealed Low to Moderate Level of Genetic Diversity in Domestic Stocks of *Catla catla*

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KA designed the idea. TA conducted the research work. MSA, SA and HA assisted in lab work and data collection. SA, SQAS and AK analyzed data. TA and HN wrote the manuscript.

Key words
Major carp, Genetic variation, Genetic differentiation, SSR markers, Domestic populations

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ABSTRACT

A total of 15 microsatellite loci in five hatchery populations of *Catla catla* were examined for the analysis of genetic diversity and genetic structure patterns. For genomic DNA isolation, dorsal muscle tissues of the sampled fish were used. In terms of the average number of alleles and observed heterozygosity, low to moderate levels of genetic variation were found in all the hatchery stocks. The total number of alleles was found to range from 2.800 to 4.000. In all the populations analyzed, F_{IS} values were found to be positive on an average basis, although some loci had negative values. No *HWE* deviation was found. The pairwise estimates of F_{ST} showed low genetic differentiation between populations. Among the individuals in the populations, much of the variance was found by applying *AMOVA*. Major cluster patterns among all the populations were measured by constructing PCA, structure, and neighborhood joining tree. In the same cluster are those populations that share the most genetic identity, while populations with the lowest genetic identity fall into a separate cluster. In solving the genetic problems associated with restocking plan and brood-stock management strategies of Catla, the present study inferences will be useful.

INTRODUCTION

Catla catla is endemic to the riverine system of Indus plain and adjoining hills of Pakistan, Bangladesh, northern India, Nepal, Laos, and Myanmar (FAO, 2014). It is one of the most important freshwater fish species in its endemic region for polyculture and consumers preferred it due to its meat quality animal protein, unsaturated fatty acid profile, a valuable source of vitamins and minerals (Hussain *et al*., 2011).

Genetic variation in a population is the measure of number and relative abundance of alleles that enables a species and population to adapt the changes in their environment (Ciftci and Okumus, 2002). Determining the genetic variability level in a population is essential for predicting their capacity to respond adaptively (Eads *et al*., 2012). It is understood that genetic diversity is strongly associated with population fitness, which has been negatively impaired by anthropogenic activities and inadequate propagation of hatchery stocks.

The most important sources of fish fauna for human consumption are the natural reservoirs but the massive hunting results on increasing demand which eventually causes genetic decline and reduces allelic diversity (Dwivedi *et al*., 2009). In order to avoid the loss of genetic resources of fish in wild environments and to manage sustainable fisheries resources, molecular genetics research is important (Ciftci and Okumus, 2002). Owing to anthropogenic activity and environmental threats such

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as overfishing, hydrological alterations, deforestation, land degradation and the introduction of invasive species, genetic diversity in fish fauna has been adversely affected during the last few decades (Abbas *et al*., 2010) and leads to extinction of many species (Ciftci and Okumus, 2002).

Long-term survival of a fish species can be increased by Integrating genetic information into aquaculture and fisheries programs (Cossu *et al*., 2021). Due to genetic degradation, the decline in fish yield has become a substantial fisheries management problem. In Pakistan, understanding of genetic problems in artificial breeding is poor. Various fish hatcheries are working through induced spawning strategies to protect the possible genetic loss of cultivated and endemic species (Bondad-Reantaso, 2007). According to Evans *et al*. (2004) the lack of technological expertise and awareness cause continuous genetic degradation.

A variety of molecular markers including DNA (nuclear and mitochondrial) and protein (allozymes) play a vital role in population genetics. Various scientific observations are obtained with the help of DNA markers which are important in aquaculture practices including species identification, genetic diversity, wild and captive populations comparison to determine the bottleneck effect (Yue *et al*., 2009). Microsatellites, also known as SSR markers, are usually small nucleotide array repeats of less or equal to six bases in length. Due to their small scale, a higher degree of polymorphism and rapid detection protocols, these markers are the most common and versatile (Chistiakov *et al*., 2006; Han *et al*., 2019).

