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RESEARCH ARTICLE

Aquatic Animal Health Management through DNA Barcoding and Morphological Identification of *Labeo rohita* and *Cirrhinus mrigala* from the River Chenab, Pakistan

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ABSTRACT

The study evaluated genetic and morphological diversity of two economically significant major carp species namely, *Labeo rohita,* and *Cirrhinus mrigala,* inhabiting the River Chenab (Chiniot Pull L1, Trimmu Barrage L2 and Head Muhammad Wala L3) Punjab, Pakistan. Fish specimens were identified structurally, and the mitochondrial *COI* gene sequence was utilized for genetic analysis. MEGA 11 and the K2P model were used to construct Maximum Likelihood (ML) phylogenetic trees. Using an online QR code generator, QR codes were made for accurate fish identification. With an average length of 664 base pairs (bp), 60 mitochondrial *COI* barcode sequences were obtained. Transitional mutations were more common than transversions. Each species showed one haplotype (H = 1), haplotype diversity (Hd) = 0.00 and nucleotide diversity (Pi) = 0.00 in the analyzed *COI* region. In nucleotide composition GC content (45.5%) was lower than the AT content (54.5%). The evolutionary tree showed degree of resemblance in the limit of 99% for sequences of *Labeo rohita* and *Cirrhinus mrigala,* which grouped in two clades because they were in the two genera but the same family. In the current study, DNA barcoding has demonstrated that it is a reliable approach for species identification. The molecular data generated in this study provides baseline information on species-level identification and intraspecific genetic variation of the examined carps, which may support future investigations of fish health observation, aquaculture management and fisheries monitoring.

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INTRODUCTION

The Chenab River, a key tributary of the Indus River system, harbors diverse freshwater fauna, including commercially important carps such as *Labeo rohita* and *Cirrhinus mrigala.* In Pakistan, carps constitute 56% of freshwater aquaculture production (Anonymous, 2023). However, escalating anthropogenic pressures in Punjab including industrial pollution, agricultural runoff, untreated urban waste and overfishing have severely impacted aquatic biodiversity (Ali *et al*., 2019; Anonymous, 2020). These stressors contribute to habitat degradation, water quality deterioration and potential genetic variation in native fish populations (Khan *et al*., 2018). Despite their economic and ecological importance, molecular information on the species-level identity and intraspecific genetic variation of these carps from the Chenab River remains limited. Establishing reliable molecular reference data can provide a useful foundation for species authentication, aquatic animal health assessment, disease related investigations, aquaculture biosecurity and the sustainable management of fisheries resources.

Commercially important species include rohu (*Labeo rohita*), mrigal carp (*Cirrhinus mrigala*), catla (*Catla catla*), striped snakehead (*Channa striatus*), wallago catfish (*Wallago attu*), giant devil catfish (*Bagarius bagarius*) and rita (*Rita rita*) (Hussain *et al.,* 2015). Cyprinid fishes are highly valued in the Indian subcontinent, where they are used in aquaculture production. According to Sarma *et al*. (2017) Cyprinid species constitute 19.6% of the fish occurrence in Asia. Within the Cyprinidae family, the genus Labeo stands out as the most dominant group. Among 105 species of fish that belong to the genus Labeo in the world, 36 species are distributed in South and Southeast Asian regions, and 69 species are distributed in Africa (Sudha *et al*., 2015).

Labeo rohita (Hamilton, 1822) is a Cyprinidae can reach a length of 2 m long and weigh 45 kilograms (Talwar

and Jhingran 1991). It is known for its fast growth, high feed conversion efficiency and nutritional value, making it one of the world's major aquaculture species. It is commonly cultured alongside Catla (*Catla catla*) and Mrigal (*Cirrhinus mrigala*) in polyculture systems (Alam *et al*., 2009; Hussain *et al*., 2021). The carp group including *Cirrhinus mrigala* dominates freshwater aquaculture, contributing 56.45% to global production, with mrigal alone accounting for 1.08% of freshwater aquaculture yield (Yu *et al*., 2019; Chowdhury *et al*., 2024).

The identification of species across many organismal groups has been made possible by the DNA barcoding approach, which has been commonly known as one of the most important genetic tools to identify the species (Kamran *et al*., 2020; Ude *et al*., 2020; Tsoupas *et al*., 2022). This method was first developed by Hebert and his colleagues at the University of Guelph, Canada using a small fragment of mitochondrial DNA to precisely determine the species (Rahman *et al*., 2019; Zafar *et al*., 2024a).

Furthermore, troubles including species mistagging and seafood mislabeling are increasing in seafood markets. To overcome such obstacles, Hebert *et al*. (2003) proposed conception of DNA barcoding, introducing technique for their recognition. This technique provides many benefits overhead conventional taxonomic paths. The current study assessed the integration of DNA barcoding in taxonomic studies of *Labeo rohita* and *Cirrhinus mrigala* to bridge morphology and genetics.

