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Morphological and Genetic Identification of Head Taunsa Barrage Fish Species

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ABSTRACT

The current study aims to construct a thorough barcoding reference database of fishes in the Head Taunsa Barrage and assess the usefulness of employing the COI gene for fish species identification. A total of 15 genera, 10 families, and 7 orders of fish were used to collect a total of 19 mitochondrial COI barcode sequences. These sequences had an average length of 800 base pairs. Within species, genera, families, orders, and classes, the average Kimura two-parameter (K2P) distances were 0.97%, 0.99%, 1.23%, and 1.26%, respectively. According to their taxonomic classification, species were commonly clustered in the K2P neighbor-joining trees based on the sequence. DNA barcoding was employed in this study to identify species with a high degree of accuracy. Moreover, it was concluded that COI sequencing can be used to recognize fish species.

Keywords: biodiversity, COI barcode sequences, genetic identification, mitochondrial genome, Taunsa Barrage, taxonomic classification

1. INTRODUCTION

Pakistan is enriched with a large number of natural water resources, such as rivers, estuaries, streams, lakes, ponds, and man-made reservoirs. The country's estimated freshwater fish fauna contains a minimum of 171 fish species [1] and a maximum of 193 fish species [2]. Head Panjnad is the conjunction point of five rivers namely Jhelum, Chenab, Ravi, Beas, and Sutlej. It is situated in District Bahawalpur, Punjab, Pakistan [3]. It was constructed with 33 bays during the years 1927-29. On the right side, a

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further 16 bays were added during 1930-31 on the commendation of the Islam Inquiry Committee of 1929 [4]. Now, a total 47 bays constitute the barrage. There are three (3) head regulators of off-taking canals from the barrage namely Panjnad canal, Abbasia canal, and Abbasia link canal or Abu Zehbi canal.

A number of studies have been conducted to explore the aquatic fish fauna of Pakistan across different regions and periods [5, 6]. These studies provide baseline information on species diversity and distribution in various places. However, they lack in a number of ways, since none of them primarily covers a genetic investigation of Head Panjnad fish species. Hence, the current research focuses on the most essential components of the COI gene, which was chosen to reveal the taxonomy, phylogenetic relationships, and genetic relatedness among fish species in Panjnad Headworks, Punjab, Pakistan.

Accurate fish identification is necessary for the fish industry and so are ecological related checks, evaluation of environmental effects, restriction of illegal trafficking, establishment of organized fish resources, and fish protecting areas [6]. Fish identification is also important for biodiversity preservation of a region [7]. In Pakistan, fish identification is generally based on a traditional morphological method that identifies a specific set of morphological characteristics of fishes, mostly present in a mature specimen. One of the foremost tasks of taxonomy includes fish identification. Usually, the fish identification is depending on observable external morphology and is completed by applying specific morphological keys [8]. Precise fish identification becomes very difficult, whereas morphological characteristics become a challenge to identify as like in trade practices, when fish is present in fillet form and during the developmental stages of fish, when morphology is incomplete. This type of situation demands an alternate method of fish identification. In this regard, DNA barcoding has proved to be a favorable alternative identification tool [9].

DNA barcoding is an effective method of specie identification [10]. Typically, morphological criteria are used to identify fish. Due to the considerable morphological plasticity and diversity of fish species, it becomes hard to identify fish and their different stages of development by observing morphological characteristics only [11]. On the other hand, basic DNA-dependent methods of identification have been established and analytically proved to be powerful [12].

DNA barcoding has proved to be the most efficient procedure for identification of fish species. It targets to have the potential of an efficient technique by applying an ordered series of specie- specific molecular tags obtained from the 5′ region of specific gene known as mitochondrial Cytochrome C Oxidase subunit I (COI) gene [13]. By investigating the new data and by comparing it with already published reference libraries, the reliable taxonomy of the created DNA barcodes can be confirmed. The authors in [14] suggested a unique process of ranking for contributing taxonomic consistency to barcodes using the Barcode of Life Database [15]

