

## DNA Barcoding of Eight Freshwater Fish Species (Order Siluriformes) From the River Ravi, Pakistan

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## Abstract

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## Keywords

COI gene, DNA Barcoding, Fish species, Siluriformes.

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## RESEARCH PAPER

# DNA Barcoding of Eight Freshwater Fish Species (Order Siluriformes) From the River Ravi, Pakistan

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## Abstract

Pakistan's freshwater bodies host significant fish species, but molecular identification is still in its infancy, necessitating the use of morphological and molecular DNA barcoding techniques. The study confirmed the species of the order Siluriformes found in the River Ravi, Pakistan, using DNA extracted from muscle tissue, sequenced for the COI gene, and obtained accession and Barcode Index Numbers from GenBank and BOLD databases. In the BLAST search, identities ranged from 99.23 to 100% and 99.52 to 100% in the GenBank and BOLD databases, respectively. The study revealed that the Kimura 2-Parameter genetic distance increased from lower to higher taxonomic levels: within species (0.00%) < within genus (15.17%) < within family (19.77%). The study estimated average nucleotide differences (104.964) and nucleotide diversity (0.16099) but found Tajima's neutrality test to be statistically insignificant. The neighbor-joining tree displayed closely linked species under a node, whereas divergent species were grouped under distinct nodes. The COI gene-based DNA barcoding aids in Pakistan's fish resource inventory, monitoring, and management, providing valuable input for traditional methods.

**Keywords:** COI gene, DNA barcoding, Fish species, Siluriformes

## 1. Introduction

Catfishes (Order: Siluriformes) are a diverse group of ray-finned fishes [1], distributed across continents [2], comprising about 4,200 valid species belonging to 450 genera under 41 families [3]. Most catfish are freshwater species. Their wide distribution and variation make them useful models for ecologists, evolutionary biologists and biogeographical researchers [4].

Ichthyologists have explored the fish fauna throughout the world. Accurate species identification has a significant impact on fisheries conservation and management through biological checks, assessment of ecological factors, control of illegal trading, systematization of fish resources and

shielding of fish habitats [5]. Taxonomists, generally identify and describe fishes through different taxonomic keys based on their morphological features [6]. These traits, however, are insufficient to identify every species due to a lack of morphological distinguishing characteristics. Taxonomic keys are only useful at specific life stages or genders. Accurate fish recognition highly challenging, especially in commercial practices, when fish are present in fillet form, and throughout the early developmental phases (when morphological features are incomplete) of fish. Such scenarios require an alternative technique for the identification of fishes. In this regard, DNA-based identification approaches (DNA Barcoding) have primarily been developed in the last decade as an alternative technique [7].

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The idea of DNA barcodes postulates that every species contains specific mitochondrial cytochrome c oxidase subunit I (COI) gene sequences, like intrinsic species tags, that may be generated by sequencing of at least 650 bp area COI [8]. DNA barcoding helps to validate the species which aids in the understanding of distribution patterns and conservation determinations. This molecular approach does not directly mitigate habitat degradation or pollution; it provides evidence-based baselines that are important for habitat protection and sustainable management of ecosystems [9]. It is applied equally as an identification tool to authenticate the species of an organism at any life stage (egg to adult) [10] and for forensic analysis and evaluating the effect of environmental changes and habitat loss on species distribution [11]. It has provided reliable results with high resolution for fish up to the species level [12] and has also been useful for the identification of species in various organism groups as reported in spiders from University of Alberta [13], mosquitoes from Canada [14], and bats from Guyana [15]. Interspecific distance should be larger than intraspecific divergence in a good barcode, which is helpful to differentiate the closely related species as well as cryptic species [16]. This technique has been used in several successful studies on fish fauna, especially in freshwater fisheries [17].

Many researchers have reported the fish fauna from Pakistan and described 193 freshwater fish species from Pakistan [18]. Their studies were based on a traditional morphological identification method. Very little work on DNA barcoding of fish has been reported in literature from Pakistan. The present study planned to identify fish species of multiple families of the order Siluriformes through a molecular approach (DNA barcoding) using the universal marker COI gene to resolve morphology-based taxonomic ambiguities and establish a reference library that will strengthen regional databases. This reference library will help in fisheries surveys and evolutionary studies in the future.

## 2. Materials and Methods

### 2.1. Sampling sites and collection

The River Ravi is one of the tributaries of the Indus River system. The fish specimens were collected from seven different stations, ranging from Ravi Syphon (31° 72' N and 74° 46' E) to Head Balloki (31° 22' N and 73° 89' E) located on the river Ravi, in Punjab, Pakistan. A variety of cast nets were used during sampling with the assistance of

professional fishermen. The specimens were identified based on morphological characteristics using a local taxonomic key as described earlier [19] and subsequently preserved in 95% ethanol for molecular identification.

### 2.2. Molecular based identification

One specimen of each species was selected as a voucher specimen for molecular based identification of species by choosing the mitochondrial gene COI. The DNA was extracted from muscle tissue using vertebrate DNA extraction methods according to Ivanova *et al.* (2006) [20]. Amplification of the COI gene was carried out using primers designed by Ivanova [21] as follows:

C\_Fish-F1t1: <sup>5</sup>TGTAACGACGGCCAGTCGACTAATCA TAAAGATATCGGCAC<sup>3</sup>

C\_FishR1t1: <sup>5</sup>CAGGAAACAGCTATGACACTTC AGGGTGACCGAAGAATCAGAA<sup>3</sup>,

The PCR products were visualized using a precast 2% agarose gel from the Invitrogen™ system and software and sequenced using M13F Tailed (Forward) and M13R Tailed (Reverse) at the Canadian Center for DNA Barcoding (CCDB). The sequences were aligned to acquire pure sequences and trace files were prepared. The voucher specimen details, including sampling ID, code, location (latitude, longitude, and elevation above sea level), date, taxonomy, and images of the specimens were deposited for DNA barcoding in the GenBank and BOLD system (<https://id.boldsystems.org/>), to obtain the accession number and Barcode Index Number (BIN). BINs were assigned when meeting compulsory quality standards (>500 bp, <1% Ns, no stop codon or contamination flag) [22].

