Pakistan J. Zool., pp 1-16, 2024. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20240131175252

Microsatellite Marker-Based Genetic Characterization of *Labeo* Species in the Riverine System of Punjab, Pakistan

Uzma Batool[1,2], Laiba Shafique[1], Muhammad Tayyab[3], Shakeela Parveen[2]*, Muhammad Hussain[4], Fouzia Tabassum[2] and Muhammad Farhan Khan[5]*

[1]*Guangxi Key Laboratory of Beibu Gulf Marine Biodiversity Conservation, Beibu Gulf University, Guangxi 535011, PR China*
[2]*Department of Zoology, Government Sadiq College Women University, Bahawalpur, Punjab, Pakistan*
[3]*Department of Zoology, Government College University, Faisalabad, Pakistan*
[4]*Department of Veterinary Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[5]*Department of Chemistry, Gomal University, Dera Ismail Khan 29050, Pakistan*

Uzma Batool and Laiba Shafique contributed equally.

Article Information
Received 31 January 2024
Revised 05 April 2024
Accepted 19 April 2024
Available online 25 November 2024 (early access)

Authors' Contribution
Uzma Batool: Conducted research. Laiba Shafique: Wrote original manuscript. Muhammad Tayyab and Muhammad Hussain: Performed statistical analysis. Fouzia Tabassum: Wrote methodology. Muhammad Farhan Khan: Wrote and revised original draft. Shakeela Perveen: Supervised and project administration. All authors reviewed the manuscript.

Key words
Labeo species, Genetic diversity, Population structure, Riverine system, Microsatellite markers

ABSTRACT

The genetic integrity of local fish populations is being compromised by overfished freshwater fisheries and inadequate stock propagation techniques. We used 10 microsatellite markers to look at the genetic diversity and population structure of three *Labeo* species (*Labeo rohita, Labeo calbasu, and Labeo gonious*) in the riverine system of Punjab, Pakistan. A total of 30 fish samples from each riverine populations were collected from the target sites. The average number of alleles in all populations of each species of *Labeo* ranged from 4.0 to 5.7. In *L. rohita*, *L. calbasu*, and *L. gonious*, the observed heterozygosity ranged from 0.323 to 0.423, 0.408 to 0.423, and 0.470 to 0.487, respectively. The average FIS varied from 0.501 to 0.616 for *L. rohita*, 0.445 to 0.491 for *L. calbasu*, and 0.381 to 0.0.415 for *L. gonious*, indicating a considerable amount of inbreeding within each *Labeo* species. The AMOVA showed the low but significant level of genetic differentiation among species (9.185%) and within species (6.097%). Based on pair wise F*ST* and unbiased genetic distance, low-to moderate level of differentiation was found within each species. Structure Bayesian clustering analysis grouped the *Labeo* species populations into 11 groups. No genetic evidence of mixing was found for pristine, original species. The *L. calbasu* species indicate a bottleneck event due to non-significant heterozygosity excess ($p<0.05$) across all loci. Conversely, the bottleneck test reveal no recent genetic bottlenecks ($p>0.05$) in *L. rohita* and *L. gonius* populations. The *Labeo* species directional relative migratory network showed that Lr.RR was the core population with the most genetic exchange with the other *L. rohita* peripheral populations. Nevertheless, no *L. calbasu and L. gonius* species population showed migration event. Unweighted pair group method with averages (UPGMA) dendrogram shown three main clusters: *L. rohita, L. calbasu and L. gonius*. Our findings make a valuable contribution to the existing scientific information about the conservation and management of dwindling populations of *Labeo* species in natural ecosystems of Pakistan.

INTRODUCTION

The conservation and maintenance of biodiversity is imperative for the regular functioning and stability of natural ecosystems (Arlinghaus *et al*., 2015). Genetic diversity plays a crucial role in ensuring the fitness of a species by offering the potential to efficiently adapt to environmental challenges and pressures from natural selection in a dynamic and evolving context. The biological diversity of a species is dependent upon the phenotypic plasticity and adaptability exhibited by its populations in response to both natural environmental

* Corresponding author: drshakeela@gscwu.edu.pk, farhankhanbgu@gmail.com
0030-9923/2024/0001-0001 $ 9.00/0

factors and manmade interventions (Nogués-Bravo *et al*., 2018). During the past few decades, the survival of fish species has been significantly impacted by a range of environmental hazards. These hazards include floods, climatic change, and human interventions such as hydrological alterations, pollution, the introduction of exotic species, the impoundment of rivers, habitat degradation, overexploitation, and overfishing (Prakash, 2021).

The *Labeo* species, *Labeo rohita* (Rohu), *Labeo calbasu* (Orange fin fish), and *Labeo gonious* (Kuria Labeo) are widely recognized members of the Cyprinidae family. These species hold significant commercial value in the field of aquaculture, making them highly desirable globally (Mosha, 2018). These *Labeo* species are native to Pakistan and widespread in Punjab riverine systems. Like many other aquaculture species in Pakistan, *L. rohita, L. calbasu, and L. gonious* face seed quality issues due to poor genetic management of brood stock (Javed and Abbas, 2018). Pakistan has minimal genetic data on these species (Díaz *et al*., 2019). In recent years, hydrological changes and human activities have destroyed natural water body spawning, breeding, and nursing sites. Dam construction disrupts several fish species migratory, which affects their life cycles (Duponchelle *et al*., 2021). Furthermore, the implications of conventional breeding operations in rivers and reductions in natural population size have been found to be negative, resulting in the loss of growth capacity, disease resistance, and stress tolerance. Since 1998, there has been a continuous annual fall of 2% in fish production from natural sources in Pakistan (Khan *et al*., 2016; Qadeer and Abbas, 2017).

Genetic diversity is essential for population fitness and adaptability to the environmental fluctuation (Hoffmann *et al*., 2017). Population geneticists have given multiple decades to establishing the significance of genetic variation within natural populations (Allendorf, 2017). Regarding the conservation of genetic resources, it is anticipated that there would be advantages associated with the preservation of a species's utmost level of genetic diversity. The reduction in genetic diversity, resulting from several factors like prolonged selective pressures, inbreeding, and isolation, has resulted in a deterioration the population's natural potential for adaptation (Bernatchez, 2016). Hence, a comprehensive understanding of the genetic composition of populations is essential in order to mitigate detrimental impacts and ensure the sustainable and efficient management of exploited stocks.

The development of molecular markers has facilitated the genetic analysis of economically significant fish species, so enabling their conservation and genetic management. Microsatellite markers have proven to be highly applicable in various research endeavors within the fields of fisheries and aquaculture, particularly in cases with modest genetic heterogeneity within and across populations (Davis *et al*., 2018). In last few decades, microsatellite markers are extensively utilized owing to their distinctive characteristics, such as a high degree of polymorphism, a rapid detection process, and a relatively compact size. These markers are being used in fisheries to aid in the identification of individuals, identify their ancestry, monitor brood stock, and assist in marker-assisted breeding operations, to conduct investigations into fluctuations in population dynamics (Wenne, 2018). These advantages made microsatellites valuable markers for examining population structure of different species.

Uncovering the fine-scale genetic structure has been long recognized as a key component in policymaking for the management of freshwater fisheries. In the present investigation, polymorphic microsatellite DNA markers already characterized for *L. rohita* by (Tripathy, 2018) were used to investigate the patterns of genetic diversity of *Labeo* species in riverine system of Punjab, Pakistan. We hypothesized that there exists substantial genetic diversity within the *Labeo* genus populations inhabiting these waters. Through the utilization of microsatellite markers, we aimed to uncover the extent of genetic differentiation, gene flow patterns, and population structure among *Labeo* species, providing valuable insights into their evolutionary history and potential adaptive responses to environmental pressures. Access to up-to-date genetic data regarding the genetic structure of *Labeo* species is crucial for determining policy decisions, particularly in relation to the conservation of genetic resources and the efficient management of natural populations.

MATERIALS AND METHODS

Sampling and DNA extraction

Total 360 specimens, 30 of each population of each species of *Labeo* (*L. rohita* (Lr), *L. calbasu* (Lc) and *L. gonious* (Lg) were collected from the four riverine sources Indus River (Head Tounsa), Chenab River (Head Trimmu), Jhelum River (Head Rasool) and Ravi River (Head Balloki) from the Province of Punjab, Pakistan (Table I, Fig. 1). The muscle tissue samples were collected from the dorsal side of the each fish. Approximately 20mg of the muscle tissue was cut and preserved in 70% ethanol and stored at -20 °C until further laboratory analysis. Total genomic DNA was isolated from the muscle tissue using the chloroform-isoamyl-alcohol method that was used following (Renshaw *et al*., 2015). We detected DNA integrity by performing 1% agarose gel electrophoresis. Additionally, we quantified the DNA concentration and

Table I. Sampling details of *Labeo* species.

