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DNA Markers Based Genetic Polymorphism in Natural Populations of *Channa marulius*

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ABSTRACT

Characterization of freshwater fish species using molecular markers is important for their management regarding the evaluation of the potential genetic effects induced by anthropogenic interventions. The genetic variability among five natural populations of *Channa marulius* was studied by using five microsatellite loci in a total of 150 individuals. The *C. marulius* population exhibited a moderate level of heterozygosity. The mean value of observed heterozygosity ranged between 0.700 – 0.833 while the expected heterozygosity varied between 0.863 – 0.868. Significant deviation from HWE ($P<0.05$) was observed in all the populations due to the deficits of heterozygotes suggesting either due to inbreeding or recent mixing of stocks. AMOVA revealed that the majority of the variation (77.21%) lied within the individuals than among the individuals within populations (15.48%). The UPGMA dendrogram based on Nei's genetic distance revealed that the *C. marulius* population was divided into two major clusters. This study would be helpful to underpin the causes of decline in genetic diversity of *C. marulius* and provide significant guidelines over the effective management and conservation strategies for sustainable fisheries of Channa species in Pakistan.

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Authors' Contribution
KA designed the idea and research layout. MJ conducted different experiments/laboratory work and wrote manuscript. SN, HA, TS and MSA assisted in experiments handling, results interpretation and technically monitor the experiments. SA and MAZ were members of supervisory committee and facilitated the author in conducting the research work.

Key words
Genetic polymorphism, DNA markers, natural populations, Catfish species, Genetic distance

INTRODUCTION

Genetic diversity provides the basis for the survival of fish species as it develops the potential to protect themselves against the risk of extinction in ever changing environmental conditions (Ashley *et al*., 2003; Banerjee *et al*., 2008). Loss of genetic variability leads to the loss of fitness in relation to vigor, fixation of genes and disease resistance causing ultimate extinction of native populations. During last several decades, human interventions like pollution, hydrological alterations, overfishing, exotic species introduction and environmental threats such as climatic changes and floods are the major elements that interrupt the survival of fish species in natural systems (Vandewoestijne *et al*., 2008).

The genetic variability of commercial fish species is devastated due to inbreeding, poor broodstock management and lack of genetic characterization. For maintenance of genetic integrity, the conservation of allelic variation is necessary for maintaining the evolutionary potential in natural populations to adapt the environmental changes as well as for captive stocks to make sure the improvement of beneficial traits (Perrier *et al*., 2011). To refrain from genetic decline resulting from over exploitation, environmental fluctuations and natural mortality, monitoring of genetic structure of the riverine fisheries stock on regular basis is critical (Islam *et al*., 2005).

The fish *Channa marulius* (Indian snakehead or sol) is a potential aquaculture fish species native to Indo-Pakistan sub-continent, better adapted to low dissolved oxygen and has a commercial value (Bhatti, 2012). The *C. marulius* population in riverine systems of Punjab has been threatened by a number of factors but genetic variability of this species is facing a continuous decline primarily as a result of anthropogenic interventions along with impoundment of rivers, habitat destruction, deterioration of water quality and overharvesting (Frankham, 2003). These interruptions have resulted in loss of breeding, spawning and nursing grounds for fish in natural water bodies. Moreover, construction of dams interrupts the

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migration patterns of this species and thus interferes with their life cycles (Agosthinho *et al*., 2008).

Molecular markers have become valuable tools in conservation biology, population genetics and evolutionary studies (Liu and Cordes, 2004). These markers are used to measure the effective population size (Cheng *et al*., 2010), historical bottlenecks and sex specific gene flow (Kucuktas *et al*., 2009). However, appropriate marker selection is beneficial to determine the genetic variation that is essential for any population genetic study (Sunnucks, 2000). Among the available molecular markers, microsatellite DNA is a reliable marker because of its unique characteristics including a high rate of mutation, wide distribution in coding as well as non-coding regions of DNA, and a quick detection protocol. These markers could be used in fisheries for individual identification, broodstock management, marker assisted breeding programs and to construct the genetic linkage map (Chistiakov *et al*., 2006).

With the decreasing flow of River Ravi and ecological alterations due to prolonged dry periods coupled with overharvesting has led to decline in fisheries resources. In Pakistan, very little work was done on stocks management and population differentiation based on molecular approaches and there is a dire need to comprehend the changes in genetic diversity of fish populations of River Ravi. Keeping in view the current situations, this study is planned to monitor the status of fish *C. marulius* in natural reservoirs as influenced by some environmental fluctuations and other unknown factors.