### 2.3. Statistical and sequences analysis

Morphological parameters were evaluated using Origin software through one-way ANOVA ( $p < 0.05$ ). BLAST searches were conducted using FASTA format sequences for each sample to identify highly similar sequences on GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the BOLD ID engine (<https://id.boldsystems.org/>). Sequence analysis was conducted using analytical tools available in the BOLD system, DnaSP 6 [23], and MEGA X software [24]. The Kimura two-parameter (K2P) distance model [25] was applied to compute the genetic distance within families, genera and species for COI. The neighbor-joining (NJ) analysis [26] was applied to generate a phylogenetic tree.

#### 2.4. Development of Quick Response (QR) codes based on COI gene sequences

A Quick Response (QR) code is a two-dimensional barcode that can be easily read using cell phone applications. This two-dimensional barcode has meaningful information more than 4000 characters in both directions, horizontal as well as vertical. DNA barcodes were developed for each of the current species using COI gene sequences through an online QR code generator.

### 3. Results

The morphometric and meristic features of each specimen were recorded, and a total of 37 specimens were identified belonging to the order Siluriformes, 5 families, 6 genera and 8 species “Fig. 1”. The typical ratios of head length to standard length, eye diameter to head length, and snout length to head length were determined “Table 1”.

All morphologically identified species were authenticated through DNA barcoding by amplification and sequencing of the partial 5' region of the mitochondrial gene (COI). The amplified sequences were 652 bp in length with no deletions, insertions, or stop codons. The results of BLAST showed 99.23 to 100% similarity in the GenBank and 99.52 to 100% similarity in the BOLD database “Table 2-3”. Molecular identification matched with the morphological feature-based identification of species. The sequences were deposited to BOLD and GenBank with all details of specimens and obtained Barcode Index Number (BIN) from BOLD “Table 2” and the accession number from NCBI GenBank “Table 3”.

All the present species were also differentiated based on genetic divergence of mitochondrial gene (COI) sequences. A summary of sequence divergence by applying Kimura 2-Parameter (K2P)

model at each taxonomic level showed that the genetic divergence increased from lower to higher taxonomic levels; within species (0.00%), within genus (15.17%, SE  $\pm$  0.26) and within families (19.77%, SE  $\pm$  0.22). In the present study, the variation in number of comparisons (n) is associated with pairwise sequence comparisons. It is not based on the number of species, genera or families “Table 4”. The minimum mean K2P generic divergence  $\pm$  SE (0.143  $\pm$  0.16) was found among *Mystus cavasius* and *M. bleekeri* and the maximum (0.214  $\pm$  0.020) among *M. vittatus* and *Heteropneustes fossilis* with the average genetic distance for entire data was computed (0.185  $\pm$  0.010) “Table 5”.

The mean GC%  $\pm$  SE was computed to be 44.69  $\pm$  0.31 and the highest 57.20  $\pm$  0.39 was recorded at codon position 1 “Table 6”. The invariable sites (431), variable sites (221), total number of mutations (305), singleton variable sites (75) and parsimony informative sites (146) were found. The transition/transversion bias (R) was projected to be 2.33. The K2P model was used to assess the substitution patterns and rates. Kimura [27] used the Kimura-2-parameter model to assess substitution patterns and rates. For this computation, the highest log likelihood was -2982.516.

The average number of pairwise nucleotide differences (k) was found to be 104.964, nucleotide diversity (Pi) 0.16099 with haplotypes (h) 8, gene diversity (Hd) 1.000, and variance of haplotype diversity 0.00391 [28]. The value of Tajima's neutrality test D (-0.59064, P > 0.10) [29], Fu and Li's D\* (-0.10707, P > 0.10) and Fu and Li's F\* test statistic values (-0.24638, P > 0.10) were estimated [30] and found statistically not significant.

A Neighbor-joining tree was constructed from the complete COI sequence dataset of the current species with a 1000 bootstrap value “Fig. 2”. Each



Fig. 1. The images of sampled fish species submitted to BOLD database.

Table 1. Mean  $\pm$  standard deviations of morphological characters of sampled fish species.

Species	TL	SL	FL	HDL	ED	SNL	HDL/SL%	ED/HDL%	SNL/HDL%
<i>Mystus cavasius</i>	17.27 $\pm$ 1.56	13.77 $\pm$ 0.864	12.53 $\pm$ 0.872	2.8 $\pm$ 0.39	0.78 $\pm$ 0.1	0.13 $\pm$ 0.05	22.42 $\pm$ 0.78	28.04 $\pm$ 1.84	4.83 $\pm$ 2.07
<i>Mystus bleekeri</i>	13.5 $\pm$ 0.691	11.27 $\pm$ 0.526	10.26 $\pm$ 0.483	2.58 $\pm$ 0.45	0.57 $\pm$ 0.14	0.15 $\pm$ 0.08	25.19 $\pm$ 1.5	21.90 $\pm$ 3.68	6.13 $\pm$ 3.67
<i>Mystus vittatus</i>	9.53 $\pm$ 0.624	8.14 $\pm$ 0.536	7.29 $\pm$ 0.531	2.06 $\pm$ 0.24	0.2 $\pm$ 0.00	0.2 $\pm$ 0.00	25.73 $\pm$ 1.15	9.82 $\pm$ 1.19	9.82 $\pm$ 1.19
<i>Sperata seenghala</i>	37.23 $\pm$ 1.57	28.33 $\pm$ 2.076	30.3 $\pm$ 2.330	27.43 $\pm$ 0.7	11.6 $\pm$ 0.19	10.3 $\pm$ 0.17	32.89 $\pm$ 19.91	51.19 $\pm$ 10.31	64.58 $\pm$ 3.31
<i>Heteropneustes fossilis</i>	7.8 $\pm$ 1.033	6.9 $\pm$ 0.427	—	1.28 $\pm$ 0.16	0.2 $\pm$ 0.00	0.1 $\pm$ 0.00	18.58 $\pm$ 0.63	15.83 $\pm$ 1.97	—
<i>Pachypterus atherinoides</i>	6.3 $\pm$ 0.00	5.1 $\pm$ 0.00	5.6 $\pm$ 0.00	0.9 $\pm$ 0.14	0.25 $\pm$ 0.07	0.2 $\pm$ 0.00	21.18 $\pm$ 1.69	12.59 $\pm$ 3.29	16.71 $\pm$ 2.6
<i>Eutropiichthys vacha</i>	18.5 $\pm$ 0.00	16.00 $\pm$ 0.000	14.0 $\pm$ 0.00	3 $\pm$ 0.00	0.7 $\pm$ 0.00	0.3 $\pm$ 0.00	20.41 $\pm$ 0.00	23.33 $\pm$ 0.00	10.0 $\pm$ 0.00
<i>Ompok bimaculatus</i>	24.2 $\pm$ 0.00	20.5 $\pm$ 0.00	21.4 $\pm$ 0.00	3.7 $\pm$ 0.00	0.8 $\pm$ 0.00	0.2 $\pm$ 0.00	18.05 $\pm$ 0.00	21.62 $\pm$ 0.00	5.41 $\pm$ 0.00