MATERIALS AND METHODS

Sample collection: The current study focused on *Labeo rohita* and *Cirrhinus mrigala* of the family *Cyprinidae*, found in the River Chenab, Pakistan. A total of 90 samples were collected between 2023 and 2025, through the assistance of indigenous fishermen from both upstream and downstream locations, namely Chiniot Pull (L1), Trimmu Barrage (L2), and head Muhammad Wala (L3) (Table 1). Sampling was conducted during the available collection periods within the study duration. Therefore, the present sampling design was intended to provide spatial representation of the two selected species and was not designed to evaluate seasonal or temporal changes in morphological and genetic variation. Fifteen samples of each species were collected from each location, resulting in 45 specimens of *Labeo rohita* and 45 of *Cirhinus mrigala* for morphological examination. For DNA barcoding, 30 specimens of each species were selected, with 10 specimens per species from each sampling location, giving a total molecular sample of 60 specimens. The molecular sampling design was intended to provide representation of both focal species across the three sampling locations and to provide replicate specimens for species level *COI* barcode assessment. Fish specimens weighing approximately 500g were obtained from these locations (L1-L3) and transported to the Department of Zoology, Government College University Faisalabad, for further process. Clove oil was used as an anesthetic and euthanasia agent due to its effectiveness, affordability and minimal physiological stress on fish. Furthermore, Clove oil was also used as a fish anesthetic because it is a natural and inexpensive product with low toxicity risks. In this study, exposure of 0.20mL of clove oil in 500mL of water for ten minutes was used for fish euthanization (Fernandes *et al*., 2017).

Subsequently, the fish samples were morphologically identified and the whole fish was preserved in absolute ethanol for molecular analysis.

Table 1: Sampling locations, fish species and sample size for morphological and molecular analysis.

Sampling Locations	Fish species	Number of Samples (n) for analysis	
		Morphological (n=90)	Molecular (n=60)
Chiniot Pull (L1)	*Labeo rohita*	15	10
(31.7157° N, 72.9936° E)	*Cirrhinus mrigala*	15	10
Trimmu Barrage (L2)	*Labeo rohita*	15	10
(31.116943°N, 72.188502°E)	*Cirrhinus mrigala*	15	10
Head Muhammad Wala (L3)	*Labeo rohita*	15	10
(30.26°N, 71.42°E)	*Cirrhinus mrigala*	15	10

Morphometric measurements: The morphometric measurements were carried out after the preservation of fish specimens to aid in the correct identification of *Labeo rohita* and *Cirrhinus mrigala* (Fig. 1 A and B) through "A Key to Fishes of Punjab" by Mirza (2003). Morphometric measurements were carried out with the assistance of a magnifying glass, scales, and Vernier caliper, ensuring precision to the nearest 0.1mM for statistical analysis. At the taxonomic level, samples were morphologically identified by measuring Total Length (TL), Standard Length (SL), Snout Length (SnL), Head Length (HL), Head Depth (HD), Fork Length (FL), Pre Dorsal Length (PrDL), Post Dorsal Length (PsDL). Meristic characters Dorsal fin rays (DFR), Anal fin rays (AFR), Caudal fin rays (CFR), Pectoral fin rayss (PecFR) and Pelvic fin rays (PelFR). Morphometric and meristic measurements were analyzed using R software because most morphometric measurements are influenced by overall body size, the morphometric variables were evaluated after accounting for size effects before assessing species-level differences. PCA analysis was used to summarize multivariate variation and to visualize the separation of species based on their morphometric characters. Pearson correlation analysis was used to describe relationships among morphometric and meristic variables and was not interpreted as direct evidence of species discrimination. The heatmap was used as a descriptive visualization of similarities and differences in the measured characters among specimens and sampling groups.

After the morphometric measurements, the samples were further processed for genetic studies.



A: *Labeo Rohita*

B: *Cirrhinus mrigala*

Fig. 1: Gross Morphology of the studied Carps (A: *Labeo rohita*, B: *Cirrhinus mrigala*).

DNA extraction, visualization and quantification: Using the "QIAamp DNA Mini Kit" procedure according to the manufacturer's instructions, the high-quality DNA was extracted from the tiny fish tissues taken from the dorsal side of the fish. Using Nanodrop values, the extracted DNA's purity and quantity were assessed (ND-1000 spectrophotometer).

PCR amplification and sequencing: The conserve region *COI* gene was amplified using the universal primers FishF1 (5`-TCAACCAACCACAAAGACATTGGCAC-3') and FishR1 (5`-TAGACTTCTGGGTGGCCAAAGAATCA-3') were commercial sequenced. These primers were previously reported in other studies (Ward *et al*., 2005; Kamran *et al*., 2020).

The total volume of a PCR reaction contained 12.5µL PCR green Master mix, 0.5µL each primer with 1µL of DNA as template and final volume makeup using the double distilled deionized water. Amplification was performed using DNA Engine Tetrad 2 Peltier Thermal Cycler, BIO-RAD. The PCR amplification was performed using the following thermal cycling conditions: initial pre-denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min to ensure complete amplification of the PCR products.

Following PCR amplification, the products underwent purification prior to Sanger sequencing using the FavorPrep PCRCleanUpMiniKit (Cat. # FAPCK001-1). Sanger sequencing was performed in both forward and reverse direction to identify the selected species. The PCR amplicons were commercial sequenced from MACROGEN, Korea and finally, deposited to GenBank for accession number and public use.

