RESEARCH ARTICLE

Microsatellite markers revealed poor genetic structure of *Wallago attu* in Punjab, Pakistan

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Manuscript details:

Received: 05.07.2016
Accepted: 25.08.2016
Published : 25.10.2016

Cite this article as:
Hafsa Basharat, Abdul Ghafoor, Arvind Chavhan, Muhammad Muddassar Zafar, Khalid Abbas, Javeria Tabasum and Razia Parveen (2016) Microsatellite markers revealed poor genetic structure of *Wallago attu* in Punjab, Pakistan, *International J. of Life Sciences*, 4 (3): 385-393.

Acknowledgement:
The authors express their sincere thanks to Mr. Tanveer Ahmad and Ms. Nadia Nazish doctorate scholars, Aquaculture Biotechnology Lab. Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad for their scholarly guidance, moral support and constant encouragement during the course of research work.

ABSTRACT

Understanding with genetic variation is important for management of wild populations and these variations are imperative for maintaining evolutionary potential and ensuring characteristics for wild populations. Genetic diversity and population structure of *Wallago attu* was assessed across its natural distribution in Punjab using five species specific microsatellite markers. A total of 177 fish samples was taken and genotypic data of all the samples were generated through PCR amplification of targeted loci. All microsatellite proved to be polymorphic, a total of 36 alleles were found in five sampled populations whose number of allele varied from 6 to 9. The mean F_{ST} value 0.0269 revealed little genetic differentiations. The mean observed heterozygosity was ranged from 0.5328 (CB) to 0.8225 (MD) and the expected heterozygosities were found in range from 0.5396 (CB) to 0.8341 (MD). Significant deviations ($p<0.05$) from Hardy-Weinberg Equilibrium (*HWE*) have been observed in all five populations. The results purposed poor genetic structuring in this valuable resource. The knowledge generated and tools used in this research work will be helpful for the future commercial development of *W. attu* in terms of selective breeding and for better understanding of effective fisheries management.

Key Words: Allelic Polymorphism, Freshwater Shark, Population Structure, Molecular Marker.

INTRODUCTION

Globally, food production has kept in front of demand for a long time; so far approximately one billion of worldwide population presently doesn't have such access. For several people today and historically for the vast majority, efforts to secure food have dictated our everyday activities of farming, gathering, ranching, hunting and fishing. Such efforts have also driven the way, but for the sake of food security we have exploited and often over-exploited our natural resources. Consequently 25% of major

fisheries have been collapsed over the last 50 years (Mullon *et al*., 2005). Over 70% of the world fisheries are under the stress of over exploitation while the rest are fully exploited (FAO, 2006).

Decline in genetic variability of a population ultimately decrease the ability of organisms to acclimatize with fluctuating ecological conditions (Allendorf and Luikart, 2007) and increase the chance of extinction for the reason that genetic diversity gives rise to different phenotypes (morphologies and behaviors) that may respond to novel ecological conditions (Frankham, 2005). At a large scale ecological interactions between and within population influenced by genetic variation, and in this manner influence the whole ecosystem dynamics (Palkovacs *et al*., 2011), because of this, genetic diversity is critical constituent of biodiversity which is integral for further consideration in natural resource management and conservation (Laikre, 2010).

Genetic monitoring of a wild population provides precious information for conservation and management. Loss of genetic resources of natural fish populations is receiving due attention and has become an important fisheries management problem. The density and size of fish available for harvesting is the main concern in fisheries management (Ward and Grewe, 1994). In programs such as selective breeding, information related to genetic characterization of various economically important fish species is obligatory to select prospective brood-stock, to get more production and for the management of fish genetic resource (Haniffa *et al*., 2006). Therefore the genetic assessment of the fish species is crucial for conserving the genetic resource and preventive for the genetic decline (Li *et al*., 2007; Peng *et al*., 2009).