2. MATERIALS AND METHOD

Samples were collected by following the rules and regulations approved by the university ethical committee. Sampling was carried out at Head Taunsa Barrage, Punjab, Pakistan (Figure 1). Fish were morphologically identified by its specific keys [16]. Fish samples were collected carefully with the help of accessible nets. These samples were transferred with care to the zoological laboratory of Government Sadiq Women College and University, Bahawalpur for further investigation. For further study, fish specimens were dissected individually in the lab. Following the optimized traditional method, genomic DNA extraction from fish muscles was done by applying standard Phenol-Chloroform Isoamyl (PCI) alcohol [17]. Confirmation was done by 1% agarose gel electrophoresis. Polymerase Chain Reaction (PCR) was performed using the universal specie-specific primer pair, that is, FISH F1 5′-TCAACCAACCACAAAGACATTGGCAC-3′ and FISH R1 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′. One set of primers was used to amplify the mitochondrial genome's COI gene which is about 658 base pairs long [17]. PCR results were checked on 2% agarose gel electrophoresis. PCR is the most accurate technique employed in DNA sequencing of specific fragments for the identification of fresh, frozen, and processed fishes. Moreover, the sequencing of purified products was done by Macrogen Korea after the completion of DNA isolation and sample amplification [18].

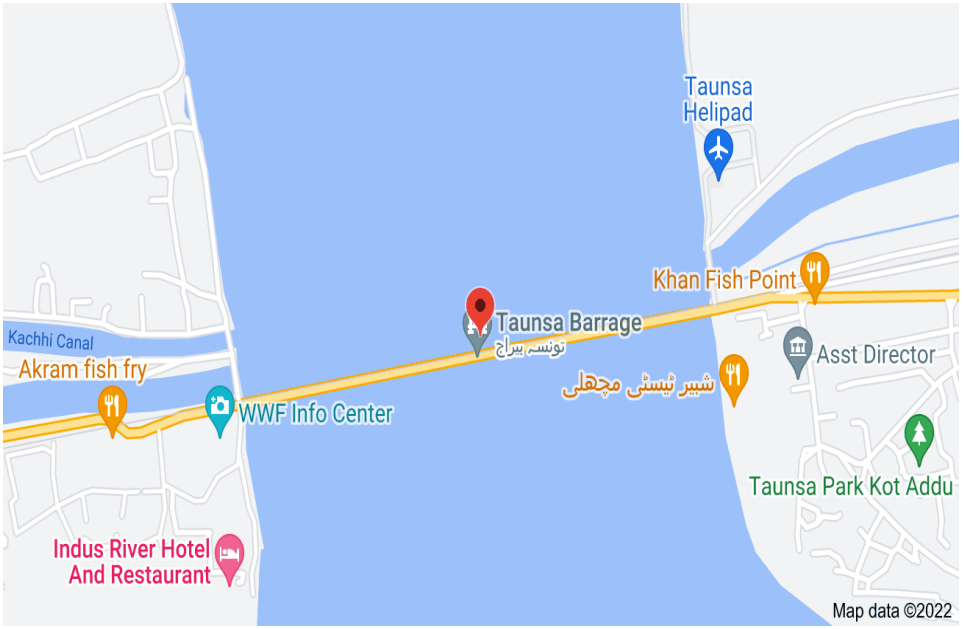

Figure 1. Map of the Study Area and Location of Sampling Sites at Head Taunsa Barrage, Punjab, Pakistan

2.1. Sequence Analysis

Nucleotide sequences were aligned through BLAST (Basic Local Alignment Search Tool). Sequencing was performed using MEGA (Molecular Evolutionary Genetics Analysis) X. By averaging pair-wise comparisons of sequence variation across all individuals, the extent of sequence variances across species was evaluated. COI gene sequences of 34 individuals were used to align to obtain a final alignment 642 base pairs. Using the software application MEGA X [19], pair-wise evolutionary distance between haplotypes was calculated via the K2P (Kimura 2-Parameter) method. The determined parameters were nucleotide diversity (pi), number of transition and transversion between species, evolutionary divergence between the sequences' nucleotide composition, and maximum likelihood estimation of substitution matrix. MEGA X was used to create a neighbor-joining (NJ) and Maximum Parsimony (MP) tree. Bootstrap analysis with 1,000 pseudo replicates was used to test the resilience of the interior nodes of NJ and MP trees [20].