Data Analysis and Blast annotation: Analysis and visualization of data were performed by using R software (Rstudio core team, 2021) and OriginPro 2021. Morphometric data were analyzed by using principal component analysis (PCA), and correlation. The *COI* sequences of samples were determined by arranging the sequences created through the forward and reverse primers to generate clean consensus sequences. The sequences were arranged and analyzed by BioEdit sequence alignment version 7.0.5.3. Other sequences were acquired from GenBank (http://www.ncbi.nlm.nih.gov/) and BOLD (Barcode of Life Data Systems, www.boldsystems.org) for verification and additional analysis. Pairwise genetic divergence between species, between species, and between genus and family members was computed using the Kimura 2-Parameter (K2P) method. MEGA 11 and the K2P model were used to create maximum likelihood (ML) trees with 1000 pseudo-duplicates plus genetic distances. The sequences were assigned GenBank accession numbers for each of the 60 *COI* sequences. DNASP 4.5 was used to estimate nucleotide diversity (π) and haplotype diversity (Hd) for each population. An online QR code generator (https://products.aspose.com/barcode/-) was used to create unique QR codes for the identification of carps.

RESULTS

Morphometric identification: In *Labeo rohita*, the dorsal region appears black while the sides take on a brownish hue upon preservation in ethanol. The fins exhibit dark gray coloration, and the iris rims display a striking red hue. Notably, the periphery of each scale is distinguished by a dark brown tinge (Fig.1A). *Cirrhinus mrigala* had a silvery ventral side and a dark gray to slightly greenish dorsal side. In a big species, the fin was pale orange in color. Compared to the abdomen, its dorsal profile was convex (Fig. 1B).

Principal component analysis: The morphometric and meristic characters were subjected to principal component analysis, which validated a considerable variability between two species sampled from selected locations of the River Chenab. Two principal components (PC1, 59.4% and PC2 27.2%) account for 86.6% of the total variance. *Cirrhinus mrigala* revealed a positive Eigen value and plotted towards the PC2 upper axis. *Labeo rohita* showed a negative Eigen value and plotted towards PC1 lower axis. AFR and PecFR were strongly related to each other and displayed a high positive Eigen value and plotted toward PC1 upper axis. FL and PsDL showed a strong negative association (Fig. 2).

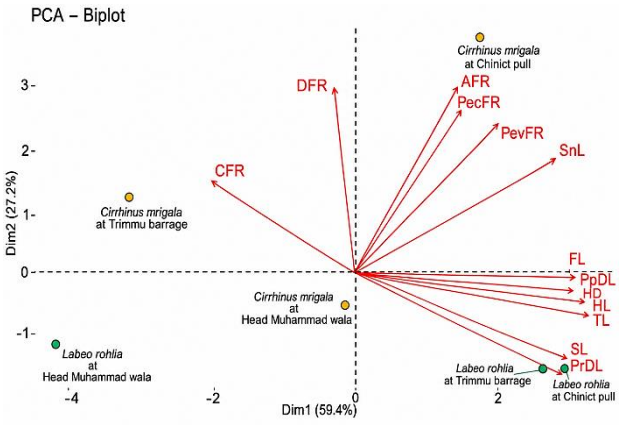


Fig. 2: Principal Component Analysis (PCA) biplot of morphometric and meristic characters for *Cirrhinus mrigala* and *Labeo rohita.*

Correlation matrix: The correlation matrix analysis revealed strong relationships among several morphometric traits of *L. rohita* and *C. mrigala.* Most morphometric characters showed significant positive correlations with each other, reflecting the expected covariation among body measurements associated with overall body size. Total length (TL) showed a strong positive correlation SL, HL, and PrDL suggesting that increases in overall body size are accompanied by proportional increases in these measurements. Several other morphometric variables such as snout length (SnL), Post dorsal length (PsDL), and Fork Length (FL) also showed moderate to strong positive correlations with major body measurements, indicating structural integration among these morphological features.

In contrast, some meristic traits exhibited trait weak or negative correlations. For instance, Dorsal fin rays (DFR), Caudal fin ray (CFR), showed negative correlations with several morphometric variables, suggesting that these meristic traits may vary independently of body size. These relationships provide valuable insights into the

morphological and meristic traits growth patterns of carp populations (Fig. 3). These relationships suggest that variation in these meristic characters was less closely associated with overall body size than the morphometric measurements. Because correlations among body measurements are expected to arise partly from common side effects, the correlation matrix was considered descriptive and was not interpreted as direct evidence of species discrimination. Species-level morphological differentiation was instead assessed using multivariate analysis.

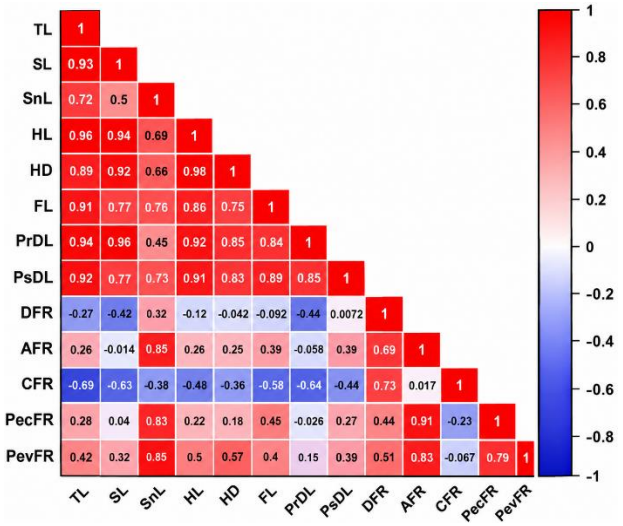


Fig. 3: Pearson correlation matrix between morphometric and meristic traits.