Based on the above-mentioned needs for the project, we planned this study to examine the patterns of genetic variability in the domestic stocks of *C. catla* using microsatellite DNA markers.

MATERIALS AND METHODS

Sampling of fish

Specimens of *Catla catla* (n = 250) were sampled with the help of seine nets from five hatcheries including Fish Seed Hatchery Lahore (LHRH, 31°2505°N 73.0439°E), Bahawalpur (BWPH, 29.3956°N 71.6836°E), Faisalabad (FSDH, 33.5983°N, 73.0441°E), Shahkot (SHKH, 31.8025°N, 74.2590°E) and Mureedke (MFH, 31.8025°N, 74.2590°E) of the Province of Punjab, Pakistan. Dorsal muscle tissues were excised at the sampling sites and kept in tagged polythene bags for identification and kept cold by placing them in crushed ice boxes for transporting to the laboratory for freezing.

DNA isolation and quantification

Dorsal muscle tissue samples were used to extract genomic DNA following Yue and Orban (2005) methods. 0.8 percent AGE in TAE buffer with loading dye (bromophenol blue) was used to test the quality of the extracted DNA. A UV spectrophotometer set at 260 nm was used to determine the concentration of DNA samples.

Amplification of microsatellite loci

Sahu *et al*. (2014) and McConnell *et al*. (2001) primer pairs were selected to amplify the targeted microsatellite loci in this study.

Microsatellite analyses

Non-denaturing polyacrylamide gels (5%) were used to separate the PCR products and visualized with the silver-staining protocol. Allelic bands were manually scored and compared to a DNA ladder (Thermo Fisher Scientific, USA) to assess the allelic band's size through gel doc.

Analyses of data

The software micro-checker 2.2.1 (Oosterhout *et al*., 2004) was used to assess the microsatellite data set for the existence of possible genotyping errors (i.e., null-alleles, high allele dropout, and stuttering bands). To quantify genetic diversity in distinct populations, the genotypic data obtained for each locus were subjected to rigorous analysis. The *FSTAT* (Version 2.9.3.2) was used to calculate some indices of genetic diversity (Goudet, 2004). Among populations, genetic differentiation (F_{ST}) was measured by the method of Weir and Cockerham (1984). Analysis of molecular variance (AMOVA) was measured by ARLEQUIN (Excoffier *et al*., 2005).

The computer-based R program (3.4.0. version) was used to applied principal component analysis (PCA) on structuring pattern among populations. Using software POPTREE2 (Takezaki *et al*., 2010), neighborhood joining tree based on Nei's genetic distance was constructed. The software structure 2.3.2 (Pritchard *et al*., 2000; Falush *et al*., 2003) was applied to determine the population structuring patterns. Evanno *et al*. (2005) was adopted to calculate k-value and genetic groups by running five independent runs with structure harvester (Earl and Vonholdt, 2012).

RESULTS

All the microsatellite loci screened in this study were found to be polymorphic at $p \leq 0.05$ in hatchery captive populations of *C. catla.* The range of average allele frequency at all loci in the studied populations of *C. catla* were observed ranging from 0.002 to 0.5492. Allele size (bp) measured in this study was measured ranging from

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Table I. Patterns of genetic diversity at various loci in hatchery stocks of *C. catla.*

