Pak. J. Agri. Sci., Vol. 54(4), 865-872; 2017
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI: 10.21162/PAKJAS/17.5736
http://www.pakjas.com.pk

MICROSATELLITE MARKERS BASED GENETIC STRUCTURE OF ROHU (*Labeo rohita)* IN SELECTED RIVERINE POPULATIONS OF PUNJAB, PAKISTAN

Iram Qadeer and Khalid Abbas*

Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad-38040, Pakistan
***Corresponding author's e-mail: dr.abbas@uaf.edu.pk**

Genetic integrity of distinct local fish populations is being compromised in face of growing anthropogenic interventions and environmental degradation. The present study examined the genetic structure of Rohu, *Labeo rohita* in riverine populations of Punjab, Pakistan, as influenced by anthropogenic interventions. Average 25 fish specimens were collected from each target site. Fifteen microsatellite markers with varying levels of polymorphism were employed for PCR amplification of target loci. The results revealed moderate level of genetic diversity in riverine populations. A total of 70 alleles with an average of 4.6 alleles per locus were detected on fifteen heterologous microsatellite loci. Observed heterozygosity varied from 0.5786 up to a maximum of 0.6293. The average value of F_{IS} ranged from 0.086 to 0.1835. About 33% of the total locus-pair combinations showed significant deviations from HWE (P<0.05, P<0.01, P<0.001) with apparent heterozygote deficits. The results of AMOVA showed that 90.40% genetic variation existed at the intra-population level while 9.12% at inter-populations level. The UPGMA dendrogram revealed that the populations clustered onto two major branches by showing their genetic relatedness partially following their geographic distribution. The dwindling populations of the species in natural resources of Pakistan demand critical efforts to conserve the genetic diversity.
Keywords: *Labeo rohita,* microsatellite, population genetics, genetic diversity

INTRODUCTION

In an ever-changing environment, genetic diversity assures the species fitness by providing potential to cope effectively with environmental challenges and natural selection pressure. Biological diversity of any species is dependent on the phenotypic plasticity and adaptability of its populations to the natural environment and anthropogenic interruptions. Environmental hazards such as floods, climatic change, and human interventions including hydrological alterations, pollution, introduction of exotic species, and overfishing are major factors effecting the survival of fish species during last several decades (Vandewoestijne *et al*., 2008).

The major carp *L. rohita*, locally known as 'Rohu' is the member of family Cyprinidae and belongs to the top ten most commercially valuable species of aquaculture in the world. It is widely distributed in riverine systems of Pakistan (Rafique and Khan, 2012). Like many other aquaculture species in Pakistan, Rohu (*L. rohita)* aquaculture is facing issues with regard to seed quality including poor genetic management of brood stock. A very little genetic information is available about this species in Pakistan. Currently, the natural seed production of *L. rohita* has been reduced with contribution level less than one percent.

The *L. rohita* population in riverine system of Punjab has been threatened by a wide range of factors but genetic diversity of this species has declined mainly due to anthropogenic interruptions that include the species introductions, impoundment of rivers, habitat degradation, water quality deterioration, and overexploitation. Anthropogenic intervention is the most important factor contributing towards loss of genetic resources of fish populations in the wild (Frankham, 2003). The hydrological alterations and increased human intervention during past years has resulted in loss of spawning, breeding and nursing grounds in natural water bodies. Dams construction interrupt the migration of many fish species and thus interfere with their life cycles (Agosthinho *et al*., 2008).

The pollution adds to the problem by reducing the rate of survival of fish spawn. Among other human interventions, are the siltation due to deforestation and merciless catching of brood fish during breeding season attributing to decline of fish populations in wild (Collares-pereira and Cowx, 2004). Moreover, the traditional breeding operations in hatcheries and natural population size reductions have detrimental genetic consequences causing loss of growth potential, disease resistance and stress tolerance. Since 1998, there has been consistent decline in fish production by 2% per year from natural sources in Pakistan (MinFAL, 2007). Wild fish stocks have been heavily overfished, resulting in a noticeable leveling of fish landing during last decade (FAO, 2014). Genetic variation is very necessary as it ensures the fitness and evolvability of population to the ever changing environment (Ullah *et al*., 2015). Therefore, knowledge on

the genetic structure of populations is important to avoid the ecological damage and to make certain the sustainable and effective management of exploited stocks.

Molecular markers have been developed, allowing the genetic analysis of economically important fish for conservation and genetic management. In fisheries and aquaculture research, microsatellite markers are suitable for versatile applications especially in limited, within and between population genetic differentiations (Yousefian and Laloei, 2011). These markers are widely used due to their unique features e.g. high level of polymorphism, fast detection protocol and relatively small size. These markers can be utilized in fisheries for individual identification, parentage identification, brood stock management; marker-assisted breeding programs, studies on population differentiation and to construct genetic linkage maps (Chistiakov *et al*., 2006). These advantages make microsatellites valuable markers for examining population structure of different species. Polymorphic microsatellite DNA markers, isolated and characterized for *L. rohita* (Naish *et al*., 1998; Barman *et al*., 2003) could be used to investigate the genetic status of the species.

The information on the current genetic status of the species would be helpful in making policy decisions, for preservation of genetic resources and effective management of natural populations.