MATERIALS AND METHODS

Sampling of fish and DNA extraction

A total of 150 samples of *C. marulius* were collected from different sites of River Ravi, Punjab including Head Balloki (HB), Head Punjnad (HP), Head Sidhnai (HS), Sidhnai-Mailsi Link Canal (SM Link-C) and Shahdra Bridge (SB) (Fig. 1). Thirty samples were taken from each sampling site including fingerlings (TL varied from 1.4-2.8cm) and mature (TL varied from 84-110cm). Dorsal muscle tissues were collected at the sampling sites and kept in marked polythene bags for DNA isolation. Samples were labeled with site code and immediately preserved in crushed icebox at temperature -20°C. By following the Sambrook and Russell (2001) standard phenol-chloroform DNA isolation technique with slight modifications, the DNA extraction from dorsal muscles tissues was carried out. Approximately 25mg of muscle tissues were cut and chopped after the scales and spikes were removed. DNA was isolated from small quantity (2mg) of frozen samples. The quality and quantity of isolated DNA was assessed by agarose gel electrophoresis and Nanodrop (Thermo Scientific), respectively.

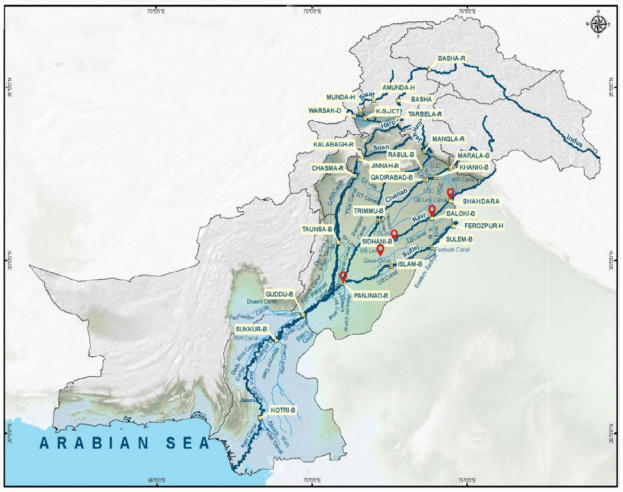

Fig. 1. Map of Punjab, showing the sampling sites of *C. marulius*.

Table I. Characteristics of microsatellite primers for *C. marulius*.

Sr. No.	Locus	Repeat motif	Sequence of Primers	T (°C)	Gene bank accession No.
1	*CA05*	(GT)18	F: ACTAATCTCTGGTCGTCTCC R: ATGAATGATAGCCTCTGGTG	56	GU253344
2	*CA07*	(GT)28	F: ATACGGTAGTTTGACGGTGG R: GTCTGACCTTCCAAAACTG	55	GU253347
3	*CA08*	(TG)28	F: CTGATGTCCAATCGTGAAGG R: CTCCCACCAACTGAGAAACT	55	GU253348
4	*CA09*	(CA)11	F: CTACACCTGGGTTTTCACAC R: CTTCACCTTCTACTTCTGGAG	58	GU253349
5	*CA10*	(CA)20	F: ACTGTGTCTTGCTCTTGTCTG R: CAGGCAAGTAAGCACAATTC	58	GU253350

Amplification of microsatellite loci

The DNA of isolated microsatellite loci was amplified by polymerase chain reaction (PCR) using species specific primers of *Channa marulius* isolated by McConnell *et al.* (2001). The characteristics of microsatellite primers are given in Table I. The PCR reaction was performed in 20µL reaction mixture containing template DNA of 50ng, 0.4µL each primer with primer sequence and PCR master mix (Thermo Scientific) 12µL in a thermocycler. The initial denaturation was carried by 5 min at 94 ℃, annealing of 30 cycles for 1 min at 94°C and final elongation at 72°C for 4 min.

Gel electrophoresis

Following the amplification, 1µL of loading dye was mixed with 5µL of PCR product and loaded on the polyacrylamide gel along with the DNA ladder. After electrophoretic resolution the gel was stained with silver nitrite solution. With the help of pUC18DNA sequencing ladder (Thermo Scientific, USA) the size of all the alleles was determined. The bands were calculated manually two bands at extreme for diploid individuals.