Wallago attu (Family: Siluridae) usually known as fresh-water shark is an important freshwater catfish which resides in various riverine systems of This fish resides in various riverine systems of South East Asia. It has elongated silver or dark grey body with large eyes and single set of extended barbels, a very large mouth that ranges back past the eyes, small forked tail and it equipped with formidable rows of teeth. The high nutritional quality and fast growth rate of its meat increase its potential as important species for aqua-culture. (Dutta-Munshi *et al.,* 1990). Habitat loss, over exploitation and cannibalism, are causes of high mortality of the *W.attu* and it has been decreasing in its natural distribution predominantly (Sahoo *et al*., 2002). According to IUCN Red List (2013) this fish species has been acknowledged as near threatened. Propagation-assisted restoration schemes and effective conservation are essential to conserve this valued resource.

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

No study has been reported with respect to microsatellites on this species up to now in Pakistan. The prime objectives of this study were to monitor the genetic status of *W. attu* in Punjab using latest molecular techniques and to make the strategies for effective management as well as conservation of this valuable fish resource.

MATERIAL AND METHODS

Sampling and DNA isolation

Total of 177 individuals of *W. attu* were collected from five major riverine sites of Punjab, Pakistan (Fig. 1) i.e. Mangla Dam (MD), Head Marala (HM), Chashma Barrage (CB), Ismail Barrage (IB) Baluki Barrage (BB). 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.

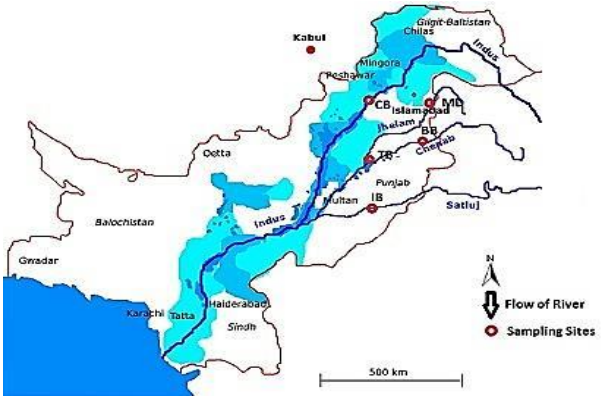

Figure 1. Geographical Map showing sampling sites

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.

PCR amplification of microsatellite loci:

Total five species specific microsatellite loci (WAM-21, WAM-23, WAM-24, WAM-29 and WAM-30) 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). Hierarchical partition of genetic diversity was assessed by AMOVA using ARLEQUIN, ver. 2.000 (Schneider, 2012b). 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).

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	JX971097	WAM-21	$(CA)_{17}$	TTCATACACGTCAAATCAAGGC	53	200-240
				CCCTCACACACCCACTCTTTAC		
2	JX971086	WAM-23	(AC)7	GGTCCCCATAAACATGAAAACA	56	129-137
				AAAGCAGTCAGAAAGACACGCT		
3	JX971112	WAM-24	$(CA)_{16}$	GTTACATCAACACGGAATGGTG	56	178-226
				GTTCTGGGAGTTTGCTCAGATG		
4	JX971094	WAM-29	(TG)9	AATTCATTACACCTCAGACCTCG	55	98-148
				TGCAGGAGTCTTTATCTGCTTG		
5	JX971092	WAM-30	(TG)9	TTGGCACTTCTCAGCTTTACTT	53	121-139
				GTTATACATCACACACGGGAAA		

Where F- forward, R- reverse.

RESULTS AND DISCUSSION

Allelic variations were evaluated by allelic richness (A_r), number of alleles (N_a) and by measuring heterozygosity of populations. Expected heterozygosity and N_a generally used to measures the genetic variations, DeWoody and Avise, (2000).

Genetic Diversity

Allelic polymorphism

A total of 36 alleles were discovered against five microsatellite loci. All microsatellite loci found to be polymorphic whose number of alleles changed between 4 (WAM-21) to 9 (WAM-29). The range of effective number of allele (N_e) was from 2.4128 to 7.6242. Mean number of alleles per population was varied from 5 (WAM-21) to 6.6 (WAM-29) as could be seen in Table 3.