2.2. Evolutionary Pathway and Phylogeny Determination

The NJ method was used to create a phylogenetic tree after eliminating non-coding areas and modifying sequences. The /maximum composite likelihood technique was also used to determine the rate of nucleotide substitution in the bases pyrimidine and purine. Resampling and tree reconstruction were replicated 500 times for the NJ method to determine the bootstrap probability of each tree. The frequency of occurrence was used to determine the bootstrap probability of each tree.

3. RESULTS

3.1. Morphological Identification

During the sampling period, each fish sample was identified morphologically with the help of local fish catchers and market sellers. Later on, standard keys were applied for the taxonomic classification of fish species. All essential body measurements of fishes were also calculated. Trained taxonomists can identify a wide range of species and have specialized competency in a particular group. Although, conceptual differences across taxonomists can restrict the repeatability of some identifications, accuracy should still be excellent. With fresh or preserved specimens, taxonomists still proven to be more helpful.

3.2. *Catla catla* (Hamilton) Thaila

It is a fish of freshwater and found in rivers, lakes, and culture ponds. Surface and bottom feeders are mainly omnivorous (Figure 2-A).

3.3. *Labeo rohita (*Hamilton) Rohu

It inhabits the freshwater rivers of South Asia. It feeds on plants (**Figure 2-B**).

3.4. *Labeo calbasu (*Hamilton) Kalbans

It inhabits the freshwater rivers of South Asia. It feeds on the dead and decaying matter present at the river bottom (Figure 2-C).

3.5. *Labeo gonius* (Hamilton) Sereeha

It spawns during the monsoon and does not breed in ponds. Fish can be cultured in ponds with other carp species (Figure 2-D).

3.6. *Hypophthalmichthys molitrix* (Valenciennes) Silver carp

The silver carp is a fish of fresh or brackish water with marked water level instabilities. It forages in lakes, flooded places, and shallow but warm backwaters (Figure 2-E).

3.7. *Cyprinus carpio* (Linnaeus) Gulfaam

It is a widespread freshwater fish of eutrophic waters in lakes and large rivers (Figure 2-F).

3.8. *Channa punctata* (Baloch) Gudo, Daula

It is a freshwater fish and mostly found in ponds, swamps, and brackish water. It feeds on worms, insects, and small fish (Figure 2-G).

3.9. *Channa marulius* (Hamilton) Soul, Snake Head Murrel

It is a freshwater fish and mostly lives in large rivers, channels, canals, and swamps where it is often associated with aquatic vegetation (Figure 2-H).

3.10. *Rita rita* (Hamilton) Desi Khagga

It is a freshwater fish and mostly lives in large rivers and lakes (Figure 2-I).

3.11. *Wallago attu* (Baloch and Schneider) Mulli

It is a specie of fresh water and mostly lives in large rivers, lakes, and tanks. It may hide in holes in riverbanks and canals. It is abundantly present during the warm season (Figure 2-J).

3.12. *Clarias batrachus* (Linnaeus) Asian catfish

It is a freshwater air breathing catfish named for its capability to walk and waggle across dry land in order to find food or suitable environment (Figure 3-A).

3.13. *Carassius auratus* (Linnaeus) Goldfish

It is a freshwater fish first found in 17% salinity in brackish water. It consumes various plant materials, insects, and crustaceans as food (Figure 3-B).

3.14. *Oreochromis mossambicus* (Peters) chirrah

It inhabits freshwater lakes and rivers. It has been generally presented for fish cultivation, bug and weed control, and game and lure purposes. Such

presentations have been related frequently with extreme natural change. Tilapias are regularly depicted as 'pioneer' species, since they flourish in upset natural surroundings, craftily moving and duplicating (Figure 3-C).

