



Assessment of genetic diversity among wild and captive-bred *Labeo rohita* through microsatellite markers and mitochondrial DNA

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Abstract

Genetic diversity serves as the basis for selecting and genetically enhancing any culturable species in aquaculture. Here, two different strains of wild (River Ravi and River Kabul) and six captive-bred strains of *Labeo rohita* from various provinces were selected, and genetic diversity among them was evaluated using three different microsatellite markers, i.e., Lr-28, Lr-29, and Lr-37, and one mitochondrial CO1 (Cytochrome c oxidase subunit 1) gene. Different strains of *L. rohita* were collected, and part of their caudal fin was cut and preserved in ethanol for DNA extraction and determination of genetic diversity among them. Results indicated that selected markers were polymorphic with polymorphic information content (PIC) content values above 0.5 with the highest in Lr-28 followed by Lr-29 and then Lr-37. The observed heterozygosity (Ho) of all strains was higher (Avg: 0.731) but less than the expected heterozygosity (He). Moreover, TMs and WRs showed the highest He, while TKs showed the lowest, He. Overall, inbreeding coefficient (FIS) values observed for all strains with selected markers were positive. The DNA barcoding with the CO1 gene revealed genetic variation among various strains, as demonstrated by the clades in the phylogenetic tree separating the strains into two distinct clusters that then divided into sub-clusters. In conclusion, TMs showed the highest heterozygosity as compared to other strains. Overall results provide the baseline data for the initiation of the genetic improvement program.

Keywords: *Labeo rohita*, Strains, CO1 gene, Microsatellite markers, Heterozygosity

Introduction

The diversity of life defines the biological world. Even maternal twins are not identical. Organisms have differences in one or

more traits and it is known as the variability that causes genetic diversity. It can be assessed through the gene count from a specific pool of genes (Ahmad et al., 2022). Generally, genetic variation results from normal cellular processes or interactions of different

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organisms with the environment (Chauhan & Rajiv, 2010). Though, variations in environmental conditions and population density can cause considerable variability, genetic drift, migration, selection, and human interference contribute more significantly (Chauhan & Rajiv, 2010). These factors act continuously, causing changes in the population's frequency of alleles as well as genetic diversity. The frequency of a few alleles is favored over others in domestication or artificial selection increasing selected alleles (Yilmaz & Boydak, 2006). Small-size populations have low genetic diversity and are also prone to genetic drift, which randomly reduces genetic variation (Lu et al., 2014).

Recently, the aquaculture sector is more focused on mitochondrial DNA (mtDNA) analysis for the assessment of deep divergence among populations or species. It is understandable because of its numerous benefits including easy collection and its inheritance to the progeny. In addition, due to the accumulation of divergence sequences, it is devoid of recombination and experiences a high rate of base substitution, which is becoming increasingly relevant in aquaculture for identifying profound divergences between populations or species (Mandal et al., 2012). Numerous researchers have reported that DNA barcodes that utilize a specific mitochondrial fragment, encompassing 600 base pairs recognized as cytochrome oxidase I (COI), are a quick, effective, and inexpensive method for gauging genetic diversity and kinship among species or populations (Hebert et al., 2004; Kamran et al., 2020). However, because of its sole maternal inheritance, a lot of researchers have expressed reservations regarding studies that rely entirely on mtDNA (Zhang & Hewitt, 2003).

Microsatellites are highly polymorphic, biparentally inherited, and found all over the euchromatic region of genomes. They have characteristics such as high variability, improved genomic coverage, high reproducibility, automation readiness, neutrality, and a lack of environmental fluctuations (Figueras et al., 2016). In aquaculture genetics, they are widely used for intrapopulation and interpopulation comparisons, identification of different strains, and genetic variability among individuals (Wattanadilokchatkun et al., 2022). However, due to the uncertainty of the ancestral information, cannot be used to deduce the genealogical patterns of relationships (Zhang & Hewitt, 2003). Thus, using both types of markers can help us to understand genetic diversity and relatedness among species or populations (Gariboldi et al., 2016).

Genetic diversity plays a vital role in providing the building blocks necessary for natural selection and facilitating the adaptation of organisms to new and challenging environments

(Gandra et al., 2021). In the last 20 years, reports have shown a decrease in the genetic diversity of wild fish due to various factors such as lower recruitment, indiscriminate exploitation, overfishing, habitat destruction, migration route blockages, and human interference (Islam & Alam, 2004; Lu et al., 2014). Moreover, inbreeding, small population size, genetic drift, and restricted gene flow in captivity, subsequently result in the reduction of genetic variation among hatchery-raised fish has been reported by many investigators (Shah, 2004).

For the sustainability of aquaculture species and the conservation of wild stock, knowledge regarding the genetic structure of the stocks is a prerequisite (Sultana et al., 2015). However, information/literature on the genetic population structure of indigenous major carp in Pakistan, including *Labeo rohita* is scarce. *L. rohita* can be found in rivers of Pakistan, India, Bangladesh, and Burma (Dahanukar, 2010; Froese et al., 2013). It has high aquaculture and commercial importance worldwide, especially in Asia, and occupies the topmost rank among Indian major carp where it is consumed owing to its fame for being a tasty fish which results in its high market value (Rahman et al., 2005). However, over the past two decades, many investigators from Bangladesh, India, and Pakistan reported a decline in the genetic diversity of wild and captive stocks of *L. rohita* (Ahammad et al., 2022; Khan, 2008; Qadeer & Abbas, 2017). Therefore, the current study was designed to evaluate the genetic variation in both wild and captive populations of the indigenous culturable species *L. rohita*, by utilizing microsatellite markers and mitochondrial marker (COI). Six fish hatcheries across Pakistan for captive-bred strains and two rivers, namely River Kabul and River Ravi for wild strains were selected to assess the genetic structure of their stock.

