

Exploiting the allelic polymorphism at microsatellite loci to underpin the genetic status of *Cirrhinus mrigala* in wild populations

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An adequate knowledge regarding the genetic issues of natural populations is imperative for developing conservation and stock improvement plans. The present research work was aimed to assess the genetic variability and structuring patterns in six natural populations of *Cirrhinus mrigala* from Punjab province, Pakistan. The PCR based amplification of targeted loci was carried out by employing a total of 12 microsatellite markers. Based on the study inferences, moderate level of genetic diversity was observed in all the populations. The average number of alleles (N_a) varied from 3.750 to 4.416 and those of effective number of alleles ranged between 3.112 to 3.856 in all the examined populations. The mean values of observed and expected heterozygosity were measured as 0.563 to 0.685 and 0.669 to 0.739, respectively. The average values of inbreeding coefficient (F_{IS}) ranged from 0.019 to 0.184. Significant deviation from HWE was detected in 9 out of 72 tests. Among all the population pairs, low to moderate level of genetic differentiation was found. After applying $AMOVA$, it was revealed that most of the variations (81.15%) lied within individuals. The $UPGMA$ dendrogram resulted in two distinct clusters. The present study inferences would be helpful for setting up effective strategies to avoid the loss of genetic variability in *C. mrigala* populations.

Keywords: Genetic diversity, population differentiation, molecular markers, riverine populations.

INTRODUCTION

Study of population genetics explores the allocation of genetic variations at various scales in populations. Genetic variability is primarily affected by the forces of selection, mutation, migration and random genetic drift. The patterns of genetic variability between different populations give effective clues about the life histories and degree of evolutionary isolation (Ciftci and Okumus, 2002). Genetic diversity is imperative for the long term survival of the species as it assures the fitness of species by providing the potential to survive under varying environmental conditions as well as natural selection pressure (Banerjee *et al.*, 2008). However, due to human interventions, poor management and lack of rehabilitation programs the genetic diversity of commercially important fish species is being devastated (Abbas *et al.*, 2010). Therefore, the assessment of genetic diversity and structure of fish stocks after few generations is indispensable to maintain a diverse gene pool for the better conservation and sustainable management of valuable fish species (Ciftci and Okumus, 2002).

The fish Mrigal, *Cirrhinus mrigala* is an economically important and culturable freshwater carp, native to the Indus riverine system of Pakistan, Bangladesh, Myanmar, Thailand and India. This species has also been successfully introduced from its natural range to other countries like China, Japan, Malaysia and Vietnam. It is highly preferable fish to be cultured under polyculture systems because of its aquaculture potential, consumer preference and high compatibility with other major carps such as *Labeo rohita* and *Catla catla* (FAO, 2006). The aquaculture production of this species was over 573, 624 metric tons, contributing 1.6% of total world fish production (FAO, 2004). However, in recent years this species showed a declining trend in wild capture fisheries and natural genetic resources due to anthropogenic interventions. Therefore, some effective measures are necessary for the conservation of the genetic integrity of this species.

In most natural water systems, gene flow and the dispersal of species are the two key components with strong influence on the evolution and demography of spatially structured populations. The genetic structure of any population is strongly influenced by gene flow, genetic drift as well as selection pressure (Banks *et al.*, 2013). Maintaining the



genetic variability and gene flow through the spread of locally adapted genes enables the populations to respond effectively to changing ecological conditions. The timing and rate of these changes may be more rapid than the potential adaptive response by the organisms possibly increasing the risk of extinction of local populations particularly for those species which have restricted movements within the riverine systems that are highly vulnerable to fragmentation (Kawecki and Ebert, 2004).

The interspecific genetic divergence through the analysis based on molecular markers provides accurate information regarding phylogenetic relations and also helps to resolve taxonomic obscurities (Rocha-Olivares *et al.*, 2000; Backer *et al.*, 2002; Rasmussen *et al.*, 2003). Among the available array of DNA markers, microsatellite DNA markers proved to be powerful tools because of their high degree of polymorphism, co-dominant nature, high rate of mutation, small locus size, abundance in genome and the relative ease of application. In fisheries genetics, these markers have been extensively used to study population genetic structure, loss of genetic variability in natural and hatchery populations as well as to plan effective breeding programs for the improvement and restoration of species in decline (Abdul-Muneer, 2014). The

aim of this study was to evaluate the level of genetic variability and genetic differentiation in riverine populations of *C. mrigala*.