Species	Site	Populations	Code	Geographical location	Date of capture	No. of samples
L. rohita	Indus River Tounsa Barrage	IR	Lr-IR	24° 18' 43.41"N 67° 45' 49.22" E	7/10/2021	30
	Chenab River Trimmu Head works	CR	Lr-CR	29°20′57″N 71°1′41″E	21-10-2021	30
	Jhelum River Rassol Barrage	JR	Lr-JR	32° 56′ 33″ N, 73° 43′ 32″ E	17-12-2021	30
	Ravi River Baloki Head Works	RR	Lr-RR	30° 34' 59.99" N 71° 48' 59.99" E	22-01-2022	30
L. gonious	Indus River Tounsa Barrage	IR	Lg-IR	24° 18' 43.41"N 67° 45' 49.22" E	12/10/2021	30
	Chenab River Trimmu Head works	CR	Lg-CR	29°20′57″N 71°1′41″E	24-10-2021	30
	Jhelum River Rassol Barrage	JR	Lg-JR	32° 56′ 33″ N, 73° 43′ 32″ E	28-12-2021	30
	Ravi River Baloki Head Works	RR	Lg-RR	30° 34' 59.99" N 71° 48' 59.99" E	20-01-2022	30
L. calbasu	Indus River Tounsa Barrage	IR	Lc-IR	24° 18' 43.41"N 67° 45' 49.22" E	22/10/2021	30
	Chenab River Trimmu Head works	CR	Lc-CR	29°20′57″N 71°1′41″E	27-10-2021	30
	Jhelum River Rassol Barrage	JR	Lc-JR	32° 56′ 33″ N, 73° 43′ 32″ E	13-12-2021	30
	Ravi River Baloki Head Works	RR	Lc-RR	30° 34' 59.99" N 71° 48' 59.99" E	23-01-2022	30

purity using the NanoDrop 2000c spectrophotometer (Thermo Scientific, US). The isolated DNA was diluted to a final concentration of approximately 50 ng/µL and then stored at -20°C for amplification with PCR.

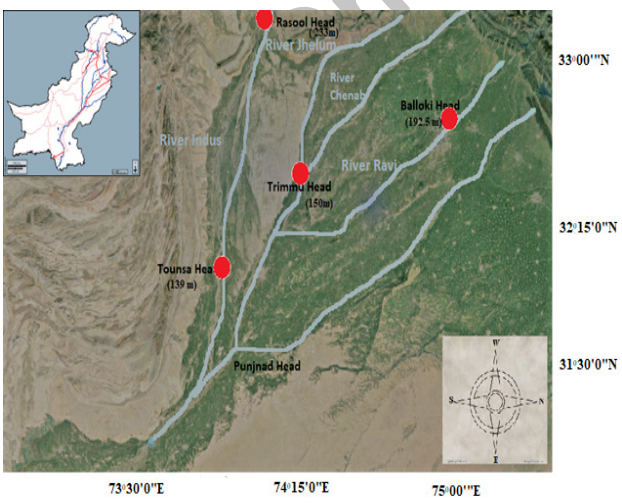

Fig. 1. Sampling locations of *Labeo* species across riverine system of Punjab, Pakistan.

Microsatellite genotyping

A total of 10 microsatellite loci were amplified with polymorphic microsatellite DNA markers already characterized for *L. rohita* by (Tripathy, 2018). The microsatellites used for the present study consisted are Lr-1, Lr-3, Lr-12, Lr-10, Lr-21, Lr-24, Lr-26, Lr-32, Lr-38, and Lr-46. The PCR amplification reactions consisted of 0.8 µl of dNTPs (10mM), 2.0 µl of 10x PCR buffer (20mM), 1.5 µl MgCl2 (20mM), 0.4 µl (2U/ µl) Taq polymerase (Sure Bio Diagnostic and Pharmaceutical),1µl forward and 1µl of reverse primer, 50ng (10mM) preserved DNA and14.1µl of ddH_2Oup to final volume of 20µl.Thermal cycling (Cycler-25 CA. 95070, USA) conditions for each locus were DNA denaturation at 94°C for 2 min, followed by 32 cycles of 94°C for 2 min, annealing temperature for 2 min, and extension at 72°C for 1 min, and then a final extension of 72°C for 1 min (Table II). Genotyping data was analyzed by using different software's.

Genetic data analysis

Genetic diversity

The assessment of genetic variation was conducted by employing fundamental summary statistics, such as expected heterozygosity (He), observed heterozygosity (Ho),

Table II. Information on microsatellite primers used in PCR.

S. No.	Locus No	Motif	Primer sequence	Gene bank accession No	Fragment size	T_a	NA
1	Lr-46	(CA)18	F-TGACGTATTGTCAACTATGGTG R- TCCACCTTCAATACCATGACTG	AM269536	190-240	58°C	4
2	Lr-32	--	F: GGC TCT CAG AAG ACC AGC G R: TCC CCT GCC GTT CTC TGA	AM231181	295 bp	60.05°C	4
3	Lr-38	(GT)12	F: ATAGCATCACCATCTGTTGGTG R: TCTGCTTCAGTCACTCAGCAC	AM269528	240-250bp	59°C	2
4	Lr-10	(CA)13	F-GATCTTCAGCGCCAGCGTG R-GAGGACCTGCCCAGCATG	AJ507523	240-250bp	60°C	4
5	Lr-21	(CA)11	F-GATCAGAGGGTCAATGTGG R-CAGCAGAGTACTATGGAAGA	AJ831436	176bp	58°C	6
6	Lr-24	(TG)17	F- CAA GGC GAA AAG TGT CCA T R-AGGAAATTGGTAAAGTGT TTC	AJ831438	170bp	56°C	5
7	Lr-26	(TG)8	F- CCAGGGAGCTGCTAAGAAT R- AGCGCTTCATGCAGTCTAC	AJ831439	151bp	56°C	5
8	Lr-1	$(TG)_{14}$	F-GACCCTTAACCCTTGACCTT R-TGGGATAATGCAGGGAAAAC	AJ507518	171	58°C	2
9	Lr-3	$(TG)_{19}$	F-ATCTGGCTGCCTATTCACC R-CATCGGCGACTGCACTGGA	AJ507520	152	58°C	5
10	Lr-12	$(CA)_{13}$	F-CACCGCTGCTGTCCATCA R-AGGTCGGCCAGATACACG	AJ507524	161	58°C	4

F, forward; R, reverse; N, no. of alleles; Ta, primer specific annealing temperature.

number of alleles (MNA), allelic richness (Ar) and inbreeding coefficient (FIS). These statistics were estimated for each population across loci, and their respective 95% confidence intervals were calculated using POPGENE (ver. 1.31) and F-statistics (Cockerham and Weir, 1993) in FSTAT ver. 2.9.3.2 (Goudet, 2002).

Population structure

The genetic divergence among populations of *Labeo* species was assessed by calculating the FST values for all pairwise comparisons between sample locations, using the method proposed by Weir and Cockerham (1984). The statistical significance of the FST estimations was evaluated through the implementation of 10,000 permutations. The approach provided by GENEPOP ver. 4.2 (Rousset and Raymond, 1995) was used to test linkage disequilibrium (LD) for all pairs of populations of *Labeo* species. The evaluation of the divergence from Hardy-Weinberg equilibrium (HWE) at each locus was conducted using POPGENE software (version 1.31) (Yeh *et al*., 1999). The significance level of deviations from HWE and LD was adjusted using the sequential Bonferroni correction method in order to control the within-sample type-I error rate at $\alpha = 0.05$ for each locus. The hierarchical partitioning of genetic diversity was estimated by doing an Analysis of Molecular Variance (AMOVA) using ARLEQUIN ver. 3.1. (Meirmans, 2012). POPGENE (ver. 1.31) was used to assess the UPGMA dendrogram based on Nei's (1973) unbiased distance (Yeh *et al*., 1999). A total of one thousand random permutations were conducted in order to evaluate the statistical significance of each pairwise comparison. The species were categorized based on their respective geographic origins (Fig. 1).

The assessment of the pattern of genetic structures among species was conducted utilizing the Bayesian technique in Structure 2.3.4 (Pritchard *et al*., 2000). This analysis employed an admixture model and correlated allele frequency. A burn-in of 100,000 iterations was used, followed by 1,000,000 Markov chain-Monte Carlo (MCMC) repetitions. To ensure consistency and identify genetic clusters, 12 independent runs were performed for each K value (ranging from 1 to 12), where K represents the number of clusters. The determination of the maximum number of clusters was achieved by adding the number of species by a single one, hence enabling the identification of potential substructure (Liu *et al*., 2017). The evaluation of the optimal K genetic cluster was conducted by determining ΔK using the Evanno method (Evanno *et al*., 2005) in structure harvester (Earl and VonHoldt, 2012). Then, Clumpp 1.1.2 (Jakobsson and Rosenberg, 2007).

The highest similarity coefficient was utilized to estimate and compare the clustering results over multiple runs for various values of K. The two-phased model (TPM) with 90% single step mutations with 1000 replications and the mode shift test were used to assess whether populations of the common carp had experienced recent bottlenecks by using Bottleneck v1.2.02 software (Yang *et al*., 2022). The bottleneck effects were estimated for each population of common carp species.

In order to analyze the gene flow patterns among populations, the relative migration rates were evaluated using the Gst statistic method proposed by (Nei, 1973). This analysis was conducted using the divMigrate-online software (Sundqvist *et al*., 2016), which can be accessed at https://popgen.shinyapps.io/divMigrate-online. Additionally, the estimation of asymmetric links between population pairs was performed with 1000 bootstrap iterations.

RESULTS

Genetic diversity

Ten microsatellite loci (Lr-1, Lr-3, Lr-12, Lr-10, Lr-21, Lr-24, Lr-26, Lr-32, Lr-38, and Lr-46) analyzed in twelve populations (30 samples were taken from each population) of *Labeo* species (*L. rohita, L. gonious* and *L. calbasu*) were found to be polymorphic, inheriting with Mendelian mode. The average number of alleles in all populations of each species of *Labeo* ranged from 4.0 to 5.7 as given in the Table III. The maximum value of A_r (5.700, 5.500, and 4.900) was found in the IR population of *L. rohita*, *L. gonious and L. calbasu*, respectively.