Data analysis

The genotypic data of each locus obtained from counting of the bands, was accurately examined by means of different genetics software and systematic programs to determine the genetic assortment of every population. Various genetic parameters like allele frequency, number of alleles (Na), allelic richness (A_r) and inbreeding coefficient (F_{IS}) were computed with program FSTAT Ver. 2.9.3.2 (Goudet, 2002).

The POPGENE software Ver.1.31 was used to measure the heterozygosity (H) and deviation from Hardy-Weinberg Equilibrium (HWE) at each locus (Yeh *et al.*, 1999). ARLEQUIN software Ver. 3.1 was used to calculate the Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 2005). The pairwise genetic differentiation of all the populations was determined and UPGMA dendrogram (Miller, 1997) based on Nei's (1972) unbiased genetic distance constructed by using TFPGA software (Weir and Cockerham, 1984).

RESULTS

A total of five microsatellite loci were amplified to measure the level of genetic polymorphism among wild populations of *Channa marulius*. All the examined microsatellite loci showed the distinct levels of polymorphism in this study. The allele frequency average values ranged from 0.020 to 0.227 while the allele size was observed ranging from 170 to 330 base pairs (bp) in the present study.

Table II. Genetic diversity at examined microsatellite loci in natural populations of *C. marulius*.

Populations/ Parameter	Locus					Average
	CA-05	*CA-07*	*CA-08*	*CA-09*	*CA-10*	
Head Sidhnai (HS)						
Na	13	13	13	13	13	13
Ar	12.998	12.999	13	12.966	12.999	12.992
Ho	0.933	0.700	0.767	0.900	0.867	0.833
He	0.852	0.862	0.821	0.905	0.890	0.866
F_{IS}	0.138	0.155	0.093	0.078	-0.074	0.078
PHWE	0.024^{NS}	0.001*	0.127^{NS}	0.058^{NS}	0.001*	-----
Head Panjnad (HP)						
Na	11	11	10	10	11	10.6
Ar	11	10.999	10	10	10.999	10.600
Ho	0.800	0.700	0.767	0.767	0.767	0.760
He	0.856	0.873	0.812	0.915	0.888	0.869
F_{IS}	0.110	0.068	0.204	0.083	0.073	0.108
PHWE	0.307^{NS}	0.0005*	0.001*	0.099^{NS}	0.085^{NS}	-----
Sidhnai-Mailsi link canal (SM link canal)						
Na	8	8	8	8	8	8
Ar	9	9	8.999	8.966	9	8.993
Ho	0.733	0.800	0.733	0.767	0.733	0.753
He	0.851	0.860	0.808	0.908	0.898	0.865
F_{IS}	0.060	0.091	0.215	0.204	0.186	0.151
PHWE	0.002*	0.155^{NS}	0.101^{NS}	0.001*	0.047^{NS}	-----
Shahdra bridge (SB)						
Na	8	8	8	7	8	7.8
Ar	8	8	7.967	8	7.999	7.993
Ho	0.733	0.767	0.767	0.667	0.700	0.727
He	0.835	0.868	0.809	0.910	0.896	0.864
F_{IS}	0.171	0.237	0.266	0.155	0.041	0.174
PHWE	0.004*	0.088^{NS}	0.002*	0.589^{NS}	0.003*	-----
Head Balloki (HB)						
Na	6	6	6	6	6	6
Ar	6	6	6	6	6	6
Ho	0.767	0.700	0.667	0.700	0.667	0.700
He	0.830	0.873	0.821	0.904	0.889	0.863
F_{IS}	0.174	0.281	0.214	0.169	0.033	0.175
PHWE	0.560^{NS}	0.238^{NS}	0.001*	0.114^{NS}	0.005*	-----

Na, number of alleles; Ar, allelic richness; Ho, observed heterozygosity; He, expected heterozygosity; Fis, inbreeding coefficient; PHWE, Hardy-Weinberg equilibrium

Genetic diversity

The mean values of number of alleles (Na) and allelic richness (Ar) ranged from 6–13 and 6–12.992, respectively. The maximum value of Na and Ar was calculated in fish

population collected from HS while, the lowest in the HB population. The value of observed heterozygosity (H_o) was noted lower than the value of expected heterozygosity (H_e) in the present study. The range of average values for H_o was observed from 0.700–0.833 while, the value of H_e was measured as 0.863–0.869. High level of heterozygosity, both H_o and H_e was seen in the fish populations of HS and HP, respectively while, the lowest in BHW population. On average, all the examined loci showed limited level of H_o in comparison to H_e. The mean F_{IS} (inbreeding coefficient) value varied from 0.078 to 0.175. Highest F_{IS} value was observed in BHW fish population while, the lowest in HS population. Out of 25 tests, 11 were found to be deviated from HWE significantly at $p<0.05$ in this study (Table II).

