RESEARCH ARTICLE

Microsatellite DNA marker based genetic polymorphism in *Wallago attu*

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ABSTRACT

Genetic diversity and structure of six Populations of *Wallago attu* were studied from Jhelum River, Pakistan using five species specific microsatellites loci. Genotypic data of all the samples were generated through PCR amplification of targeted loci. A total of 23 alleles were found in 177 fish samples. All the microsatellite loci were found to be polymorphic. The mean F_{ST} value (0.0248) showed little genetic differentiation. The average observed heterozygosity values were measured in range from 0.3450 to 0.4400 whereas average expected heterozygosity ranging from 0.5363 to 0.5777 were recorded in all the population. Majority of examined loci significantly deviated *(P<0.05)* from Hardy Weinberg Equilibrium *(HWE*). Analysis of Molecular Variance (AMOVA) indicated that majority of the variation lies within the individuals (81.10%) than among the individuals with in populations (16.4%).The results showed genetic decline in this valuable fish resource. The findings of the present study will be helpful for management, conservation as well as least kinship selective breeding programs for *W. attu*.

Key words: Genetic Variation, Freshwater Shark, Population Structure, Molecular Marker.

INTRODUCTION

Genetic diversity of freshwater fish has been declining due to ecological reasons and various anthropogenic activities, which result in extirpated thousands of fish species (Ciftci and Okumus, 2002). *Wallago attu* that usually known as freshwater shark is an important fresh-water catfish that resides in various riverine systems of South East Asia. Due to high nutritional quality of its meat and fast growth rate increase its potential as important species for aquaculture (Dutta-Munshi *et al.,* 1990). Habitat loss, over exploitation and cannibalism, are the causes of its declining in its natural distribution (Sahoo *et al*., 2002).

Population genetics is the science of how genetic variation is dispersed among species, individuals and populations. Fundamentally, it is concerned with how the distribution of genetic diversity is affected by the selection, evolutionary forces of mutation, migration and random genetic drift. The evidences to the population degree of evolutionary isolation and life histories can be provided by patterns of genetic diversity and variations among populations (Okumus and Çiftci, 2003).

Microsatellites markers are tendomly repeated stretches, generally specific and conserved that are proven to be very beneficial in genetic study of animals because of their vast applications such as marker assisted selection, mapping and evaluation of genetic variation (Wang *et al.,* 2009).

Finally, the authors would like to draw the reader's attention before this fact that species *W. attu* and all of its subpopulations has been acknowledged as near threatened species (IUCN, 2014). The situation is so overwhelming; that any further declining event will deprive its integrity from the natural ecosystem. Globally, so trivial has documented and so much leftovers to be discovered about this valuable resource. No study has been reported with respect to microsatellites on this species up to now in Pakistan. The prime objective of this study is to monitor the genetic status of *W. attu* in Jhelum River using molecular techniques to make the strategies for effective management as well as conservation of this valuable fish resource.

MATERIALS AND METHODS

Sampling and DNA isolation

Total of 177 individuals of *W. attu* were collected from six different sites i.e. of Jhelum River (Fig. 1) Mangla Dam (MD), Jhelum Bridge (JM), Rasul Barrage (RB), Khushab Bridge (KB), Trimmu Barrage (TB). The populations were named after the initial letters of sampling localities. Fish samples were placed in crushed ice boxes by keeping them polyethylene bags which were tagged according to sampling site for identification. Fish samples containing boxes were transported to Aquaculture Biotechnology Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture Faisalabad for freezing at -20 ^{o}C.