Table III. Genetic diversity parameters of four populations at ten microsatellite loci of *L. rohita, L. gonious and L. calbasu.*

Species	Pop.	Parameters	Lr-46	Lr-32	Lr-38	Lr-10	Lr-21	Lr-24	Lr-26	Lr-1	Lr-3	Lr-12	Average
L. rohita	IR	N_a	7	4	5	7	5	7	6	5	6	5	5.7
		A_r	7	4	5	7	5	7	6	5	6	5	5.7
		H_o	0.5	0.367	0.4	0.533	0.367	0.467	0.4	0.4	0.367	0.433	0.423
		H_e	0.869	0.767	0.802	0.875	0.716	0.798	0.826	0.76	0.836	0.803	0.805
		F_{IS}	0.465	0.565	0.505	0.465	0.492	0.419	0.559	0.514	0.565	0.464	0.501
		Ne	6.622	3.92	4.724	6.7	3.377	4.639	5.035	3.875	5.607	4.74	4.925
	CR	N_a	5	6	3	6	5	5	4	4	5	5	4.8
		A_r	5	6	3	6	5	5	4	4	5	5	4.8
		H_o	0.433	0.367	0.267	0.367	0.3	0.367	0.4	0.333	0.4	0.367	0.36
		H_e	0.79	0.824	0.633	0.806	0.746	0.778	0.755	0.757	0.794	0.803	0.769
		F_{IS}	0.495	0.559	0.583	0.549	0.602	0.533	0.475	0.564	0.501	0.585	0.545
		Ne	4.279	5.263	2.651	4.825	3.75	4.255	3.887	3.913	4.568	4.595	4.199
	JR	N_a	7	5	4	6	5	5	4	5	5	5	5.1
		A_r	7	5	4	6	5	5	4	5	5	5	5.1
		H_o	0.333	0.3	0.233	0.4	0.267	0.333	0.333	0.367	0.333	0.333	0.323
		H_e	0.81	0.784	0.708	0.826	0.79	0.788	0.769	0.782	0.793	0.799	0.785
		F_{IS}	0.63	0.701	0.674	0.558	0.666	0.581	0.609	0.574	0.584	0.587	0.616
		Ne	4.778	4.032	3.29	5.097	4.477	4.444	3.976	4.184	4.534	4.675	4.348
	RR	N_a	5	3	5	6	4	4	4	4	5	5	4.5
		A_r	5	3	5	6	4	4	4	4	5	5	4.5
		H_o	0.4	0.3	0.367	0.367	0.267	0.333	0.3	0.367	0.367	0.433	0.35
		H_e	0.779	0.683	0.796	0.824	0.735	0.75	0.751	0.716	0.812	0.75	0.76
		F_{IS}	0.528	0.608	0.544	0.559	0.68	0.56	0.643	0.492	0.628	0.468	0.571
		Ne	4.132	2.925	4.603	5.263	3.541	3.813	3.738	3.377	4.625	3.617	3.963
L. gonious	IR	N_a	6	5	7	6	6	5	7	5	4	4	5.5
		A_r	6	5	7	6	6	5	7	5	4	4	5.5
		H_o	0.414	0.414	0.367	0.4	0.414	0.414	0.433	0.379	0.5	0.4	0.413

Table contibued on next page.....

Species	Pop.	Parameters	Lr-46	Lr-32	Lr-38	Lr-10	Lr-21	Lr-24	Lr-26	Lr-1	Lr-3	Lr-12	Average
		H_e	0.829	0.748	0.824	0.814	0.782	0.769	0.823	0.779	0.714	0.758	0.784
		F_{IS}	0.52	0.471	0.559	0.513	0.499	0.491	0.478	0.539	0.365	0.476	0.491
		Ne	5.391	3.779	5.278	5.013	4.312	4.092	5.247	4.258	3.35	3.921	4.464
	CR	N_a	6	6	6	4	5	4	6	4	4	5	5
		A_r	6	6	6	4	5	4	6	4	4	5	5
		H_o	0.448	0.414	0.429	0.448	0.379	0.517	0.483	0.433	0.414	0.448	0.441
		H_e	0.825	0.796	0.803	0.711	0.728	0.673	0.809	0.737	0.755	0.733	0.757
		F_{IS}	0.481	0.504	0.507	0.386	0.506	0.275	0.431	0.461	0.471	0.423	0.445
		Ne	5.272	4.595	4.722	3.317	3.511	2.956	4.875	3.636	3.875	3.571	4.033
	JR	N_a	4	5	5	3	5	4	3	5	5	4	4.3
		A_r	4	5	5	3	5	4	3	5	5	4	4.3
		H_o	0.433	0.367	0.345	0.367	0.393	0.433	0.414	0.379	0.483	0.467	0.408
		H_e	0.732	0.807	0.8	0.677	0.763	0.752	0.652	0.804	0.794	0.731	0.751
		F_{IS}	0.412	0.55	0.587	0.463	0.522	0.428	0.382	0.548	0.418	0.365	0.468
		Ne	3.564	4.838	4.685	2.995	3.989	3.838	2.78	4.764	4.558	3.55	3.956
	RR	N_a	6	3	5	4	3	5	5	4	5	4	4.4
		A_r	6	3	5	4	3	5	5	4	5	4	4.4
		H_o	0.414	0.448	0.433	0.345	0.467	0.4	0.379	0.414	0.464	0.467	0.423
		H_e	0.829	0.668	0.786	0.759	0.64	0.785	0.789	0.728	0.747	0.761	0.749
		F_{IS}	0.521	0.354	0.453	0.565	0.274	0.495	0.539	0.45	0.431	0.391	0.447
		Ne	5.391	2.915	4.401	3.939	2.698	4.379	4.449	3.511	3.76	3.973	3.941
L. calbasu	IR	N_a	6	5	5	5	7	4	4	4	5	4	4.9
		A_r	6	5	5	5	7	4	4	4	5	4	4.9
		H_o	0.5	0.567	0.467	0.533	0.467	0.467	0.5	0.467	0.433	0.467	0.487
		H_e	0.787	0.799	0.798	0.79	0.843	0.764	0.74	0.684	0.787	0.75	0.774
		F_{IS}	0.442	0.331	0.458	0.329	0.489	0.431	0.365	0.322	0.489	0.418	0.407
		Ne	4.17	4.485	4.438	4.488	5.532	3.875	3.548	3.056	3.346	3.696	4.163
	CR	N_a	5	4	4	5	5	5	4	5	6	4	4.7
		A_r	5	4	4	5	5	5	4	5	6	4	4.7
		H_o	0.467	0.433	0.5	0.567	0.433	0.533	0.467	0.433	0.467	0.500	0.480
		H_e	0.75	0.758	0.738	0.8	0.788	0.798	0.755	0.797	0.838	0.758	0.778
		F_{IS}	0.382	0.471	0.36	0.329	0.491	0.374	0.386	0.496	0.483	0.379	0.415
		Ne	3.805	3.771	3.571	4.583	4.301	4.438	3.887	4.521	5.496	3.84	4.221
	JR	N_a	5	4	4	3	4	4	5	4	4	5	4.2
		A_r	5	4	4	3	4	4	5	4	4	5	4.2
		H_o	0.467	0.467	0.5	0.567	0.4	0.467	0.433	0.467	0.4	0.533	0.470
		H_e	0.758	0.762	0.742	0.674	0.759	0.759	0.788	0.709	0.707	0.772	0.743
		F_{IS}	0.425	0.391	0.33	0.202	0.477	0.428	0.454	0.384	0.438	0.351	0.388
		Ne	3.796	3.982	3.696	2.836	3.938	3.771	4.444	3.191	3.278	3.976	3.691
	RR	N_a	6	4	3	5	4	3	4	3	5	3	4
		A_r	6	4	3	5	4	3	4	3	5	3	4
		H_o	0.433	0.4	0.533	0.533	0.467	0.433	0.533	0.433	0.467	0.467	0.470
		H_e	0.805	0.743	0.66	0.727	0.681	0.666	0.773	0.666	0.819	0.673	0.721
		F_{IS}	0.502	0.503	0.232	0.27	0.353	0.353	0.388	0.389	0.507	0.31	0.381
		Ne	4.659	3.617	2.757	3.425	2.966	2.898	3.91	2.826	4.839	2.955	3.485

Na, number of alleles per locus; Ar, Allelic Richness per locus and population; Ne, Effective number of alleles; Ho, Observed Heterozygosity; He, Expected Heterozygosity; FIS, Coefficient of Inbreeding. For details of populations see, Table I.

Table IV. Species deviation from Hardy-Weinberg Equilibrium at all loci.

Species	Populations	Locus									
		Lr-46	Lr-32	Lr-38	Lr-10	Lr-21	Lr-24	Lr-26	Lr-1	Lr-3	Lr-12
L. rohita	IR	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	CR	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	JR	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	RR	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
L. gonious	IR	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000
	CR	0.000	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.000
	JR	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
	RR	0.000	0.003	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000
L. calbasu	IR	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.004	0.000	0.000
	CR	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	JR	0.000	0.000	0.000	0.526	0.000	0.000	0.000	0.002	0.000	0.000
	RR	0.000	0.000	0.104	0.000	0.017	0.012	0.000	0.415	0.000	0.099

For details of populations see, Table I.

The average observed values of heterozygosity H_o in *L. rohita* ranged from 0.323(JR) to 0.423(IR). While in *L. gonious* these values ranged from 0.408 (JR) to 0.423(RR). And in *L. calbasu* these values of Ho ranged from 0.470(RR) to 0.487(IR). H_o at all loci in overall populations of three species was lower than the corresponding He. The mean values of FIS in *L. rohita* varied from 0.501 to 0.616, in *L. gonious* from 0.445 to 0.491, and in *L. calbasu* from 0.381 to 0.415. The JR population had the highest FIS value in *L. rohita*, whereas the IR population had the highest FIS value in *L. gonious*, and the CR population had the highest FIS value in *L. calbasu* (Table III).