Materials and Methods

Study population and strains

For the current study, six different strains of captive and two wild rohu (*L. rohita*) were collected from hatcheries and rivers across the country. The collection of strains was performed with the active collaboration of various collaborators at eight locations across Pakistan while local fishermen assisted in the collection of wild stocks from the Ravi and Kabul Rivers. The strains along with the study area given in Fig. 1 and for ease, the locations are also mentioned in the following abbreviated form.

- USs (Upper Sindh Fish Hatchery, Province, Sindh; 25° 23'00" N

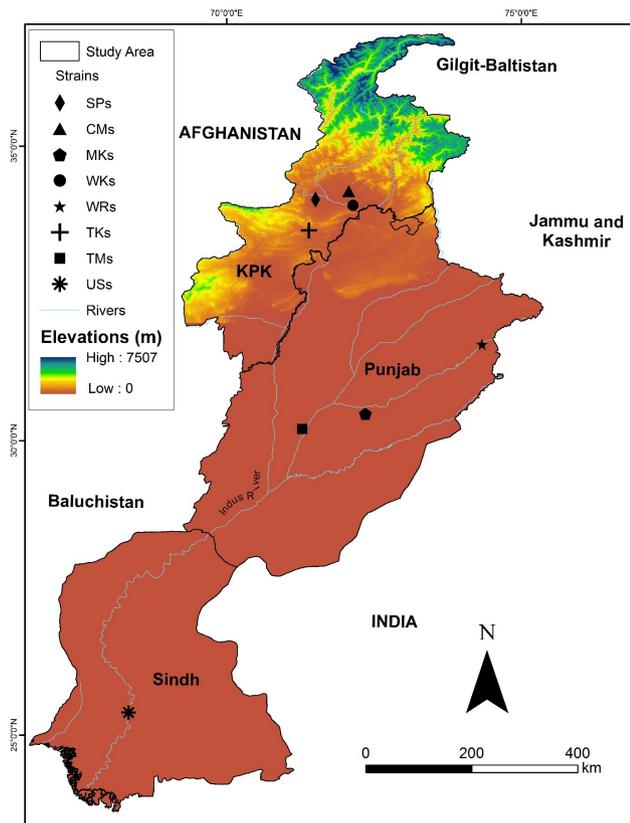


Fig. 1. Collection sites of strains used in the study.

68°20'05" E)

- SPs (Carp Hatchery and Training Center, Peshawar, Province, Khyber Pakhtunkhwa 34.0933° N, 71.5105° E)
- WKs (Wild Strain from River Kabul; 33° 55'0"N 72°13'56"E)
- WRs (Wild Strain from River Ravi; 31° 29' 2.2272"N 74° 9' 46.4292"E)
- TMs (Tawakkal Fish Hatchery Muzaffargarh, Province Punjab; 30° 11' 57"N 71°17' 07" E)
- MKs (Govt. Fish Hatchery, Mianchannu, Province Punjab; 30° 26' 20.1732"N 72° 21' 18.198"E)
- CMs (Govt. Fish Hatchery, Charbanda, Mardan, Province Khyber Pakhtunkhwa; 34° 21' 06" N 71° 58' 22" E)
- TKs (Tanda Govt. Fish Hatchery Kohat, Province Khyber Pakhtunkhwa 33° 34' 07"N 71° 24' 01"E)

From all locations, twenty fish per population/strain were collected and their part of the caudal fins was removed, washed with 75% alcohol, and stored in 90% ethanol at a temperature of 4°C to enable future DNA extraction (Lutz et al., 2023).

Extraction of DNA

A modified salt extraction method reported by Kumar et al. (2007) was employed to isolate the DNA from the caudal fin of each specimen. Briefly, the fin was cut into small pieces and dried on filter paper. Afterwards, 20 mg of macerated fin was placed in a 5 mL tube containing 1.5 mL lysis buffer (200 mM Tris-HCl (pH 8.0); 250 mM NaCl, 100 mM EDTA, 60 µL of 20% SDS, 10 µL Proteinase K (20 mg/mL). The contents were mixed well and incubated at 48°C for 3 hours in a water bath. Then, 800 µL of phenol-chloroform-isoamyl alcohol (25:24:1 ratio) was added to the lysed contents, and tubes were shaken manually for 5 min. The mixture was then centrifuged for 10 min at 10,000 rpm, and the supernatant was collected in a tube containing 800 µL of chloroform-isoamyl alcohol (24:1 ratio). The mixture was mixed well manually and again centrifuged, and the supernatant was collected in a new tube. Afterwards, DNA was precipitated using sodium acetate (NaAc, pH 4.8) and isopropanol and dissolved in 35 µL of ultrapure water. The amount and quality of the DNA obtained were evaluated using NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA).