Genetic structure

The population genetic differentiation increased as the geographical distance increased. The estimates of pair-wise F_{ST} indicated the low genetic differentiation among all the studied populations of *C. marulius*. Minimum level of differentiation (0.0054) was observed for the HS and SM-Link Canal while, the highest (0.0128) was observed for the HP-SB population pairs (Table III). Among pairs of populations, the unbiased genetic distance indicated considerable variation ($P<0.05$) in magnitude. The maximum genetic distance was observed 0.0947 for the HP-SB while, the minimum 0.0391 was noted for the HS and SM-Link Canal, population pairs. The highest genetic identity 0.9617 was observed for the HS and SM-Link Canal while, the lowest was noted 0.9097 for the HP-SB, population pairs (Table IV).

Table III. Pair wise population differentiation among the wild populations of *C. marulius*.

Population	HS	HP	SM-link canal	SB	HB
HS	-				
HP	0.0075*	-			
SM-link canal	0.0054*	0.0074*	-		
SB	0.0118*	0.0128*	0.0096*	-	
HB	0.0085*	0.0127*	0.0086*	0.0058*	-

*Significant at $P<0.05$. For details of population see Table II.

Analysis of Molecular Variance (*AMOVA*) for five microsatellite loci revealed that majority (77.21%) of the variation was present within individuals and 15.48% among individuals within populations (Table V). An unweighted pair group method with arithmetic mean (UPGMA) analysis was followed to examine the genetic relatedness among entire natural fish populations. Two major clusters were observed by the construction of phylogenetic tree (Fig. 2). The cluster one included only HP population whereas, the second cluster is divided into two sub-clusters containing SB, HB, HS and SM-Link Canal fish populations.

Table IV. Pair-wise genetic distance (below diagonal) and genetic identity (above diagonal) among the natural populations of *C. marulius*.

Population	HS	HP	SM-link canal	SB	HB
HS	-	0.9378	0.9617	0.9194	0.9346
HP	0.0642	-	0.9483	0.9097	0.9112
SM-link canal	0.0391*	0.0531	-	0.9252	0.9322
SB	0.0840	0.0947	0.0777	-	0.9547
HB	0.0677	0.0930	0.0703	0.0464*	-

*Significant at $P<0.05$. For details of population see Table II.

Table V. Analysis of molecular variance (AMOVA) for natural populations of *C. marulius*.

Source of variance	Df	MSS	Variance	% Variation
Among populations	4	19.5	0.26	7.31
Among individuals within populations	173	2.16	0.17	15.48
Within individuals	179	1.87	1.98	77.21

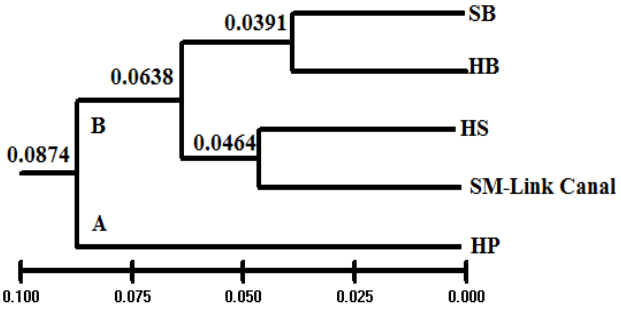

Fig. 2. UPGMA dendrogram pattern based on genetic distance among natural populations of *C. marulius*.

DISCUSSION

The genetic diversity of natural fish populations is being devastated due to the anthropogenic interventions. Maintaining the genetic diversity is critical because the presence of genetic variation determines the capacity for genetic improvement and fitness of a population (Schaal *et al*., 1991). It is imperative to know about the level

of genetic diversity in natural populations not only for their conservation but also for the sustainability of other aquaculture species.