Clustered heat map: The Clustered heatmap was made for the detection of effect of different morphometric and meristic characters of *Labeo rohita* and *Cirrhinus mrigala*. S1L2 was strongly and positively influenced by CFR and S1L1 by AFR and PecFR (Fig. 4).

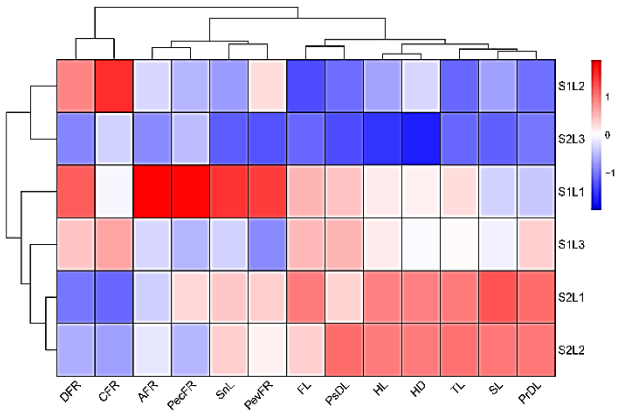


Fig. 4: Clustered heatmap among morphometric and meristic traits of both studied fishes (S) and locations (L).

Nucleotide frequency: In current study, 60 *COI* gene sequences were examined, amplified, and deposited into the BOLD and GenBank databases (Table 2). The sequences of mitochondrial *COI* gene of investigated specimens were aligned to create a final sequence ranged as 664 bp. No stop codon, deletions, or insertions were established in any of the aligned sequences. Blast analysis showed that the *COI* sequences obtained from the studied specimens shared high sequence identity (99-100%) with reference sequences available in public databases. The high identity values supported the assignment of the analyzed specimens to *L. rohita* and *C. mrigala* consistent with their morphological identification (Table 2). The observed BLAST identities were therefore interpreted as confirmation of species assignment rather than evidence of novel genetic signatures or substantial genetic differentiation.

Table 2: Accession No. and NCBI blast sequence results with percentage identity of selected carps

Query sequence ID and location	Accession No.	Query length	Percentage identity	E-Value	Query Cover
L. rohita L1	PP434517	658	100%	0.0	100%
L. rohita L1	PP434521	657	100%	0.0	100%
L. rohita L1	PP278380	642	100%	0.0	100%
L. rohita L1	PP434524	660	100%	0.0	100%
L. rohita L1	PP434530	656	100%	0.0	100%
L. rohita L1	PP434531	656	100%	0.0	100%
L. rohita L1	PP434532	661	100%	0.0	100%
L. rohita L1	PP434808	541	100%	0.0	100%
L. rohita L1	PP434811	563	100%	0.0	100%
L. rohita L1	PP437208	616	100%	0.0	100%
L. rohita L2	PP437248	610	100%	0.0	100%
L. rohita L2	PP437244	628	100%	0.0	100%
L. rohita L2	PP437267	590	100%	0.0	100%
L. rohita L2	PP437487	612	100%	0.0	100%
L. rohita L2	PP437480	602	100%	0.0	100%
L. rohita L2	PP495135	540	100%	0.0	100%
L. rohita L2	PP495150	620	100%	0.0	100%
L. rohita L2	PP495221	576	100%	0.0	100%
L. rohita L2	PP495256	598	100%	0.0	100%
L. rohita L2	PP495261	599	100%	0.0	100%
L. rohita L3	PP495311	550	100%	0.0	100%
L. rohita L3	PQ817734	552	100%	0.0	100%
L. rohita L3	PQ817735	550	100%	0.0	99%
L. rohita L3	PQ817737	510	100%	0.0	100%
L. rohita L3	PQ817738	422	100%	0.0	100%
L. rohita L3	PQ817740	444	100%	0.0	100%
L. rohita L3	PQ817739	407	100%	0.0	100%
L. rohita L3	PQ817750	418	100%	0.0	100%
L. rohita L3	PQ817747	404	100%	0.0	100%
L. rohita L3	PQ817748	401	100%	0.0	100%
C. mrigala L1	PQ821060	644	100%	0.0	100%
C. mrigala L1	PQ821062	524	100%	0.0	100%
C. mrigala L1	PQ821064	592	100%	0.0	100%
C. mrigala L1	PQ836514	455	100%	0.0	100%
C.mrigala L1	PQ826278	446	100%	0.0	100%
C. mrigala L1	PQ826279	512	100%	0.0	100%
C. mrigala L1	PQ826286	424	100%	0.0	100%
C. mrigala L1	PQ836515	469	99.36%	0.0	100%
C. mrigala L1	PQ836529	414	100%	0.0	100%
C. mrigala L1	PQ836545	493	100%	0.0	100%
C. mrigala L2	PQ836564	446	100%	0.0	100%
C. mrigala L2	PQ836565	359	100%	0.0	100%
C. mrigala L2	PQ836566	482	100%	0.0	100%
C. mrigala L2	PQ836567	482	100%	0.0	100%
C. mrigala L2	PQ836568	381	100%	0.0	100%
C. mrigala L2	PQ836570	511	100%	0.0	100%
C. mrigala L2	PQ836572	435	100%	0.0	100%
C. mrigala L2	PQ836573	386	100%	0.0	100%
C. mrigala L2	PQ836574	392	100%	0.0	100%
C. mrigala L2	PQ836575	360	100%	0.0	100%
C. mrigala L3	PQ836576	441	100%	0.0	100%
C. mrigala L3	PQ836577	353	100%	0.0	100%
C. mrigala L3	PQ836578	408	100%	0.0	100%
C. mrigala L3	PQ836579	464	100%	0.0	100%
C. mrigala L3	PQ836580	505	100%	0.0	100%
C. mrigala L3	PQ836582	468	100%	0.0	100%
C. mrigala L3	PQ836583	542	100%	0.0	100%
C. mrigala L3	PQ836584	584	100%	0.0	100%
C. mrigala L3	PQ836585	574	100%	0.0	100%
C. mrigala L3	PQ836586	583	100%	0.0	100%