Amplification of DNA and PCR conditions

Three heterologous microsatellite primers used by Patel et al. (2010) for cross-species amplification of 34 rohu microsatellite loci in *Labeo bata* and some other cyprinids and a CO1 gene primer designed and used by Ward et al. (2005) for the identification of 207 fish species and assessment of different stocks of farmed *L. rohita* (Kamran et al., 2023) were selected (Table 1). Primers were synthesized from Macrogen (Macrogen, Seoul, Korea). A 30 µL mixture containing 14 µL of Thermo Scientific PCR master mix (0.05 U/µL Taq DNA polymerase,

Table 1. Sequence of all primers and their PCR conditions used for the analysis in eight strains of *Labeo rohita*

S.no	Primer name	Sequence	Tm (°C)
1	Lr-28	F: TTCACGGACAGATTTGACCCAG R: AGTCTTTTCAGGAGATTAGCAG	58
2	Lr-29	F: ACGTAAAGGTCACAAGCTGAAG R: AGCACGGTGTGTTGTGTGCGAG	60
3	Lr-37	F: TGAGATGTTGAGCAGGAGCTC R: GAGCGTGCAGTGGCGTTTC	60
CO1 gene marker			
1	FishF1	F: TCAACCAACCACAAAGACATTGGCAC R: TAGACTTCTGGGTGGCCAAAGAATCA.	46.6

reaction buffer, 4 mM MgCl₂, 0.4 mM of each dNTP), 11 µL PCR water, 1 µL of each primer (forward and reverse) (0.2 µM in a 1 µL volume) (Thermo Fisher Scientific, Waltham, MA, USA) and 3 µL DNA was used in a gradient thermal cycler PCR (BIO-RAD T100™ thermal cycler, Hercules, CA, USA). The following were the conditions used for COI PCR: 1) An initial denaturation at 94°C for 1 minute, 2) Denaturation at 94°C for 60 seconds, 3) Annealing at 54°C for 45 seconds, iv) Elongation at 72°C for 60 seconds, succeeded by 7 minutes at 72°C. There were 35 cycles for denaturation, annealing, and extension steps. Additionally, for amplifying microsatellite primers, the PCR conditions were: i) A 5-minute initial denaturation, ii) Denaturation at 95°C for 1 minute, iii) Annealing of Lr-28 at 58°C, Lr-29 and Lr-37 at 60°C for 45 seconds, iv) Elongation at 72°C for 1 minute and then final elongation at 72°C for 10 minutes. In this PCR procedure, a total of 30 cycles were performed. Finally, the end products were kept at 4°C.

Gel electrophoresis

To analyze the amplified products, 1.5% agarose gel (100 mL) solution was prepared by dissolving 1.5 g of agarose powder, 20 mL of 5X TBE buffer (45 mM Tris-borate, 1 mM EDTA), and ethidium bromide was added at a concentration of 0.5 µg/mL visualize the DNA bands. Subsequently, gel electrophoresis was conducted by loading a DNA ladder in one well of each comb, while samples containing 1 µL bromophenol blue dye were loaded in the rest of the wells. After electrophoresis, a few excellent-quality bands were sent to Macrogen (Macrogen, Seoul, Korea) for sequencing.

Analysis of microsatellite data

Manual scoring of the alleles represented as bands on the microsatellite loci was performed, and band sizes were calculated using the DNA fragment program (version 3.03) following the procedure followed by Nash (1991). Determination of various particular-sized alleles at the locus was carried out through genotype scoring for specialized locus.

Bioinformatics analysis

The sequences underwent alignment via the BioEdit software (Version 7.2.5) (Thompson et al., 1997) and were subsequently identified on the NCBI platform utilizing the BLAST algorithm.

Analysis of molecular variance (AMOVA)

AMOVA was carried out using DnaSP5.exe for the examination

of the spatial structure of strains. For both microsatellite and mitochondrial markers, population-wise pairwise FST statistics were computed. The significance levels ($p < 0.05$) were determined.

Statistical analysis

To produce a map of the research location, ArcGIS version 10.8 was utilized. GENEPOP version 4.0 (Rousset, 2008) was used to compute several factors, including the frequency and number of alleles/locus (NA), Ho and He, as well as conformity with Hardy-Weinberg Equilibrium (HWE). Allelic richness (Ar) and significance of the FIS within the population and other loci were calculated using FSTAT version 2.9.3.2 (Goudet, 1995) while the difference between and within populations known as FIS was calculated using Genetix version 4.05 software. Moreover, genetic relatedness and variation among strains were determined by constructing a UPGMA dendrogram (Nei, 1972) using the R programming language (version: 3.6.1).

Results

Genetic variability of microsatellite markers

Lr-28, Lr-29, and Lr-37 primers were effectively amplified by PCR, and all three microsatellite loci were identified as polymorphic, with a PIC value exceeding 0.5 (PIC > 0.5). The Lr-28 loci had a PIC value of 0.79, while the Lr-37 loci had a PIC value of 0.59. A total of 12 alleles were recognized at the three loci, with 5 alleles at the Lr-28 loci, 3 alleles at the Lr-29 loci, and 4 alleles at the Lr-37 loci. The number of alleles ranged from 3 to 5, having an average of 4.0 alleles per locus. Overall selected loci (Table 2), allelic size and frequency differed from sample to sample.

Genetic diversity between strains and Hardy-Weinberg Equilibrium

The genetic variation observed among different strains is presented in Table 2. The Ho ranged from 0.671 to 0.791 for all strains, with TMs having the highest Ho and TKs having the lowest. Likewise, the He ranged from 0.758 to 0.860, with WKs having the highest He, followed by TMs and TKs having the lowest He. TMs showed the highest genetic variability, while TKs showed the lowest compared to other strains, based on the overall mean value of all loci. The FIS value ranged from 0.103 to 0.325, with the lowest average FIS value for TMs followed by WRs and WKs, while TKs showed the highest average FIS value. Moreover, 12 of the 24 tests showed significant deviations from HWE.