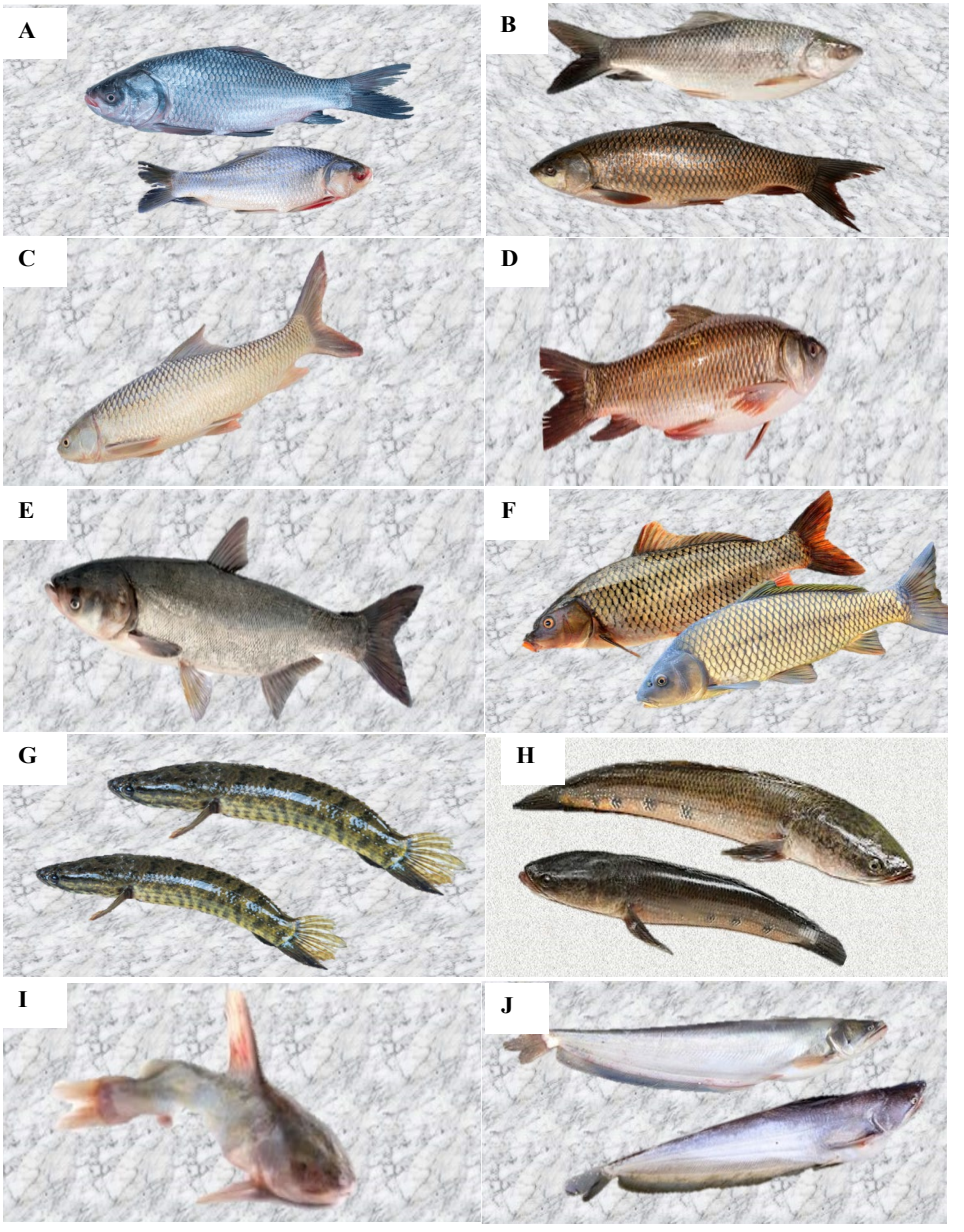

Figure 2. Morphological identification of studied fish species. **A:** *Catla*

catla; **B:** *Labeo rohita*; **C:** *Labeo Calbasu*; **D:** *Labeo gonius*; **E:** *Hypophthalmichthys molitrix*; **F:** *Cyprinus carpio*; **G:** *Channa punctata*; **H:** *Channa marulius*; **I:** *Rita rita*; **J:** *Wallago attu*

3.15. *Bagarius bagarius* (Hamilton) Mujahid, faujikhagga, Gonch

It prevents large and medium-sized rivers from having fast or rocky pools. It consumes insects, tiny fish, frogs, and shrimps for food (Figure 3-D).