MATERIALS AND METHODS

Fish sampling: A total of 210 individuals (35 individuals per population) of the proposed fish i.e., *C. mrigala* were collected from six natural sites (important fisheries points of River Indus basin) of Punjab province, Pakistan. The riverine populations were sampled from Baloki Headworks located on River Ravi (BHW), Khanki Headworks located on River Chenab (KHW), Trimmu Headworks located on River Jhelum/Chenab (THW), Sulemanki Headworks located on River Sutlej (SHW), Chashma Barrage located on River Indus (CB) and Taunsa Barrage located on River Indus (TB) (Fig. 1). After sampling, fish specimens were identified by their morphological features and named according to their site codes. The specimens were placed in tagged polythene bags and preserved in crushed dry ice boxes for transportation to the laboratory where they were stored at -20°C.

DNA isolation and quantification: The genomic DNA was isolated from frozen muscle tissues by following the

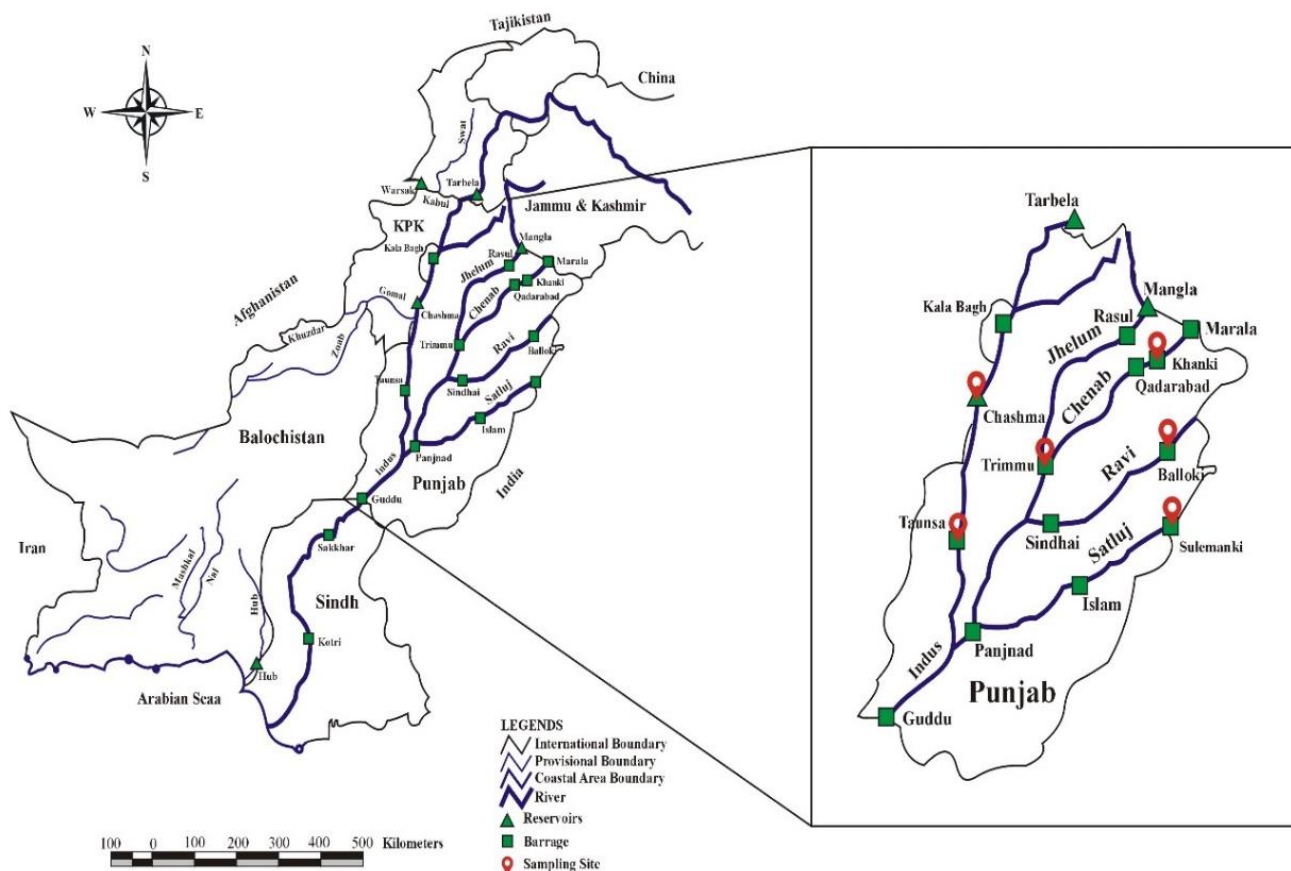


Figure 1. Map showing the riverine sampling sites of *C. mrigala* from Punjab province, Pakistan

Proteinase-K and standard Phenol/Chloroform/Isoamyl alcohol method of Sambrook and Russel (2001) with slight modifications. The agarose gel electrophoresis (0.8 %) incorporated in TAE buffer with bromophenol blue as loading dye was conducted to confirm the quality of isolated DNA and the concentration of DNA was checked by using NanoDrop at 260 nm (Thermo Scientific).