MATERIALS AND METHODS

***Sampling and DNA extraction*:** Total 25 individuals of *L. rohita* were sampled from each riverine population (Fig. 1). Samples of the fish were preserved in ice boxes and transferred to Aquaculture Biotechnology laboratory, University of Agriculture, Faisalabad. To extract genomic DNA from muscle tissues, Chloroform-Isoamyl-Alcohol method was used following Yue and Orban (2005).

***Microsatellite loci amplification*:** The characteristics of the microsatellite DNA markers used in the present study have been summarized in the Table 1. For the fifteen *L. rohita*-specific microsatellite loci, the oligo primers *Lr1*, *Lr3*, *Lr6*, *Lr10*, *Lr12*, *Lr21*, (Das *et al*., 2005), *Lr22*, *Lr27*, *Lr28*,*Lr34*, *Lr35*, *Lr38*, *Lr40*, *Lr43*, *Lr46* (Patel *et al*., 2009) were purchased from the company Gene-link, USA and used to amplify the target loci from each individual (Table 1).

The PCR reaction was carried out in a 20µl reaction mixture, which included 0.8µl of each primer set (10µM), 0.4 µl of dNTPs (10mM), 1.5 µl MgCl2 (20mM), 2.0 µl of 10x PCR

Table 1- Characteristics of *L. rohita*-specific microsatellite loci[1].

Sr. No	Locus	Accession No.	SSR motif	Primers (5′-3′)	Annealing temperature (˚C)	Size[2)] (bp)	No. of alleles
1	[a]*Lr1*	AJ507518	$(TG)_{14}$	F-GACCCTTAACCCTTGACCTT R-TGGGATAATGCAGGGAAAAC	58°C	167-171	2
2	[a]*Lr3*	AJ507520	$(TG)_{19}$	F- ATCTGGCTGCCTATTCACC R- CATCGGCGACTGCACTGGA	58°C	141-165	5
3	[a]*Lr6*	AJ507522	$(TG)_{16}$	F- TATCCTGGCTGAAAACTTTG R- CCCCTACAGGAACAACCAT	56°C	158-162	3
4	[a]*Lr10*	AJ507523	$(CA)_{13}$	F-GATCTTCAGCGCCAGCGTG R-GAGGACCTGCCCAGCATG	60°C	240-250	4
5	[a]*Lr12*	AJ507524	$(CA)_{13}$	F-CACCGCTGCTGTCCATCA R-AGGTCGGCCAGATACACG	58°C	155-170	4
6	[a]*Lr21*	AJ831436	$(CA)_{11}$	F-GATCAGAGGGTCAATGTGG R-CAGCAGAGTACTATGGAAGA	58°C	140-148	6
7	[b]*Lr22*	AM285342	$(TG)_{19}$	F- GATCTGTGTGTGTGTGTGC R- GGTGGCGACACAACAAATG	58°C	140-155	4
8	[b]*Lr27*	AM231176	$(AC)_{12}$	F-TGGAAATCGAAGGCGTTTCCAC R- AGCACTTACAGTCCATTGGCTC	60°C	158-175	3
9	[b]*Lr28*	AM231177	$(AC)_{18}$	F- TTCACGGACAGATTTGACCCAG R- AGTCTTTTCAGGAGATTAGCAG	60°C	173-178	8
10	[b]*Lr34*	AM269524	$(CA)_{15}$	F- TGAGTGGTAGTGCATATGCAG R- CCGGGGCATCTGATACCGT	57°C	171-174	3
11	[b]*Lr35*	AM269525	$(CA)_{13}$	F- TGTGAACATGCAAGCTCTCAG R- CTAGTCCCACTCTAGTCAGCA	50°C	140-160	3
12	[b]*Lr38*	AM269528	$(GT)_{12}$	F- ATAGCATCACCATCTGTTGGTG R- TCTGCTTCAGTCACTCAGCAC	59°C	141-153	2
13	[b]*Lr40*	AM269530	$(GT)_{15}$	F- GATCAATCTTACAGTAATCTTG R- AGACGGAGATATGATGAACTG	54°C	170-174	2
14	[b]*Lr43*	AM269533	$(GT)_{20}$	F- GATCCCAGCAGAGGCTGTG R- AGTCTGTGCTCTCTGGAGTG	57°C	170-190	3
15	[b]*Lr46*	AM269536	$(CA)_{18}$	F- TGACGTATTGTCAACTATGGTG R- TCCACCTTCAATACCATGACTG	58°C	190-240	4

1) [a]- (Das *et al.,* 2005); [b]- (Patel *et al.,* 2009); F– forward; R – reverse; N- no. of alleles 2) Allele size (bp) ranges observed in current study

buffer (20mM), 0.4 µl (2U/ µl) Taq polymerase (Sure Bio-Diagonistic and Pharmaceutical), and approximately 50ng of template DNA using gradient thermal cycler (Multigene Optimax, LabNet, USA). The PCR cycles were as follows: five minutes at 94°C, 32 cycles of 1 minute at 94°C, 30sec at a primer-specific annealing temperature, 1 minute at 72°C, and final elongation for 4 minutes at 72°C.