Populations/ Parameters	Range at studied locus	Average
LHRH		
Na	2.000 – 5.000	4.0000
Ar	2.000 – 5.000	3.9882
Ne	1.814 – 4.117	2.8586
Ho	0.280 – 0.620	0.4973
He	0.4488 – 0.760	0.6342
1-Ho/He	-0.003 – 0.376	0.2134
F_{IS}	-0.006 – 0.356	0.1920
PHWE	0.009^{NS} – 1.00^{NS}	NS
BWPH		
Na	2.000 – 4.000	3.0000
Ar	2.000 – 4.000	2.9952
Ne	1.980 – 3.429	2.5185
Ho	0.420 – 0.660	0.5226
He	0.480 – 0.7084	0.5973
1-Ho/He	0.044 – 0.257	0.1251
F_{IS}	-0.032 – 0.299	0.0872
PHWE	0.042^{NS} – 1.00^{NS}	NS
FSDH		
Na	2.000 – 5.000	3.4666
Ar	2.000 – 4.937	3.4618
Ne	1.999 – 3.358	2.6579
Ho	0.340 – 0.640	0.5186
He	0.4758 – 0.7022	0.6139
1-Ho/He	-0.002 – 0.3349	0.1536
F_{IS}	-0.032 – 0.299	0.1275
PHWE	0.074^{NS} – 1.00^{NS}	NS
SHKH		
Na	2.000 – 4.000	2.8000
Ar	2.000 – 4.000	2.7998
Ne	1.885 – 3.000	2.4401
Ho	0.400 – 0.700	0.488
He	0.4608 – 0.659	0.5797
1-Ho/He	-0.089 – 0.324	0.1552
F_{IS}	-0.178 – 0.313	0.1238
PHWE	0.016^{NS} – 1.00^{NS}	NS
MFH		
Na	2.000 – 4.000	3.0000
Ar	2.000 – 3.978	2.9980
Ne	1.814 – 3.073	2.4120
Ho	0.300 – 0.640	0.4620
He	0.4550 – 0.6746	0.5750
1-Ho/He	0.043 – 0.373	0.1970
F_{IS}	0.022 – 0.351	0.1610
PHWE	0.008^{NS} – 1.00^{NS}	NS

Na, Number of alleles; *Ar*, Allelic richness; *Ne,* Effective number of alleles; *Ho*, Observed heterozygosity; *He*, Expected heterozygosity; F_{IS}, Inbreeding coefficient; *PHWE,* Hardy-Weinberg equilibrium probability value. NS, Non-significant; *, Significant; **, Highly significant.

66 to 206 base pairs. At various loci, the zero (0) value indicated missed alleles in all studied populations.

Genetic diversity

The micro-checker software was applied to the genotypic data that indicated no stuttering bands and null alleles occurrence at all the loci employed for genotyping in this study.

The number of alleles (*Na*) per locus ranging from 2 to 6 with an average 2.800 to 4.000 were noted in this study. The highest number of allele average value was observed in the LHRH and minimum in SHKH population. The maximum average values of A_r were observed 3.9882 in the LHRH while, minimum 2.7998 in SHKH population. The effective number of alleles (*Ne*) average values was observed ranged from 2.412-2.8586. Heterozygosity (*H*) level observed in all studied populations of *C. catla* in the present study was observed low-to-moderate. The average values of observed heterozygosity (H_o) were measured ranged from 0.462 to 0.5226. The fish population collected from BWPH revealed the maximum H_o as compared to others. Expected heterozygosity (H_e) average values observed in this study were recorded ranged from 0.62 to 0.67. Highest H_e value was observed for LHRH population while, lowest for MFH. The values of 1-*Ho/He* were found positive at all loci with some exception at some loci where negative values are also observed in the present study. The inbreeding coefficient (F_{IS}) values ranged from 0.0872 to 0.192 was recorded in this study. Highest F_{IS} value was measured for LHRH and lowest for BWPH populations of *C. catla*. Out of 75 tests, no test was found to deviate from *HWE* significantly in this study after applying multiple test correction (Table I).

Population genetic structure

Considerable variation *(P<0.05*) in magnitude among pairs of populations were indicated unbiased genetic distance. The maximum genetic distance was noted 0.0530 between the population pairs of LHR-MFH while, the minimum 0.0154 between the SHKH-MFH. Pairwise estimates of F_{ST} indicated low genetic differentiation among the *C. catla* populations in this study. The maximum value of genetic differentiation (0.0218) was seen between the population pairs of LHR-MFH and lowest (-0.0011) between the SHKH-MFH populations (Table II).