Testing was done to observe HWE (Table IV) and genotypic LD (Table V) deviations. The $\chi 2$ results indicate significant variations in most loci across all populations. The majority of tests showed considerable variance. All populations demonstrated non-significant departures from HWE at some loci. Half of the ten microsatellite loci pairs in all populations showed significant LD values ($P<0.05$) out of 45 comparisons using GENEPOP v.4.2 (Rousset, 2008) with Markov chain parameters (dememorization 10,000, batches 1000, iterations 10,000 per batch). A sequential Bonferroni correction was performed to HWE and genotypic LD tests to adjust for multiple comparisons. Any locus-pair combination had no significant LD after Bonferroni correction for multiple testing.

Population structure and differentiation

The population genetic differentiation was analyzed by pair-wise comparison of each population as given in the Table VI. The value of F_{ST} among all the populations of *Labeo* species ranged from 0.027 (between Lr-CR and Lc-IR) to 0.225 (between Lc-RR and Lg-JR). The highest value of F_{ST} *was* observed between Lc-RR and Lg-JR (F_{ST}=0.225). While the lowest value was observed between Lr-CR and LC-RR (F_{ST}=0.027). Nei's standard unbiased genetic distances between pairs of populations of *Labeo* species varied from 0.089 to 0.456. The smallest distance was observed between Lc.IR and Lc.CR of *L.calbasu* and the largest was between populations Lg.JR and Lg.RR of *L.gonious* (Table VII).

The UPGMA dendrogram grouped the twelve populations into two major clusters as given in the Figure 2. The first major cluster includes the populations of *L. rohita* and *L. Calbasu*. While the second cluster include the populations of *L. gonious*. Cluster one is further subdivided into 2 sub clusters. 1st sub cluster include the four populations of *L. rohita* and the 2nd sub cluster include the populations of *L. calbasu* and also the JR population of *Labeo gonious*. While the cluster 2 includes the IR, CR and RR populations of *L. gonious*.

AMOVA results (Table VIII) show that 9.185% variation among the species, 6.097% variation among populations or within species and 84.718% variation was found within populations of *Labeo* species. The results proved that the majority of variations were from the intra-population diversity. The logarithm probabilities, denoted as Ln p (X/K), obtained from the initial structure run and associated with various genetic cluster numbers K, were computed using a structure analysis of 360 individuals. The results indicated that the highest value

Table V. Chi square tests for *LD* for all the ten microsatellite loci of *L .rohita, L. calbasu* and *L. gonious.*

Loci	*L. rohita*				*L. calbasu*				*L. gonious*			
	Populations											
	IR	CR	JR	RR	IR	CR	JR	RR	IR	CR	JR	RR
Pair(0, 1)	0.243 ns	0.164 ns	0.657 ns	0.249 ns	0.271 ns	0.609 ns	0.526 ns	0.574 ns	0.283 ns	0.523 ns	0.250 ns	0.000*
Pair(0, 2)	0.854 ns	0.424 ns	0.433 ns	0.035 ns	0.429 ns	0.009*	0.456 ns	0.001*	0.185 ns	0.673 ns	0.544 ns	0.077 ns
Pair(1, 2)	0.721 ns	0.022*	0.185 ns	0.536 ns	0.603 ns	0.435 ns	0.032*	0.771 ns	0.586 ns	0.544 ns	0.744 ns	0.643 ns
Pair(0, 3)	0.217 ns	0.000*	0.045*	0.392 ns	0.067 ns	0.943 ns	0.332 ns	0.752 ns	0.865 ns	0.509 ns	0.337 ns	0.457 ns
Pair(1, 3)	0.001*	0.209 ns	0.103 ns	0.033*	0.605 ns	0.060 ns	0.371 ns	0.109 ns	0.460 ns	0.386 ns	0.707 ns	0.400 ns
Pair(2, 3)	0.163 ns	0.035*	0.056 ns	0.532 ns	0.256 ns	0.374 ns	0.006*	0.961 ns	0.509 ns	0.481 ns	0.259 ns	0.385 ns
Pair(0, 4)	0.268 ns	0.157 ns	0.134 ns	0.220 ns	0.001*	0.185 ns	0.201 ns	0.187 ns	0.334 ns	0.777 ns	0.136 ns	0.958 ns
Pair(1, 4)	0.492 ns	0.000*	0.511 ns	0.284 ns	0.061 ns	0.256 ns	0.619 ns	0.503 ns	0.825 ns	0.058 ns	0.654 ns	0.804 ns
Pair(2, 4)	0.379 ns	0.025*	0.018*	0.100 ns	0.006*	0.005*	0.309 ns	0.492 ns	0.091 ns	0.002*	0.379 ns	0.886 ns
Pair(3, 4)	0.597 ns	0.133 ns	0.726 ns	0.409 ns	0.360 ns	0.047*	0.505 ns	0.039*	0.002*	0.128 ns	0.011*	0.260 ns
Pair(0, 5)	0.194 ns	0.364 ns	0.106*	0.494 ns	0.002*	0.209 ns	0.067 ns	0.218 ns	0.747 ns	0.033*	0.879 ns	0.537 ns
Pair(1, 5)	0.394 ns	0.019*	0.897 ns	0.071 ns	0.190 ns	0.827 ns	0.575 ns	0.044*	0.644 ns	0.309 ns	0.747 ns	0.574 ns
Pair(2, 5)	0.051 ns	0.786 ns	0.366 ns	0.064 ns	0.045*	0.379 ns	0.463 ns	0.147 ns	0.862 ns	0.420 ns	0.647 ns	0.835 ns
Pair(3, 5)	0.072 ns	0.499 ns	0.577 ns	0.011*	0.766 ns	0.547 ns	0.103 ns	0.779 ns	0.283 ns	0.337 ns	0.491 ns	0.867 ns
Pair(4, 5)	0.068 ns	0.000*	0.005*	0.007*	0.010*	0.188 ns	0.731 ns	0.941 ns	0.026*	0.910 ns	0.228 ns	0.773 ns
Pair(0, 6)	0.019*	0.206 ns	0.012*	0.141 ns	0.416 ns	0.587 ns	0.017*	0.011*	0.583 ns	0.644 ns	0.064 ns	0.113 ns
Pair(1, 6)	0.590 ns	0.637 ns	0.632 ns	0.001*	0.529 ns	0.010*	0.846 ns	0.046*	0.572 ns	0.485 ns	0.828 ns	0.141 ns
Pair(2, 6)	0.922 ns	0.022*	0.134 ns	0.003*	0.152 ns	0.717 ns	0.380 ns	0.345 ns	0.002*	0.093 ns	0.069 ns	0.194 ns
Pair(3, 6)	0.504 ns	0.037*	0.187 ns	0.009*	0.241 ns	0.014*	0.209 ns	0.051 ns	0.501 ns	0.337 ns	0.653 ns	0.887 ns
Pair(4, 6)	0.965 ns	0.001*	0.055 ns	0.175 ns	0.457 ns	0.014*	0.495 ns	0.733 ns	0.902 ns	0.022*	0.037*	0.079 ns
Pair(5, 6)	0.001*	0.123 ns	0.001*	0.008*	0.012*	0.664 ns	0.794 ns	0.031*	0.033*	0.338 ns	0.286*	0.565 ns
Pair(0, 7)	0.388 ns	0.527 ns	0.187 ns	0.793 ns	0.391 ns	0.040*	0.440 ns	0.072 ns	0.233 ns	0.588 ns	0.011*	0.000*
Pair(1, 7)	0.019*	0.822 ns	0.058 ns	0.474 ns	0.203 ns	0.035*	0.086 ns	0.042*	0.882 ns	0.001*	0.004*	0.150 ns
Pair(2, 7)	0.002*	0.000*	0.004*	0.314 ns	0.460 ns	0.313 ns	0.000*	0.426 ns	0.259 ns	0.442 ns	0.957 ns	0.457 ns
Pair(3, 7)	0.154 ns	0.099 ns	0.002*	0.000*	0.000*	0.062*	0.288 ns	0.099 ns	0.698 ns	0.015*	0.283 ns	0.204 ns
Pair(4, 7)	0.422 ns	0.037*	0.016*	0.817 ns	0.408 ns	0.392 ns	0.209 ns	0.909 ns	0.085 ns	0.122 ns	0.537 ns	0.307 ns
Pair(5, 7)	0.508 ns	0.462 ns	0.013*	0.510 ns	0.423 ns	0.853 ns	0.107 ns	0.288 ns	0.282 ns	0.488 ns	0.167 ns	0.483 ns
Pair(6, 7)	0.055 ns	0.007*	0.139 ns	0.632 ns	0.520 ns	0.597 ns	0.091 ns	0.185 ns	0.194 ns	0.381 ns	0.280 ns	0.154 ns
Pair(0, 8)	0.356 ns	0.608 ns	0.627 ns	0.009*	0.554 ns	0.063 ns	0.966 ns	0.112 ns	0.306 ns	0.672 ns	0.538 ns	0.042*
Pair(1, 8)	0.001*	0.065 ns	0.623 ns	0.002*	0.208 ns	0.620 ns	0.565 ns	0.365 ns	0.020*	0.666 ns	0.084 ns	0.028*
Pair(2, 8)	0.705 ns	0.611 ns	0.056 ns	0.376 ns	0.007*	0.135 ns	0.001*	0.016*	0.495 ns	0.047*	0.995 ns	0.427 ns
Pair(3, 8)	0.177 ns	0.279 ns	0.659 ns	0.288 ns	0.025*	0.718 ns	0.111 ns	0.710 ns	0.981 ns	0.405 ns	0.069 ns	0.795 ns
Pair(4, 8)	0.244*	0.101 ns	0.289 ns	0.413 ns	0.687 ns	0.023*	0.465 ns	0.148 ns	0.614 ns	0.648 ns	0.093 ns	0.796 ns
Pair(5, 8)	0.036*	0.105 ns	0.582 ns	0.033*	0.057 ns	0.432 ns	0.042*	0.863 ns	0.149 ns	0.010*	0.020*	0.080 ns
Pair(6, 8)	0.178 ns	0.016*	0.067 ns	0.227 ns	0.082 ns	0.120 ns	0.869 ns	0.607 ns	0.669 ns	0.471 ns	0.903 ns	0.473 ns
Pair(7, 8)	0.074 ns	0.751 ns	0.009*	0.890 ns	0.489 ns	0.293 ns	0.026*	0.781 ns	0.006*	0.793 ns	0.000*	0.786 ns
Pair(0, 9)	0.057 ns	0.292 ns	0.522 ns	0.036*	0.001*	0.645 ns	0.755 ns	0.302 ns	0.359 ns	0.110 ns	0.217 ns	0.573 ns
Pair(1, 9)	0.373 ns	0.498 ns	0.000*	0.459 ns	0.379 ns	0.515 ns	0.097 ns	0.310 ns	0.026*	0.119 ns	0.883 ns	0.385 ns
Pair(2, 9)	0.330 ns	0.050*	0.020*	0.442 ns	0.623 ns	0.029*	0.843 ns	0.239 ns	0.103 ns	0.441 ns	0.649 ns	0.364 ns
Pair(3, 9)	0.198 ns	0.617 ns	0.003*	0.609 ns	0.300 ns	0.568 ns	0.589 ns	0.089 ns	0.475 ns	0.397 ns	0.909 ns	0.155 ns
Pair(4, 9)	0.027*	0.198 ns	0.005*	0.010*	0.262 ns	0.028*	0.884 ns	0.065 ns	0.005*	0.558 ns	0.286 ns	0.987 ns
Pair(5, 9)	0.111 ns	0.114 ns	0.447 ns	0.901 ns	0.025*	0.171 ns	0.233 ns	0.020*	0.370 ns	0.568 ns	0.173 ns	0.134 ns
Pair(6, 9)	0.006*	0.194 ns	0.001*	0.134 ns	0.016*	0.321 ns	0.328 ns	0.055 ns	0.816 ns	0.216 ns	0.101 ns	0.386 ns
Pair(7, 9)	0.213 ns	0.078 ns	0.029*	0.032v	0.491 ns	0.118 ns	0.861 ns	0.013*	0.059 ns	0.902 ns	0.544 ns	0.816 ns
Pair(8, 9)	0.091 ns	0.234 ns	0.629 ns	0.030*	0.378 ns	0.162 ns	0.629 ns	0.528 ns	0.276 ns	0.588 ns	0.613 ns	0.076 ns