E value: E value is the statistical significance of the alignment between query sequence and sequences in the database.

Analysis of nucleotide base composition: The frequencies of nucleotide were investigated on 60 sequences, comprising positions of all codons (1st, 2nd, 3rd) as well as noncoding areas. In each dataset nuclear positions were excluded by pairwise deletion. Mean value of total nucleotide bases for both investigated fishes were A (26.2%), T (28.3%), G (17.8%), C (27.7%). Mean AT (54.5%) was greater than GC (45.5%) contents in nucleotide base structure analysis of mitochondrial *COI* gene sequences (Fig. 5). Transversionsal (sv=16.82) were less than Transitional substitutions (si=83.16) having R (si/sv) ratio of 4.94 for the database (Table 3).

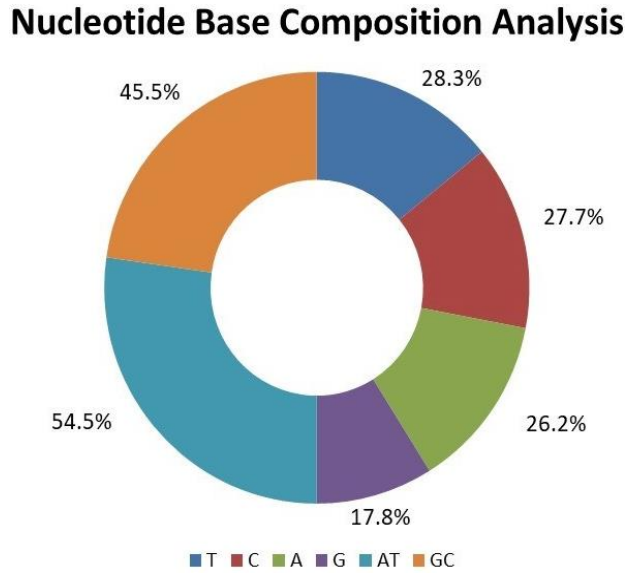


Fig. 5: Average Nucleotide Composition of *Labeo rohita* and *Cirrhinus mrigala.*

Table 3: The highest composite likelihood estimate of the nucleotide substitution pattern

	A	T	C	G
A	-	*2.38*	*2.16*	22.02
T	*2.18*	-	15.62	*1.69*
C	*2.18*	17.16	-	*1.69*
G	28.36	*2.38*	*2.16*	-

Transitional substitutions = bold, Transversionsal substitutions = *Italic*

Nucleotide pair frequency analysis: A nucleotide pair frequency analysis was done through Mega 11 software. Total 664 sites were noted. From the 664 sites 586 conserved (cs), 74 variable, 66 parsimony informative (pis) and 8 singleton (sv) sites were assessed (Fig.6).

DNA Sequence Polymorphism: For C. mrigala, analysis of 30 COI sequences revealed a single haplotype (H=1), with a haplotype diversity (Hd) of 0.00, indicating no detectable haplotype variation among the examined specimens. For L. rohita, the 30 analyzed COI sequences represented a haplotype (H)=1 and haplotype diversity (Hd) of 0.00. The results of both species indicate that no detectable haplotype variation was observed among the examined specimens within the analyzed COI region. Because no segregating variation was detected, Tajima's D and Fu and Li's D* and F* were not calculated for these species.

Evolutionary Analysis: The maximum likelihood analysis based on the *COI* sequences grouped the examined specimens according to their species identity. The Maximum Likelihood (ML)/ evolutionary tree was formulated by using 73 sequences (60 examined sequences and 13 reference sequences from NCBI) through Mega 11 software. The branch length of tree was 0.1 with 1000 bootstrap duplicates and *Sperata seenghala* as outgroup to provide a phylogenetically distinct reference for rooting the tree. It was selected because it belongs to the order Siluriformes, whereas the focal species *L. rohita* and *C. mrigala*, belong to order Cypriniformes. Thus, *S. seenghala* represents a taxonomically distinct lineage outside the focal cyprinid group and provides an appropriate external reference for interpreting the branching pattern of the analyzed sequences. This analysis clearly divided the carps into three distinct clades on the basis of relationship among fishes.