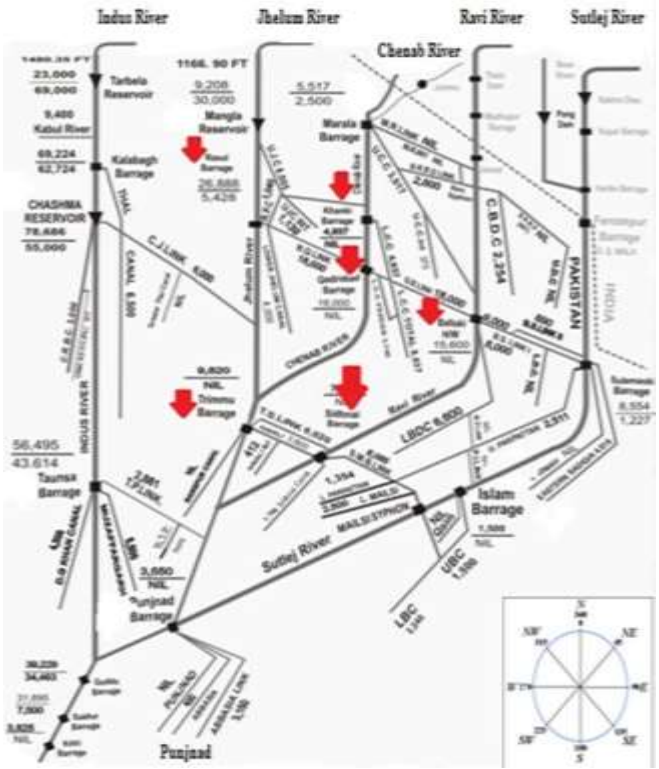

Figure 1. Hydrographic map of riverine populations of *L. rohita* in the Punjab Province, Pakistan.

***Gel electrophoresis*:** Following the PCR amplification, the amplicons were confirmed for the successful amplification through 5% agarose gel electrophoresis. In case some DNA samples failed to amplify, PCR was repeated for the very missing individuals. Five µl of the PCR product of each individual was mixed with 1µl loading dye (Bromophenol Blue) and loaded on to the 8% non-denaturing polyacrylamide gel along with DNA ladder (marker). The electrophoresis was done in a vertical gel electrophoresis chamber (MV20DSYS, Major Science, USA) at constant voltage (286Volts) for 90 minutes. After electrophoretic resolution of PCR products, the gel was removed and washed for ethidium bromide staining (Fig.2).

***Genotypic data analyses*:** The allele frequency, allelic richness (*Ar*), observed heterozygosity (*Ho*), expected heterozygosity (*He*) (Nei, 1987) were calculated with FSTAT ver. 2.9.3.2 (Goudet, 2002) and POPGENE (ver. 1.31) (Yeh *et al*., 1999). Linkage disequilibrium (*LD*) between all pairs of loci was tested using the procedure implemented by GENEPOP ver. 4.2 (Raymond and Rousset, 1995). The deviation from HWE across each locus was assessed using POPGENE (ver. 1.31) (Yeh *et al*., 1999). The statistical significance of deviations from HWE and *LD* was adjusted using the sequential Bonferroni correction to maintain a within sample type-I error rate of $\alpha = 0.05$ for each locus.

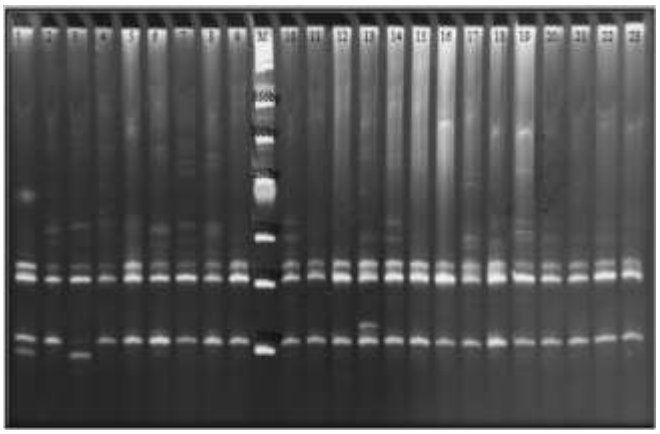

Figure 2. Radiograph showing pattern of genotypes over locus *Lr38* for the Rasul Barrage population of *L. rohita*.

Inbreeding coefficient (F_{IS}) and level of population subdivision per population over loci were estimated by unbiased F-statistics (Weir and Cockerham, 1984) by using FSTAT ver. 2.9.3.2 (Goudet, 2002). Genetic divergence among subdivisions F_{ST} for all pair comparisons between sampling locations were inferred by calculating Weir and Cockerham's (1984). The significance of the estimates of F_{ST} was assessed using 10,000 permutations. Analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) was conducted using ARLEQUIN ver. 3.1 to estimate the hierarchical partitioning of genetic diversity.

UPGMA dendrogram based on Nei's (1972) unbiased distance was analyzed using TFPGA ver. 1.3 software (Miller, 1997).

RESULTS

***Genetic diversity*:** The locus specific pattern of the allelic polymorphism as measured with reference to the DNA ladder is shown in the sample radiograph (Fig. 2). The genetic diversity statistics across each riverine population has been summarized in Table 2. All the loci were found to be polymorphic following the $P_{0.95}$ allele frequency criteria. The number of alleles ranged between 2 to 8 throughout the populations. The polymorphism was observed for all the alleles and allelic size varied between 140-250 bp across all the loci. Two private alleles $Lr3_{170bp}$ and $Lr35_{155bp}$ were

Table 2. Microsatellite loci diversity indices for the riverine populations of *L. rohita*.