AMOVA indicated low variation percentage (22.6%) between populations and revealed that most of the variation (41.7%) lies within the individuals (Table III).

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Table II. Unbiased genetic distance (above diagonal) and population differentiation (F_{ST}) (below diagonal) among *C. catla* populations.

Populations	LHRH	BWPH	FSDH	SHKH	MFH
LHRH	-	0.0386*	0.0520	0.0416*	0.0530
BWPH	0.0119	-	0.0315*	0.0292*	0.0398*
FSDH	0.0172	0.0084	-	0.0345*	0.0353*
SHKH	0.0149	0.0083	0.0110	-	0.0154*
MFH	0.0218	0.0153	0.0117	-0.0011	-

*Genetic distance significant at (P<0.05).

Table III. AMOVA table for *C. catla* populations.

SOV	df	MSS	Variance	% Variation
Between populations	4	22.153	0.17217	22.6285
Between samples within populations	245	4.9363	0.3635	35.6616
Within individuals	250	4.2092	4.2092	41.719
Total	499	31.299	4.7448	100

Across the 5 autonomous runs for each k value, consistent results were seen and highest average value and Δk was measured for k= 2 (Figs. 1, 2). Figure 3 showed the two major clades/clusters among the populations that are genetically related. The results of structure and PCA analyses indicated four distinct genetic clusters among the hatchery populations (Fig. 4).

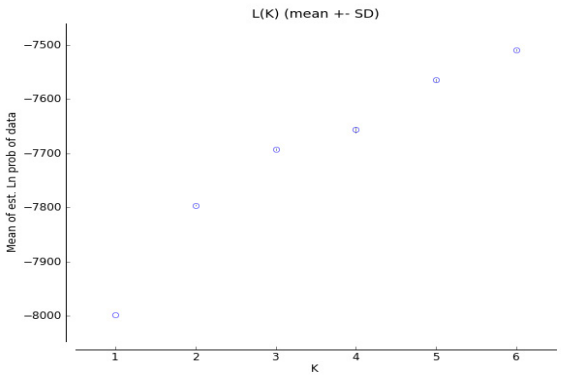

Fig. 1. Log data likelihood L(K) for each k values ranging from 1 to 5 for the model of admixture and correlated frequencies (averaged over six separate sequences) for the *C. catla* populations.

DISCUSSION

It is understood that genetic diversity is strongly associated with population fitness that has been severely

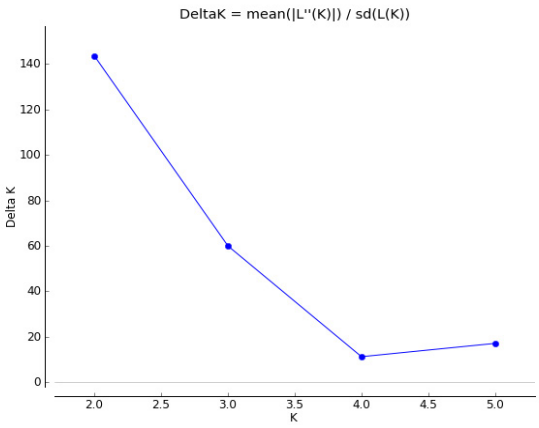

Fig. 2. Delta k values for each of the k inferred clusters of *C. catla* with a maximum value obtained at k = 2.