TL = Total length, SL= Standard Length, FL= Fork Length, HDL= Head length, ED = Eye Diameter, SNL = Snout Length.

Table 2. Barcode index numbers and identification results of COI sequences of sampled fish species on BOLD database.

Sample ID	Species	Process ID On BOLD	BIN issued by BOLD	COI-5P Seq. Length	Overlap (bp)	Match (%)	Match Species	Sample ID
PUL-HMA-Fish-745	<i>Mystus cavasius</i>	HMA045-22	BOLD:ADX0539	652	479	100	<i>Mystus cavasius</i>	BOLD:ADX0539
PUL-HMA-Fish-746	<i>Mystus bleekeri</i>	HMA046-22	BOLD:AAZ9696	652	476	100	<i>Mystus bleekeri</i>	BOLD:AAZ9696
PUL-HMA-Fish-747	<i>Mystus vittatus</i>	HMA047-22	BOLD:AAZ3611	652	480	99.85	<i>Mystus vittatus</i>	BOLD:AAZ3611
PUL-HMA-Fish-749	<i>Sperata seenghala</i>	HMA049-22	BOLD:AAZ3699	652	476	100	<i>Sperata seenghala</i>	BOLD:AAZ3699
PUL-HMA-Fish-750	<i>Heteropneustes fossilis</i>	HMA050-22	BOLD:ACR4875	652	490	100	<i>Heteropneustes fossilis</i>	BOLD:ADZ7088
PUL-HMA-Fish-751	<i>Pachypterus atherinoides</i>	HMA051-22	BOLD:ABX1827	652	480	99.52	<i>Pachypterus atherinoides</i>	BOLD:ABX1827
PUL-HMA-Fish-752	<i>Eutropiichthys vacha</i>	HMA052-22	BOLD:ABX1828	652	482	99.85	<i>Eutropiichthys vacha</i>	BOLD:ABX1828
PUL-HMA-Fish-753	<i>Ompok bimaculatus</i>	HMA053-22	BOLD:AAA9421	652	481	100	<i>Ompok bimaculatus</i>	BOLD:AAA9421

Table 3. Accession numbers and identification results of COI sequences of sampled fish species on NCBI GenBank.

Species	GenBank accession #	Query Cover (%)	Similarity (%)	Matched Species	Matched accession #
<i>Mystus cavasius</i>	OP575615	100	99.23	<i>Mystus cavasius</i>	KX946715.1
<i>Mystus bleekeri</i>	OP575613	100	100	<i>Mystus bleekeri</i>	MK029805.1
<i>Mystus vittatus</i>	OP575616	100	99.85	<i>Mystus vittatus</i>	KX177968.1
<i>Sperata seenghala</i>	OP575633	100	100	<i>Sperata seenghala</i>	KT364786.1
<i>Heteropneustes fossilis</i>	OP575596	99	100	<i>Heteropneustes fossilis</i>	GQ466395.1
<i>Pachypterus atherinoides</i>	OP575621	97	99.53	<i>Pachypterus atherinoides</i>	JN628909.1
<i>Eutropiichthys vacha</i>	OP575592	100	99.85	<i>Eutropiichthys vacha</i>	MK572196.1
<i>Ompok bimaculatus</i>	OP575617	100	100	<i>Ompok bimaculatus</i>	JX983413.1

Table 4. Genetic distance of fish species within taxa.

Label	n	Taxa	Comparisons	Min. Dist. (%)	Max. Dist. (%)	Mean Dist. (%) ± SE
Within species	0	0	0	0.00	0.00	0.00 ± 0.00
Within genus	3	1	3	14.31	16.17	15.17 ± 0.26
Within Family	4	1	3	18.87	20.46	19.77 ± 0.22

Table 5. Evolutionary divergence among sampled fish species.

Species	1	2	3	4	5	6	7	8
1. <i>Mystus cavasius</i>								
2. <i>Mystus bleekeri</i>	0.143							
3. <i>Mystus vittatus</i>	0.162	0.150						
4. <i>Sperata seenghala</i>	0.200	0.189	0.205					
5. <i>Heteropneustes fossilis</i>	0.212	0.191	0.214	0.205				
6. <i>Pachypterus atherinoides</i>	0.169	0.146	0.174	0.170	0.193			
7. <i>Eutropiichthys vacha</i>	0.188	0.188	0.210	0.201	0.173	0.179		
8. <i>Ompok bimaculatus</i>	0.180	0.187	0.202	0.207	0.180	0.183	0.172	

species that shared a similar morphology or closely related was grouped under the same node in the tree that represented the evolutionary relationship, whereas distant species were grouped under other nodes. The species belonging to the same family were under the closeness cluster, while other families were under different clusters. *Sperata seenghala* clustered in a separate node from other species of family Bagridae, as it showed more distant divergence (0.189 to 0.205). *H. fossilis* and *Eutropiichthys vacha* clustered under the same node expressed that Heteropneustidae and Schibeidae were closely

related families. The family Horabagridae is distantly related, as *Pachypterus atherinoides* bunched distinctly with respect to the species of other family fish species. The tree discriminated the species as genetic divergence decreased from higher to lower taxonomic levels. The reference sequences of *Labeo rohita* belonging to the order Cypriniformes were downloaded from NCBI GenBank with accession numbers (MH102309) and considered as the outgroup. The outgroup clustered separately with respect to the current species of the order Siluriformes.