Microsatellite loci amplification: The targeted microsatellite loci were cross amplified by using 12 primer pairs developed for *Labeo rohita* (Das *et al.*, 2005, Sr. no. 1-9) and *Cyprinus carpio* (Lal *et al.*, 2004, Sr. no. 10-12). The characteristics of each primer pair are given in Table 1. The PCR amplification was accomplished in a 20 µL reaction mixture, comprised of DNA template (50 ng), 10 X reaction buffer (1 µL), each of the dNTPs (0.25 µM), each of the primer (2 µM), MgCl₂ (1.5 mM) and *Taq* polymerase (1 unit) by using the gradient thermal cycler (Multigene Optimax, Labnet USA). The initial DNA denaturation temperature was kept 94°C for 3 minutes following 35 cycles at 94°C for 30 seconds, at primer specific annealing temperature for 30 seconds and final elongation at 72°C for 5 minutes.

Electrophoretic separation of the amplified products and visualization: The amplified PCR products were resolved on 8% non-denaturing polyacrylamide gel electrophoresis containing acrylamide: bis-acrylamide, stained and visualized

following the silver-staining protocol (Sanguinetti *et al.*, 1994). The gel imaging was done in the gel documentation system (Major Science, USA) and the genotypic data was obtained by allelic scoring of gel images with reference to standard DNA ladder (Thermo Fisher Scientific, USA).

Data analysis: The obtained allelic data was analyzed with specialized data packages to infer the different parameters of fish population genetics. The genotypic data was analyzed in Micro-Checker for indicating any scoring errors related to large alleles, stuttering bands and null alleles (Oosterhout *et al.*, 2004). The number of alleles (N_a), effective allele number (N_{ae}), observed heterozygosity (H_o), expected heterozygosity (H_e) and deviation from Hardy-Weinberg equilibrium (HWE) at each locus was estimated by using POPGENE Version 1.31 (Yeh *et al.*, 1999).

Genetic differentiation among populations was assessed through F_{ST} (Weir and Cockerham's, 1984). The program FSTAT Version 2.9.3.2 was used to calculate allele frequency, allelic richness and inbreeding coefficient (Goudet, 2002). The UPGMA dendrogram based on Nei's (1972) unbiased distance was constructed with the help of TFPGA Version 1.3 (Miller, 1997). The analysis of molecular variance (AMOVA) was conducted by using ARLEQUIN, Version 3.1 (Excoffier *et al.*, 2005). The evidence for genetic bottleneck was tested under infinite allele model (IAM), two-

Table 1. Characteristics of microsatellite loci for *C. mrigala*

Sr. No.	Locus	Repeat Unit	Primer Sequence (5'-3')	GenBank accession number	Ta (°C)
1	<i>Lr1</i>	(TG)14	F-GACCCTTAACCCTTGACCTT R-TGGGATAATGCAGGGAAAAAC	AJ507518	58°C
2	<i>Lr3</i>	(TG)19	F-ATCTGGCTGCCTATTACCC R-CATCGGCGACTGCACTGGA	AJ507520	58°C
3	<i>Lr6</i>	(TG)16	F-TATCCTGGCTGAAAACCTTG R-CCCCTACAGGAACAACCAT	AJ507522	56°C
4	<i>Lr10</i>	(CA)13	F-GATCTTCAGCGCCAGCGTG R-GAGGACCTGCCAGCATG	AJ507523	60°C
5	<i>Lr12</i>	(CA)13	F-CACCGCTGCTGTCCATCA R-AGGTCGGCCAGATACACG	AJ507524	58°C
6	<i>Lr20</i>	(CA)9	F-GCCCGCTGCCGTCTGAC R-CAATAACTCAGCATGTGGAG	AJ831435	58°C
7	<i>Lr21</i>	(CA)11	F-GATCAGAGGGTCAATGTGG R-CAGCAGAGTACTATGGAAGA	AJ831436	58°C
8	<i>Lr23</i>	(TG)12	F-GGAAATTACAAATGTGCTGATG R-AGCGCTGCTGTCACTTCTGT	AJ831437	60°C
9	<i>Lr24</i>	(TG)17	F-CAAGGCCAAAAGTGTCAT R-AGGAAATTGGTAAAGTGTTTC	AJ831438	56°C
10	<i>MFW 1</i>	(CA)	F-GTCCAGACTGTTTCATCAGGAG R-GAGGTGTACTGAGTCACGC	EF144118	57°C
11	<i>MFW 2</i>	(CA)	F-CACACCGGGCTACTGCAGAG R-GTGCAGTGCAGGCAGTTTGC	EF144119	55°C
12	<i>MFW 17</i>	(CA)	F-CAACTACAGAGAAATTTTCATC R-GAAATGGTACATGACCTCAAG	EF144122	51°C