Observed (H_o) and expected (H_e) heterozygosity

The fundamental parameter to assess the genetic diversity was heterozygosity. The H_o and H_e for each locus were calculated for five populations minimum observed heterozygosity (H_o) was found at Wam-21 0.5328 in (CB) population whereas maximum was at WAM-30 0.8225 in (MD) population, Tables 3.

F-coefficients and Gene flow (N_m)

F_{ST}, F_{IT}, F_{IS} coefficients and N_m for the total population consisting of 5 subpopulations were given in Table 2. A significant F_{ST} measure of 0.0269 at over all loci is an indication of little genetic differentiation is existing among five *W. attu* sampled populations. F_{IS} and F_{IT} measures that show deviations from Hardy-Weinberg equilibrium indicate a great deficiency of heterozygotes as revealed by positive F_{IS} and F_{IT} values.

Population Differentiation (*D*)

The F_{ST} estimates show low to moderate level of genetic differentiation between populations. The minimum pairwise population differentiation was observed between population MD and HM i.e. 0.0112. The highest pairwise F_{ST} value 0.0925 was observed between MD and IB that revealed population pair is moderately differentiated, little gentic differentiation was found between rest of the populations pair which shows that the all the populations are genetically distinct from each other Table 4.

Graphical representation

The scatter plot was plotted to show the relationship between geographical distances of sampling locations and genetic distance (Figure 2) the graph explained positive relationship between these two variables which means that the genetic distance increased as the geographical distance increased.

Hardy-Weinberg Tests

It was noticed that all five microsatellite loci in all populations were deviated from Hardy-Weinberg Equilibrium. 23 out of 25 population-locus combinations were showed statistically significant ($p<0.05$) of significance and two locus combination were significant ($p<0.01$) Table 3. All deviations were in favor of homozygotes.

Table 2: The *F* coefficients and N_m at five microsatellite loci

Locus Name	F_{IS}	F_{IT}	F_{ST}	N_m
WAM-21	0.4394	0.4473	0.0113	21.8541
WAM-23	0.6670	0.6868	0.0514	4.6175
WAM-24	0.5555	0.5609	0.0087	28.5044
WAM-29	0.5608	0.5860	0.0420	5.7071
WAM-30	0.5547	0.5648	0.0151	16.3476
Mean	0.5554	0.5691	0.0269	9.0474

N_m = Gene flow estimated from $F_{ST} = 0.25(1 - F_{ST})/F_{ST}$.

Table 3. Genetic diversity, Observed and Expected Heterozygosity

Locus Name	*Parameters*	MD	HM	BB	CB	IB	Average
WAM-21	***Na***	6	5	5	4	5	5
	Ar	5.998	4.958	4.997	4.000	4.999	4.990
	Ho	0.6661	0.6620	0.6101	**0.5328**	0.5884	0.6118
	He	0.6758	0.6709	0.6180	**0.5396**	0.5981	0.6204
	Fis	0.412	0.572	0.464	0.354	0.411	0.443
	PHW	0.0399*	0.0012*	0.0092*	0.1666	0.0034*	------
WAM-23	***Na***	5	5	7	5	5	5.4
	Ar	5.000	4.966	6.877	5.000	5.000	5.374
	Ho	0.7319	0.6706	0.7531	0.7897	0.7362	0.7375
	He	0.7422	0.6797	0.7627	0.7997	0.7483	0.7465
	Fis	0.741	0.724	0.805	0.597	0.487	0.671
	PHW	0.0000*	0.0000*	0.0000*	0.0000*	0.0344*	-----
WAM-24	***Na***	7	5	5	7	6	6
	Ar	6.923	4.992	4.937	6.933	6.000	5.957
	Ho	0.7319	0.7008	0.6478	0.7175	0.7352	0.7066
	He	0.7422	0.7102	0.6560	0.7266	0.7472	0.7164
	Fis	0.405	0.633	0.584	0.625	0.572	0.563
	PHW	0.0643*	0.0000*	0.0000*	0.0000*	0.0000*	-----
WAM-29	***Na***	7	6	5	6	9	6.6
	Ar	6.974	5.956	4.983	5.996	8.966	6.581
	Ho	**0.8225**	0.7036	0.6134	0.7728	0.8148	0.7454
	He	**0.8341**	0.7130	0.6212	0.7826	0.8281	0.7558
	Fis	0.537	0.671	0.48	0.524	0.614	0.565
	PHW	0.3642	0.0000*	0.0087*	0.0033*	0.0040*	-----
WAM-30	***Na***	5	5	5	7	7	5.8
	Ar	4.996.	4.992	4.997	6.756	7.000	5.7482
	Ho	0.7242	0.6707	0.6769	0.6897	0.7911	0.7105
	He	0.7344	0.6796	0.6860	0.6986	0.8045	0.7206
	Fis	0.360	0.693	0.466	0.526	0.672	0.543
	PHW	0.0266*	0.0000*	0.0000*	0.0010*	0.0140*	-----