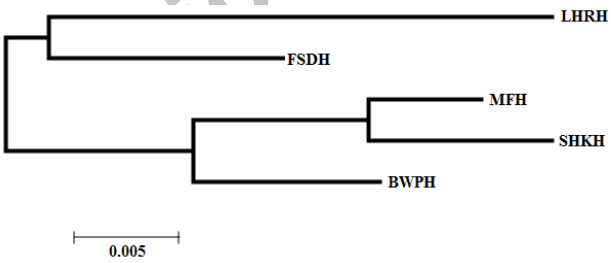

Fig. 3. Neighbour joining tree base on Nei's genetic distance showing the relationship and clustering patterns between hatchery captive populations of *C. catla.*

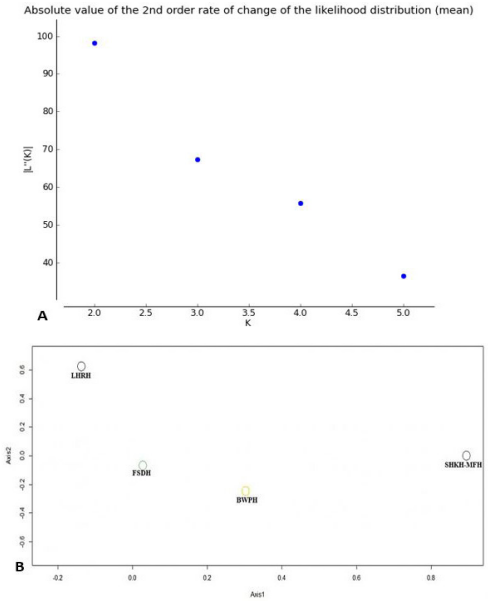

Fig. 4. Patterns of genetic structuring among hatchery stocks of *C. catla* (A) as revealed by PCA analysis (B).

affected by anthropogenic action, including overfishing, habitat loss, hydrological changes and improper propagation of hatchery stock that annually erodes million-dollar fisheries. Fisheries and aquaculture sectors rely on healthy fish stocks that could be achieved by both genetic conservation and management practices.

Genetic diversity

To maintain the fitness and the evolutionary potential, genetic diversity preservation is imperative for both cultivated and wild fish species. In all the captive populations of *C. catla,* all the fifteen microsatellite loci screened in this study were found to be polymorphic and no scoring errors related to large allele, stuttering bands and null alleles presence at all these loci were found by applying the Micro-checker software. In hatchery populations, similar frequencies were observed for the microsatellite alleles that occur most commonly at different locus, but differences in allele frequencies were also observed between samples representing different derivatives of the same population in this study. Expected heterozygosity is based on mostly allele frequency in a population.

This study showed low to moderate level of genetic diversity in terms of an average number of alleles and heterozygosity in captive *C. catla* populations. The number of alleles is the fundamental tool for the measurement of genetic diversity among populations. Fish population collected from LHRH revealed highest while SHKH showed lowest mean value of allelic richness and number of alleles. Number of alleles depends on the effective population size (Nei, 1972). The values of effective alleles were measured lower than number of alleles suggesting loss of alleles during captive spawning. Comparable results were found and reported by Barroso *et al*. (2005) in hatchery stock of *Brycon opalinus*, by Thai *et al*. (2007) in the cultured stocks of *Cyprinus carpio*, Kaczmarczyk and Zuchowska (2011) in the hatchery populations of *Labeo calbasu*, Rahman *et al*. (2009) in hatchery-reared and wild captured *C. catla* and Gu *et al*. (2020) in the Gudgeon (*Sarcocheilichthys sinensis*) using the microsatellite markers.

Heterozygosity (*H*) level was noted low-to-moderate. BWPH population revealed the highest average value of H_o as compared to others. Expected heterozygosity (H_e) values were recorded higher in comparison to H_o. The null alleles presence at a locus may be the reason of a deficiency in the observed heterozygosity (Ede and Crawford, 1995; Pemberton *et al*., 1995). Excess of expected heterozygosity at all the loci in a population suggests that the population has faced a recent bottleneck. Even when no new broodstock is introduced, cross-fertilization happens frequently in farmed fish which results in low genetic diversity and survival rate, and increased disease susceptibility (Kim *et al*., 2018). In the present study, 1-*Ho/He* values were found positive mostly at different locus in all the hatchery populations while at some locus negative values are also observed. Positive values of 1-*Ho/He* at all loci in captive stock specified heterozygosity deficit that could be the result of kinship due to preferential mating while, negative values of 1-*Ho/He* at some loci in various populations specified heterozygosity excess. Comparable findings were reported regarding the level of heterozygosity in domestic *L. rohita* stocks by Sultana *et al*. (2015); Ahmed and Abbas (2018) in *C. catla* (wild and cultured).