Where F= forward primer, R= reverse primer, Ta= specific annealing temperature of the respective primer pair

phase model (TPM) and stepwise-mutation model (SMM) by sign and Wilcoxon test using the BOTTLENECK program (Piry *et al.*, 1999).

The STRUCTURE Version 2.3.2 was used to analyze the population genetic structure (Pritchard *et al.*, 2000; Falush *et al.*, 2003) by using a burn-in length of 50,000 to 100,000 Monte Carlo-Markov Chain (MCMC) iterations. For each K value, five independent runs were conducted and the number of genetic clusters were specified according to Evanno *et al.* (2005) with STRUCTURE HARVESTOR (Earl and Vonholdt, 2012).

RESULTS

Genetic diversity: The indices of genetic diversity for each riverine population have been summarized in Table 2. In the present study, all the examined microsatellite loci were found to be polymorphic in all the populations. There was no evidence of scoring errors related to large alleles, stuttering bands and null alleles at all the screened microsatellite loci. The allelic size was observed in the range from 144-252 bp and the number of alleles varied from 2 to 8 at the various loci analyzed. The average number of alleles (N_a) varied from

Table 2. Microsatellite loci based genetic diversity in the wild populations of *C. mrigala*

Populations		Loci												Average
	Parameters	Lr1	Lr3	Lr6	Lr10	Lr12	Lr20	Lr21	Lr23	Lr24	MFW1	MFW2	MFW17	
BHW	Na	2.000	5.000	3.000	5.000	5.000	4.000	5.000	3.000	5.000	3.000	4.000	4.000	4.000
	Ar	2.000	5.000	3.000	5.000	5.000	4.000	5.000	3.000	5.000	3.000	4.000	4.000	4.000
	Nae	1.789	4.231	2.906	4.076	4.351	3.629	4.512	2.913	4.246	2.892	3.035	3.245	3.485
	Ho	0.485	0.628	0.514	0.628	0.485	0.600	0.571	0.542	0.657	0.571	0.571	0.514	0.563
	He	0.447	0.774	0.665	0.765	0.781	0.735	0.789	0.666	0.775	0.663	0.68	0.701	0.703
	1-Ho/He	-0.085	0.189	0.228	0.18	0.380	0.184	0.276	0.186	0.152	0.139	0.161	0.267	0.188
	F _{IS}	-0.086	0.169	0.23	0.181	0.382	0.186	0.279	0.187	0.135	0.141	0.162	0.244	0.184
	PHWE	0.604 ^{NS}	0.012 ^{NS}	0.013 ^{NS}	0.027 ^{NS}	0.000*	0.082 ^{NS}	0.005 ^{NS}	0.169 ^{NS}	0.021 ^{NS}	0.000*	0.067 ^{NS}	0.000*	-----
KHW	Na	2.000	5.000	3.000	5.000	4.000	4.000	6.000	4.000	7.000	2.000	4.000	4.000	4.166
	Ar	2.000	4.971	3.000	5.000	4.000	4.000	6.000	4.000	7.000	2.000	4.000	4.000	4.164
	Nae	1.724	3.293	2.806	4.605	2.941	3.792	4.382	3.315	5.361	1.581	3.592	3.673	3.422
	Ho	0.485	0.685	0.800	0.714	0.571	0.714	0.685	0.714	0.657	0.428	0.685	0.600	0.644
	He	0.426	0.706	0.653	0.794	0.669	0.747	0.783	0.708	0.825	0.373	0.732	0.738	0.679
	1-Ho/He	-0.138	0.030	-0.225	0.101	0.146	0.044	0.125	-0.008	0.204	-0.147	0.064	0.187	0.031
	F _{IS}	-0.142	0.030	-0.255	0.102	0.148	0.044	0.101	-0.008	0.206	-0.162	0.064	0.190	0.026
	PHWE	0.385 ^{NS}	0.265 ^{NS}	0.011 ^{NS}	0.038 ^{NS}	0.124 ^{NS}	0.262 ^{NS}	0.098 ^{NS}	0.002 ^{NS}	0.022 ^{NS}	0.340 ^{NS}	0.290 ^{NS}	0.259 ^{NS}	-----