Population	Parameter	Loci															Mean
		Lr1	*Lr3*	*Lr6*	*Lr10*	*Lr12*	*Lr21*	*Lr22*	*Lr27*	*Lr28*	*Lr34*	*Lr35*	*Lr38*	*Lr40*	*Lr43*	*Lr46*	
RB	*Na*	3.000	6.000	4.000	3.000	4.000	4.000	6.000	3.000	8.000	4.000	6.000	4.000	3.000	3.000	5.000	4.400
	Ar	3.000	5.999	4.000	3.000	4.000	4.000	5.875	3.000	7.986	4.000	6.000	4.000	3.000	3.000	5.000	4.390
	Ho	0.400	0.680	0.520	0.560	0.720	0.640	0.760	0.560	0.760	0.680	0.800	0.560	0.480	0.600	0.640	0.624
	He	0.679	0.794	0.751	0.680	0.762	0.749	0.808	0.678	0.872	0.745	0.846	0.760	0.613	0.677	0.778	0.746
	FIS	0.399	0.126	0.294	0.160	0.035	0.128	0.040	0.158	0.111	0.069	0.035	0.248	0.201	0.095	0.161	0.150
HQ	*Na*	3.000	6.000	4.000	2.000	4.000	4.000	4.000	3.000	7.000	4.000	4.000	4.000	3.000	3.000	5.000	4.000
	Ar	3.000	6.000	4.000	2.000	4.000	4.000	4.000	3.000	7.000	4.000	4.000	3.999	3.000	3.000	5.000	3.999
	Ho	0.640	0.680	0.520	0.440	0.680	0.680	0.560	0.560	0.760	0.680	0.520	0.560	0.600	0.560	0.640	0.605
	He	0.678	0.797	0.659	0.510	0.758	0.762	0.752	0.672	0.851	0.754	0.761	0.718	0.664	0.654	0.782	0.718
	FIS	0.037	0.129	0.195	0.120	0.085	0.089	0.240	0.150	0.089	0.079	0.303	0.204	0.078	0.126	0.165	0.139
HK	*Na*	3.000	7.000	4.000	3.000	4.000	4.000	5.000	3.000	7.000	4.000	6.000	4.000	3.000	3.000	5.000	4.330
	Ar	3.000	7.000	4.000	3.000	4.000	4.000	4.986	3.000	6.907	4.000	6.000	4.000	3.000	3.000	5.000	4.326
	Ho	0.360	0.680	0.560	0.280	0.560	0.760	0.640	0.600	0.600	0.520	0.720	0.640	0.520	0.560	0.680	0.579
	He	0.654	0.837	0.741	0.482	0.745	0.739	0.674	0.656	0.805	0.722	0.830	0.728	0.675	0.674	0.806	0.718
	FIS	0.438	0.171	0.229	0.408	0.233	-0.050	0.030	0.066	0.239	0.266	0.115	0.103	0.214	0.152	0.139	0.184
HT	*Na*	3.000	7.000	4.000	3.000	4.000	4.000	4.000	3.000	7.000	4.000	6.000	4.000	3.000	3.000	5.000	4.260
	Ar	3.000	7.000	3.987	3.000	4.000	4.000	4.000	3.000	6.860	4.000	6.000	4.000	3.000	3.000	5.000	4.256
	Ho	0.480	0.640	0.560	0.400	0.640	0.640	0.520	0.560	0.560	0.640	0.640	0.840	0.680	0.560	0.640	0.600
	He	0.644	0.817	0.702	0.575	0.734	0.715	0.764	0.754	0.656	0.656	0.750	0.745	0.662	0.660	0.728	0.704
	FIS	0.240	0.201	0.186	0.290	0.110	0.242	0.087	0.306	0.128	0.004	0.130	-0.151	-0.048	0.134	0.103	0.131
HS	*Na*	3.000	7.000	4.000	3.000	4.000	4.000	4.000	3.000	4.000	4.000	6.000	4.000	3.000	3.000	5.000	4.060
	Ar	3.000	6.953	3.987	3.000	4.000	4.000	4.000	3.000	4.000	4.000	5.988	4.000	3.000	3.000	4.955	4.058
	Ho	0.480	0.640	0.720	0.400	0.600	0.760	0.760	0.680	0.720	0.680	0.600	0.640	0.560	0.520	0.680	0.629
	He	0.582	0.810	0.682	0.678	0.745	0.760	0.680	0.684	0.781	0.692	0.724	0.751	0.659	0.636	0.749	0.708
	FIS	0.159	0.194	-0.077	0.398	0.178	-0.020	-0.141	-0.113	0.060	-0.002	0.155	0.130	0.133	0.166	0.073	0.086
HB	*Na*	3.000	6.000	3.000	3.000	4.000	4.000	4.000	3.000	6.000	4.000	6.000	4.000	3.000	3.000	5.000	4.070
	Ar	3.000	6.000	3.000	3.000	4.000	4.000	4.000	3.000	6.000	4.000	5.994	4.000	3.000	3.000	5.000	4.066
	Ho	0.440	0.640	0.440	0.360	0.840	0.640	0.600	0.560	0.680	0.640	0.880	0.640	0.480	0.560	0.640	0.603
	He	0.667	0.818	0.680	0.672	0.719	0.757	0.762	0.654	0.807	0.664	0.746	0.747	0.654	0.667	0.809	0.721
	FIS	0.327	0.202	0.340	0.453	-0.192	0.137	0.196	0.126	0.141	0.016	-0.204	0.126	0.251	0.143	0.193	0.150