3.16. *Notopterus notopterus* (Pallas) But Pari

It inhabits mostly the lower parts of rivers, tributes, and swamps. It moves towards flooded forest areas to spawn and feeds on small fishes, crustaceans, and other invertebrates (Figure 3-E).

3.17. *Chitala chitala* (Hamilton-Buchanan) Cheetal Pari

It is a specie of freshwater which inhabits rivers and freshwater lakes. It has the ability to withstand in respect to availability of food items in different habitats (Figure 3-F).

3.18. *Parambassis ranga (Hamilton)* Sheesha

It is a specie of freshwater which inhabits rivers and freshwater lakes. It is also found in standing and sluggish water and feeds on crustaceans, annelids, and worms (Figure 3-G).

3.19. *Mastacembelus armatus* (Lacepede) Baam, Marmahi

In rivers, lakes, still waterways, and lowland wetlands, nocturnal fish are the most prevalent. They are typically found during the summer season. This particular species forages on larvae, earthworms, black worms, and plunged plant material (Figure 3-H).

3.20. *Hypophthalmicthys nobilis* (Valenciennes) Bighead carp

It is a freshwater fish (Figure 3-I).

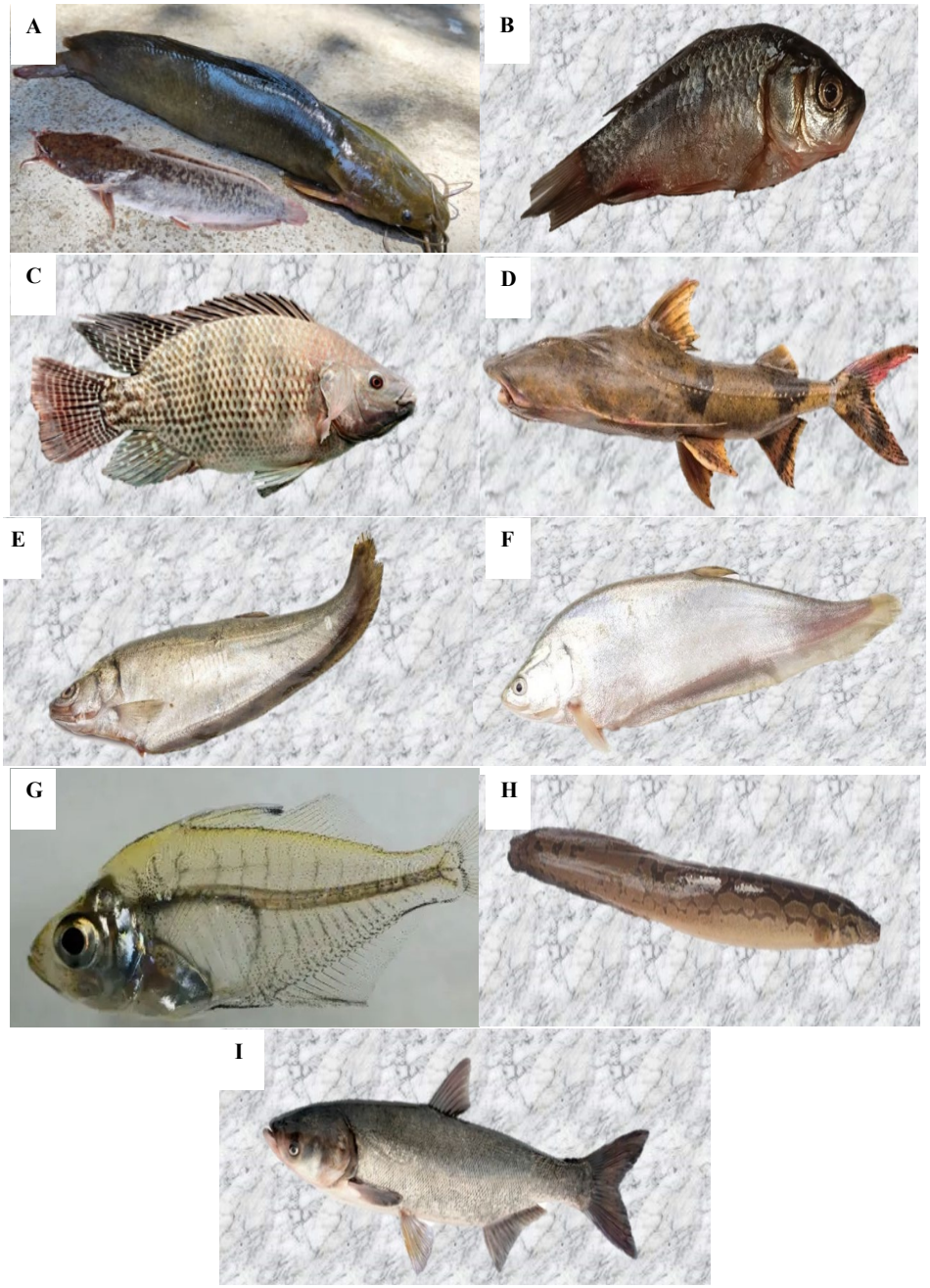

Figure 3: Morphological identification of studied fish species. **A:** *Clarias batrachus*; **B:** *Carassius auratus*; **C:** *Oreochromis mossambicus*; **D:**

Bagarius bagarius; **E:** *Notopterus notopterus*; **F:** *Chitala chitala*; **G:** *Parambassis ranga*; **H:** *Mastacembelus armatus*; **I:** *Hypophthalmichthys nobilis*

3.21. Morphological Identification of Fish Found at Head Taunsa Barrage, Punjab, Pakistan