On the average base, F_{IS} values were found positive in all the screened populations, although some loci in all the captive populations showed negative values. Highest F_{IS} value was observed in LHRH while, lowest in BWPH populations of *C. catla*. Positive values of F_{IS} in a population confirm the homozygosity excess (Schneider *et al*., 2012) and significant deviation from the *HWE*. In *Sparus aurata*, Brown *et al*. (2005) reported high inbreeding coefficient.

Genetic structure

Among populations, species and higher taxa, genetic diversity is distributed non-randomly. This distribution of genotypes and alleles in time or in space is often stated to as the genetic structure of a population (Nevo, 1998).

Low genetic differentiation between captive *C. catla* populations was indicated by the pairwise estimates of F_{ST} as revealed by Wright (1978). F_{ST} value was found higher between the LHRH-MFH and the lowest between SHKH-MFH population pairs. The smallest value indicated the similar genetic origin of populations. Secondly, it may be due to the exchange of brooders among various hatcheries. Low genetic differentiation among captive population also indicated a small domestic history of hatchery populations and secondly hatchery operator collect brooder for spawning mostly from rivers/natural populations. The highest genetic distance value was noted among the pair of LHRH-MFH populations while, the smallest between the SHKH-MFH. Shortest genetic distance values suggest both populations underwent similar genetic adaptations and have similar genetic backgrounds. Equivalent inferences were observed by Yousefian (2011) in *C. carpio*; Singh *et al*. (2011) in *L. calbasu*; Kolangi-Miandare and Askari (2014) in *Oxynoemacheilus argyrogramma.*

Population genetic structure and the genetic similarity and differentiation between the populations were measured by AMOVA which indicated the smaller percentage of variation between the populations and revealed majority of the variation lies within the individuals in the populations. Chaturvedi *et al*. (2011) and Gopalakrishnan *et al*. (2009)

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reported similar genetic variation patterns in some freshwater fishes.

Structure harvester admixture model inferences showed highest estimated log-likelihood average value and Δk value for K= 2 by the method of Evanno *et al.* (2005). The analyses of structure and PCA exhibited that the hatchery populations revealed four genetic clusters. The populations of SHKH and MFH lie in the same cluster, while the populations of LHRH, BWPH, and FSDH lie in the different separate cluster.

Two major clusters or clades were seen based on the genetic distance computed by Nei's (1972) that predicted a close relationship between the populations in both clusters. In cluster first two studied populations (LHRH and FSDH) of *C. catla* were present while, in the second cluster the remaining three populations, i.e., SHKH, MFH and BWPH were present. Among populations, NJ tree clustering patterns reflect the relationships, those populations that share the highest genetic identity, indicating the closest genetic relationship. While populations showed the lowest genetic identity and their genetic relationship was the farthest.

CONCLUSION

In conclusion, the microsatellite DNA marker has proved to be an effective for investigating the genetic status of *C. catla* populations in captivity. This study represents the basic information about the present genetic status of *C. catla* in hatcheries and would be useful for further studies to evaluate the population genetic relationships. To formulate a good management strategy, further such kind of research in various aquatic reservoirs inhibiting *C. catla* across Pakistan is needed.

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IRB approval and ethical statement

The present study was approved by the Institutional Biosafety and Bioethics committee of University of Agriculture, Faisalabad and was conducted following institutional guidelines for ethical conduct.

Statement of conflict of interest

The authors have declared no conflict of interest.

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