DNA Extraction:

dorsal muscle tissues of freezed fish samples were used for the total genomic DNA extraction by using traditional proteinase-K digestion and phenol/chloroform isolation method of Yue and Orban (2005). The isolated genomic DNA was confirmed through 0.8% agarose gel in TAE buffer. The DNA solutions that showed positive results were made subjected to polymerase chain reaction (PCR) for amplification

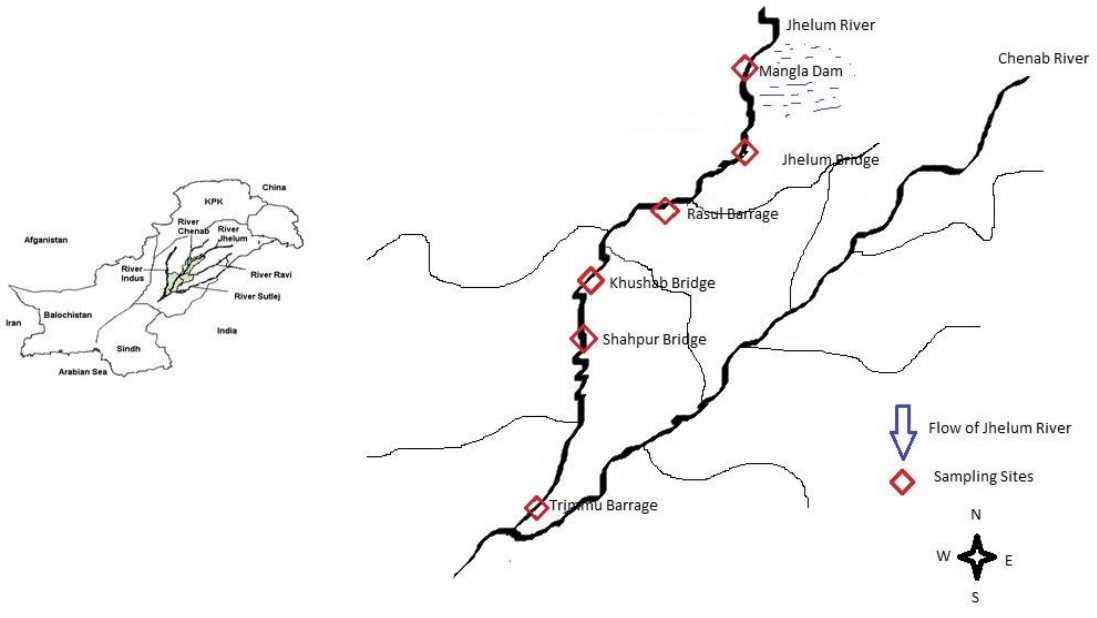

Figure 1. Geographical Map showing six sampling sites

Table 1. Microsatellite loci of *W. attu* with their characteristics

Serial No.	GenBank Accession No.	Locus	Repeat Motif in the clone	Primer Sequence (5′ to 3′)	Ta (°C)	Size Range
1	JX971060	WAM-8	$(AG)_{11}$	F: ATTTTACAGATCGAGTCACGGA	57	181-201
				R: CAGCGTGTTCTCCTTACATCAG		
2	JX971071	WAM-17	$(TG)_{10}$	F: CGATGAGTGGAAACGAGAGAC	56	201-229
				R: ACCTAATTCCTTTGTTGATGCC		
3	JX971097	WAM-21	$(CA)_{17}$	F: TTCATACACGTCAAATCAAGGC	53	200-240
				R: CCCTCACACACCCACTCTTTAC		
4	JX971112	WAM-24	$(CA)_{16}$	F: GTTACATCAACACGGAATGGTG	56	178-226
				R: GTTCTGGGAGTTTGCTCAGATG		
5	JX971099	WAM-28	$(GT)_{11}$	F: TCTACAGAGGATGGTGAGAGCC	57	188-230
				R: GATAGGAGGAAAGCAGGAGGAG		

Where F- forward, R- reverse.

PCR amplification of microsatellite loci:

Total five species specific microsatellite loci (WAM-8, WAM-17, WAM-21, WAM-24 and WAM-28) developed by Singh *et al*. (2013) were amplified through PCR (Table 1). The primers were synthesized by e-oligos, Gene Link™, New York. The PCR amplification was carried out in 20µL reaction mixture that contained template DNA (approximately 50ng), *Taq* polymerase (0.4µL), dNTPs (0.4µL) , 0.4µL of each primer and reaction buffer that include tris HCl, gelatin- 0.01%, MgCl2 and KCl (2.0µL) in a thermocycler. The denaturation was carried out at 94°C for 5min, 32 cycles of 1min at 94°C and elongation for 4min at 72°C.