From 15 species, 10 families, and 7 orders of fish, a total of 19 mitochondrial COI barcode series were obtained (Table 1). After excision, the accord length of all barcode sequences was 800bp, and no stop codons, insertions, or deletions were detected in any of the sequences. All examined sequences were 800bp. An analysis of all the dataset nucleotide pair frequencies showed that 3 of the 845 sites were conserved. Of the 845 sites, 831 were variable and parsimony informative, while 18 of 845 sites were singletons. The average number of alike pairs (ii) was 26, while transversional pairs (sv = 31) were found to be more frequent than transitional pairs (si = 17)), with a si/sv (R) ratio of 0.54 for the dataset. The third codon location (si = 58 and sv = 10) was where both transitional and transversional pairings were the most prevalent. T (22.8%), C (25.3%), A (31.7%), and G (20.3%) were the average nucleotide base frequencies found for these sequences. The average A content was the maximum and the average G content was the minimum in the base composition of the COI sequence, while the AT content (54.5%) was greater than the GC content (45.6%) (Table 2).

Table 1. Cataloging of Species Examined for COI Sequences

Order	Family	Species	Common name
Siluroformes	Siluriidae	*Wallago attu*	Mulli
	Bagridae	*Rita rita*	Dasi khagga
	Clariidae	*Clarias batrachus*	Asian catfish
	Sisoridae	*Bagarius bagarius*	Fauji Khagga
Cypriniformes	Cyprinidae	*Cyprinus carpio*	Gulfam
		Catla catla	Thaila
		Labeo rohita	Rohu
		Labeo gonius	
		Labeo calbasu	Kalbans
		Carassius auratus	Goldfish
		Hypophthalmichthys molitrix	Silver carp
		Hypophthalmicthys nobilis	Bighead carp

Order	Family	Species	Common name
Anabantiformes	Channidae	*Channa punctata*	Gudo
		Channa marulius	Sool
Osteoglossiformes	Notopteridae	*Notopterus notopterus*	But pari
		Chitala chitala	Cheetal pari
Perciformes	Ambassidae	*Parambassis ranga*	Glass perch
Synbranchiformes	Mastacembelidae	*Mastacembelus armatus*	Zig zag eel
Cichliformes	Cichlidae	*Oreochromis mossambicus*	Chirra