Table 2. Genetic variations in *Labeo rohita* populations at Lr-28, Lr-29, and Lr-37 locus

Strains	Parameters	Lr-28	Lr-29	Lr-37	Overall mean
TMs (20)	PIC	0.79	0.63	0.59	0.67
	Alleles (bp)	175-168	170-166	161-155	167.3
	Ar	05.00	03.00	04.00	4.00
	Ho	0.891	0.791	0.691	0.791
	He	0.913	0.813	0.824	0.85
WRs (20)	FIS	0.011	0.148	0.152	0.103
	HWE	0.004**	0.083	0.093	
	Ho	0.843	0.784	0.673	0.766
	He	0.892	0.797	0.799	0.829
	FIS	0.017	0.167	0.310	0.144
MKs (20)	HWE	0.084	0.064	0.088	
	Ho	0.769	0.719	0.671	0.723
	He	0.872	0.839	0.844	0.828
	FIS	0.106	0.237	0.325	0.152
	HWE	0.071	0.005**	0.076	
CMs (20)	Ho	0.791	0.721	0.652	0.721
	He	0.831	0.811	0.822	0.821
	FIS	0.162	0.197	0.372	0.223
	HWE	0.081	0.051*	0.003**	
	Ho	0.736	0.636	0.643	0.671
TKs (20)	He	0.829	0.750	0.695	0.758
	FIS	0.347	0.363	0.266	0.245
	HWE	0.052*	0.002**	0.004**	
	Ho	0.753	0.673	0.661	0.695
	He	0.862	0.810	0.773	0.815
SPs (20)	FIS	0.201	0.289	0.292	0.201
	HWE	0.076	0.043*	0.047*	
	Ho	0.796	0.740	0.664	0.733
	He	0.881	0.857	0.781	0.839
	FIS	0.157	0.183	0.331	0.223
USs (20)	HWE	0.098	0.001**	0.049*	
	Ho	0.854	0.743	0.670	0.755
	He	0.897	0.892	0.793	0.860
	FIS	0.013	0.186	0.297	0.148
	HWE	0.083	0.075	0.043*	

PIC, polymorphic information content; Ar, allelic richness; Ho, observed Heterozygosity; He, expected Heterozygosity; FIS, fixation index (inbreeding coefficient), and conformity to HWE.

*Significant ($p < .05$) and ** highly significant ($p < .01$) departure from HWE.

HWE, Hardy-Weinberg Equilibrium.

Genotype frequencies at all twelve loci across 24 tests showed an overall deviation from HWE (Table 2). CMs, TKs, SPs, and USs showed deviation at two loci each, TMs, MKs, and WKs at one

locus, while WRs did not show deviation from HWE at any loci.

Genetic differentiation and inter-strain genetic structure

The study conducted pairwise comparisons between each strain (Table 3) and found significant differences in their genetic differentiation. The F_{ST} value indicated that all strains showed differentiation. The F_{ST} value for all strains had an average range of 0.002 to 0.059, indicating a moderate to low level of population structure. The study revealed that the gene flow was highest between WKs and WRs and lowest between CMs and MKs. The highest F_{ST} value between CMs and MKs indicated that both strains do not share the allele and are remarkably different ($F_{ST} = 0.059$) while the lowest F_{ST} value between TMs and MKs demonstrated maximum gene flow between both strains ($F_{ST} = 0.002$). Similarly, the genetic distance analysis based on the COI gene revealed the highest gene flow between WKs and WRs (0.004) while the minimum gene flow between WRs and USs (0.068).

Analysis of molecular variance (AMOVA) for Microsatellites and Mitochondrial Marker (COI) gene

The molecular variance analysis presented in Table 4 revealed a minor genetic differentiation among strains using microsatellites ($F_{ST} = 0.03524, p = 0.000$) and COI gene ($F_{ST} = 0.04464, p = 0.000$). However, the observed genetic variation in the populations (microsatellite marker, 68.98%, and COI gene, 59.36% are at the individual level within the strains).

Relatedness through UPGMA dendrogram

The UPGMA dendrogram, illustrated in (Fig. 2) and based on Nei's genetic distance (1972), demonstrated the genetic relatedness among all strains. The formation of two major clusters was depicted in Fig. 2A and 2B based on both microsatellite and mtDNA markers. One cluster comprised three strains, while the other consisted of five strains. Sub-clusters were further divided into each cluster. In the first sub-cluster, only the USs strain was present while TMs and MKs were in the other sub-cluster. The second sub-cluster was further divided into two clusters. One cluster had two strains, WRs, and WKs, while the other three strains, CMs, TKs, and SPs, were in the other.

Figures S1 to S4 (supplementary material) show gel electrophoresis images of 680 bp PCR products of mtDNA COI and PCR products of microsatellite markers. The result indicated no modifications (insertion or deletion).