THW	Na	3.000	5.000	3.000	4.000	5.000	4.000	6.000	3.000	7.000	3.000	4.000	4.000	4.250
	Ar	3.000	5.000	3.000	4.000	5.000	4.000	6.000	3.000	7.000	3.000	4.000	4.000	4.250
	Nae	2.283	4.909	2.657	3.810	4.069	3.746	5.030	2.845	5.946	1.949	3.751	3.576	3.714
	Ho	0.457	0.685	0.628	0.685	0.714	0.714	0.657	0.600	0.742	0.400	0.657	0.657	0.633
	He	0.570	0.807	0.632	0.748	0.765	0.743	0.812	0.658	0.843	0.494	0.744	0.730	0.712
	1-Ho/He	0.198	0.151	0.006	0.084	0.066	0.039	0.191	0.088	0.120	0.190	0.117	0.100	0.112
	F _{IS}	0.201	0.129	0.007	0.085	0.046	0.040	0.194	0.055	0.121	0.193	0.118	0.072	0.105
	PHWE	0.005 ^{NS}	0.001*	0.608 ^{NS}	0.002 ^{NS}	0.063 ^{NS}	0.084 ^{NS}	0.011 ^{NS}	0.282 ^{NS}	0.003 ^{NS}	0.000*	0.027 ^{NS}	0.101 ^{NS}	-----
SHW	Na	2.000	5.000	3.000	4.000	4.000	4.000	5.000	3.000	6.000	2.000	3.000	4.000	3.750
	Ar	2.000	5.000	3.000	4.000	4.000	4.000	5.000	3.000	6.000	2.000	3.000	4.000	3.750
	Nae	1.90	3.480	2.948	3.27	3.085	3.194	4.090	2.976	4.351	1.960	2.825	3.266	3.112
	Ho	0.428	0.657	0.657	0.600	0.685	0.685	0.600	0.628	0.714	0.457	0.628	0.657	0.616
	He	0.48	0.723	0.670	0.704	0.685	0.696	0.766	0.673	0.781	0.496	0.655	0.703	0.669
	1-Ho/He	0.108	0.091	0.019	0.148	0.000	0.016	0.216	0.067	0.086	0.079	0.041	0.065	0.078
	F _{IS}	0.110	0.072	0.020	0.150	0.000	0.016	0.202	0.068	0.087	0.081	0.042	0.04	0.074
	PHWE	0.515 ^{NS}	0.011 ^{NS}	0.943 ^{NS}	0.024 ^{NS}	0.061 ^{NS}	0.005 ^{NS}	0.028 ^{NS}	0.231 ^{NS}	0.211 ^{NS}	0.631 ^{NS}	0.204 ^{NS}	0.357 ^{NS}	-----
CB	Na	3.000	5.000	3.000	4.000	4.000	4.000	6.000	4.000	8.000	3.000	5.000	4.000	4.416
	Ar	3.000	5.000	3.000	4.000	4.000	4.000	6.000	4.000	8.000	3.000	5.000	4.000	4.416
	Nae	2.825	4.430	2.980	3.678	3.822	3.678	4.851	3.465	5.946	2.941	3.983	3.673	3.856
	Ho	0.485	0.600	0.657	0.771	0.828	0.857	0.685	0.628	0.685	0.571	0.685	0.685	0.678
	He	0.655	0.785	0.674	0.738	0.749	0.738	0.805	0.721	0.843	0.669	0.759	0.738	0.739
	1-Ho/He	0.260	0.235	0.025	-0.044	-0.105	-0.161	0.149	0.129	0.187	0.146	0.097	0.071	0.082
	F _{IS}	0.262	0.221	0.026	-0.045	-0.108	-0.163	0.150	0.131	0.165	0.148	0.099	0.049	0.077
	PHWE	0.000*	0.027 ^{NS}	0.930 ^{NS}	0.186 ^{NS}	0.075 ^{NS}	0.009 ^{NS}	0.11 ^{NS}	0.000*	0.016 ^{NS}	0.000*	0.077 ^{NS}	0.373 ^{NS}	-----
TB	Na	2.000	6.000	3.000	4.000	5.000	4.000	7.000	3.000	7.000	3.000	4.000	4.000	4.333
	Ar	2.000	6.000	3.000	4.000	5.000	4.000	7.000	3.000	6.971	3.000	4.000	4.000	4.330
	Nae	1.942	5.147	2.689	3.746	3.926	3.656	5.361	2.743	5.396	2.340	3.388	3.816	3.679
	Ho	0.542	0.657	0.742	0.685	0.771	0.771	0.800	0.771	0.742	0.371	0.685	0.685	0.685
	He	0.492	0.817	0.637	0.743	0.756	0.737	0.825	0.644	0.826	0.581	0.715	0.748	0.710
	1-Ho/He	-0.102	0.196	-0.165	0.078	-0.02	-0.046	0.030	-0.197	0.102	0.361	0.042	0.084	0.030
	F _{IS}	-0.104	0.198	-0.213	0.079	-0.021	-0.082	0.031	-0.200	0.103	0.341	0.042	0.051	0.019
	PHWE	0.536 ^{NS}	0.018 ^{NS}	0.349 ^{NS}	0.786 ^{NS}	0.303 ^{NS}	0.417 ^{NS}	0.008*	0.154 ^{NS}	0.090 ^{NS}	0.017 ^{NS}	0.989 ^{NS}	0.870 ^{NS}	-----