Na- Number of alleles per locus; *Ar*- Allelic Richness per locus and population; *Ho*- Observed Heterozygosity; *He*- Expected Heterozygosity; F_{IS}- Coefficient of Inbreeding

observed in the HT and HS populations, respectively. The average number of alleles was maximum (4.4) in the RB population while minimum (4.0) in HQ population (Table 2). Various indices of genetic variability measured for each riverine population are explained in the following section.

***Heterozygosity and inbreeding coefficient*:** Moderate level of heterozygosity was noted for all the loci. Among riverine populations, the maximum average observed heterozygosity (0.6293) was in HS population and minimum in HK population (0.5786). The range of average expected heterozygosity was between 0.7040 (RB) and 0.7460 (HT). The values for observed heterozygosity over all the loci in all the populations were lower than the expected heterozygosity for all the loci except for the locus *Lr21* in the HK population, *Lr38* and *Lr40* in HT population, *Lr6*, *Lr21*, *Lr22*, *Lr27* and *Lr34* in HS population and *Lr12* and *Lr35* in HB population, respectively.

The maximum value of inbreeding coefficient F_{IS} (0.1835) was observed for HK population and minimum value (0.0860) for HS population. In all the riverine populations, F_{IS} values were positive across all the fifteen examined loci except for locus *Lr21* in HK population, loci *Lr38* and *Lr40* in HT population, loci *Lr6, Lr21, Lr22, Lr27* and *Lr34* in HS population and loci *Lr12* and *Lr35* in HB population, respectively. Negative values of F_{IS} indicated possibility of recent outbreed occurring across said loci in these populations (Table 2).

***Deviation from HWE and LD*:** To observe the deviations from Hardy-Weinberg Equilibrium (HWE) and the genotypic linkage disequilibrium (*LD*) tests were performed. The χ^2 values clearly suggested significant deviations over most of the loci in all the populations. Out of 90 tests, significant deviation was observed in 30 tests. The loci *Lr6, Lr40* and *Lr43* showed non-significant deviations from HWE in all the populations.

The test for the *LD* over each pair of the fifteen microsatellite loci in all the populations gave four significant values (P<0.05) out of 105 comparisons in GENEPOP v.4.2 (Raymond and Rousset, 1995) with Markov chain parameters (demamorization 10,000, batches 1000 and iterations 10,000 per batch). To correct for multiple comparisons, a sequential Bonferroni correction was applied to both HWE and genotypic *LD* tests. After Bonferroni correction for multiple tests, no significant linkage disequilibrium was observed in any locus-pair combination.

***Genetic differentiation and inter-population genetic structure*:**

***Population differentiation*:** The population genetic differentiation was analyzed by pair-wise comparison of each population as given in the Table 3. The values of F_{ST} indicated that all the populations were not homogenous. The maximum value of F_{ST} was observed in the population pair HS-HK and the minimum value between HB and HS populations.

Table 3. Pair-wise population differentiation (F_{ST}) among the six populations of *L. rohita* across fifteen microsatellite loci.

Population	RB	HQ	HK	HT	HS	HB
RB	-----					
HQ	0.0118	-----				
HK	0.0176	0.0101	-----			
HT	0.0308	0.0205	0.0238	------		
HS	0.0295	0.0234	0.0340	0.0094	-----	
HB	0.0181	0.0146	0.0205	0.0087	0.006	-----

The higher value of F_{ST} indicated that two populations HK and HS were genetically more isolated from one another and shared less common genes (F_{ST} = 0.0340) and the least value between HB and HS populations (F_{ST} =0.006), indicated the genetic relatedness of these two populations.

***Genetic distance*:** Unbiased genetic distance between the population pairs indicated considerable variation (Table 4) but most were significant (P<0.05). The maximum genetic distance was found to be 0.0960 (HS-HK) followed by 0.090 (HT-RB), 0.0872 (HS-RB) decreasing by small intervals to the minimum value of 0.0020 (HB-HS). The maximum geographical distance is present between the population pair HS-RB (350 Km) and HS-HK (311 Km). In the riverine populations, the genetic distance measurement inferred maximum genetic isolation between HS and RB populations while the lowest value of genetic distance was observed between HS and HB populations following the hypothesis of isolation by distance.

Table 4. Comparison of all the six populations, based on the genetic distance (below diagonal) and identity (above diagonal).