Gel electrophoresis:

After amplification of microsatellite loci, 5µl of the PCR product was mixed with 1µl DNA loading dye. The mixture was loaded onto polyacrylamide gel for resolution at standard conditions. The bands in gel was be produced by silver staining and visualized in UV trans-illuminator for gel imaging. The bands were scored manually

Data analyses:

The genotypic data of each locus was subjected to rigorous analysis to compute allele frequency, population differentiation, heterozygosity, linkage disequilibrium, deviation from Hardy-Weinberg Equilibrium (*HWE*), and inbreeding coefficient (F_{IS}). Software FSTAT Ver.2.9.3.2 (Goudet, 2002) was used to analyze allele frequency, heterozygosity allelic richness and inbreeding coefficient by the F-statistics. The program GENEPOP 1.2. (Raymond and Rousset, 1995) was used for testing the linkage disequilibrium and deviation from *HWE*. The population differentiation was determined and dendrogram constructed by the TFPGA (Weir and Cockerham, 1984). A multivariate ordination was conducted to visualize the genetic relationships among populations by Principal components analysis (PCA) carried out by EIGENANALYSIS 2.4.1. (Patterson *et al*., 2006).

RESULTS AND DISCUSSIONS

Genetic variations were analyzed by determination of number of alleles (N_a), allelic richness (A_r) and heterozygosity measures among populations. Expected heterozygosity and the number of alleles are usually applied as the measures of genetic diversity as described by DeWoody and Avise, 2000).

Number of Alleles (N_a) and Allelic Richness (A_r)

Total 23 alleles were found at five studied microsatellite loci in a total 177 samples from six *W. attu* populations. All microsatellite loci were found to be polymorphic with number of alleles ranged between 3 (WAM-8, WAM-17) to 9 (WAM-28) were recorded and the mean allelic richness per population were between 3.8 (KB) to 4.6 (TB), whereas mean number of allele per Population was between 3.8 (KB) to 4.8 (TB). Allele frequencies of five microsatellite loci in each of six *W. attu* populations could be seen in Tables 2. The difference in A_r values indicated the geographical variations among all the populations. (Table 3).

Observed (H_o) and expected (H_e) heterozygosity

Heterozygosity (H) is the central parameter to assess the genetic diversity. The observed and expected heterozygosities for each locus were calculated for all the studied populations (Tables 3). Average expected heterozygosities (H_e) for each population was observed in the range between 0.5363 (RB) to 0.5777 (JM) whereas the average observed heterozygosity (H_o) was measured in range from 0.3450 (RB) and 0.4400 (MD).

Deviation from Hardy-Weinberg Equilibrium (*HWE*) were noticed at all loci for all the studied populations of 30 population-locus combinations significant ($p<0.01$), from which 20 loci deviated statistically at $p<0.05$ (Table 4).

Population Differentiation (D)

A poor genetic structure is observed in pairwise differentiation of *W. attu* populations. The F_{ST} estimates show low to moderate level of genetic differentiation between populations. The minimum pairwise population differentiation was observed between population JM and KB i.e. 0.0013. The highest pairwise F_{ST} value 0.0725 was observed between KB and TB that revealed population pair is moderately differentiated, mild to moderate level of population differentiation was found between rest of the populations pair which shows that the all the populations are genetically distinct from each other (Table 4).

Table 2. Frequency of alleles at five microsatellites loci in all six populations of *W. attu*