Table 3. Pairwise FST (below diagonal) and p-values (above diagonal) between eight strains of *Labeo rohita* populations across all loci

	Microsatellite								mtDNA							
	CMs	TKs	MKs	WRs	TMs	SPs	USs	WKs	CMs	TKs	MKs	WRs	TMs	SPs	USs	WKs
CMs	-	0.001	0.017	0.002	0.001	0.016	0.002	0.001	-	0.017	0.017	0.001	0.013	0.022	0.011	0.003
TKs	0.011	-	0.0021	0.013	0.001	0.019	0.001	0.011	0.007	-	0.001	0.020	0.001	0.023	0.002	0.012
MKs	0.059	0.057	-	0.003	0.001	0.017	0.001	0.002	0.067	0.061	-	0.001	0.002	0.013	0.011	0.001
WRs	0.037	0.033	0.052	-	0.006	0.014	0.007	0.012	0.038	0.032	0.064	-	0.001	0.019	0.004	0.021
TMs	0.048	0.049	0.004	0.042	-	0.013	0.003	0.015	0.063	0.061	0.005	0.051	-	0.017	0.006	0.018
SPs	0.010	0.017	0.056	0.032	0.056	-	0.001	0.001	0.009	0.008	0.061	0.036	0.061	-	0.003	0.002
USs	0.053	0.045	0.020	0.049	0.021	0.045	-	0.013	0.067	0.063	0.050	0.068	0.040	0.061	-	0.016
WKs	0.020	0.020	0.052	0.002	0.048	0.022	0.019	-	0.035	0.031	0.059	0.004	0.057	0.034	0.055	-

FST values are shown below the diagonal and p-values are shown above the diagonal. Significant values at $p < .01$ (for microsatellite loci and mtDNA). MtDNA, mitochondrial DNA.

Table 4. Analysis of molecular variance (AMOVA) within and among eight strains

Variation source	Variance component	Variation (%)	Fixation parameters	Significance (p-value)
Microsatellite markers				
Among populations	0.030	4.51	FST = 0.03524	0.000 ± 0.000
Among individuals within populations	0.621	26.56	FIS = 0.17987	0.000 ± 0.000
Within individuals	1.115	68.98	FIT = 0.47154	0.000 ± 0.000
mtDNA				
Among populations	0.038	7.63	FST = 0.04464	0.000 ± 0.000
Among individuals within populations	0.700	33.01	FIS = 0.18811	0.000 ± 0.000
Within individuals	1.323	59.36	FIT = 0.34312	0.000 ± 0.000

MtDNA, mitochondrial DNA.

Discussion

To establish efficient conservation methods, improved knowledge of population dynamics, and implementation of a genetic improvement plan for any species and assessment of genetic diversity is a crucial first step. In recent years, microsatellite markers and mtDNA have become frequently used for this aim (Costa-Urrutia et al., 2012). In the current study, three microsatellite markers and an mtDNA marker provided valuable information about the diversity among two wild (WKs & WRs) populations caught from River Kabul and River Ravi respectively, and 6 captive-bred (TMs, TKs, CMs, MKs, SPs, and USs) populations of indigenous culturable species *L. rohita* collected from different fish hatcheries across Pakistan.

Detecting polymorphisms is crucial for selecting molecular markers. The informativeness and usefulness of a genetic marker in genetic diversity analysis are often measured using the PIC

value (Serrote et al., 2020). The selected microsatellite markers in this study were found to be polymorphic, with 12 alleles across three loci and a PIC value of more than 0.5 (0.59–0.79) (Table 2). Genetic evaluation studies frequently prefer microsatellites with 3-4 alleles per locus and a PIC value greater than 5 (Lie et al., 2010). These results suggest that the microsatellite markers used in this study are meaningful for assessing the genetic diversity of *L. rohita* strains. Previous research has used Lr family markers in combination with other markers to examine population structure and genetic diversity in both wild and farmed *L. rohita* populations, and similar patterns of genetic variation have been found (Alam et al., 2009; Hussain et al., 2021).

Maintaining genetic variation in a population is the most important in conservation and genetic improvement programs. Genetic diversity allows the population to face environmental challenges in the future and respond to long-term selection, either natural or artificial for desirable traits (Sharma et al., 2016).

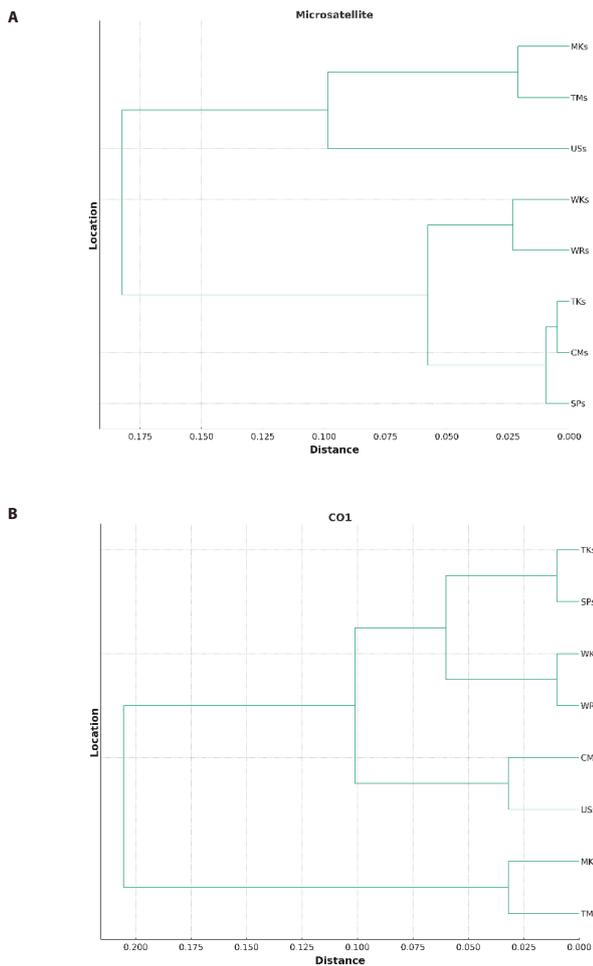


Fig. 2. Illustration of the genetic relationships among eight strains (TMs, WRs, MKs, CMs, TKs, SPs, USs, and WKs). (A) microsatellite markers based on genetic distances with clustering patterns reflecting relatedness. (B) mtDNA sequences of CO1 gene based on genetic distances with clustering patterns reflecting relatedness.