Table 3. Pairwise population differentiation (above diagonal) and genetic distance (below diagonal) across all the loci between the wild populations of *C. mrigala*

Populations	BHW	KHW	THW	SHW	CB	TB
BHW	-----	0.0482*	0.0261*	0.0240*	0.0244*	0.0250*
KHW	0.1571	-----	0.0503	0.0360*	0.0356*	0.0577
THW	0.1074	0.1640	-----	0.0335*	0.0278*	0.0203*
SHW	0.0903	0.1126	0.1140	-----	0.0325*	0.0446*
CB	0.1079	0.1275	0.1193	0.1146	-----	0.0151*
TB	0.1021	0.1832	0.0896	0.1415	0.0792	-----

*Significant at $p < 0.05$ **Table 4. Analysis of molecular variance (AMOVA) for the wild populations of *C. mrigala***

Source of variation	df	SS	Variance components	% variation
Among populations	5	341.824	0.73368	4.72
Among individuals within populations	204	3469.457	2.19643	14.13
Within individuals	210	2649.000	12.61429	81.15

Table 5. Analysis of genetic bottleneck for the *C. mrigala* populations

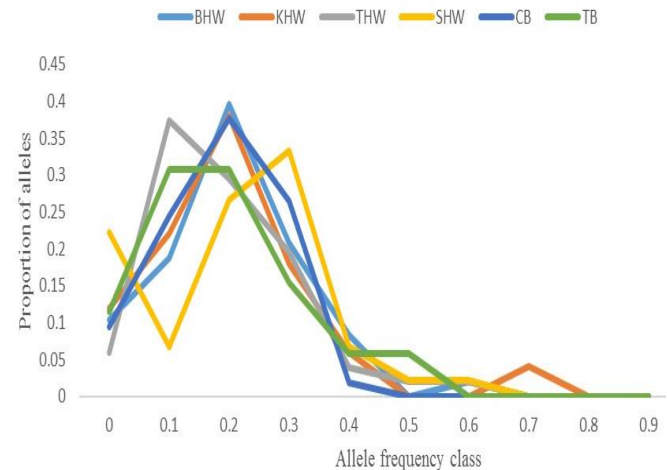
Populations	Sign test			Wilcoxon test		
	IAM	TPM	SMM	IAM	TPM	SMM
BHW	0.000688*	0.001489*	0.001488*	0.000122*	0.000122*	0.000122*
KHW	0.000534*	0.001170*	0.001083*	0.000122*	0.000122*	0.000122*
THW	0.000816*	0.001681*	0.001924*	0.000122*	0.000122*	0.000122*
SHW	0.000542*	0.001183*	0.001293*	0.000122*	0.000122*	0.000122*
CB	0.000797*	0.001883*	0.001964*	0.000122*	0.000122*	0.000122*
TB	0.000660*	0.001412*	0.001564*	0.000122*	0.000122*	0.000122*

*Significant at $p < 0.05$

3.750 in SHW population to 4.416 in CB population. The mean values of effective number of alleles (N_{ae}) was observed in a range from 3.112 to 3.856. The average values of observed heterozygosity (H_o) ranged from 0.563 to 0.685 and those of expected heterozygosity (H_e) from 0.669 to 0.739. The highest value of H_o was measured in TB and the lowest in the BHW population. Whereas, the lowest value of H_e was in SHW and highest in CB population. The range of coefficient of inbreeding (F_{IS}) values was observed from 0.019 to 0.184. After applying multiple test correction, 9 out of 72 tests were significantly deviated from HWE at $P < 0.05$.