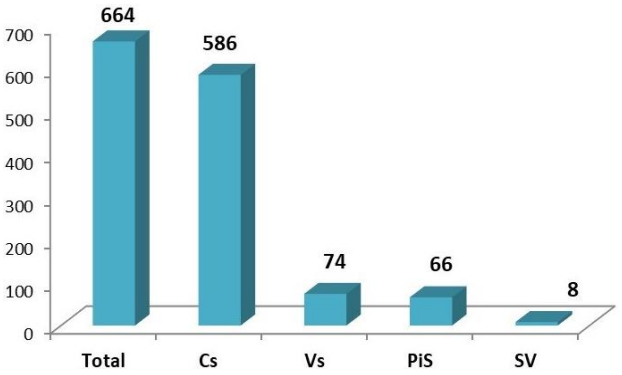


Fig. 6: Nucleotide pair frequency analysis of Labeo rohita and Cirrhinus mrigala based on COI gene sequences. The Y-axis represents the number of nucleotide sites [Conserved sites (cs), Variable sites (Vs), parsimony informative sites (Pis), Singleton Variable sites (SV)].

Distinct species resided in distinct nodes, but the same species were grouped together in one node. All specimens of *L. rohita* including reference sequences formed one clade having 99% bootstrap value, *C. mrigala* in another clade with 99% bootstrap. It is genetically distant enough to serve an external reference for rooting the tree. The outgroup of *S. seenghala* showed a separate clade and these observations disclosed meaningful perceptions into the phylogenetic history and genetic relationships among the carps, as examined by *COI* sequence analysis (Fig. 7). The *COI* phylogeny therefore supported species-level discrimination of the examined specimens and demonstrated the utility of the marker for DNA barcoding.

QR code generation: As a supplementary practical application, the *COI* barcode sequences generated in the present study were converted into QR code representations. The QR codes provide a conventional digital format for storing and sharing the sequence information associated with the two examined carp species. This application was intended as a simple information assessment tool rather than as an independently validated species-identification system. No field validation, database integration, diagnostic accuracy testing or comparison with established digital fish identification platforms was performed. Therefore, the QR code component is presented only as a supplementary demonstration of how the generated barcode information could be digitally represented and accessed (Fig. 8).

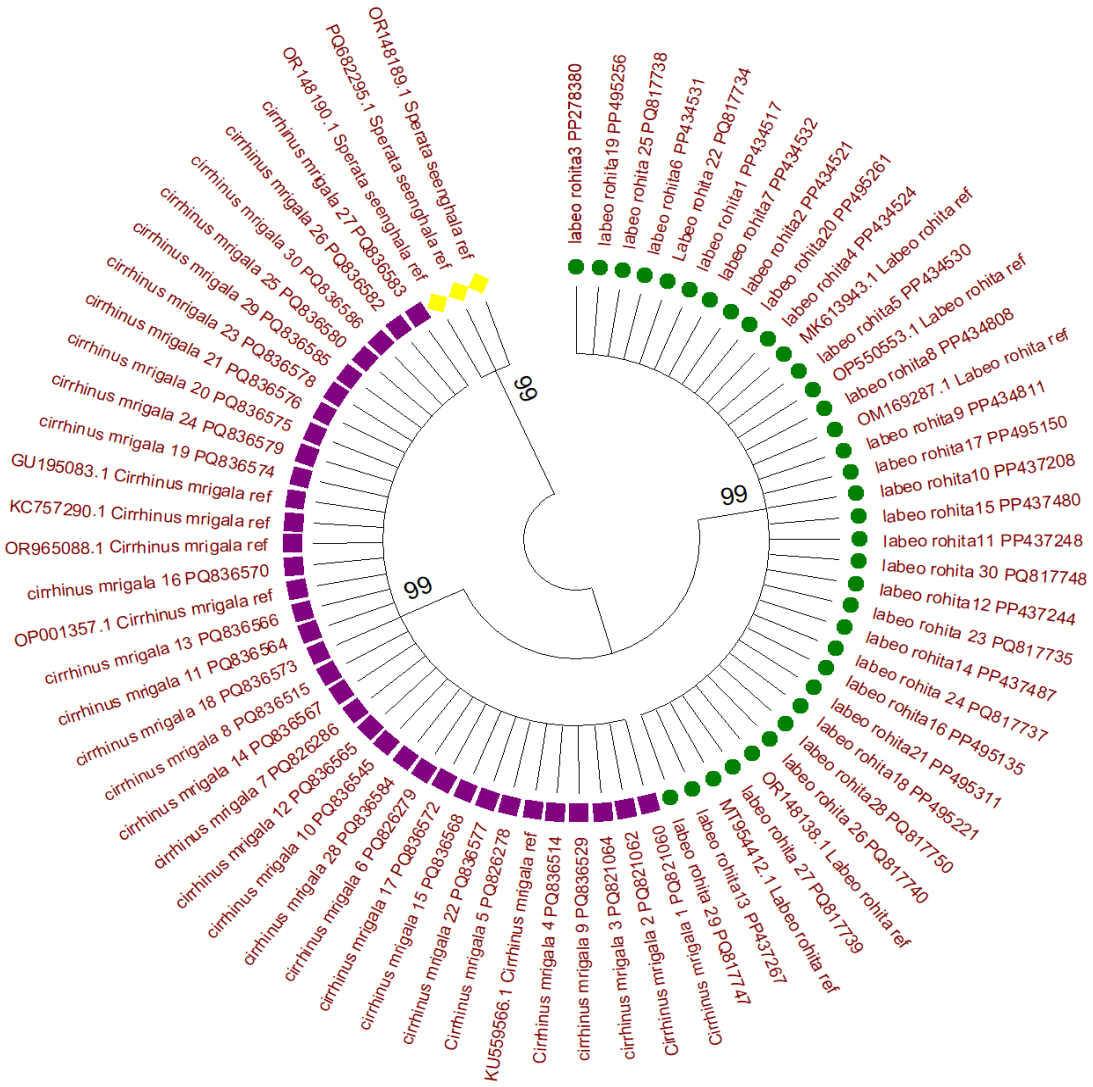


Fig. 7: Phylogenetic tree based on *COI* sequences from *Labeo rohita* (green circles), *Cirrhinus mrigala* (purple squares), and *Sperata seenghala* (yellow diamonds) with reference sequences from GenBank illustrating the evolutionary relationships.