*P < 0.05 and NS, Not Significant. For details of populations see, Table I.

Table VI. Pairwise measures of population differentiation (F_{ST}) between populations of *Labeo* species across all the ten loci.

Species/Pop.		*L. rohita*				*L. gonious*				*L. calbasu*			
		IR	CR	JR	RR	IR	CR	JR	RR	IR	CR	JR	RR
L. rohita	IR	0											
	CR	0.027*											
	JR	0.043*	0.039*	0									
	RR	0.042*	0.047*	0.313	0								
L. gonious	IR	0.180	0.190	0.189	0.199	0							
	CR	0.181	0.192	0.192	0.200	0.044*	0						
	JR	0.195	0.208	0.209	0.221	0.079	0.081	0					
	RR	0.115	0.120	0.110	0.122	0.079	0.090	0.099	0				
L. calbasu	IR	0.139	0.140	0.123	0.141	0.189	0.195	0.200	0.045*	0			
	CR	0.131	0.132	0.111	0.126	0.184	0.185	0.198	0.055	0.016	0		
	JR	0.148	0.144	0.134	0.141	0.193	0.178	0.217	0.088	0.091	0.089	0	
	RR	0.146	0.148	0.148	0.153	0.203	0.193	0.225	0.085	0.080	0.081	0.083	0

*P < 0.05 and NS, Not Significant. For details of populations see, Table I.

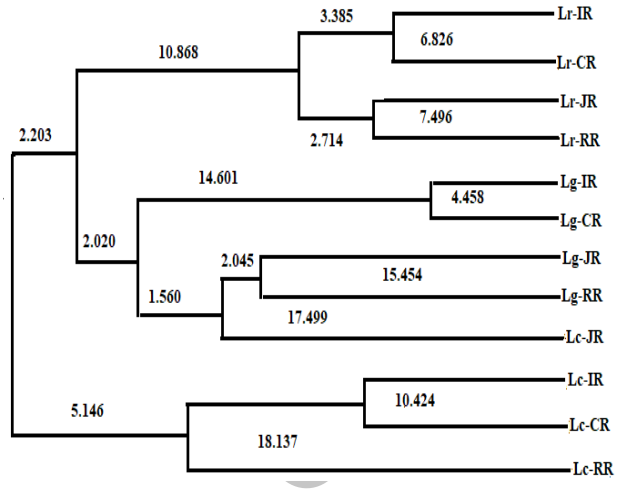

Fig. 2. UPGMA dendrogram based on the genetic distance computed by Nei (1978) between populations of *L. rohita, L. gonious* and *L. calbasu* species

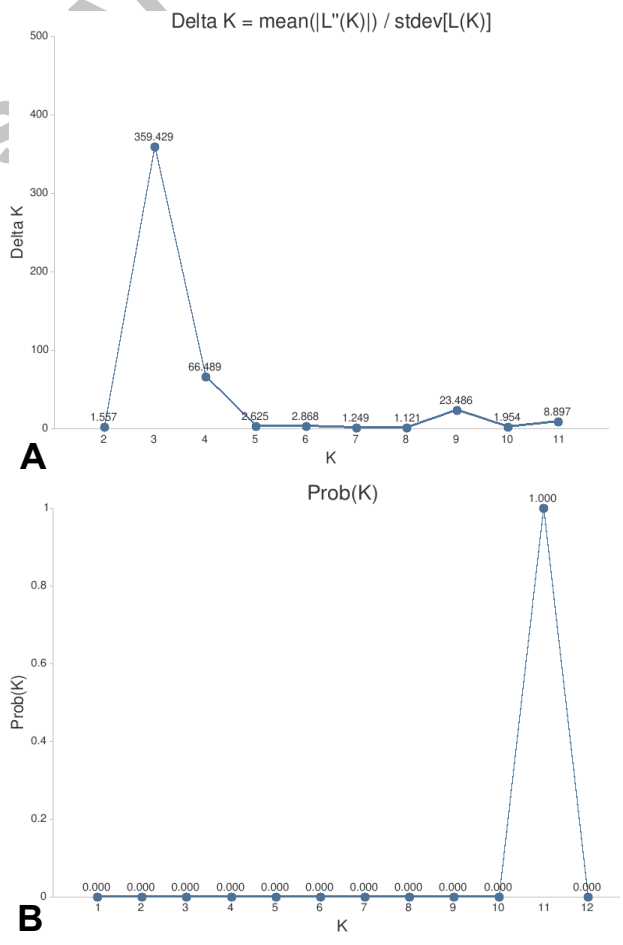

Fig. 3. A, Optimal K by Evanno; and B, using median values of Ln (Pr Data) the k for which Pr (K= k) is highest: 11.

was observed at K = 3 (Fig. 3A), followed by K = 11 (Fig. 3B). The observed dissimilarities among individuals from different species were found to be statistically significant, as indicated by the value of K = 11 (Fig. 3B).

Bottleneck effect and contemporary gene flow

The impacts of the bottleneck were evaluated for each population of *Labeo* species using the two-phased model (TPM) with 90% single step mutations and 1000 replications as well as the mode shift test (Table IX). In some *Labeo* species populations, a high Ho compared to He level

Table VII. Comparison of all the four populations of *Labeo* species, based on the genetic distance and identity.

Species compared	Populations	Biased		Unbiased	
		Dist.	Ident.	Dist.	Ident.
L. rohita	IR vs. CR	0.165	0.847	0.136	0.872
	IR vs. JR	0.258	0.772	0.227	0.796
	IR vs. RR	0.229	0.795	0.200	0.818
	CR vs. JR	0.214	0.806	0.187	0.829
	CR vs. RR	0.226	0.797	0.201	0.817
	JR vs. RR	0.176	0.838	0.149	0.860
L. gonious	IR vs. CR	0.235	0.789	0.208	0.811
	IR vs. JR	0.403	0.668	0.376	0.686
	IR vs. RR	0.353	0.702	0.326	0.721
	CR vs. JR	0.383	0.681	0.358	0.698
	CR vs. RR	0.423	0.654	0.398	0.671
	JR vs. RR	0.480	0.618	0.456	0.633
L. calbasu	IR vs. CR	0.116	0.890	0.089	0.914
	IR vs. JR	0.439	0.644	0.414	0.660
	IR vs. RR	0.340	0.711	0.316	0.728
	CR vs. JR	0.422	0.655	0.397	0.672
	CR vs. RR	0.353	0.702	0.329	0.719
	JR vs. RR	0.330	0.718	0.309	0.734

For details of populations see Table I.