Table 2. Nucleotide Pair Frequency Analysis

Fish Species	T(U)	C	A	G	Total
Labeo rohita	30.7	19.8	35.9	13.6	800
Catla catla	30.8	21.5	32.5	15.3	800
Cyprinus carpio	24.5	27.3	23.6	24.6	800
Labeo calbasu	30.3	21.6	32.9	15.3	800
Labeo gonius	19.6	26.8	31.5	22.1	800
Hypophthalmichthys molitrix	19.4	27.4	31.0	22.3	800
Oreochromis mossambicus	20.5	28.0	29.6	21.9	800
Bagarius bagarius	20.1	26.3	32.8	20.9	800
Notopterus notopterus	19.8	27.4	31.6	21.3	800
Chitala chitala	20.8	26.3	32.1	20.9	800
Parambassis ranga	21.9	27.6	29.9	20.6	800
Mastacembelus armatus	21.4	27.6	31.6	19.4	800
Clarias batrachus	20.5	26.8	31.8	21.0	800
Wallago attu	21.5	25.8	32.6	20.1	800
Rita rita	20.8	23.4	36.5	19.4	800
Channa marulius	20.3	26.9	32.4	20.5	800
Channa punctata	20.9	25.9	32.5	20.8	800
Carassius auratus	32.5	15.9	31.1	20.5	800
Hypophthalmichthys nobilis	19.5	27.1	30.8	22.6	800
Avg.	22.8	25.3	31.7	20.3	800

The Kimura-2-parameter model is suggested by the Consortium for the Barcode of Life (CBOL) for computing genetic distance [21]. K2P model was employed in this study to calculate genetic distances within and between fish species. According to Table 3, the K2P distance within species

in this study ranged from 0.0706% to 2.1245%, with an average distance of 0.97%. The mean genetic distance across the genera was 0.99%, while the range was from 0.0907% to 2.1365%. The average genetic distance between families was 0.1245%, while the mean genetic distance between orders was 0.1554%, with both ranges averaging 1.26%. In comparison to the genetic distance within species, the mean genetic distance across families and orders was greater than 1%. Only the genetic distance between species and genus was less than 1%. According to the findings, the genetic distance (K2P) increased less sharply and remained small at higher taxonomic levels than it did at lower taxonomic levels, that is, above the level of the species.

Table 3. Genetic Variation at Different Taxonomic Levels

Comparison within	Taxa	Mean %	Minimum %	Maximum %	SE
Species	19	0.97	0.0706	2.1245	0.01
Genus	15	0.99	0.0907	2.1365	0.01
Family	10	1.23	0.1245	2.6834	0.02
Order	7	1.26	0.1554	2.8435	0.02

In the phylogenetic tree, *Hypophthalmicthys molitrix* and *Wallago attu* are clustered under the same node (Figure 21). *Hypopthalmicthys molitrix* belongs to order cypriniformes and family cyprinidae. On the other hand, *Wallago attu* belongs to order siluriformes and family siluridae. Distantly related species cluster under a different node, whilst closely related species are grouped under the same node. A total of 7 orders were identified which included siluriformes, cypriniformes, anabantiformes, osteoglossiformes, perciformes, synbranchiformes, and cichliformes.