However, loss of genetic diversity in the captive population due to poor management, lack of technical knowledge, and genetic awareness among fish seed-producing persons is the major issue in Pakistan (Riaz et al., 2023). Though genetic variability of the wild population forms the hub for the selection of stock for the selective breeding program, however, the natural stocks of most of the fish species are under threat due to anthropogenic intervention (Islam & Alam, 2004). Generally, genetic degradation resulted in a decline in fish and consequently affected the sustainability of the aquaculture business. Thus, it is imperative to address genetic problems, genetically characterize the population,

and provide baseline data for the initiation of the selective breeding program and supply of quality fish seed.

The most effective approach to measuring genetic variation in populations is the H_o per locus which has a non-random variation not only at the loci level but also at the species and population level (Allendorf & Utter, 1979). The mean value of H_o for all strains ranged from 0.671 to 0.791, with TMs displaying the highest H_o and TKs exhibiting the lowest. Similarly, the mean value of H_e ranged from 0.758 to 0.860, with WKs demonstrating the highest H_e , followed by TMs, and with TKs having the lowest value. Here the H_o and H_e values 0.755–0.766 and 0.829–0.860 respectively of the wild population collected from River Ravi and River Kabul were higher than $H_o = 0.655–0.705$, $H_e = 0.702–0.725$ reported by Nabeela et al. (2014) in the wild population of *L. rohita* collected from three major rivers (Halda, Jamuna, and Padma) of Bangladesh, indicating higher heterozygosity. Nonetheless, the H_e (0.5624) recorded in *L. rohita* specimens collected from three distinct regions of the Chenab River by Hussain et al. (2021) was inferior to the value reported in the current investigation.

In captive populations, small population size and negative selection are the major factors contributing to inbreeding depression and genetic diversity (Sah et al., 2018). However, in the present study, the observed and expected heterozygosity of all populations collected from different fish hatcheries were $H_o = 0.67–0.79$, $H_e = 0.758–0.85$ with the highest in TMs and the lowest in TKs, indicating a somewhat similar level of heterozygosity in some captive population. However, Sultana et al. (2015) reported a low level of $H_o = 0.42–0.7$ and $H_e = 0.661–0.717$ in *L. rohita* samples collected from six fish hatcheries other than those reported here. In the present study, samples were collected across Pakistan while Sultana et al. (2015) selected hatcheries from only one province. The variation in genetic variability among captive stock may indicate the level of genetic awareness, technicality, and the number of broodstock used for induced breeding. It is a personal observation that TMs had a healthy broodstock and educated/trained staff and are in the practice of rotation of broodstock compared to Tanda Hatchery. The lower H_o than H_e in both wild and captive populations in the current study, indicating a departure from HWE and the possibility of inbreeding in successive generations, may be due to non-random mating (Sharma et al., 2016). These deviations were not systematic, detected in 12 out of 24 tests and at different loci for different populations. For instance, WRs did not show deviation at any loci while others deviated at one locus (TMs,

MKs, WKs), others at three loci (CMs, SPs, USs), or all loci (TKs). Departure from HWE of a somewhat low magnitude (41.66) has been reported by Alam et al. (2009) in three wild populations while Ullah et al. (2015) detected 61.11% deviation as compared to the observed (50%) in the present study. The difference may be attributed to variable genetic structures (Hussain et al., 2021).

The observed difference between H_o and H_e was also evident in the positive FIS value in analysis based on microsatellite markers which revealed variation among strains as it ranged from 0.103 (TMs)–0.325 (TKs). Moreover, except TMs all hatchery stocks showed high FIS (0.192–0.325) compared to wild stocks (0.164–0.165). The high positive FIS value is attributed to non-random mating resulting in inbreeding. Like present observations, some other studies also indicate a similar level of FIS value and loss of heterozygosity in the Pakistani stocks of *L. rohita* (Qadeer & Abbas, 2017; Sultana et al., 2015) and confirming the outcomes of the present study.

The FST analysis between different population pairs based on both microsatellite and mtDNA analysis indicated the low genetic differentiation among different strains and ranged from 0.002 to 0.059 and 0.004 to 0.068 respectively. Both markers indicated the lowest genetic difference and the highest gene flow among the wild population (WKs and WRs). This could be due to the physical connectivity of both rivers as River Kabul meets River Indus at Attock, Punjab, and River Ravi meets River Indus at Mithankot, Rajanpur, Punjab which enables the genetic mixing of the populations despite having a geographical distance in between. Moreover, the highest genetic difference among the hatchery-reared population could be due to the minimum gene flow between these strains because of captive breeding or may be related to geographical isolation (Alam et al., 2009). However, the low genetic distance between MKs and TMs and among CMs, TKs, and SPs could be related to the sharing of brooders between the hatcheries, as Tawakkal Fish Hatchery and Mianchannu Fish Hatchery are in the same Province, Punjab. Similarly, Carp Hatchery and Training Center, Peshawar, Government Fish Hatchery, Charbanda, Mardan, and Tanda Government Fish Hatchery Kohat are situated in the Province of Khyber Pakhtunkhwa. There is a common practice of sharing brooders between hatcheries of the same province instead of another province because of geographical distance. The present investigation affirms the correlation between genetic and geographical separation, which is in line with the previous research conducted by Sultana et al. (2015) and Kamran et al. (2023), who observed a comparable degree of genetic resemblance

among populations from three separate hatcheries located in the same province (Faisalabad, Farooqabad, and Mianchannu hatcheries). The UPGMA dendrogram classified the eight strains into two primary clusters based on genetic distance, which were subsequently divided into sub-clusters, indicating the genetic proximity of seven strains. Nevertheless, the USs were genetically different from all other strains.