Population	RB	HQ	HK	HT	HS	HB
RB	-----	0.9611	0.9450	0.9140	0.9165	0.9424
HQ	0.0397	------	0.9676	0.9442	0.9365	0.9554
HK	0.0565	0.0330	------	0.9356	0.9085	0.9393
HT	0.0900	0.0574	0.0666	------	0.9744	0.9746
HS	0.0872	0.0656	0.0960	0.0259	------	0.9980
HB	0.0594	0.0456	0.0626	0.0257	0.0020	------

***AMOVA analysis*:** The AMOVA revealed two major components of variation, within population genetic variation (90.40%) and among populations genetic variation (9.12%) and among groups variation was 0.48 %. The values for global fixation indices, F_{ST}, F_{SC} and F_{CT} were 0.0177, 0.0340 and 0.4540, respectively (Table 5).

Table 5. Hierarchal AMOVA of riverine populations.

Source of variation	Df	Sum of square	Variance component	% Variation
Among groups	2	12.464	0.0177	0.48
Among populations	5	110.04	0.0340	9.12
Within populations	143	900.00	0.4540	90.4
Total	150	1022.50	0.5057	

***UPGMA dendrogram analysis*:** The UPGMA was done by using the software package TFPGA to explore the underlying genetic structure of riverine populations of *L. rohita*. The dendrogram showed that the populations clustered onto two major branches. The upper cluster included the HS, HB and HT populations while the lower cluster showed the HQ, HK and RB populations together. The highest genetic identity was found among HS, HB and HT populations, with similarity coefficients, 0.9980 (genetic distance, 0.00200) in HB-HS pair and 0.9746 (genetic distance, 0.0257) in HB-HT population pair, respectively. The lowest similarity index was between HS and HK populations with the similarity coefficient, 0.9085 (genetic distance, 0.0960). The first cluster further diverged to show HB-HS populations together while the HQ and HK populations clustered together corresponding to their genetic relatedness (Fig. 3).

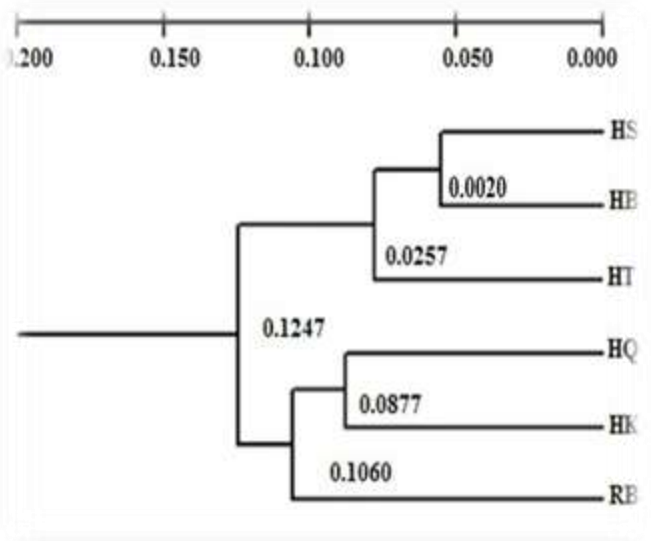

Figure 3. UPGMA dendrogram based on the genetic distance computed by Nei (1972) in the riverine populations of *L. rohita*

DISCUSSION

A considerable amount of research work has been done on the nutrition, toxicity, physiology, growth performance, and culture of *L. rohita* in Pakistan but genetic issues pertaining to the subject species remained unattended. The available genetic data on population genetics of *L. rohita* owes to limited studies confined to Bangladesh and India. Recently, a preliminary assessment of genetic diversity in selected populations of *L. rohita* on a limited scale has been reported in Punjab in a couple of studies using RAPD markers (Bhatti *et al.*, 2014) and SSR markers (Sultana *et al.*, 2015). The reasons obviously include the lack of knowledge about the genetic issues related to the fisheries management and of course the expertise required for the genetic analyses. To assess the impact of stocking programs and other human interventions in the natural fish stocks, this study is the first comprehensive attempt in Pakistan to evaluate the genetic structure of *L. rohita* in rivers of Punjab.

The results obtained from microsatellite loci revealed moderate level of genetic diversity for the subject species in riverine populations. Following 0.95 percent allele frequency criteria, all the microsatellite loci were polymorphic. This is consistent with the results obtained by Alam *et al*. (2009) who employed four microsatellite loci (*Lr3, Lr12, Lr14a* and *Lr21*) to examine the genetic structure of *L. rohita* collected from three major rivers in Bangladesh. They reported that all the loci were polymorphic. Patel *et al*. (2009) developed 21 polymorphic microsatellite loci from total 25 individuals of *L. rohita* collected from river Ganga and reported the polymorphic nature of microsatellite loci in *L. rohita*. In all the studied populations, the sizes and number of alleles showed variations comparing to the originally characterized microsatellite loci for *L. rohita*. Except for some populations, the allele size ranges were consistent to those reported by Das *et al*. (2005) and Patel *et al*. (2009). The variations could be attributed to the differences in sample size and sampling locations.

The maximum level of inbreeding was observed in HK population while the minimum in HS population. The lowest value of inbreeding coefficient found in the HS population was also in conformity to high level of genetic diversity assessed from heterozygosity for the same population. F_{IS} values were positive across all the fifteen examined loci except for locus *Lr21* in HK population, loci *Lr38* and *Lr40* in HT population, loci *Lr6, Lr21, Lr22, Lr27* and *Lr34* in HS population and loci *Lr12* and *Lr35* in HB population, respectively. Negative values of F_{IS} indicated possibility of recent outbreeding occurring across said loci in these populations. Basak *et al.* (2014) analyzed the population genetic structure of three riverine populations of *Catla catla* from the brood bank of Fisheries department in Bangladesh using microsatellite markers. They also observed negative values of F_{IS} and defined them as non-significant values.