Table VIII. Hierarchal AMOVA analysis of populations of *L. rohita, L. gonious* and *L. calbasu species.*

Source of variation	Sum of squares	Variance component	Percentage variation
Among species	264.937	0.424	9.185
Among populations or within species	197.313	0.281	6.097
Within Populations	3003.517	3.911	84.718
Total	3465.767	4.616	

raises the hypothesis that some populations may be experiencing a bottleneck. Under the two-phase mutation model, the presence of notable but non-significant heterozygosity excess ($p<0.05$) in the *L. calbasu* populations (Lc-JR, Lc-JR Lc-JR and Lc-RR) across all loci indicates that these populations have also undergone a bottleneck event. However, conversely, according to the bottleneck test, there was notable significant ($p>0.05$) excess of heterozygosity seen in the *L. rohita* and *L. gonius* populations indicated that no recent genetic bottleneck had occurred in any population. The observed ratio (10:0) exhibited a notable deviation from the anticipated ratio (1:1) just in the context of non-bottlenecked, equilibrium populations, as determined by the Wilcoxon test (Sign test: P = 0.133; Wilcoxon test: One tail for Hex, P= 0.042) in Lc-JR and (Sign test: P= 0.128; Wilcoxon test: one tail for Hex, P = 0.043) in Lc-RR population. In contrast, the datasets obtained from the CR and JR populations of *L. gonious* did not exhibit a significant excess of heterozygosity (Sign test: P = 0.280 and Wilcoxon test: one tail for Hex, P = 0.067 in Lg-CR; Sign test: P = 0.250 and Wilcoxon test: one tail for Hex, P = 0.063 in Lg-JR) deviated toward an excess of heterozygosity, as expected for bottlenecked populations (nearly significant). The data sets from the Lr-IR and Lr-CR populations of *L. rohita* have significant excess of heterozygosity (Hex/Hd=5/5).

Table IX. Heterozygosity excess under two-phase mutation model at ten microsatellite loci in each of the twelve populations of *Labeo* species.

Species/ Populations		Hex/Hd	Sign test P	Wilcoxon test P (One tail for Hex)
L. rohita	IR	5/5	0.420	0.942
	CR	5/5	0.400	0.938
	JR	6/4	0.340	0.921
	RR	7/3	0.330	0.900
L. gonious	IR	7/3	0.310	0.070
	CR	8/2	0.280	0.067
	JR	9/1	0.250	0.063
	RR	8/2	0.200	0.060
L. calbasu	IR	9/1	0.200	0.035
	CR	9/1	0.150	0.038
	JR	10/0	0.133	0.042
	RR	10/0	0.128	0.043

P; probability, Hex; heterozygosity excess, Hd; heterozygosity deficiency.

The directed relative migratory network was established by utilizing a dataset consisting of 12 populations from three distinct species of *Labeo*, as outlined in Table X. The analysis of the directed relative migration network for the *Labeo* species under investigation revealed that the Lr-RR population served as the central hub, exhibiting a substantial degree of genetic interchange with other populations, namely Lr-CR, Lr-JR, Lr-RR, and Lr-IR. This is evident from the presence of directional migration connections in the relative migration networks. There was no discernible presence of an asymmetric migration pattern, as indicated by Figure 5. The lowest observed relative migration was recorded between Lg-JR and Lg-RR, with a value of 0.001. Conversely, the highest relative migration value was seen between Lr-RR and Lr-CR, with a value of 1.00.

Table X. The directional relative migration of 12 populations of three species of *Labeo*.

Species population		*L. rohita*				*L. gonious*				*L. calbasu*			
		IR	**CR**	**JR**	**RR**	**IR**	**CR**	**JR**	**RR**	**IR**	**CR**	**JR**	**RR**
L. rohita	IR	0.000	0.137	0.082	0.023	0.005	0.018	0.004	0.003	0.008	0.01	0.008	0.013
	CR	0.256	0.000	0.103	0.090	0.011	0.016*	0.006	0.010	0.023	0.028	0.021	0.042
	JR	0.138	0.030	0.000	0.031	0.004	0.011	0.003	0.004	0.02	0.026	0.020	0.021
	RR	0.928*	1.000*	0.871*	0.000	0.004	0.015*	0.002	0.005	0.016	0.047	0.030	0.048
L. gonious	IR	0.004	0.003	0.003	0.003	0.000	0.059	0.02	0.109	0.003	0.003	0.002	0.004
	CR	0.005	0.004	0.004	0.003	0.051	0.000	0.012	0.02	0.002	0.002	0.002	0.003
	JR	0.003	0.003	0.001	0.001	0.051	0.049	0.000	0.043	0.007	0.003	0.003	0.003
	RR	0.007	0.006	0.005	0.007	0.189	0.019	0.016	0.000	0.002	0.004	0.002	0.007
L. calbasu	IR	0.010	0.010	0.011	0.010	0.004	0.004	0.012	0.003	0.000	0.054	0.022	0.025
	CR	0.013	0.012	0.021	0.013	0.003	0.005	0.003	0.002	0.260	0.000	0.020	0.020
	JR	0.012	0.012	0.012	0.012	0.004	0.004	0.004	0.003	0.028	0.031	0.000	0.110
	RR	0.019	0.019	0.016	0.019	0.007	0.009	0.005	0.007	0.068	0.075	0.115	0.000

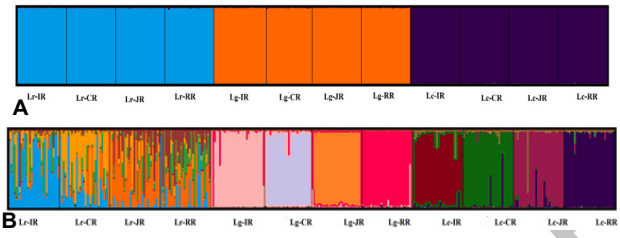

Fig. 4. Clustering of individuals by Bayesian algorithm and 10 microsatellites for K = 3 (A) and for K = 11 (B) (each cluster is indicated by a unique color). Each vertical column represents one population (A) and one one individual (B) and the separation of the column into 3 (A) or 11 colors (B) represents the estimated coefficient of membership to each species.

DISCUSSION

The fishing sector in Pakistan has experienced a sudden decline as a result of human interventions, such as habitat degradation, excessive exploitation of fish resources, hydrological modifications, and the detrimental impacts of hatchery practices on wild populations. The preservation of evolutionary potential necessitates and the maintenance of allelic differences in order to achieve its ultimate objective. This phenomenon not only facilitates the adaptation of *Labeo* species populations to potential environmental changes, but also serves as a assurance for the enhancement of selective breeding operations in hatchery populations. In the present study, 30 individuals for each *L. rohita*, *L. calbasu* and *L. gonious* species were collected from Indus River, Chenab River, Jhelum River and Ravi River of Punjab, Pakistan to determine their genetic diversity and population structure by using the 10 microsatellite markers.

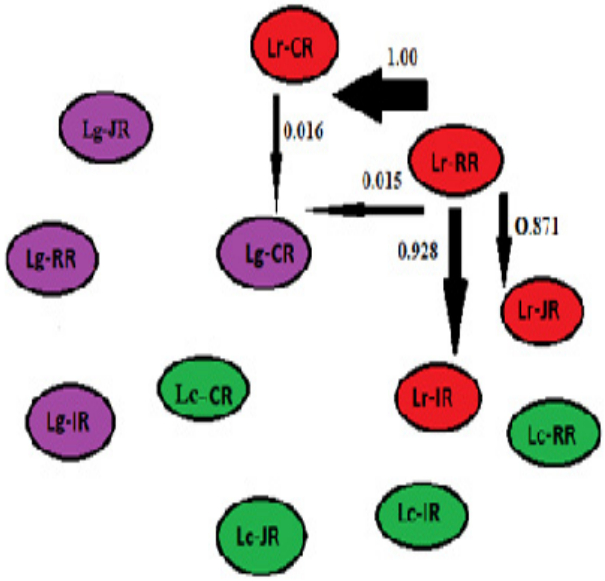

Fig. 5. Contemporary directional relative migration network inferred using divMigrate software.

Genetic diversity

In this study, a total of 10 different microsatellite DNA loci derived were utilized to conduct a genetic analysis of three fish species, namely *L. rohita, L. gonious*, and *L. calbasu*. Based on the criterion of a 0.95 percent allele frequency, it was observed that all of the microsatellite loci exhibited polymorphism. This is similar with the findings of

Alam *et al*. (2009), who used four microsatellite loci (Lr3, Lr12, Lr14a, and Lr21) to analyze the genetic makeup of *L. rohita*, a species that was collected from three significant rivers in Bangladesh (River Halda, River Jamuna, and river Padma). It was revealed that all of the loci exhibited polymorphism. According to the study conducted by Wang *et al*. (2021) the use of microsatellite markers provide a significant and comprehensive insights in the context of fish populations management. Sahoo *et al*. (2022a) successfully isolated and characterized the microsatellite loci in *L. rohita*. These researchers also effectively amplify these loci across different species of carps, demonstrating the polymorphism characteristics of the microsatellite loci employed in this particular investigation. In another study, from a total of 25 individuals of *L. rohita*, Patel *et al*. (2009) produced 21 polymorphic microsatellite loci and documented the polymorphism nature of microsatellite loci in *L. rohita.*

In the present study, the number of alleles ranged from 3 to 7 for Indus River, Chenab River, Jhelum River and Ravi River populations of *L. rohita, L. gonious* and *L. gonious* species. The maximum average number of alleles per locus across all the populations were 5.7 in *L. rohita*, 5.5 in *L. gonious* and 4.9 in *L. calbasu*. Except for some populations, the range of potential allele sizes was comparable to the information acquired from the first established loci (Sahoo *et al*., 2022b). The variation in the size and number of alleles scored in the present study could be attributed to the difference in populations and the sampling. In the present study, the average Ho ranged between 0.323 - 0.423 in *L. rohita*, 0.408-0.423 in *L. gonious* and 0.470-0.487 in *L. calbasu* that is comparable with the Ho (0.4466–0.7761) reported by Singh *et al*. (2012). Over all, the values of the Ho in RR populations were found lower than those in the IR populations of *L. rohita, L. gonious* and *L. calbasu* species.