Table 6. Nucleotides composition in sequences of sampled fish species.

Specie	G %	C %	A %	T %	GC %	GC % at 1st Codon	GC % at 2nd Codon	GC % at 3rd Codon
<i>Mystus cavasius</i>	17.64	26.69	25.31	30.37	44.33	56.68	42.86	33.49
<i>Mystus bleekeri</i>	17.02	27.61	25.77	29.6	44.63	58.06	42.86	33.03
<i>Mystus vittatus</i>	18.1	28.07	23.77	30.06	46.17	56.68	42.86	38.99
<i>Sperata seenghala</i>	18.25	26.23	25.46	30.06	44.48	55.76	42.86	34.86
<i>Heteropneustes fossilis</i>	17.18	25.92	26.53	30.37	43.1	57.6	42.86	28.9
<i>Pachypterus atherinoides</i>	17.48	26.69	26.23	29.6	44.17	56.22	42.86	33.49
<i>Eutropiichthys vacha</i>	18.71	26.53	25.61	29.14	45.25	57.14	42.86	35.78
<i>Ompok bimaculatus</i>	18.1	27.3	26.23	28.37	45.4	59.45	42.86	33.94
Min.	17.02	25.92	23.77	28.37	43.1	55.76	42.86	28.9
Max	18.71	28.07	26.53	30.37	46.17	59.45	42.86	38.99
Mean ± SE	17.81 ± 0.19	26.88 ± 0.24	25.61 ± 0.28	29.70 ± 0.23	44.69 ± 0.31	57.20 ± 0.39	42.86 ± 0.00	34.06 ± 0.94

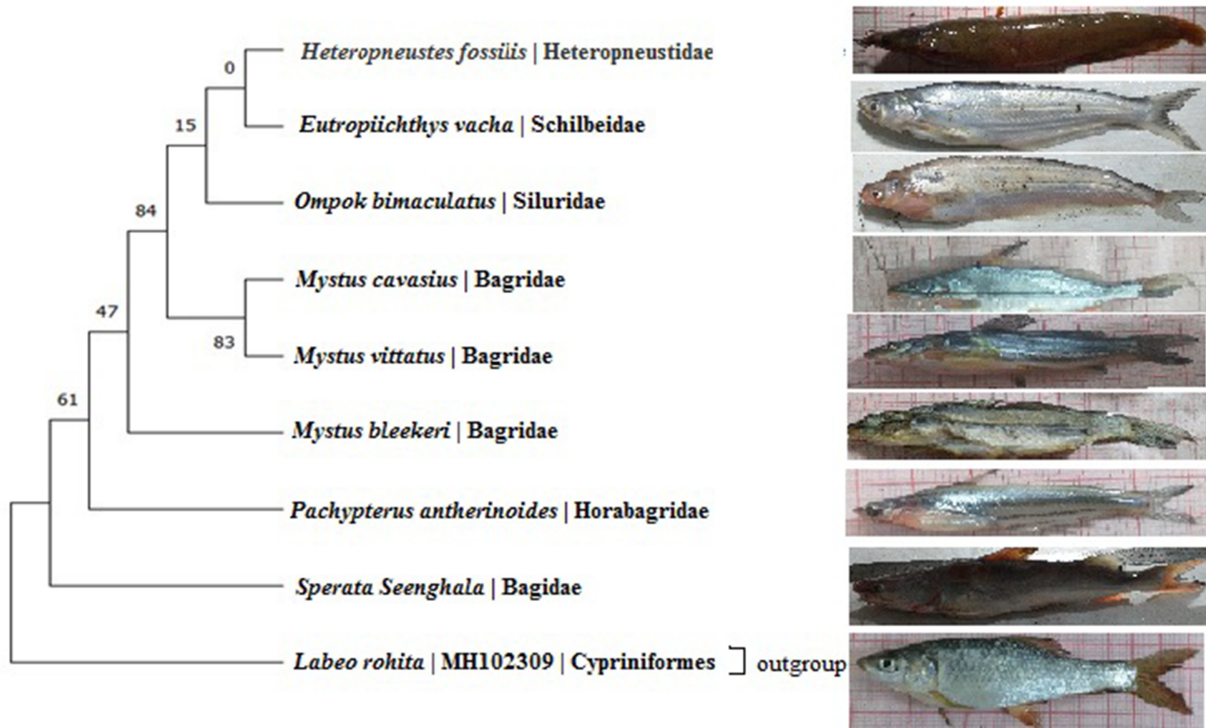


Fig. 2. Neighbor-joining phylogenetic relationship of fish species of order Siluriformes with *Labeo rohita* (accession numbers: MH102309) order Cypriniformes as an outgroup by using MEGA X software.

In the present study QR codes were generated and named at the molecular level based on DNA sequences for the discrimination of current species. These codes could be scanned by online or cell phone applications “Fig. 3”.