The deviation from HWE was highly significant for 33% of the riverine populations. Significant deviations from HWE had been reported in a wide range of freshwater fishes (Castric *et al*., 2002; Yue *et al*., 2004). Non-random sampling, inbreeding, intra-population structure, genetic drift, fishing pressure or collectively all aforementioned factors could be the reason of the heterozygote deficit in natural populations (Bergh and Getz, 1989; Abbas *et al*., 2010). In the present study the departures from HWE could mainly be attributed to the deficits of heterozygotes. Alam and Islam (2005) evaluated the genetic variation in three riverine and one hatchery populations of *C. catla* using eight microsatellite loci and reported significant deviations from HWE due to heterozygote deficiency. Keeping in view the vast scale hydrological alterations in the riverine system of Pakistan, during the second half of the last century, there has been a startling decline in fish biodiversity in the rivers of Pakistan. Migration-drift disequilibrium as a result of hydrological alterations in natural water bodies might be the reason of heterozygote deficiency in all the riverine populations.

To understand the connectivity among populations and to develop conservation strategies, reliable estimates of population differentiation are required. The microsatellite analysis revealed a moderate level of population differentiation among the riverine populations of *L. rohita*. The values of F_{ST} indicated that all the populations were not homogenous. The lowest level of population differentiation was observed between HB and HS populations conforming to a physical connectivity between these two riverine populations. Alam *et al*. (2009) analyzed the genetic structure of *L. rohita* using microsatellite markers in three major riverine populations in Bangladesh. They observed low levels of genetic differentiation in a pair of riverine populations while in other sets of populations, there existed non-significant population differentiation. They concluded that lack of physical connectivity in riverine population results in ongoing gene flow leading to low differentiation among the populations.

The AMOVA revealed that within-population variation was more than among-populations in all the riverine populations. The analysis demonstrated significant genetic structuring in sampled populations, where majority of variance existed within populations.

The UPGMA dendrogram revealed two major clusters of the six riverine populations; HS, HB and HT in one cluster while HQ, HK and RB in the other cluster (Fig. 3). The populations HT, HB and HS are geographically distant but the genetic similarity among these populations was high. This could have resulted from restocking from a single source during the recent years. Further, HB and HS are populations from headworks on the same River Ravi. The floods in the River Ravi during last years could have caused the flood-induced gene flow between these populations (IPD, 2011). Recurrent flooding events and gene flow between the rivers and

alternatively, open water fish stocking and escaping of fish from farms have been resulted in homogenization of gene pools.

The lowest similarity coefficient was observed between HS and HK populations while the geographical distance was high in between these two populations. These populations were collected from the locations at head works on two major rivers, River Ravi and River Chenab, respectively. Head Sidhnai is next to Bhureki and is also located in Punjab, Pakistan. The Chenab River discharges through Trimmu-Sidhnai-link canal to Sidhnai barrage at River Ravi with an apparent geological isolation from HK population. The genetic differentiation between the distant riverine populations is caused by the non-migratory behavior of the species and the presence of non-crossable barriers.

To get away the threats of local extinction and implement conservation measures, studies on morphology, reproduction and molecular genetic aspects of the *L. rohita* are suggested. Reinforcement of the existing fisheries act and fresh legislation in this connection could be useful. Employment of a composite panel of different polymorphic marker systems and extensive sampling over a wide hydrographic scale may reveal a finer genetic structure of the *L. rohita* populations and suspected intra-specific hybridization/introgression among major carps. Based on the sound genetic database, population management units could be defined for effective genetic resource management of *L. rohita* and other commercially important fishes in Pakistan.

***Acknowledgement*:** The author feels grateful to Higher Education Commission of Pakistan for awarding the doctoral scholarship (PIN NO. 112-27128-2Bm1-281).

REFERENCES

Abbas, K., X. Zhou, Y. Li, Z. Gao and W. Wang. 2010. Microsatellite diversity and population genetic structure of yellowcheek, *Elopichthys bambusa* (Cyprinidae) in the Yangtze River. Biochem. Syst. Ecol. 38:806-812.

Agosthinho, A.A., F.M. Pelicice and L.C. Gomes. 2008. Dams and the fish fauna of the Neotropical region: impacts and management related to diversity and fisheries. Braz. J. Biol. 68:1119-1132.

Alam, M.S. and M.S. Islam. 2005. Population genetic structure of *Catla catla* (Hamilton) revealed by microsatellite DNA markers. Aquaculture 246:151-160.

Alam, M.S., M. Jahan, M.M. Hossain and M.S. Islam. 2009. Population genetic structure of three major river populations of Rohu, *Labeo rohita* (Cyprinidae: Cypriniformes) using microsatellite DNA markers. Genes Genom. 31:43-51.

Barman, H.K., A. Barat, M. Yadav, S. Banerjee, P. K. Meher, P.V.G.K. Reddy and R.K. Jana. 2003. Genetic variation between four species of Indian major carps as revealed by random amplified polymorphic DNA assay. J. Aquacult. 217:115-123.