In present study, for 30% of the riverine the population, the departure from HWE was highly significant. Numerous species of freshwater fishes were found to deviate significantly from their HWE (Inoue and Berg, 2017). The lack of heterozygotes in natural populations may be the result of non-random sampling, inbreeding, intra-population structure, genetic drift, fishing pressure, or any combination of the aforementioned reasons (Qadeer and Abbas, 2017). In the current investigation, departures from HWE could mostly be attributable to deficits in the number of heterozygotes. Alam *et al*. (2009) evaluated the genetic variation in three riverine populations and one hatchery population of *C. catla* was assessed by employing eight microsatellite loci. The findings of this study revealed notable deviations from HWE, primarily attributed to heterozygote deficiency. In light of the extensive environmental dangers and anthropogenic activities in the riverine system of Pakistan, a significant decrease in fish biodiversity in the country's rivers has been observed throughout the latter part of the previous century. The potential cause of heterozygote deficit in riverine populations could be attributed to migration-drift disequilibrium, which arises from environmental dangers and hydrological modifications in natural water bodies. The values of FIS indicated that all the populations were not homogenous. The highest rate of inbreeding was observed in JR population of *L. rohita*, IR population of *L. gonious* and CR population of *L. calbasu* and the lowest in IR population of *L. rohita*, CR population of *L. gonious* and RR population of *L. calbasu*.

Genetic differentiation and population structure

We found a low level of genetic differentiation among *L. rohita* populations (Fst < 0.05), which provided evidence of widespread gene flow across the sampling populations. While F_{ST} values for *L. gonius L. calbasu* are found to be non-significant which indicated lack of heterogeneity in the populations of these species. The low pairwise FST values (0.027) obtained for Lr-CR in this study were likely the concomitant result of high heterozygosity detected within populations. The findings of the current study correspond with the research conducted by Napora-Rutkowski *et al*. (2017), which investigated the extent of genetic variation in various species of common carp in Bangladesh through the utilization of microsatellite DNA markers. AMOVA analysis demonstrated significant genetic structure of *L. rohita, L .gonious and L. calbasu* in the sample populations. AMOVA demonstrated that the level of genetic diversity within populations exceeded that observed between populations in all of the riverine populations. This study also revealed a notable presence of genetic structure among the populations that were investigated, with the majority of the observed variance being attributed to differences between groups.

The UPGMA dendrogram based on genetic distance revealed genetic relation of the sample populations. As evident from the Figure 2, it produced two major clusters, categorizing the populations into two population groups. The populations IR, and CR appeared as one group while the JR and RR clustered into the second group in *L. rohita* and *L. calbasu* species, while in *L. gonious* IR, CR and RR clustered in one group and JR population appeared to be separate.

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. The results of the structure-based analysis (Fig. 4A) revealed that the largest ΔK value was observed at

K=3 indicating significant differentiation across *Labeo* species. Significant differences were also seen among individuals from different species, as determined by the value of K= 11 (Fig. 4B). It is widely recognized that the preservation of a stable polymorphism is contingent upon the presence of a heterozygous advantage, specifically an overweight effect. The obtained outcome can serve as empirical support for a comparatively substantial genetic diversification among typical *Labeo* species.

Genetic bottleneck and contemporary gene flow

Genetic bottlenecks are likely to occur from the loss of genetic variation, reduced adaptive potential, and probability of persisting in particular populations because of habitat fragmentation and increasing insularization. Once Ho exceeds anticipated heterozygosity, a population bottleneck may be observed. The examination of mutation-drift equilibrium as a means of identifying genetic bottlenecks indicated a population reduction among the species. Populations with a notable surplus of heterozygosity were predominantly those that have experienced the most severe and extensively documented bottlenecks.

The Ho decreases in *L. calbasu* populations are likely attributable to a combination of deterministic causes, including but not limited to excessive harvesting, contamination of water, loss of habitat, and alterations to water bodies through engineering activities. The presence of notable non-significant heterozygosity excess ($p<0.05$) in the *L. calbasu* populations (Lc-JR, Lc-JR Lc-JR and Lc-RR) across all loci indicates that these populations have also undergone a bottleneck event. However, conversely, according to the bottleneck test, there was notable significant ($p<0.05$) excess of heterozygosity seen in the *L. rohita* and *L. gonius* populations indicated that no recent genetic bottleneck had occurred in any population. The findings of the current study align with the study conducted by Saha *et al*. (2010) which investigated the bottleneck phenomenon in the endangered kalibaus, *L. calbasu* (cyprinidae: cypriniformes) populations in Bangladesh using microsatellite DNA markers. Fazzi-Gomes *et al*. (2021) also found a loss of genetic diversity and high inbreeding rates in farmed populations of the fish *Arapaima gigas* throughout the Amazon basin due to genetic bottlenecks caused by the domestication process and founding effects.

Gene flow increases the genetic variation within a population; however, it tends to make populations genetically similar to each other (Dudu *et al*., 2015) which is driven by individual movement. The directional relative migration network for the studied *Labeo* species indicated that Lr-RR is core population that had a high level of genetic exchange with other populations (Lr-JR and Lr-RR, Lr-IR) (i.e., migration in directional relative migration networks). No significant asymmetric migration pattern was detected. The lowest relative migration found between Lg-JRand Lg-RR (0.001) and the highest value between Lr-RR and Lr-CR (1.00). As other riverine cyprinid species, *L. rohita* migrates upstream in riverine system for spawning and downstream for feeding and nursing. Zhu *et al*. (2022) described the different relative directional migration rates (0.23–1.00) in bighead carps populations of the Yangtze River indicating the presence of both directional gene flows.

Regarding the increasing significance of aquaculture to the availability of aquatic products, ensuring genetic diversity of aquatic organisms has become a key concern. In the current study, the utilization of microsatellite markers demonstrated their efficacy and dependability as tools for assessing genetic diversity in *L. rohita*, *L. gonius*, and *L. calbasu*. The current research study's findings would serve as foundational data for the genetic management of *Labeo* species in the populations of the Indus River, Chenab River, Jhelum River, and Ravi River. The necessity of maintaining a pollution-free environment and preventing inbreeding has been widely acknowledged as essential for ensuring the genetic sustainability of the riverine system in Punjab, Pakistan. The simultaneous advancement in both areas will offer essential maintaining of *Labeo* species' population from genetic deterioration, so ensuring the conservation of genetic variability and attaining sustainable fisheries stocks.

CONCLUSIONS

The study suggests that *Labeo* species in Punjab, Pakistan's riverine system had low to moderate genetic diversity, giving a baseline for conservation and exploitation. We successfully characterized genetic differences within and across *Labeo* species using microsatellite loci. The average number of alleles in all populations of each species of *Labeo* ranged from 4.0 to 5.7. In *L. rohita*, *L. calbasu*, and *L. gonious*, the observed heterozygosity ranged from 0.323 to 0.423, 0.408 to 0.423, and 0.470 to 0.487, respectively. Overall, *Labeo* species exhibit low to moderate level of genetic diversity. Structure clustered the *Labeo* species were into 11 categories using Bayesian analysis. Genetic evidence of mixing was absent in pure species. There is significant gene flow among *L. rohita* populations. The *L. calbasu* showed a bottleneck event due to non-significant heterozygosity excess ($p<0.05$) at all loci. The bottleneck test shows no recent genetic bottlenecks in *L. rohita* and *L. gonius* ($p>0.05$). We recommend using larvae from

licensed native breeding farms and an effective number of parents for enhancement and restocking to reduce anthropogenic effects e.g. genetic shift and inbreeding) on natural resources. Due to the slight but significant genetic divergence between some population combinations, we propose cross-breeding for genetic enhancement and output, but oppose releasing offspring into native rivers. Working with more polymorphic markers and performing broad geographically diverse sample could help us better understand the genetic problems relating to *L. rohita, L. calbasu*, and *L. gonious* species. The application of molecular markers in this instance could act as a standard for the genetic maintenance of other aquaculture species.

DECLARATIONS

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

IRB approval

This experimental study was approved and reviewed by the Department of Animal Welfare and Ethical Committee of Department of Zoology, Government Sadiq College Women University, Bahawalpur 63100, Pakistan.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Alam, M., Jahan, M., Hossain, M. and Islam, M., 2009. Population genetic structure of three major river populations of rohu, *Labeo rohita* (Cyprinidae: Cypriniformes) using microsatellite DNA markers. *Genes Genom.,* **31**: 43-51. https://doi.org/10.1007/BF03191137

Allendorf, F.W., 2017. *Genetics and the conservation of natural populations: Allozymes to genomes:* Wiley Online Library. https://doi.org/10.1111/mec.13948

Arlinghaus, R., Lorenzen, K., Johnson, B.M., Cooke, S.J. and Cowx, I.G., 2015. Management of freshwater fisheries: Addressing habitat, people and fishes. *Freshw. Fish. Ecol.*, pp. 557-579. https://doi.org/10.1002/9781118394380.ch44

Bernatchez, L., 2016. On the maintenance of genetic variation and adaptation to environmental change: considerations from population genomics in fishes. *J. Fish Biol.,* **89**: 2519-2556. https://doi.org/10.1111/jfb.13145

Cockerham, C.C. and Weir, B., 1993. Estimation of gene flow from F-statistics. *Evolution,* **47**: 855-863. https://doi.org/10.1111/j.1558-5646.1993.tb01239.x

Davis, C.D., Epps, C.W., Flitcroft, R.L. and Banks, M.A., 2018. Refining and defining riverscape genetics: How rivers influence population genetic structure. *Wiley Interdis. Rev. Water,* **5**: e1269. https://doi.org/10.1002/wat2.1269

Díaz, S., Settele, J., Brondízio, E.S., Ngo, H.T., Agard, J., Arneth, A. and Chan, K.M., 2019. Pervasive human-driven decline of life on earth points to the need for transformative change. *Science*, **366**: eaax3100. https://doi.org/10.1126/science.aax3100