#### 4. Discussion

The study identified 37 fish specimens, including 8 species from the order Siluriformes, 5 families, and 6 genera, based on morphological characteristics. The study of morphological characteristics is

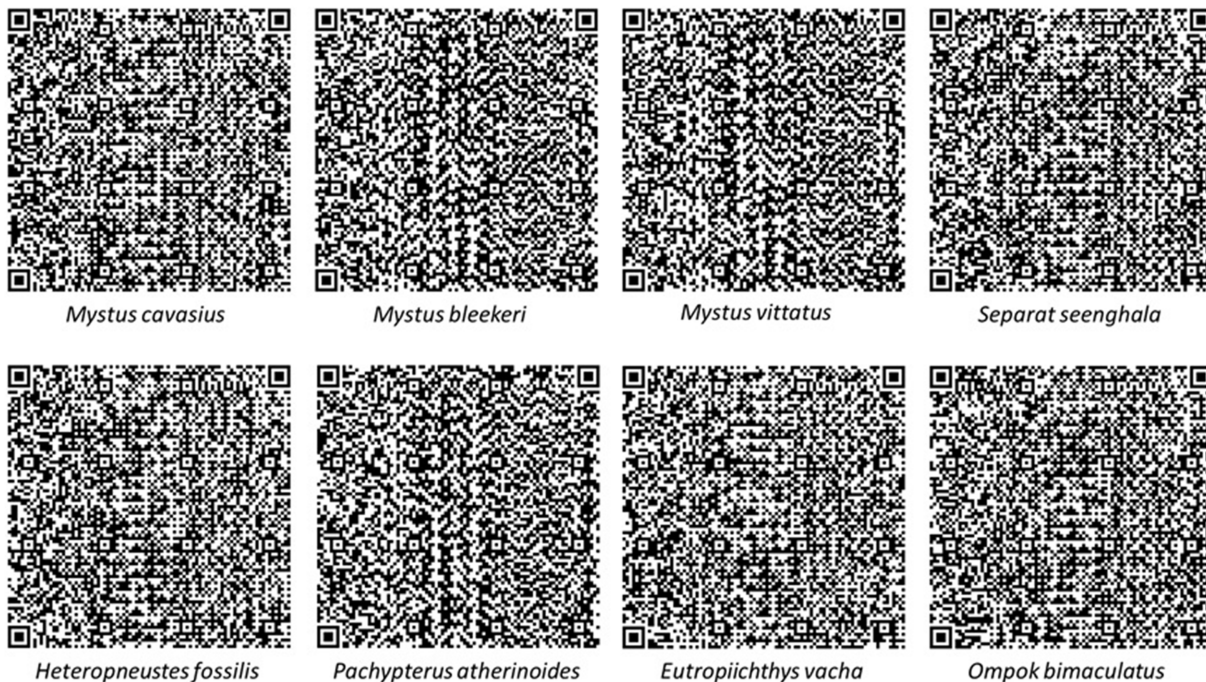


Fig. 3. QR codes generated using FASTA sequences of COI gene for sampled fish specie.

vital for assessing the correlation between different fish body parts. Morphological exploration was vital for assessing various parameters such as fish growth, development, and the correlation between body parts, as well as for detecting disparities influenced by ecological factors [31]. Morphometric analysis, a traditional fisheries research practice since the 1980s, accurately calculates the standard error of the mean by converting measurements to a percentage of the standard length [27]. Morphological features showed constructive growth with fish length, whereas meristic counts were invariable with different body lengths, indicating that meristic counts are independent of body length [28]. The values of SL, HDL, ED, and SNL were different in different fish species in the present study. Similarly, Sajjad *et al.* [32] studied and reported that all relationships between TL, HDL, SL, SNL, FL, ED, PrDL, and PoDL were extremely significant ( $p < 0.05$ ), indicating significant interrelationships between length-length relationships. The morphometrics studies are helpful to identify fishes and useful tools to discriminate the fish populations [33]. Meristic characters in fishes like *Nematalosa nasus* [34], *Pseudobagrus ichikawai* [35] vary based on body size, with changes in longer body length.

The authenticated identification and classification of fish have a major impact on the administration and preservation of freshwater fisheries. Accurately identifying fish species is essential before using them in any sector. To authenticate the identification, the sequences of the present species were aligned with reference sequences available on both GenBank and BOLD collections using BLAST. The five out of eight sequences of sampled fish species (*Mystus cavasius*, *Mystus bleekeri*, *Sperata seenghala*, *Heteropneustes fossilis*, *Eutropiichthys vacha* and *Ompok bimaculatus*) revealed 100% homology with reference sequences available on the BOLD system and a minimum of 99.52% was recorded for *Pachypterus atherinoides*. The four species (*Mystus bleekeri*, *Sperata seenghala*, *Heteropneustes fossilis* and *Ompok bimaculatus*) showed 100% similarity with GenBank sequences and a minimum match of 99.23% was observed for *Mystus cavasius*. Yang *et al.* [36] described that the specimens were identified with the name of the species that showed their COI gene similarity of 98 to 100% with the available reference sequences at GenBank or BOLD; this supports current results. Similar studies have been reported by earlier researches, such as, Panprommin *et al.* [37] identified 174 samples into 52 species with a 97 to 100% similarity index from

GenBank and BOLD databanks. Azmir *et al.* [38] identified 40 fish species through DNA barcoding with 91 to 100% genetic similarity to GenBank. Our findings are supported by previous studies that used DNA barcoding to distinguish between species worldwide, including freshwater fishes in India [39], north European shelf fish fauna [40], species diversity in fish from Kwan Phayao, Thailand [37], *Wallago attu* from Indus River Pakistan [32], edible fish species of Pakistan [41], and fishes from Pakistani coast of the Arabian Sea reveals [42].

The average composition of nucleotide bases showed GC content ( $44.69 \pm 0.31\%$ ), while at 1st, 2nd and 3rd codon positions have GC content ( $57.20 \pm 0.39$ ,  $42.86 \pm 0.00$  and  $34.06 \pm 0.94\%$ ), respectively. Our results are in line with earlier findings that described the average GC content (45.2%) while at 1st, 2nd, and 3rd codon positions, was 42.4%, 49.3%, and 43.8% as reported for catfishes of order Siluriformes [43]. Patil *et al.* [44] reported the average GC percentage (44.6) for ornamental fishes of order Siluriformes. Other researchers also reported the percentage of GC content was ( $45.04 \pm 0.18\%$ ) for the fishes of the Narmada [45], 47.03% for coastal fish species from Pakistan [46], and 46.2% GC content with COI gene amplification in *Wallago attu* [32]. These studies support our findings. The genetic distance was computed between taxonomic levels of current sequences using the K2P model. The mean hereditary distance within species (0.00%), within genus ( $15.17 \pm 0.26\%$ ) and within families ( $19.77 \pm 0.22\%$ ). The genetic divergence increases with increasing taxonomic level (within species < genus < family). Overall, genetic divergence consistently increased depicting taxonomic resolution among the analyzed fish species. Our results are similar to the outcomes reported in earlier studies [12]. The value of Tajima's neutrality test D [29], Fu and Li's D\* and Fu and Li's F test statistic [30] (0.59064,  $-0.10707$  and  $-0.24638$ ;  $p > 0.10$ ) was computed in the present study, respectively. The negative value of Fu's F<sub>s</sub> test indicates an excess of rare haplotypes, as would be expected under neutrality. Similar outcomes such as Tajima D ( $-0.957$ ,  $p > 0.10$ ), FLD (1.05942,  $p > 0.10$ ) and FLF (0.55331,  $p > 0.10$ ) were reported by Akhtar and Ali [46]. Their findings expressed that genetic variances among populations were not neutral.