The AMOVA analysis carried out on microsatellite markers and mtDNA indicated that the molecular diversity among the eight strains was mainly due to individual differences within the population and had less to do with variations between populations. Similar findings were reported by Luhariya et al. (2012) in the wild population of rohu. Both these studies observed that the variations were primarily due to within-individual variations for microsatellite markers and mtDNA gene, respectively. In scattered populations, especially in freshwater species, a higher degree of genetic differentiation is typically expected (Habib, 2010). However, the relatively low genetic diversity observed among the eight *L. rohita* strains in this study may be attributed to the sharing of ancestral gene pools.

Conclusion

In conclusion, based on both microsatellite and mtDNA markers, all the strains showed intra and inter-strains genetic variation among wild and captive-bred populations of *L. rohita*, however, the overall variations are low to moderate, reflecting the proper management strategy. Moreover, the results of the current study provide the baseline data for the initiation of a genetic program.

Supplementary Materials

Supplementary materials are only available online from: <https://doi.org/10.47853/FAS.2023.e67>

Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Ethics approval and consent to participate

This study was conducted following the guidelines outlined by Pakistan's Society for the Prevention of Cruelty to Animals (SPCA). Additionally, permission for the use of animals in scientific research was granted by the 'Bioethical Committee of the Faculty of Biological Sciences, Quaid-i-Azam University' under reference number BEC-FBS-92-QAU-2020.

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References

- Ahammad AKS, Hasan NA, Bashar A, Haque MM, Abualreesh MH, Islam MM, et al. Diallel cross application and histomolecular characterization: an attempt to develop reference stock of *Labeo ariza*. *Biology*. 2022;11:691.
- Ahmad M, Zuberi A, Ali M, Sherzada S, Noorullah M. Identification and determination of phylogenetic relationship among *Labeo rohita*, *Labeo catla* and their reciprocal hybrids by traditional and molecular tools. *Aquac Res*. 2022;53:2686-96.
- Alam MS, Jahan M, Hossain MM, Islam MS. Population genetic structure of three major river populations of rohu, *Labeo rohita* (Cyprinidae: Cypriniformes) using microsatellite DNA markers. *Genes Genom*. 2009;31:43-51.
- Allendorf FW, Utter FM. Population genetics. *Fish Physiol*. 1979;8:407-54.
- Chauhan T, Rajiv K. Molecular markers and their applications in fisheries and aquaculture. *Adv Biosci Biotechnol*. 2010;1:281-91.
- Costa-Urrutia P, Abud C, Secchi ER, Lessa EP. Population genetic structure and social kin associations of Franciscana dolphin, *Pontoporia blainvillei*. *J Hered*. 2012;103:92-102.
- Figueras A, Robledo D, Corvelo A, Hermida M, Pereiro P, Rubiolo JA, et al. Whole genome sequencing of turbot (*Scophthalmus maximus*; *Pleuronectiformes*): a fish adapted to demersal life. *DNA Res*. 2016;23:181-92.
- Gandra M, Assis J, Martins MR, Abecasis D. Reduced global genetic differentiation of exploited marine fish species. *Mol Biol Evol*. 2021;38:1402-12.
- Gariboldi MC, Túnez JI, Failla M, Hevia M, Panebianco MV, Paso Viola MN, et al. Patterns of population structure at microsatellite and mitochondrial DNA markers in the franciscana dolphin (*Pontoporia blainvillei*). *Ecol Evol*. 2016;6:8764-76.
- Goudet J. FSTAT (version 1.2): a computer program to calculate F-statistics. *J Hered*. 1995;86:485-6.
- Habib A. Possible economic impact on coastal fish stock resources in Bangladesh in the case of climate change [M.S. thesis]. Norway: University of Tromsø; 2010.
- Hebert PD, Stoeckle MY, Zemplak TS, Francis CM. Identification of birds through DNA barcodes. *PLoS Biol*. 2004;2:e312.
- Hussain M, Naqqash T, Yaseen G, Amin Q, Shabir G, Babar M. Genetic diversity of rohu, *Labeo rohita* (Hamilton, 1822) from Chenab river and its reservoirs. *J Biotechnol Res*. 2021;12:177-85.
- Islam MS, Alam MS. Randomly amplified polymorphic DNA analysis of four different populations of the Indian major carp, *Labeo rohita* (Hamilton). *J Appl Ichthyol*. 2004;20:407-12.
- Kamran M, Razaq H, Noorullah M, Ahmad M, Zuberi A. Comparative analysis of genetic diversity, growth performance, disease resistance and expression of growth and immune related genes among five different stocks of *Labeo rohita* (Hamilton, 1822). *Aquaculture*. 2023;567:739277.
- Kamran M, Yaqub A, Malkani N, Anjum KM, Awan MN, Paknejad H. Identification and phylogenetic analysis of Chan-na species from riverine system of Pakistan using COI gene as a DNA barcoding marker. *J Bioresour Manag*. 2020;7:10.
- Kumar NP, Rajavel AR, Natarajan R, Jambulingam P. DNA barcodes can distinguish species of Indian mosquitoes (Diptera: Culicidae). *J Med Entomol*. 2007;44:1-7.
- Lie HC, Simmons LW, Rhodes G. Genetic dissimilarity, genetic