Genetic structure: The genetic differentiation (F_{ST}) was found low-to-moderate among the wild populations of *C. mrigala*. The highest value (0.0577) of genetic differentiation was observed between the KHW and TB population pair while, the lowest (0.0151) between CB-TB pair (Table 3). Among the pairs of populations, the genetic distance values showed considerable differences in magnitude at $P < 0.05$. The genetic distance between the population pair of KHW and TB was found to be maximum (0.1832) while, that of CB and TB was minimum (0.0792). The Analysis of molecular variance (AMOVA) revealed that 81.15% differentiation was contributed due to variation within individuals. While, minor variations were observed (4.72%) among populations and among individuals within populations (14.13%) (Table 4).

The tests for detecting genetic bottleneck under IAM, TPM and SMM mutation models detected a recent genetic bottleneck (Table 5, Fig. 2).

**Figure 2. Proportion of allele frequencies in the wild populations of *C. mrigala***

The UPGMA dendrogram resulted in two distinct clusters, showing the close relationship between the populations in each cluster. The first cluster was further subdivided into two

subclusters, encompassing CB, TB and THW in first subcluster while, BHW and SHW in the other subcluster. Whereas, the second cluster did not show any further subdivision and comprised only KHW population (Fig. 3). The genetic relationship among the wild populations of *C. mrigala* was further investigated by STRUCTURE grouping algorithm method. The consistent results were obtained over 7 independent runs and the maximum estimated log likelihood mean value and delta k value was observed for K=2 (Fig. 4).

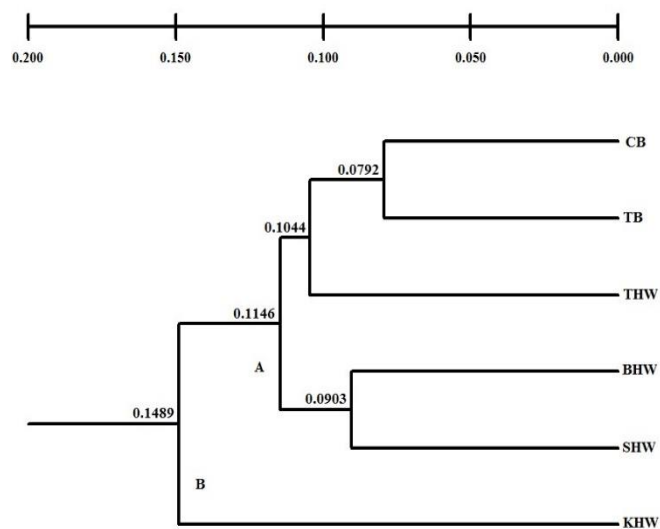


Figure 3. UPGMA dendrogram among the wild populations of *C. mrigala* based on Nei's standard genetic distance

DISCUSSION

Despite having large biodiversity of freshwater fishes in Pakistan, there are limited studies available on the genetic status of natural populations. The fish, *Cirrhinus mrigala* is an important freshwater fish species of Pakistan significantly contributing to the economy of the country but facing a decline in its population due to human interventions as well as captive breeding programs. Few studies are available on the genetic status of this species (Lal *et al.*, 2004; Das *et al.*, 2005; Chauhan *et al.*, 2007; Masih *et al.*, 2014). However, this study is the first to report the extent of genetic variability in wild populations of *C. mrigala* from Punjab province, Pakistan.

Genetic diversity: In this study, moderate level of genetic diversity was observed in the wild populations of *C. mrigala*. The observed number of alleles at various microsatellite loci extended from 2 to 8. The maximum values of observed number of alleles, allelic richness and effective number of alleles was examined in CB population while, the lowest in SHW population.



Figure 4. Genetic structuring between the wild populations of *C. mrigala* as identified by STRUCTURE. The individuals are represented by vertical bars and each column is representing one individual. The distinct colors of the columns indicate the estimated probability of belonging to one or the other population. Different colors in the same individual represents the percentage of shared genome.

Allelic diversity and heterozygosity are the two key components which should be considered in studies related to genetic variations. However, prior to heterozygosity, the most important is the number of alleles (N_a) which is more influenced by the effective size of population and strongly depends on the past evolutionary history of a population (Nei *et al.*, 1975). Moreover, the observed number of alleles as well as their frequency are influenced by the sample size and the molecular marker system used (Maqsood and Ahmad, 2017). The range of average values of observed heterozygosity was measured from 0.563 to 0.685. Whereas, the average values of expected heterozygosity ranged from 0.669 to 0.739, which were higher than the values of observed heterozygosity.