N_a: number of alleles, A_r: allelic richness, H_o: observed heterozygosity, H_e: expected heterozygosity, *PHW*: Hardy-Weinberg *P*-value

Table 4: pairwise F_{ST} values (below diagonal) and P-value for multiple comparisons measure of population differentiation is (above diagonal)

MD	HM	BB	CB	IB	
MD	******				
HM	0.0112*				
BB	0.0292*	0.0462*			
CB	0.0577**	0.0513**	0.0350*		
IB	0.0962**	0.0441*	0.0607**	0.0845**	******

A "*" indicates significance of population differentiation whereas"**"indicates moderate genetic differentiation between Populations.

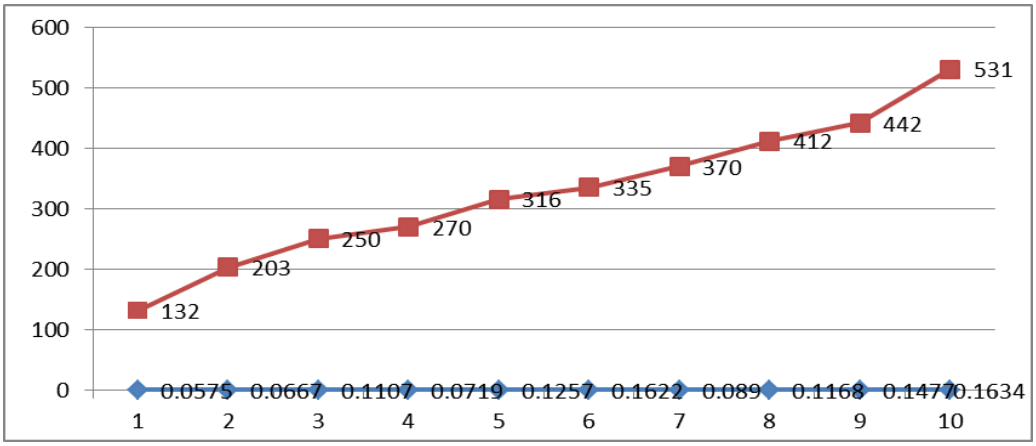

Figure 2 Graphical representations of genetic distance at X-axis and geograpgic distance at Y-axis (km)

Table 5 AMOVA

Source of variation	*d.f.*	*Sum of squares*	*Variance components*	*% age of variation*
Among populations	*4*	*97.410*	*0.29323Va*	*12.58*
Among individuals within populations	*172*	*373.850*	*0.1482 Vb*	*16.36*
Within individuals	*177*	*334.500*	*1.88983 Vc*	*81.06*
Total	*353*	*805.760*	*2.33128*	*100*