Dudu, A., Georgescu, S.E. and Costache, M., 2015. Evaluation of genetic diversity in fish using molecular markers. *Mol. Approaches Genet. Diversity*, pp. 165-193. https://doi.org/10.5772/60423

Duponchelle, F., Isaac, V.J., Rodrigues C.D.C., Van Damme, P.A., Herrerar, G.A., Anderson, E.P. and Agudelo, E., 2021. Conservation of migratory fishes in the Amazon basin. *Aquat. Conserv. Mar. Freshw. Ecosyst.,* **31**: 1087-1105. https://doi.org/10.1002/aqc.3550

Earl, D.A. and VonHoldt, B.M., 2012. Structure harvester: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.,* **4**: 359-361. https://doi.org/10.1007/s12686-011-9548-7

Evanno, G., Regnaut, S. and Goudet, J., 2005. Detecting the number of clusters of individuals using the software structure: A simulation study. *Mol. Ecol.,* **14**: 2611-2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Fazzi-Gomes, P.F., Aguiar, J.D.P., Marques, D., Fonseca C.G., Moreira, F.C., Rodrigues, M.D.N. and Santos, S., 2021. Novel microsatellite markers used for determining genetic diversity and tracing of wild and farmed populations of the Amazonian giant fish *Arapaima gigas*. *Genes* (Basel), **12**: 1324. https://doi.org/10.3390/genes12091324

Goudet, J., 2002. FSTAT (version 2.9. 3.2): A program to estimate and test gene diversities and fixation indices. http://www.unil.ch/izea/software/fstat.html.

Hoffmann, A.A., Sgrò, C.M. and Kristensen, T.N., 2017. Revisiting adaptive potential, population size, and conservation. *Trends Ecol. Evol.,* **32**: 506-517. https://doi.org/10.1016/j.tree.2017.03.012

Inoue, K. and Berg, D.J., 2017. Predicting the effects of climate change on population connectivity and genetic diversity of an imperiled freshwater

mussel, *Cumberlandia monodonta* (Bivalvia: Margaritiferidae), in riverine systems. *Glob. Change Biol.,* **23**: 94-107. https://doi.org/10.1111/gcb.13369

Jakobsson, M. and Rosenberg, N.A., 2007. CLUMPP: A cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics,* **23**: 1801-1806. https://doi.org/10.1093/bioinformatics/btm233

Javed, M. and Abbas, K., 2018. Inland fisheries and aquaculture in Pakistan. *Developing Sustainable Agriculture in Pakistan*, CRC Press. pp. 543-559. https://doi.org/10.1201/9781351208239-25

Khan, M.N., Shahzad, K., Chatta, A., Sohail, M., Piria, M. and Treer, T., 2016. A review of introduction of common carp *Cyprinus carpio* in Pakistan: Origin, purpose, impact and management. *Croat. J. Fish.,* **74**: 71-80. https://doi.org/10.1515/cjf-2016-0016

Liu, X., Cao, Y., Xue, T., Wu, R., Zhou, Y., Zhou, C. and Wu, X., 2017. Genetic structure and diversity of *Nodularia douglasiae* (Bivalvia: Unionida) from the middle and lower Yangtze River drainage. *PLoS One,* **12**: e0189737. https://doi.org/10.1371/journal.pone.0189737

Meirmans, P.G., 2012. AMOVA-based clustering of population genetic data. *J. Hered.,* **103**: 744-750. https://doi.org/10.1093/jhered/ess047

Mosha, S., 2018. A review on significance of Azolla meal as a protein plant source in finfish culture. *J. Aquacult. Res. Dev.,* **9**. https://doi.org/10.4172/2155-9546.1000544

Napora-Rutkowski, Ł., Rakus, K., Nowak, Z., Szczygieł, J., Pilarczyk, A., Ostaszewska, T. and Irnazarow, I., 2017. Genetic diversity of common carp (*Cyprinus carpio* L.) species breed in Poland based on microsatellite, AFLP, and mtDNA genotype data. *Aquaculture,* **473**: 433-442. https://doi.org/10.1016/j.aquaculture.2017.03.005

Nei, M., 1973. Analysis of gene diversity in subdivided populations. *Proc. natl. Acad. Sci.,* **70**: 3321-3323. https://doi.org/10.1073/pnas.70.12.3321

Nogués-Bravo, D., Rodríguez-Sánchez, F., Orsini, L., de Boer, E., Jansson, R., Morlon, H. and Jackson, S.T., 2018. Cracking the code of biodiversity responses to past climate change. *Trends Ecol. Evol.,* **33**: 765-776. https://doi.org/10.1016/j.tree.2018.07.005

Patel, A., Das, P., Swain, S., Meher, P., Jayasankar, P. and Sarangi, N., 2009. Development of 21 new microsatellite markers in *Labeo rohita* (Rohu). *Anim. Genet.,* **40**: 253-254. https://doi.org/10.1111/j.1365-2052.2008.01834.x

Prakash, S., 2021. Impact of climate change on aquatic ecosystem and its biodiversity: An overview. *Int. J. Biol. Innov.,* **3**: 312-317. https://doi.org/10.46505/IJBI.2021.3210

Pritchard, J.K., Stephens, M., Rosenberg, N.A. and Donnelly, P., 2000. Association mapping in structured populations. *Am. J. Hum. Genet.,* **67**: 170-181. https://doi.org/10.1086/302959

Qadeer, I. and Abbas, K., 2017. Microsatellite markers based genetic structure of rohu (*Labeo rohita*) in selected riverine populations of Punjab, Pakistan. *Pak. J. agric. Sci.,* **54**. https://doi.org/10.21162/PAKJAS/17.5736

Renshaw, M.A., Olds, B.P., Jerde, C.L., McVeigh, M.M. and Lodge, D.M., 2015. The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol–chloroform–isoamyl alcohol DNA extraction. *Mol. Ecol. Resour.,* **15**: 168-176. https://doi.org/10.1111/1755-0998.12281

Rousset, F. and Raymond, M., 1995. Testing heterozygote excess and deficiency. *Genetics,* **140**:1413-1419.

Rousset, F., 2008. Genepop'007: A complete re-implementation of the genepop software for Windows and Linux. *Mol. Ecol. Resour.,* **8**: 103-106. https://doi.org/10.1111/j.1471-8286.2007.01931.x

Saha, D., Nahiduzzaman, M., Akter, S., Islam, M.N., Hossain, M.A.R. and Alam, M.S., 2010. Bottleneck in the endangered kalibaus, *Labeo calbasu* (Cyprinidae: Cypriniformes) populations in Bangladesh revealed by microsatellite DNA marker analysis. *Genes Genom.,* **32**: 47-53. https://doi.org/10.1007/s13258-010-0798-7

Sahoo, L., Das, S., Bit, A., Patnaik, S., Mohanty, M., Das, G. and Das, P., 2022a. De novo assembly, transcriptome characterization and marker discovery in Indian major carp, *Labeo rohita* through pyrosequencing. *Genetica,* **150**: 59-66. https://doi.org/10.1007/s10709-021-00141-7

Sahoo, L., Das, S., Bit, A., Patnaik, S., Mohanty, M., Das, G. and Das, P., 2022b. De novo assembly, transcriptome characterization and marker discovery in Indian major carp, *Labeo rohita* through pyrosequencing. *Genetica*, pp. 1-8. https://doi.org/10.1007/s10709-021-00141-7

Singh, R.K., Lal, K.K., Mohindra, V., Punia, P., Sah, R.S., Kumar, R. and Ayyappan, S., 2012. Genetic diversity of Indian major carp, *Labeo calbasu* (Hamilton, 1822) populations inferred from microsatellite loci. *Biochem. Syst. Ecol.,* **44**: 307-316. https://doi.org/10.1016/j.bse.2012.02.002

Sundqvist, L., Keenan, K., Zackrisson, M., Prodöhl,

P. and Kleinhans, D., 2016. Directional genetic differentiation and relative migration. *Ecol. Evol.,* **6**: 3461-3475. https://doi.org/10.1002/ece3.2096

Tripathy, S.K., 2018. Broad spectrum utilities of microsatellite in fish and fisheries. *J. Biotechnol. Res.,* **4**: 29-45.

Wang, W., Ma, C., Ouyang, L., Chen, W., Zhao, M., Zhang, F. and Zhang, H., 2021. Genetic diversity and population structure analysis of *Lateolabrax maculatus* from Chinese coastal waters using polymorphic microsatellite markers. *Sci. Rep.,* **11**: 15260. https://doi.org/10.1038/s41598-021-93000-6

Weir, B.S. and Cockerham, C.C., 1984. Estimating F-statistics for the analysis of population structure. *Evolution,* **1**:1358-1370.

Wenne, R., 2018. Single nucleotide polymorphism markers with applications in aquaculture and assessment of its impact on natural populations. *Aquat. Living Resour.,* **31**: 2. https://doi.org/10.1051/alr/2017043

Yang, X., Ye, J. and Wang, X., 2022. *Factorizing knowledge in neural networks.* Paper presented at the European Conference on Computer Vision. https://doi.org/10.1007/978-3-031-19830-4_5

Yeh, F., Yang, R., Boyle, T. and Mao, J., 1999. *PopGene: Microsoft window-based freeware for population genetic analysis, version 1.31.* University of Alberta, Edmonton, Canada.

Zhu, W., Fu, J., Luo, M., Wang, L., Wang, P., Liu, Q. and Dong, Z., 2022. Genetic diversity and population structure of bighead carp (*Hypophthalmichthys nobilis*) from the middle and lower reaches of the Yangtze River revealed using microsatellite markers. *Aquacult. Rep.,* **27**: 101377. https://doi.org/10.1016/j.aqrep.2022.101377