Comparable results about moderate level of heterozygosity were reported in wild populations of *Labeo calbasu* (Singh *et al.*, 2012), *Gara rufa* (Shabani *et al.*, 2013) and *Labeo rohita* (Tonny *et al.*, 2014) by employing microsatellite markers. In the case of wild fish, the H_e is directly related to the effective size (N_e) of the population. The occurrence of homozygosity at a locus or population facing genetic bottleneck could be attributed to the excess of H_e and smaller values of H_o in fish populations (Pemberton *et al.*, 1995). The reason behind lower H_o as compared to the H_e might also be postulated by the stress due to overfishing of natural resources, deterioration in breeding environments and sampling error.

Positive mean values of $1-H_o/H_e$ and F_{IS} were found in all the populations. Related inferences were presented by Hasanat *et al.* (2015) in wild and captive populations of *Cirrhinus cirrhosus* which revealed that all the studied populations were deficient in heterozygosity. Biba *et al.* (2017) also found positive values of inbreeding coefficient in natural populations of *Cyprinus carpio*. After applying multiple test correction, a total of 9 out of 72 tests deviated significantly from Hardy-Weinberg equilibrium and the deviated loci are considered to be heterozygote deficient. Non-random sampling, inbreeding, Wahlund effect/intra-population structure, genetic drift, overexploitation and a combination of all the aforementioned factors are presumably responsible for the deviation from HWE (Bergh and Getz, 1989; Gopalakrishnan *et al.*, 2009; Abbas *et al.*, 2010).

Genetic structure: Reliable estimates of population differentiation are required for better understanding the connectivity among populations and to develop conservation strategies. The genetic differentiation can be divided into different levels viz; weak (0.00-0.05), moderate (0.50-0.150), strong (0.150-0.250) and above 0.250 indicates very high level of genetic differentiation (Wright, 1978). Based on the above mentioned assumptions, low-to-moderate level of genetic differentiation was found among the wild populations of *C. mrigala*. The highest value of genetic differentiation was observed as 0.0577 between the population pair of KHW and TB and the lowest as 0.0151 between the CB-TB pair. The minimum value shows a close relationship with the sharing of genetic material while, the maximum value specifies that the

populations are genetically isolated with less sharing of alleles. The population genetic structure of freshwater fish is reinforced by the distribution of their riverine systems (Nagarajan *et al.*, 2006). The genetic differentiation is also influenced by various physical and biological factors including overexploitation, habitat fragmentation, hydrological alterations, contamination of native gene pool (inbreeding and introgression) and bottleneck effect (Koizumi *et al.*, 2006). By using microsatellite markers, Ahmed and Abbas (2018) found the same pattern of genetic differentiation in wild and hatchery stocks of *Catla catla* in Punjab province, Pakistan. In contrast, Chauhan *et al.* (2007) and Nesa *et al.* (2018) evaluated low level of genetic structuring in wild populations of *Cirrhinus mrigala* and *Cirrhinus cirrhosus*, respectively. The analysis for genetic bottleneck revealed a recent genetic bottleneck under various mutation models. The evidences of genetic bottleneck in natural populations despite having moderate genetic diversity could be related to the pre-bottleneck size of the populations being large enough or the populations are subjected to demographic recovery. Furthermore, the overexploitation, habitat degradation and population fragmentation have also been the possible reason of bottleneck in many natural populations.

The AMOVA revealed that most of the variation lied within the individuals of wild populations. Similar pattern of genetic structuring was observed by Qadeer and Abbas (2017) in riverine *Labeo rohita* inferred from microsatellite markers. The UPGMA dendrogram showed two main clusters comprising the CB, TB, THW, BHW and SHW in first cluster excluding KHW population alone in the second cluster. The reasons behind such kind of homogenization between the natural populations could be attributed to the open water stocking of farmed fish from the same source, aquaculture practices and natural calamities. The genetic relationship was further investigated by STRUCTURE grouping algorithm method which proposed the presence of two distinct genetic clusters. The maximum estimated log likelihood mean value and delta k value was observed for K=2. The accurate determination of the number of groups present among populations is dependent on the total number of individuals genotyped and the number of markers utilized. Evanno *et al.* (2005) described that calculating delta k by using a minimum of five microsatellite markers can allow the precise determination of the actual number of groups.

For the sustainable management of freshwater fish species, it is a prerequisite to make persistent use of available resources and to maintain a high level of genetic variations. The most suitable approach is to regularly assess the fish stocks in natural waters, avoid the overexploitation of fish populations, setting up adequate number of brooders in fish hatcheries, discourage the stocking of natural waters with genetically depauperated fish seeds and improve the breeding and feeding grounds by regulating the riverine water flow.

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