Labeo rohita *Cirrhinus mrigala*

Fig. 8: DNA-Based QR Codes for Carps Identification.

DISCUSSION

DNA barcoding, a powerful tool for species identification, generates genetic "barcodes" for species across different geographic regions. Factors such as genetic drift, ecological pressures, and adaptive development drive population diversification and the emergence of new species, resulting in genetic and physical variations among

species from distinct areas. The current study provided a molecular perspective on fish biodiversity, conservation, and new genetic data for carps (*L. rohita* and *C. mrigala)* from the River Chenab (Chiniot Pull (L1), Trimmu Barrage (L2), and Head Muhammad Wala (L3) in Punjab.

In current study, sum of 90 samples including *L. rohita* (N=45) and *C. mrigala* (N=45), were collected from both upstream and downstream locations, namely Chiniot Pull (L1), Trimmu Barrage (L2), and Head Muhammad Wala (L3) from the River Chenab, Pakistan. At taxonomic level, samples were identified by measuring Total Length (TL), Standard Length (SL), Snout Length (SnL), Head Length (HL), Head Depth (HD), Fork Length (FL), Pre Dorsal Length (PrDL), and Post Dorsal Length (PsDL), Dorsal fin ray (DFR), Anal fin ray (AFR), Caudal fin ray (CFR), Pectoral fin ray (PecFR) and Pelvic fin ray (PelFR). The Principal Component Analysis (PCA) identified the key variables contributing most of the variance highlighting the morphometric and meristic variables. Total PC1 and PC2 were observed as 86.6%. All of these factors' results agreed with the early researchers' findings of Hossain *et al.* (2010) who studied Landmark-based morphometric and meristic variations of the endangered carp, kalibaus (*L. calbasu*); Mir *et al*., 2013, who assessed *L. rohita*'s stock structure of Ganga basin (India) and Zafar *et al.* (2024b), who examined the morphological variations of the family Cyprinidae based on landmarks in River Indus, Pakistan.

Correlation matrix analysis revealed strong relationships among several morphometric traits of *L. rohita* and *C. mrigala.* Most morphometric characters showed significant positive correlations with eachother, such as TL showed a strong positive correlation with SL, HL, and PrDL suggesting that increases in overall body size are accompanied by proportional increases in these measurements. These findings are consistent with previous studies that reported significant relationships among morphometric characters in different freshwater fishes, suggesting that these measurements play an important role in describing phenotypic variation and population structure (Rahman *et al*., 2019).

The similarity in external morphology among freshwater carps (*L. rohita* and *C. mrigala*) species makes it difficult to distinguish between them. In the present study, an examination of morphometric characters revealed no distinct traits that could be used to differentiate the species accurately. This complexity in evolutionary changes, both morphological and physiological, has led to controversies in phylogenetic trees constructed on morphology alone. Recent advances, however, have seen the introduction of mitochondrial DNA (mtDNA) genetic analysis to resolve these taxonomic controversies (Erguden *et al*., 2010).

DNA barcoding, which has recently attracted attention as a highly effective method of species identification, especially for fish, uses the *COI* gene as a marker (Knebelsberger *et al*., 2015). Because of its distinctive variation pattern, which demarcates evident divergence and paucity of overlapping area between genetic distances within and between species, *COI* was chosen as a standard barcode gene (Hebert *et al*., 2003).

Generally, average genetic distance between species was noticed as 0.044. Mean distances between two species calculated as 0.00-0.086, and this gap expands by the expansion to taxonomic level. Present observations counterpart with findings of Indian freshwater fishes having distance of 0.8 within species, 9.06 within genus and 15.35 within family (Modeel *et al*., 2024). The present observations also corroborate with the study of Marnis *et al*. (2024), who obtained similar results with fish diversity of Batanghari River in Indonesia. Freshwater fishes of the Poso River, Indonesia showed genetic distance range from 0.038 to 0.065 (Wibowo *et al*., 2025).

No apparent stop codons, insertion or deletions were identified in present study, supporting their suitability for DNA barcoding analysis and the average length of consensus sequences in barcode was 664 base pairs. The mean nucleotide base composition of the species of carp fish that had been studied was GC 45.5 and AT 54.5%. In general, the average GC content was less than the average AT content (Fig. 5). The same average composition of nucleotide bases was found in *Wallago attu* (Sajjad *et al*., 2023). The analysis of the base composition of freshwater fishes in the Beas River revealed the same as the outcomes of the present research (Modeel *et al*., 2024). The obtained mean frequencies of the nucleotides were like those previously obtained with GC 35.74 and AT 54.22 that were found in freshwater fish in Enugu and Anambra states of Nigeria (Ude *et al*., 2020).