Locus: Name	Allele Size (bp)	Populations						
		MD	JM	RB	KB	SB	TB	Average
Locus: WAM-8	185	0.794	0.803	0.808	0.795	0.766	0.809	0.796
	189	0.088	0.066	0.077	0.077	0.078	0.059	0.074
	200	0.118	0.132	0.115	0.128	0.156	0.132	0.130
Locus: WAM-17	203	0.786	0.662	0.763	0.718	0.750	0.778	0.742
	210	0.171	0.284	0.188	0.179	0.141	0.167	0.189
	217	0.043	0.054	0.050	0.103	0.109	0.056	0.068
Locus: WAM-21	238	0.400	0.382	0.438	0.410	0.422	0.472	0.421
	219	0.443	0.461	0.425	0.462	0.484	0.347	0.436
	221	0.157	0.158	0.138	0.128	0.094	0.153	0.139
	201	0.000	0.000	0.000	0.000	0.000	0.028	0.005
Locus: WAM-24	181	0.157	0.184	0.250	0.308	0.281	0.042	0.205
	195	0.514	0.579	0.663	0.654	0.641	0.278	0.557
	210	0.243	0.171	0.050	0.038	0.078	0.514	0.180
	223	0.086	0.066	0.038	0.000	0.000	0.167	0.059
Locus: WAM-28	188	0.029	0.026	0.066	0.053	0.065	0.057	0.049
	192	0.086	0.092	0.092	0.039	0.065	0.086	0.077
	190	0.214	0.237	0.211	0.224	0.274	0.157	0.219
	200	0.114	0.026	0.079	0.158	0.113	0.143	0.105
	209	0.229	0.224	0.276	0.276	0.242	0.157	0.235
	223	0.086	0.105	0.092	0.079	0.097	0.114	0.096
	228	0.114	0.145	0.105	0.171	0.129	0.100	0.128
	199	0.129	0.145	0.079	0.000	0.016	0.129	0.084
	201	0.000	0.000	0.000	0.000	0.000	0.057	0.010
	r= null alleles	2	2	2	4	3	Zero	2.17

Table 3. Individual microsatellite locus statistics for the *W.attu* populations

Population	Parameters	WAM-8	WAM-17	WAM-21	WAM-24	WAM-28	Average
MD	*Na*	3	3	3	4	8	4.1
	Ar	3.000	2.999	3.000	4.000	7.988	4.1
	Ho	0.3429	0.1143	0.4286	0.6286	0.6857	0.4400
	He	0.3445	0.3565	0.6282	0.6538	0.8559	0.5678
	Fis	0.000	0.683	0.321	0.039	0.201	0.226
	PHW	0.2954	0.0000	0.0006	0.5148	0.0016	-----
JM	*Na*	3	3	3	4	8	4.1
	Ar	2.999	3.000	3.000	4.000	7.936	4.1
	Ho	0.3321	0.1579	0.3421	0.4737	0.4474	0.3526
	He	0.3386	0.4768	0.6256	0.6053	0.8421	0.5777
	Fis	-0.011	0.668	0.456	0.220	0.472	0.393
	PHW	0.2190	0.0000	0.0004	0.0106	0.0000	-----
RB	*Na*	3	3	3	4	8	4.1
	Ar	3.000	2.998	3.000	3.988	8.000	4.1
	Ho	0.3250	0.1750	0.3500	0.3250	0.5500	0.3450
	He	0.3256	0.3858	0.6168	0.5009	0.8525	0.5363
	Fis	-0.002	0.550	0.436	0.354	0.318	0.346
	PHW	0.2750	0.0001	0.0000	0.0091	0.0000	-----
KB	*Na*	3	3	3	3	7	3.8
	Ar	3.000	3.000	3.000	2.993	6.994	3.8
	Ho	0.3077	0.2564	0.3077	0.3333	0.6410	0.3692
	He	0.3503	0.4476	0.6101	0.4825	0.8258	0.5433
	Fis	0.123	0.430	0.499	0.312	0.199	0.315
	PHW	0.0151	0.0007	0.0000	0.0533	0.0000	-----
SB	*Na*	3	3	3	3	8	4.2
	Ar	3.000	3.000	7.000	3.000	8.000	4.2
	Ho	0.3833	0.2500	0.2500	0.3438	0.5938	0.3688
	He	0.3894	0.4122	0.5878	0.5124	0.8383	0.5480
	Fis	-0.044	0.397	0.579	0.333	0.267	0.322
	PHW	0.1676	0.0091	0.0000	0.0136	0.0000	-----
TB	*Na*	3	3	4	4	9	4.8
	Ar	2.999	3.000	3.982	3.998	9.000	4.6
	Ho	0.3056	0.1944	0.3333	0.5556	0.4722	0.3722
	He	0.3142	0.3693	0.6412	0.6381	0.8920	0.5710
	Fis	0.019	0.477	0.484	0.131	0.458	0.343
	PHW	0.1430	0.0004	0.0000	0.0524	0.0000	-----