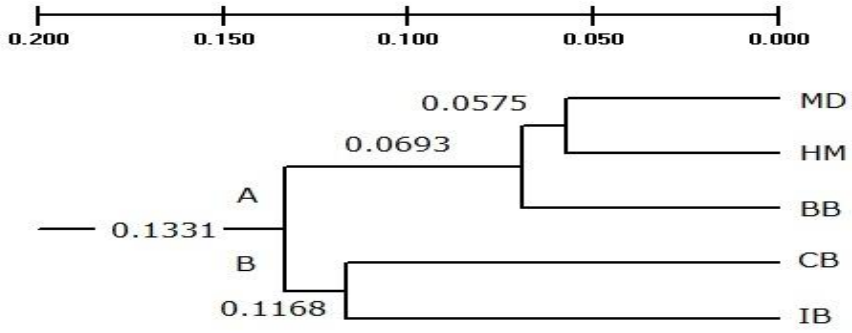

Figure 3 UPGMA dendrogram analysis of five *W. attu* populations

Analysis of Molecular Variance

Hierarchical partition of genetic diversity was assessed by AMOVA using ARLEQUIN, ver. 2.000. This analysis estimates the population structure at different levels i.e., within population, among sub populations and within individuals. The AMOVA analysis of genetic variations showed that variation with in individuals was high (81.06%), while lower among individuals within populations (16.36%) and among populations was very low (12.58%) Table 5.

UPGMA Dendrogram Analysis

The UPGMA dendrogram obtained from cluster analysis of similar indices of five *W. attu* populations, and exhibited two distinct clusters correspondingly that are represented by A and B (Figure 3). The cluster (A) encompassed three population that are MD, HM, BB whereas cluster (B) included two populations i.e. CB and IB. The UPGMA demonstrated that there is no gene flow between both A and B clusters from decades.

DISCUSSION

The population structure is an unstable phenomenon and it is exposed to change over time. Over-exploitation of natural fish population has the potential to change allele frequencies, this could alter the effects of local adaptation and long term evolutionary potential of a population, the effects of these impacts are important to monitor. Such impacts on gene level diversity could result from stock enhancement measures, therefore enhancement measures need to consider the current genetic structure of a population. Future impacts of changing climate and the effects of future outbreaks of environmental catastrophe on the level of genetic variation and the population structure could also be monitored in this manner. It is important to note, that not all of the genetic changes are anthropogenic in nature, an assessment of the genetic structure over time could also reveal the effects of chaotic mechanisms on the stability of genetic structure.

The allelic polymorphism and measure of heterozygosity are widely using parameters for evaluation of population genetic structure. Genetic variations are essential for fitness of population and ensuring the evolutionary potential (Frankham, 2003). The current study exhibited a poor genetic structure pertaining to N_a and H_e. A total of 36 alleles discovered at five microsatellite loci. The average 5.8 alleles per locus and their sizes 186bp to 240bp were similar to the findings of average freshwater catfish population genetics. The N_a was lowermost in HM population i.e. 6 with lowest *Ne* (2.4128), whereas IB population showed maximum N_a i.e. 9 with highest *Ne* (7.6242). The *Ne* is considerably less than the actual N_a showing that frequencies are unequal for all alleles.

Differences in N_a have been studied in catfish by many authors, e.g., Lee *et al*. (2014) studied *L. obesus* (Bull-head torrent catfish) a mean 7.83 alleles yielded against 12 microsatellites, Jin *et al*. (2012) studied *G. laosensis* and observed the number of alleles for each locus varied from 2 to 7, Guo, *et al.* (2009) characterized 14 microsatellite *Glyptosternum maculatum* (sisorid catfish). The number of alleles was observed from 2 to 9, Gopalakrishnan *et al*. (2006) studied *H. brachysoma* (Bagridae, Siluriformes) the number of alleles for each locus varied from 3 to 7. The number of alleles observed in this study ranged from 6 to 9 was consistent as reported in average freshwater catfish species, but higher than reported for *M. bleekeri* (Sheath catfish), Phongkaew *et al.* (2011).

However, the mean N_a, H_o and H_e found in this study were lesser than those reported Singh *et al*. (2013). In each case H_e*s* were higher than the respective H_o. *He* is considered as gene diversity for the reason that it is predicted probability of any individual to be heterozygous at a certain locus and it depends on the number of allele and their relative frequencies at that locus, Hale *et al.* (2012). Inbreeding causes reduction in H_o but it doesn't disturb the allelic frequency and thus the H_e of a population. The present study revealed, observed heterozygosity changed between 0.5328 (CB) and 0.8225(MD) whereas expected heterozygosity changed between 0.5396 (CB) and 0.8341 (MD). The findings are in agreement with range reported about catfish by other authors.