The Neighbor-joining tree was clustered in the present study to express the phylogenetic relationship of the sampled fish species and authenticate the morphological identification with non-significant disparity at taxonomic levels. The species within the

same family showed a close relationship while the species related to other families were grouped under isolated nodes showing divergence. In the present study, *Sperata seenghala*, which belongs to the family Bagridae, is separated from the other species within this family in the phylogenetic tree. This separation might be associated with its distinct morphological adaptation and historical isolation of this species within the South Asian river system. The isolated position within the Bagridae family in the phylogenetic tree reflects the taxonomic and evolutionary uniqueness of *Sperata seenghala*. Similarly, the evolutionary relationship for fishes of 15 families reported that the species of a family bunched together in a tree [47]. Naeem and Hasaan [48] described that species with low genetic divergence were grouped under the same cluster, while divergent species were under separate nodes. Similarly, the evolutionary analysis performed for 447 COI sequences reported that the COI gene was suitable to authenticate the species identification [49].

In the present study, QR codes were created at the molecular level using COI gene sequences for the authentication of current species. QR codes can be scanned using a cell phone application. Similarly, QR codes were generated for *Wallago attu* [32] and for edible fish species [41], which supported our results. Yang *et al.* [50] used DNA sequence based codes for the first time, which vary from the current approach.

This study sheds light on the importance of DNA barcoding for fish identification through the use of barcodes. The current research could be expanded to create a catalog of the fish fauna found in the river systems of Pakistan, which could have practical implications for efficient management plans and the preservation of commercially valuable fisheries resources. As there has been no previous DNA-based study on fish species of the order Siluriformes from the River Ravi, Pakistan.

## 5. Conclusion

In the present study, the fish species from the River Ravi were identified based on the traditional method along with the molecular based technique (DNA Barcoding) using COI gene. DNA barcoding through COI gene has adequate disparities, and it was helpful to estimate various population genetics factors. The study of biodiversity indices depends upon authenticated identification of fishes. The identification of fishes through DNA barcoding is recommended along with traditional methods to validate the fish diversity in Pakistan.

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## Ethical statement

This study has been approved by Advance Studies & Research Board, with reference No. D/10077/Acad, dated 29-12-2020.

## Conflict of interest

The authors declare that they have no competing interests.

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## References

- [1] J.S. Nelson, T.C. Grande, M.V. Wilson, *Fishes of the World*, fifth ed., John Wiley & Sons, Hoboken. 2016 <https://doi.org/10.1002/9781119174844>.
- [2] R. Diogo, M. Chardon, P. Vandewalle, Osteology and myology of the cephalic region and pectoral girdle of *Batrochoglanis raninus*, with a discussion on the synapomorphies and phylogenetic relationships of the pseudopimelodinae and pimelodidae (Teleostei: Siluriformes), *Anim. Biol.* 54 (3) (2004) 261–280, <https://doi.org/10.1163/1570756042484728>.
- [3] G. Arratia, R.E. Reis, An introduction to catfishes: a tale of evolutionary success, in: *Catfishes, a Highly Diversified Group*, CRC Press. 2025, pp. 3–17, <https://doi.org/10.1201/9781003361336-2>.
- [4] L.R. Malabarba, M.C. Malabarba, Phylogeny and classification of neotropical fish, in: *Biology and Physiology of Freshwater Neotropical Fish*, Academic Press. 2020, pp. 1–19, <https://doi.org/10.1016/B978-0-12-815872-2.00001-4>.
- [5] T. Silva, I. Figueiredo, A. Neves, P.D. Muñoz, M.M. Coelho, L.S. Gordo, Molecular barcoding of north-east Atlantic deep-water sharks: species identification and application to fisheries management and conservation, *mar, Freshwater Res.* 59 (2008) 214–223, <https://doi.org/10.1071/MF07192>.
- [6] R.D. Ward, DNA barcode divergence among species and genera of birds and fishes, *Mol. Ecol. Resources.* 9 (4) (2009) 1077–1085, <https://doi.org/10.1111/j.1755-0998.2009.02541.x>.
- [7] F. Teletchea, C. Maudet, C. Hänni, Food and forensic molecular identification: update and challenges, *Trends Biotechnol.* 23 (7) (2005) 359–366, <https://doi.org/10.1016/j.tibtech.2005.05.006>.
- [8] P.D. Hebert, S. Ratnasingham, J.R. deWaard, Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species, *Proc. R. Soc. London, Ser. A or B* 270 (suppl\_1) (2003) 96–99, <https://doi.org/10.1098/rsbl.2003.0025>.