- diversity, and mate preferences in humans. *Evol Hum Behav.* 2010;31:48-58.
- Lu YF, Goldstein DB, Angrist M, Cavalleri G. Personalized medicine and human genetic diversity. *Cold Spring Harb Perspect Med.* 2014;4:a008581.
- Luhariya RK, Lal KK, Singh RK, Mohindra V, Punia P, Chauhan UK, et al. Genetic divergence in wild population of *Labeo rohita* (Hamilton, 1822) from nine Indian rivers, analyzed through MtDNA cytochrome b region. *Mol Biol Rep.* 2012;39:3659-65.
- Mandal A, Mohindra V, Singh RK, Punia P, Singh AK, Lal KK. Mitochondrial DNA variation in natural populations of endangered Indian Feather-Back fish, *Chitala chitala*. *Mol Biol Rep.* 2012;39:1765-75.
- Nabeela F, Azizullah A, Bibi R, Uzma S, Murad W, Shakir SK, et al. Microbial contamination of drinking water in Pakistan—a review. *Environ Sci.* 2014;21:13929-42.
- Nash JHE. DNAfrag, program version 3.03. Ottawa: Institute for Biological Sciences, National Research Council of Canada; 1991.
- Nei M. Genetic distance between populations. *Am Nat.* 1972;106:283-92.
- Okumuş İ, Çiftci Y. Fish population genetics and molecular markers: II-molecular markers and their applications in fisheries and aquaculture. *Turk J Fish Aquat Sci.* 2003;3:51-79.
- Patel A, Das P, Barat A, Meher PK, Jayasankar P. Utility of cross-species amplification of 34 rohu microsatellite loci in *Labeo bata*, and their transferability in six other species of the cyprinidae family. *Aquac Res.* 2010;41:590-3.
- Qadeer I, Abbas K. Microsatellite markers based genetic structure of rohu (*Labeo rohita*) in selected riverine populations of Punjab, Pakistan. *Pak J Agric Sci.* 2017;54:865-72.
- Rahman MA, Mazid MA, Rahman MR, Khan MN, Hossain MA, Hussain MG. Effect of stocking density on survival and growth of critically endangered mahseer, *Tor putitora* (Hamilton), in nursery ponds. *Aquaculture.* 2005;249:275-84.
- Riaz R, Junaid M, Rehman MYA, Iqbal T, Khan JA, Dong Y, et al. Spatial distribution, compositional profile, sources, ecological and human health risks of legacy and emerging per- and polyfluoroalkyl substances (PFASs) in freshwater reservoirs of Punjab, Pakistan. *Sci Total Environ.* 2023;856:159144.
- Rousset F. Genepop'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Mol Ecol Resour.* 2008;8:103-6.
- Sah U, Mukhiya Y, Wagle SK. Comparative evaluation of genetically improved and farmed rohu (*Labeo rohita*) on growth and yield at initial stage of rearing. *Int J Fish Aquat Stud.* 2018;6:47-50.
- Serrote CML, Reiniger LRS, Silva KB, dos Santos Rabaiolli SM, Stefanel CM. Determining the polymorphism information content of a molecular marker. *Gene.* 2020;726:144175.
- Shah MS. Management improvement of hatchery and brood stocks of Indian major carps, rohu (*Labeo rohita*), mrigal (*Cirrhinus cirrhosus*) and catla (*Catla catla*). Bangladesh: University Grant Commission; 2004.
- Sharma P, Tang S, Mayer GD, Patiño R. Effects of thyroid endocrine manipulation on sex-related gene expression and population sex ratios in Zebrafish. *Gen Comp Endocrinol.* 2016;235:38-47.
- Sultana F, Abbas K, Xiaoyun Z, Abdullah S, Qadeer I, Husnain R. Microsatellite markers reveal genetic degradation in hatchery stocks of *Labeo rohita*. *Pak J Agric Sci.* 2015;52:775-81.
- Thompson KG, Bergersen EP, Nehring RB, Bowden DC. Long-term effects of electrofishing on growth and body condition of brown trout and rainbow trout. *N Am J Fish Manag.* 1997;17:154-9.
- Ullah A, Basak A, Islam MN, Alam MS. Population genetic characterization and family reconstruction in brood bank collections of the Indian major carp *Labeo rohita* (Cyprinidae: Cypriniformes). *Springerplus.* 2015;4:774.
- Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN. DNA barcoding Australia's fish species. *Philos Trans R Soc B Biol Sci.* 2005;360:1847-57.
- Wattanadilokchatkun P, Panthum T, Jaisamut K, Ahmad SF, Dokkaew S, Muangmai N, et al. Characterization of microsatellite distribution in siamese fighting fish genome to promote conservation and genetic diversity. *Fishes.* 2022;7:251.
- Yaqub A, Kamran M, Malkani N, Anjum KM, Faheem M, Iqbal M, et al. Mitochondrial COI gene based molecular identification and phylogenetic analysis in exotic fish (*Oreochromis mossambicus*) of Pakistan. *J Anim Plant Sci.* 2019;29:1501-8.
- Yılmaz A, Boydak E. The effects of cobalt-60 applications on yield and yield components of cotton (*Gossypium barbadense L.*). *Pak J Biol Sci.* 2006;9:2761-9.
- Zhang DX, Hewitt GM. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Mol Ecol.* 2003;12:563-84.