Table 4. Pairwise F_{ST} values (below diagonal)

	MD	JM	RB	KB	SB	TB
MD	******					
JM	0.0096	******				
RB	0.0028	0.0062	******			
KB	0.0056	0.0013	0.0112	******		
SB	0.0014	0.0032	0.0136	0.0160	******	
TB	0.0130	0.0351	0.0607**	0.0725**	0.0610**	******

A "*" indicates population differentiation is poor whereas"**"indicates moderate genetic differentiation between Populations.

Table 5. N_m, R_{ST}, F_{ST} **index of five microsatellite loci in six populations for *W. attu***

	WAM-8	WAM-17	WAM-21	WAM-24	WAM-28	**Mean**
R_{ST}	0.011	0.009	0.021	0.014	0.003	**0.0116**
F_{ST}	0.017	0.019	0.027	0.024	0.037	**0.0248**
N_m	14.973	12.947	9.121	10.164	6.552	**10.752**

Table 6. Analysis of Molecular Variance

Source of variation	*d.f.*	Sum of squares	Variance components %	age of variation
Among populations	5	97.5	0.29	12.5
Among individuals within populations	171	373.5	0.15	16.4
Within individuals	177	335.0	1.89	81.10
Total	353	805.0	2.33	100

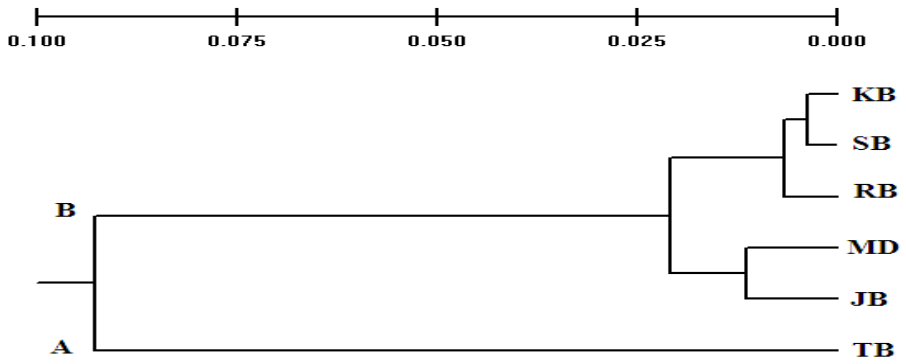

Figure 2. UPGMA dendrogram based on genetic distance

Number of migrants (N_m)

Number of migrants after correction for size was 1.96121. Both F_{ST} and R_{ST} for all populations across all loci show that there was insignificant population structuring among all populations.

Analysis of Molecular Variance

Hierarchical partition of genetic diversity was assessed by AMOVA using ARLEQUIN, which estimates the population structure at different levels i.e. within population, among sub populations. The results of AMOVA showed structuring in the sample populations. So, prominent diversity existed within populations (Table 6) percentage of variation among populations is 12.5% which was lower than variation within populations (16.4%).

UPGMA dendrogram anaylsis

The UPGMA dendrogram obtained from cluster analysis exhibited two distinct clusters corresponding to six populations that are represented by A and B (Figure 2). The cluster (A) encompassed only one population that is TB whereas cluster (B) included all remaining five populations. The UPGMA demonstrated that there is no gene flow between A and B clusters from decades. The largest value for genetic distance was 0.1268, found between KB and TB, whereas the smallest value of genetic distance was 0.0038, obtained from KB and SB. The UPGMA dendrogram depicted the underlying structure of population differentiation. It demonstrated two distinct groups however the genetic relatedness among the population was revealed by microsatellites analysis.

The line graph was plotted to show the relationship between geographical distances of sampling locations and genetic distances (Figure 3) the graph explained positive relationship between these two variables which means that the genetic distances increased as the geographical distance increased.