All the genetic parameters studied in the present research indicated that the level of genetic diversity in all the wild populations of *C. marulius* was low-to-moderate. The number of alleles (Na) at different loci varied from 6 to 13. The highest mean values of Na and Ar was detected in fish population captured from HS which demonstrated that elevated level of genetic diversity is present among HS populations as compared to other populations. Allelic richness (Ar) is the basic parameter to measure the level of genetic diversity in all populations. The calculation of Ar is a complex process dependent upon the effective population size (Steven, 2004). In this study, the values of number of alleles and allelic richness were noted limited that are mainly affected by the effective size of populations. Related results about low level of genetic diversity were reported by Yang *et al*. (2012) in riverine population of Chinese Catfish *Leiocassis longirostris* and Abdul *et al*. (2009) in wild stocks of Yellow Catfish, *Horabagrus brachysoma*. The average values of observed heterozygosity (Ho) and expected heterozygosity (He) ranged from 0.700 to 0.833 and 0.863 to 0.868, respectively. The He values were observed higher as compared to Ho values that could be attributed to the effect of overfishing and deterioration in breeding grounds of natural fish populations. Consistent results were explained by Musammilu *et al*. (2014) who observed moderate level of heterozygosity in wild *Gonoproktopterus curmuca* populations. The low level of genetic diversity among riverine *Channa striata* populations may be due to the bottleneck effect as revealed by Jamaluddin *et al*. (2011). F_{IS} (inbreeding coefficient) measures the frequencies of observed heterozygosity compared to expected heterozygosity and effective population size is reduced due to the high rate of inbreeding in natural populations. On average, the maximum F_{IS} values were found positive in BHW population that indicated the excess level of homozygosity in that population. Because of inbreeding, genetic diversity is lost in populations of fish species (Ramstad *et al*., 2004). Thai *et al*. (2007) described the similar results about loss of genetic diversity because of inbreeding in natural populations of *Cyprinus carpio* by using SSR markers.

Out of 25 tests ($P<0.05$), 11 were found to be deviated from HWE after applying the multiple test corrections in natural populations of *C. marulius*. This deviation from HWE was caused by several factors like non random sampling, inbreeding and random mating. Significant departure from HWE was also observed in another study by Zhou *et al*. (2004) on microsatellite diversity in Chinese Common Carp. Several factors, e.g., null alleles, inbreeding, bottleneck effect and random genetic drift are the main reason of deviation from HWE stated by Castric *et al*. (2002).

Understanding the genetic structure of a species is crucial for developing the conservation and management strategies for natural as well as threatened fish species. In this study, positive correlation between F_{ST} and geographical distance was noted. Some dissimilarity was also observed due to the poor data of isolation by distance in which populations SB and HB deviated from straight line. Delimited dispersal of populations due to geographical distance was reported by Rahim *et al*. (2012) in *Channa striata* populations. Lack of genetic differentiation among adjacent populations is expected but when distant populations show less genetic differences, then demographic and historical explanations is the main reason to justify such less genetic differentiation that might include the ancient connectivity among populations (Steven, 2004). The population genetic distance increased as the geographical distance increased and sharing of genes decreased among populations with the minimum gene flow (Li *et al*., 2011). The pair-wise values of population differentiation determine the unbiased genetic distance and identity between the pairs of populations. The maximum level of genetic distance was found among the HP and SB while the minimum genetic distance was observed among the population pair of HS-SM-Link Canal suggesting that both populations probably share the same genetic origin. The results of genetic identity were found antagonistic to that of genetic distance in the present study. Parallel results were reported about population genetic structure of *C. carpio* by Yousefian (2011).

AMOVA as a suitable means for determination of population structure and between population variations showed that majority of variation was related to within population (93%) than the between populations (7%). The small variation between populations is also confirmed by F_{ST} values. Similar findings related to AMOVA were also observed by Hussain *et al*. (2019) in riverine *C. marulius*. In this study, two major clusters were found among the wild populations by constructing the UPGMA dendrogram to examine the genomic resemblance among the populations. In first cluster only HP population was included while, the second cluster was divided into two sub-clusters containing SB, HS, HB and SM-Link Canal. Both the populations on the same clade revealed the recent expansion among *C. marulius* populations and suggested thatthe existence of non-crossable barriers and the species' non-migratory habit induce the genetic difference across distant riverine populations.

CONCLUSIONS

The present study inferences revealed that habitat destruction due to industrial activities, agriculture runoff and construction of dams on River Ravi is the main reason of limited connectivity among *C. marulius* populations to reduce the effective population size and increase inbreeding depression. These anthropogenic activities must be carefully managed and effective management strategies should be implemented to avoid further habitat fragmentations in order to conserve and maintain the populations of *C. marulius*. Moreover, hatchery operations and open water restocking need to be checked to avoid the genetic contamination of natural populations. The findings of this study would be helpful in formulating the efficient management plans in fisheries department at government and semi-government level.

Statement of conflict of interest

The authors have declared no conflict of interest.

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