- [9] M.A.A. Odah, Unlocking the genetic code: exploring the potential of DNA barcoding for biodiversity assessment, *AIMS Mol. Sci.* 10 (4) (2023) 263–294, <https://doi.org/10.3934/molsci.2023016>.
- [10] R. Hanner, R. Floyd, A. Bernard, B.B. Collette, M. Shivji, DNA barcoding of billfishes, *Mitochondrial DNA 22 (suppl\_1)* (2011) 27–36, <https://doi.org/10.3109/19401736.2022.5968>.
- [11] P.F. Thomsen, E. Willerslev, Environmental DNA—An emerging tool in conservation for monitoring past and present biodiversity, *Biol. Conserv.* 183 (2015) 4–18, <https://doi.org/10.1016/j.biocon.2014.11.019>.
- [12] N. Hubert, R. Hanner, E. Holm, N.E. Mandrak, E. Taylor, M. Burridge, D. Watkinson, P. Dumont, A. Curry, P. Bentzen, J. Zhang, Identifying Canadian freshwater fishes through DNA barcodes, *PLoS One* 3 (6) (2008) e2490, <https://doi.org/10.1371/journal.pone.0002490>.
- [13] R.D.H. Barret, P.D.N. Hebert, Identifying spiders through DNA barcodes, *Can. J. Zool.* 83 (2005) 481–491, <https://doi.org/10.1139/z05-024>.
- [14] A. Cywinska, F.F. Hunter, P.D.N. Hebert, Identifying Canadian mosquito species through DNA barcodes, *Med. Vet. Entomol.* 20 (2006) 413–424, <https://doi.org/10.1111/j.1365-2915.2006.00653.x>.
- [15] L.E. Clare, B.K. Lim, M.D. Engstrom, J.L. Ege, P.D.N. Hebert, DNA barcoding of neotropical bats: species identification and discovery within Guyana, *Mol. Ecol. Notes* 7 (2007) 184–190, <https://doi.org/10.1111/j.1471-8286.2006.01657.x>.
- [16] M. Hajibabaei, G.A. Singer, P.D. Hebert, D.A. Hickey, DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics, *Trends Genet.* 23 (4) (2007) 167–172, <https://doi.org/10.1016/j.tig.2007.02.001>.
- [17] R.D. Ward, T.S. Zemlak, B.H. Innes, P.R. Last, P.D. Hebert, DNA barcoding Australia's fish species, *Phil. Trans. Biol. Sci.* 360 (1462) (2005) 1847–1857, <https://doi.org/10.1098/rstb.2005.1716>.
- [18] A.N. Abro, B. Waryani, T.N. Narejo, S. Ferrando, A.S. Abro, R.A. Abbasi, H. Ul-Hassan, Diversity of freshwater fish in the lower reach of Indus River, Sindh province section, Pakistan, Egypt, *J. Aquat. Biol. Fish.* 24 (6) (2020) 243–265, <https://doi.org/10.21608/EJABF.2020.111114>.
- [19] H.M. Ashraf, H.A. Shakir, J.I. Qazi, Fish abundance and diversity during low and high flow seasons of River Ravi, Punjab, Pakistan, *Pakistan J. Zool.* 56 (5) (2024) 2143–2156, <https://doi.org/10.17582/journal.pjz/20230218100231>.
- [20] N.V. Ivanova, J.R. Dewaard, P.D. Hebert, An inexpensive, automation-friendly protocol for recovering high-quality DNA, *Mol. Ecol. Notes* 6 (4) (2006) 998–1002, <https://doi.org/10.1111/j.1471-8286.2006.01428.x>.
- [21] N.V. Ivanova, T.S. Zemlak, R.H. Hanner, P.D. Hebert, Universal primer cocktails for fish DNA barcoding, *Mol. Ecol. Notes* 7 (4) (2007) 544–548, <https://doi.org/10.1111/j.1471-8286.2007.01748.x>.
- [22] S. Ratnasingham, P.D. Hebert, A DNA-based registry for all animal species: the Barcode Index Number (BIN) system, *PLoS One* 8 (7) (2013) e66213, <https://doi.org/10.1371/journal.pone.0066213>.
- [23] J. Rozas, A. Ferrer-Mata, J.C. Sánchez-DelBarrio, S. Guirao-Rico, P. Librado, S.E. Ramos-Onsins, A. Sánchez-Gracia, DnaSP 6: DNA sequence polymorphism analysis of large data sets, *Mol. Biol. Evol.* 34 (12) (2017) 3299–3302, <https://doi.org/10.1093/molbev/msx248>.
- [24] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, Mega X: molecular evolutionary genetics analysis across computing platforms, *Mol. Biol. Evol.* 35 (6) (2018) 1547–1549, <https://doi.org/10.1093/molbev/msy096>.
- [25] M. Kimura, A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, *J. of Molecular Evol.* 16 (1980) 111–120, <https://doi.org/10.1007/BF01731581>.
- [26] N. Saitou, M. Nei, The Neighbor-joining method: a new method for reconstructing phylogenetic trees, *Mol. Biol. Evol.* 4 (4) (1987) 406–425, <https://doi.org/10.1093/oxfordjournals.molbev.a040454>.
- [27] E.K. Balon, Origin and domestication of the wild carp, *Cyprinus carpio*: from roman gourmets to the swimming flowers, *Aquaculture* 129 (1–4) (1995) 3–48, [https://doi.org/10.1016/0044-8486\(94\)00227-F](https://doi.org/10.1016/0044-8486(94)00227-F).
- [28] F.L. Bookstein, Creases as Morphometric Characters, *Systematics Association Special Volume*, 2002, pp. 139–174, <https://doi.org/10.1007/978-3-662-08865-4>.
- [29] F. Tajima, Statistical method for testing the neutral mutation hypothesis by DNA polymorphism, *Genetics* 123 (1989) 585–595, <https://doi.org/10.1093/genetics/123.3.585>.
- [30] Y.X. Fu, W.H. Li, Statistical tests of neutrality of mutations, *Genetics* 133 (1993) 693–709, <https://doi.org/10.1093/genetics/133.3.693>.
- [31] A.K. Dwivedi, K. De, Role of morphometrics in fish diversity assessment: status, challenges and future prospects, *Natl. Acad. Sci. Lett.* 47 (2024) 123–126, <https://doi.org/10.1007/s40009-023-01323-x>.
- [32] A. Sajjad, F. Jabeen, M. Ali, S. Zafar, DNA barcoding and phylogenetics of *Wallago attu* using mitochondrial COI gene from the River Indus, *J. King Saud Univ. Sci.* 35 (6) (2023) 102725, <https://doi.org/10.1016/j.jksus.2023.102725>.
- [33] J. Palma, J.P. Andrade, Morphological study of *Diplodus sargus*, *Diplodus puntazzo*, and *Lithognathus mormyrus* (sparidae) in the eastern Atlantic and Mediterranean Sea, *Fish. Res.* 57 (1) (2002) 1–8, [https://doi.org/10.1016/S1385-1101\(97\)00018-X](https://doi.org/10.1016/S1385-1101(97)00018-X).
- [34] H. Laj, Variations in meristic characters of *Nematalosa nasus* from Iraqi and Kuwaiti waters, *Jpn. J. Ichthyol.* 33 (4) (1987) 422–425, <https://doi.org/10.11369/jji1950.33.422>.
- [35] K. Watanabe, Meristic variation in the endangered Bagrid catfish, *Pseudobagrus ichikawai*, *Ichthyological Research* 45 (1998) 99–104, <https://doi.org/10.1007/BF02678581>.
- [36] L. Yang, Z. Tan, D. Wang, L. Xue, M.X. Guan, T. Huang, R. Li, Species identification through mitochondrial rRNA genetic analysis, *Sci. Rep.* 4 (1) (2014) 4089, <https://doi.org/10.1038/srep04089>.
- [37] D. Panprommin, K. Soontornpravit, S. Tuncharoen, S. Pithakpol, J. Keereelang, DNA barcodes for the identification of species diversity in fish from Kwan Phayao, Thailand, *J. Asia Pac. Bus.* 12 (3) (2019) 382–389, <https://doi.org/10.1016/j.japb.2019.05.003>.
- [38] A. Azmir, Y. Esa, S.M.N. Amin, I.S. Md-Yasin, F.Z. Md-Yusof, Identification of larval fish in mangrove areas of Peninsular Malaysia using morphology and DNA barcoding methods, *J. Appl. Ichthyol.* 33 (5) (2017) 998–1006, <https://doi.org/10.1111/jai.13425>.
- [39] M. Chakraborty, S.K. Ghosh, An assessment of the DNA barcodes of Indian freshwater fishes, *Gene* 537 (1) (2014) 20–28, <https://doi.org/10.1016/j.gene.2013.12.047>.
- [40] T. Kneibelsberger, M. Landi, H. Neumann, M. Kloppmann, A.S. Sell, P.D. Campbell, S. Laakmann, M.J. Raupach, G.R. Carvalho, F.O. Costa, A reliable DNA barcode reference library for the identification of the north European shelf fish fauna, *Mol. Ecol. Resour.* 14 (5) (2014) 1060–1071, <https://doi.org/10.1111/1755-0998.12238>.
- [41] M.Z. Ghouri, M. Ismail, M.A. Javed, S.H. Khan, N. Munawar, A.B. Umar, S.O. Aftab, S. Amin, Z. Khan, A. Ahmad, Identification of edible fish species of Pakistan through DNA barcoding, *Front. Mar. Sci.* 7 (2020) 554183, <https://doi.org/10.3389/fmars.2020.554183>.
- [42] S.A. Amir, B. Zhang, R. Masroor, Y. Li, D.X. Xue, S. Rashid, N. Ahmad, S. Mushtaq, J.D. Durand, J. Liu, Deeper in the blues: DNA barcoding of fishes from Pakistani coast of the Arabian Sea reveals overlooked genetic diversity, *Mar. Biodivers.* 52 (4) (2022) 37, <https://doi.org/10.1007/s12526-022-01272-6>.
- [43] H. Limbu, D. Rajbanshi, L. Khanal, W. Hui, R.C. Adhikari, S. Thapa, J.Q. Yang, C. Li, DNA barcoding of catfishes (Order: siluriformes) with new records of two species from Eastern Nepal, *J. Appl. Ichthyol.* 2024 (1) (2024) 4907672, <https://doi.org/10.1155/2024/4907672>.
- [44] G.S. Patil, N. Pinto, R. Nath, M. Goswami, Decoding the molecular phylogenetics of ornamental catfishes