All five populations of *W. attu* revealed significant deviations from Hardy-Weinberg Equilibrium (*HWE*) due to reduction in heterozygosity. Results were consistent with Singh *et al.,* (2013). A total of 23 out of 25 population-locus combinations were showed statistically significant deviations at 5% level of significance (Table 3). All deviations were in favor of homozygotes. The particularly possible explanation about bottleneck and *HWE* deviation might relate with events of over-exploitation and environment degradation because these are the major causes of decline in freshwater diversity and increasing level of heterozygote scarcity as described by (Liao *et al*., 2006; Wang *et al*., 2007; Abbas *et al*., 2010; Zhao *et al*., 2011).

A total of 2 out of 25 population-locus combinations was in Hardy-Weinberg Equilibrium, *HWE* may be due to various aspects such as reduction in effective population size due to over exploitation, differences in mating probabilities (Schneider *et al.*, 2012), selection pressure on specific locus (Ferguson, 1995; Garcia de Leon *et al*., 1997), Wahlund effect (Hartl and Clark, 1997) and predilection of the individuals to mate with physically nearer individuals. Reduction in effective breeding individual is the most expected reason that results in nonconformity to *HWE*. Moreover, all microsatellite loci deviated from *HWE.*

The increasing level of heterozygote scarcity reflected by positive F_{IS} values which ranging from 0.36 to 0.724 that were higher than those reported by Schneider *et*

al. (2012). F_{IS} is a measure of the heterozygote frequencies compared to that expected when the populations are in *HWE*. The F_{ST} between the population's pairs was in range of 0.0112 to 0.0962. This level of population differentiation concerning the pairs of populations considered as low (Balloux and Lugon-Moulin, 2002). The lowermost value of F_{ST} (0.0112) between MD and HM population showed relatively closer relation between these populations.

The UPGMA dendrogram obtained from cluster analysis of similar indices of five *W. attu* populations, and exhibited two distinct clusters correspondingly that are represented by A and B (Figure 3). The cluster (A) encompassed three populations that are MD, HM, BB whereas cluster (B) included two populations i.e. CB and IB. The UPGMA demonstrated that there is no gene flow between both A and B clusters from decades.

There was mild to moderate genetic distances observed amongst the populations. The largest value for genetic distance was 0.1634, found between IB and HM, which may be due to larger geographical gap between these populations whereas the smallest value of genetic distance was 0.0575, obtained from MD and HM populations which may be due to smaller distance between these two populations i.e. 132 km, interconnected canals, long distance migratory behavior of this species and anthropogenic activities like selective breeding programs. This species does not have any selection programs in Pakistan and no significant bottlenecks revealed in any examined population in this study, so other above mentioned causes may be associated. However, the genetic relatedness among the population was revealed by microsatellites analysis.

Hierarchical partition of genetic diversity was assessed by AMOVA using ARLEQUIN, ver. 2.000. This analysis estimates the population structure at different levels i.e., within population, among sub populations, within river basins and among river basins. The results of AMOVA showed structuring in the sample populations. So, prominent diversity existed within populations Percentage of variation among populations was 12.58%, which was lower than variation within populations (16.36%). In conclusion IB population revealed better structuring and proves to be superior too other four populations concerning the allelic richness and effective number of alleles.

Genetic structure amongst populations is a well-defined phenomenon, but little attention has been paid to genetic structure within population. This overall lack of familiarity is a central limitation to existing management of *W. attu* population and for predicting changes that are expected to be affected excessively by climate change. The authors hope that the results, tools and techniques presented in this document would be helpful for the effective fisheries management policies, conservational community and evolutionary biology in conserving the biodiversity of our (still) green planet and specifically the seriously threatened genetic resource of *W. attu*.

This document defines the genetic computational methodologies and their practices in learning about genetic structure and more customarily about the demographic account of population. The population structure stated in this document can inform monitoring efforts and it establishes a base line for future comparisons

Conflicts of interest: The authors stated that no conflicts of interest.

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