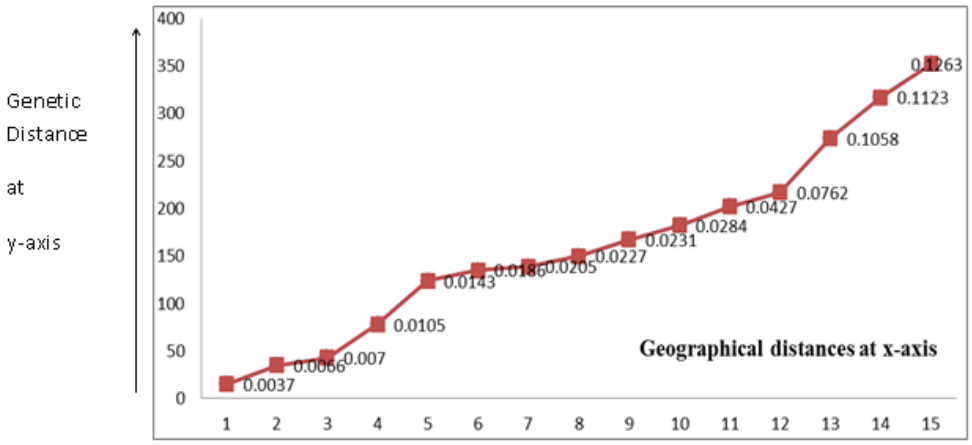

Fig. 3: Graphical representations between geographical distances and genetic distances.

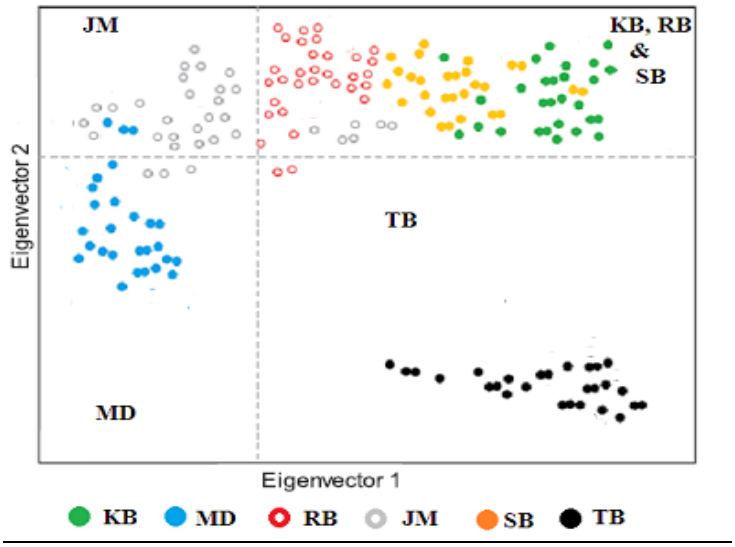

Fig. 4: Plots of eigenvectors 1 versus 2, Principal components analysis of six *W. attu* populations.

Principal components analysis (PCA)

A multivariate ordination was conducted to visualize the genetic relationships among populations by Principal components analysis (PCA) carried out by EIGENANALYSIS 2.4.1. This approach not only useful to demonstrate population structure, but also delivers a formal approach for allocating statistical significance to population subdivision (Patterson *et al*., 2006). The results support four discontinuous populations with gene flow. The results were more are less same as revealed by UPGMA.

DISCUSSION

The genetic structure of any population is not a fixed phenomenon and it is exposed to change over time. The extent and trends of the variations principally depend on the intensity of anthropogenic interventions. The present study identified a reducing trends in the effective population size (N_e) of *W. attu* in the Jhelum river as revealed by insufficiency in heterozygotes and enhancing inbreeding (F_{IS} values). Genetic diversity of larger populations decline at a slower rate than that of a smaller population and loss of genetic diversity is thought to be the loss of suitability of a population.

Several previous studies on genetic variation revealed that narrow distribution or small population size tend to maintain low degree of genetic variability due to the impact of genetic drift, the founder effect, directional selection and with high levels of inbreeding (Hamrick and Godt, 1996). Several natural factors and

anthropogenic involvements, for example fluctuating environment, habitats destruction and severe exploitation have caused an intense decline in freshwater populations (Fu *et al*., 2003).