Basak, A., A. Ullah, M.N. Islam and M.S. Alam. 2014. Genetic characterization of brood bank stocks of *Catla catla* (Hamilton) (Cyprinidae:Cypriniformes) collected from three different rivers of Bangladesh. J. Anim. Plant Sci. 24:1786-1794.

Bergh, M.O. and W.M. Getz. 1989. Stability and harvesting of competing populations with genetic variation in life history strategy. Genetics 113:939-965.

Bhatti, E.M., N.A. Qureshi, F. Rasool, M. Siddique and A.B. Saddiqi. 2014. Cluster analysis in *Channa punctatus* fish revealed by molecular markers in Punjab-Pakistan. Pak. J. Sci. 66:317-324.

Castric, V., L. Bernatchez, K. Belkhir and F. Bonhomme. 2002. Heterozygote deficiencies in small lacustrine populations of brook charr Salvelinus Fontinalis Mitchill (Pisces, Salmonidae): A test of alternative hypotheses. Heredity 89:27-35.

Chistiakov, D.A., B. Hellemans and F.A.M. Volckaert. 2006. Microsatellites and their genomic distribution, evolution, function and applications: A review with special reference to fish genetics. Aquaculture 255:1-29.

Collares-pereira, M.J. and I.G. Cowx. 2004. The role of catchment scale environmental management in freshwater fish conservation. Fish. Mang. Ecol. 11:303-312.

Das, P., A. Barat, P.K. Meher, P.P. Ray and D. Majumdar. 2005. Isolation and characterization of polymorphic microsatellite in *Labeo rohita* and their cross species amplification in related species. Mol. Ecol. Notes 5:231-233.

Excoffier, L., P.E. Smouse and J.M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics 96:479-491.

FAO. 2014. The status of world fisheries and aquaculture. Rome, Italy.

Frankham, R. 2003. Genetics and conservation biology. C.R. Biologies 326:22-29.

Goudet, J. 2002. FSTAT Version 2.9.3.2. A program to estimate and test gene diversities and fixation indices.Institute of ecology and evolution, University of Lausanne, Switzerland.

IPD. 2011. Flood fighting plan. Government of Punjab, Pakistan. pp.1-5.

Miller, M. P. 1997. Tools for population genetic analyses (TFPGA) v 1.3: a windows program for the analysis of allozyme and molecular genetic data. Flagstaff: Northern Arizona, University.

MINFAL. 2007. Agricultural Statistics of Pakistan 2006-07. Ministry of Food, Agriculture and Live Stock, Government of Pakistan, Islamabad, Pakistan.

Naish, K.A. and D.O.F. Skibinski. 1998. Tetranucleotide microsatellite loci for Indian major carp. J. Fish. Biol. 53:886-889.

Nei, M. 1972. Genetic distance between populations. Am. Nat. 106:283-292.

Nei, M. 1987. Genetic distance and molecular phylogeny. In: N. Ryman and F.M. Utter (eds.), Population Genetics and Fishery Management. University of Washington, Washington. pp.193-224.

Patel, A., P. Das, S.K. Swain, P.K. Meher, P. Jayasankar and N. Sarangi. 2009. Development of 21 new microsatellite markers in *Labeo rohita* (Rohu). Anim. Genet. 40:253-254.

Rafique, M. and N.U.H. Khan. 2012. Distribution and status of significant freshwater fishes of Pakistan. Rec. Zool. Surv. Pak. 21:90-95.

Raymond, M. and F. Rousset. 1995. GENEPOP, version 1.2. Population genetics software for exact tests and ecumenicism. Heredity 86:248-249.

Sultana, F., K. Abbas, Z. Xiaoyun, S. Abdullah, I. Qadeer and R.U. Hussnain. 2015. Microsatellite markers reveal genetic degradation in hatchery stocks of *Labeo rohita*. Pak. J. Agri. Sci. 52:775-781.

Ullah, A., A. Basak, M.N. Islam and M.S. Alam. 2015. Population genetic characterization and family reconstruction in brood bank collections of the Indian major carp, *Labeo rohita* (Cyprinidae:Cypriniformes). Springerplus 4:774.

Vandewoestijne, S., N. Schtickzelle and M. Baguette. 2008. Positive correlation between genetic diversity and fitness in a large, well-connected metapopulation. BMC Biol. 6:46.

Weir, B.S. and C.C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38:1358-1370.

Yeh, F.C., R.C. Yang and T. Boyle. 1999. POPGENE VERSION 1.31: Microsoft windows-based free software for population genetic analysis. Journal Vol Pages

Yousefian, M. and F. Laloei. 2011. Genetic variations and structure of common carp (*Cyprinus carpio*) populations by use of biochemical, mitochondrial and microsatellite markers. J. Sci. Res. 7:339-345.

Yue, G.H., Y. Li, L.C. Lim and L. Orban. 2004. Monitoring the genetic diversity of three Asian arowana (*Scleropages formosus*) captive stocks using AFLP and microsatellites. Aquaculture 237:89-102.

Yue, G.H. and L. Orban. 2005. A simple and affordable method for high-throughput DNA extraction from animal tissues for polymerase chain reaction. Electrophoresis 26:3081-3083.