- (Siluriformes) of North East India using DNA barcoding approach, *Mol. Biol. Rep.* 51 (1) (2024) 528, <https://doi.org/10.1007/s11033-024-09487-5>.
- [45] G.D. Khedkar, R. Jamdade, S. Naik, L. David, D. Haymer, DNA barcodes for the fishes of the Narmada, one of India's longest rivers, *PLoS One* 9 (7) (2014) e101460, <https://doi.org/10.1371/journal.pone.0101460>.
- [46] T. Akhtar, G. Ali, DNA barcoding of *Schizothorax* species from the Neelum and Jhelum Rivers of Azad Jammu and Kashmir, *Mitochondrial DNA Part B* 1 (1) (2016) 934–936, <https://doi.org/10.1080/23802359.2016.1258337>.
- [47] F.S. Ali, M. Ismail, W. Aly, DNA barcoding to characterize biodiversity of freshwater fishes of Egypt, *Mol. Biol. Rep.* 47 (2020) 5865–5877, <https://doi.org/10.1007/s11033-020-05657-3>.
- [48] M. Naeem, S. Hassan, Molecular approach for identification of *Labeo bata* based on COI gene sequence from Pakistan, *Mitochondrial DNA Part B* 4 (1) (2019) 244–246, <https://doi.org/10.1080/23802359.2018.1547131>.
- [49] M. Kochzius, C. Seidel, A. Antoniou, S.K. Botla, D. Campo, A. Cariani, E.G. Vazquez, J. Hauschild, C. Hervet, S. Hjörleifsdottir, G. Hreggvidsson, K. Kappel, M. Landi, A. Magoulas, V. Marteinson, M. Nölte, S. Planes, F. Tinti, C. Turan, M.N. Venugopal, H. Weber, D. Blohm, Identifying fishes through DNA barcodes and microarrays, *PLoS One* 5 (9) (2010) e12620, <https://doi.org/10.1371/journal.pone.0012620>.
- [50] H. Yang, K.C. Wu, L.Y. Chuang, H.W. Chang, Decision theory-based COI-SNP tagging approach for 126 scombriformes species tagging, *Front. Genet.* 10 (2019) 259, <https://doi.org/10.3389/fgene.2019.00259>.