All five microsatellite loci were found to be polymorphic and showed multiple alleles in all populations of *W. attu*. Two loci, WAM-8 and WAM-17 showed very low diversity (N_a= 3) whereas locus WAM-28 exhibited maximum diversity (N_a= 9) among samples. The mean number of alleles per locus were varied between 3.8 (JM) to 4.8 (TB). A total of 23 alleles were discovered against five microsatellite loci the effective number of alleles varied between 1.5117 to 6.6461. More or less all six populations shared the most of the alleles at each locus. The average allelic richness (A_r) at each locus varied from 2.991 to 8.420 with grand mean of 4.33, the difference in A_r values indicates geographic variations among all populations. The results of this study were in corroboration with Yue *et al*. (2003) who studied the population structure of *Clarias batrachus*.

In term of H_o and N_a results were lower than that reported generally for freshwater fish species i.e. H_o = 0.54 ± 0.25, and N_a = 9.1 ± 6.1 and anadromous fish species N_a = 10.8 ± 7.2, (Dewoody and Avise, 2000). Prrior to heterozygosity, the N_a is used as central parameter to assess the diversity of a species in genetic monitoring, for the reason that N_a may be lost more rapidly than heterozygosity, and frequency of alleles have little influences on overall heterozygosity (Lind et al., 2009). The genetic drift and bottleneck are significant factors which contribute in reduction of N_a without having apparent effect on H_o and H_e (Lundrigan et al., 2005). Both the heterozygosity and the number of allele are indicative of population structure, but the allele number is much more reliant on population effective size (N_e) than heterozygosity (Nei et al., 1975). Thus, N_a is more reliable to evaluate genetic diversity of a population for conservation, selection and enhancement programmes (Diz and Presa, 2009).

The results revealed that the mean observed heterozygosity was lesser than mean Expected heterozygosity (Table 3). The high *Nm* values, error in manual alleles scoring and a small sample size are the few causes for a low H_o *as* described (Li et al., 2009)
Even though there were no significant statistical differences in number of allele but this study revealed that there was significantly higher diversity in TB population than that of other five populations. The largest geographical distance was between TB population and MD populations i.e. 352km. The differentiation may result from different selection procedures, different environment and difference in geographical area. There was no genetic differentiation between KB and the SB populations. It may result from large number of population sizes or little distance between of these two sites which was almost 17 Km.

Deviations to *HWE* may be due to high $\boldsymbol{N_m}$ values, low number of specimens, selection programs, inbreeding propagation and genetic drift, (Lucentini et al., 2006, Dahle et al., 2006). This species do not have any selection programs in Pakistan and no significant bottlenecks revealed in any exmined population in this study, so the deviation from the *HWE* may be associated to the causes mentioned previous.

In summary, the present research work is the first report on population genetics of *W. attu* from the Jhelum river. Microsatellites maker used in this study proved very useful analyzing instruments in genetic monitoring. All the five microsatellite loci were polymorphic (100%). Therefore, these primers are recommended for other researcher to explore the genetic structure of *W. attu*. Results revealed that diversity of *W. attu* was rather high in the TB (intra population), but genetic variation among six populations was extremely low (12.5%) due to certain reasons like high gene flow, overfishing, habitats degradation, expansion of industries near the river and wastewater pollution.

This document defines the genetic computational methodologies and their practices in learning about genetic structure and more customarily about the demographic account of populations. The present-day *W. attu* populations still maintain high degree of intra-population genetic diversity and exhibit low levels of inter-population differentiation as evinced by the present microsatellite analysis. According to results this species might have suffered heavy losses of genetic diversity from a current habitat loss and a decline in population size. Hence, if it continues to decline at present rate, this fish will be endangered species soon; it is quite possible that the process of habitat isolation will lead to a loss of genetic diversity by dramatically increasing mating opportunities

between relatives and intra-clones within small populations. Appropriate conservation strategies are required for the long-time survival of this species.

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