The observed Transitional substitutions (si=83.16) were higher than transversionsal (sv=16.82) having R (si/sv) ratio of 4.94 for the database (Table 3). Comparable to findings in *Chana striata*, the detected transitional mutations were ubiquitous than the transversional values (Boonkusol and Tongbai, 2016) and the labeo genus (Zafar *et al*., 2024a). Similarly, rate of transitional substitutions observed greater (52.25) than the transversional substitutions (47.74) in *Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis* in Bangladesh (Afrin *et al*., 2024).

The Nucleotide pair frequency analysis was detected through the Mega 11 software. Total 664 sites were observed. Out of 664 sites, 586 conserved(cs), 74 variable(vs), 66 parsimony informative(pis) and 8 singleton(sv) have been detected (Fig. 6). Out of the analyzed sites, 150 were constant, while 470 presented variability, along with 251 parsimony informative and 219 singletons within the fishes of the brackish water lake in South Asia (Agneeswaran *et al*., 2023). This finding supports the flying fish barcoding research according to Rathipriya *et al*. (2019).

The Haplotype analysis of sequences consisting of both investigated carps separately for *L. rohita* and *C. mrigala* showed haplotype (H=1), haplotype diversity (Hd=0.00), and nucleotide diversity (Pi=0.00). The computation results of freshwater fishes of Nigeria agreed well with the current study (Ude *et al*., 2020). Tajima's D test was reported as 4.303 ($P<0.001$). A high level of selection and little population growth within the species under study were indicated by a positive result for D. For this reason, the study examined the selective neutrality hypothesis. The findings are like the freshwater genus Labeo in the River Indus as reported by Zafar *et al*. (2024a). Larson *et al*., 2016 reported 33 haplotypes and 149 polymorphic sites in 83 samples of four species of *Pacifastacus.* Similarly, 36 haplotypes and 56 polymorphic sites were identified from 43 sequences of *Steindachneridion scriptum* (Paixao *et al*., 2018).

To better comprehend the evolutionary links between species are essential for investigating their origins, diversification, and ecological interactions an evolutionary tree was built with the use of K2P. With 99 bootstrap values, the tree's topology showed three main clades, making it evident that these two carps are members of the same family (Fig. 7). While an out-group appeared in a different clade, it indicated that it belonged to a different ancestor. Similar findings were investigated in a few earlier studies (Ude *et al*., 2020; Lyiola *et al*., 2018; Zafar *et al*., 2024a). The *COI*-based phylogenetic analysis provided clear species-level clustering of the examined *L. rohita* and *C. mrigala* specimens, supporting the morphological identification and sequence-based alignments. These findings demonstrate the usefulness of *COI* as a DNA-barcoding marker for distinguishing the two examined carp species.

QR codes have grown in popularity as a quick and easy way to store and share information. In recent years, QR codes' adaptability has spread to scientific research, where they are used to encode complex data such as genetic sequences. This study leveraged the versatility of QR codes to encode DNA sequences for the precise identification of species. These DNA based QR codes of studied species were generated from the selected river (Fig. 8) of the study area. Previously, similar QR codes were created by Yang *et al*. (2019) Sajjad *et al*. (2023) and Zafar *et al*. (2024a).

Fish diversity is being threatened on a global scale (Tickner et *al*., 2020). Many inorganic chemicals, pesticides, and other toxic materials alter the genetic composition of fishes (Orun *et al*., 2014). Organic waste and other human activities have the potential to seriously harm the aquatic ecology (Guzel *et al*., 2022). For this reason, each year the number of biologically significant species decreases (Tickner *et al*., 2020). Thus, DNA barcoding is a flexible and precise method for managing fish resources, evaluating fish biodiversity, and promoting fish protection (Jaonalison *et al*., 2022).

Unfortunately, the Chenab River's fish diversity has lately decreased in some areas for a number of causes. The recent floods have also led to an imbalance in the variety of fish. Numerous fish species have been studied and used in Pakistan for a variety of reasons, including food, medicine, ornament, and laboratory study. Conventional fish management monitoring is typically costly and time-consuming. Nonetheless, barcode databases are becoming more effective and useful for misidentification in the conservation and evaluation of fish variety. The River Chenab was the site of a molecular study for the conservation of the fishes under study, and the data from this area will be easier to access than before.

Conclusions: DNA barcoding provided molecular evidence supporting the species-level identification of *L. rohita* and *C. mrigala* from the sampled locations along the River Chenab, Pakistan. The *COI* based identification was consistent with the morphological identification of the studied carps, supporting the use of DNA barcoding as a complementary approach to morphological and meristic methods. The present study was not designed to assess population abundance, recruitment, effective population size, habitat degradation, fishing pressure, disease status or temporal changes in genetic variation; therefore, the findings should not be interpreted as direct evidence for conservation status or fishing management strategies. Instead, the *COI* sequences generated in this study provide baseline molecular reference information for the two examined carp species from the River Chenab. These data may support future investigations involving broader geographic and temporal sampling, population-level genetic assessment, species authentication and evidence-based conservation research.

Ethical statement: The study was approved by the Ethical Review Committee (ERC) of Government College University Faisalabad on animal experimentation with Ref. No. GCUF/ERC/24/038.

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