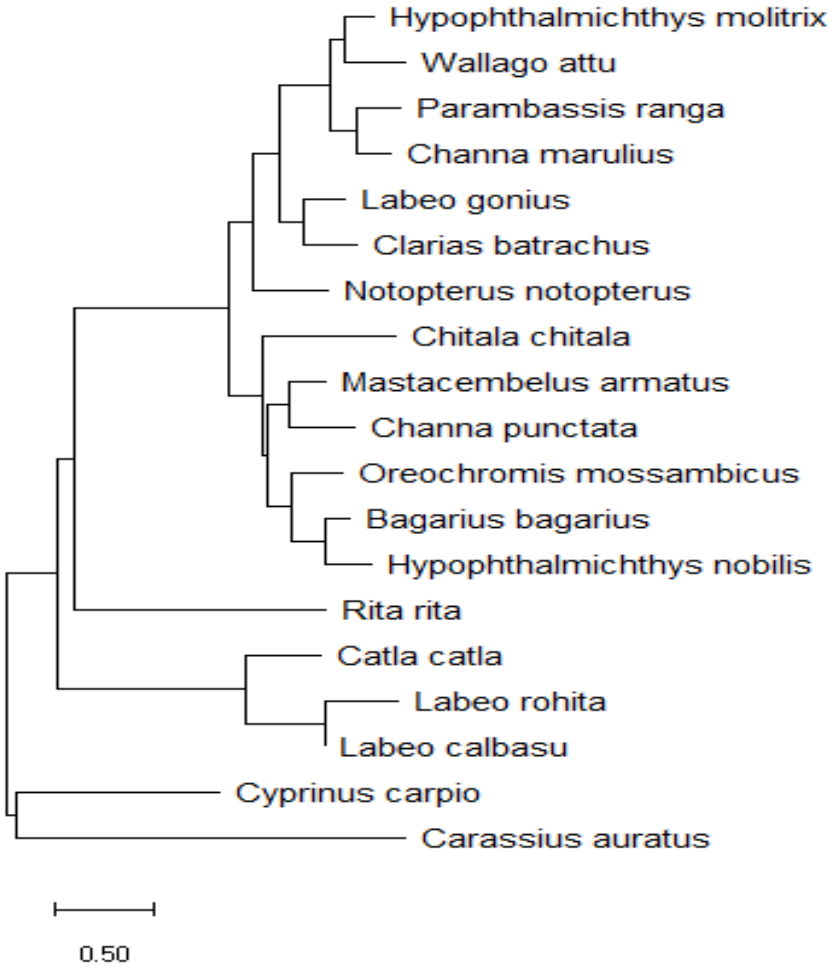

Figure 21. Phylogenetic Tree (Neighbour-joining) of COI Sequences using K2P Distances

4. DISCUSSION

Species identification is made possible through DNA barcoding technology which makes use of a segment of DNA sequence shared by organisms with significant interspecies diversity. This method allows for the informatization and standardization of species identification by overcoming the over-reliance on the skills and proficiencies of individual

taxonomists in traditional morphological categorization. Since it exhibits a high level of conservation within species and moderate level of genetic variability between different species, the mitochondrial COI gene is generally utilized as species barcode. Its excellent efficacy in species identification has been documented in studies on Japanese marine fishes [22], Indian freshwater fishes [23], Taiwan ray finned fishes [24], and Mediterranean fishes [25]. In this work, the COI barcode sequences were effectively augmented for 89 marine fish species. The fact that the primer pairs employed in this study were able to amplify the target region without causing any deletions or insertions suggests that DNA barcoding might become a universally accepted method for classifying fish species.

A nucleotide pair frequency study resulted in 3 conserved sites, 831 variable sites, 813 bp parsimony informative sites and no singleton sites. There were more transversional pairs (sv = 31) than transitional pairs (si = 17). The nucleotide pair frequencies that were found were comparable to those found in fish studies conducted in Turkey. The third codon position had the highest proportion of synonymous mutations and transitional and transversional pairings (58 and 10, respectively, for si and sv). Fish populations may undergo demographic shifts as a result of the level of variety found in mitochondrial DNA. The results of the base composition analysis of the COI sequence were comparable to those observed in Australian genomes in that AT content (54.5%) was greater than GC content (45.6%) [26], Canadian [27] and Cuban fish species [28].

The efficiency of species identification through DNA barcoding depends on both interspecific divergence and intraspecific divergence. Barcode analysis attempts to identify the boundaries to delineate species, which corresponds to the divergence between the nearest neighbors within a group [23]. There is still no established universal standard threshold for interspecies delineation, nevertheless. Recently, the barcoding gap was defined by the difference between the lowest congeneric and maximum conspecific divergence, and this difference was more effective than the mean of intra- and interspecific sequence variability [29]. In this work, the average intraspecific K2P distance was 0.97%, compared with 0.99% for species within genera. The mean interspecific distance was found to be 31-fold higher than the mean intraspecific distance, which was similar to the 25-fold difference observed in Australian marine fishes [26] and the 26.2-fold difference observed in Canadian mesopelagic and upper bathypelagic

marine fishes [30]; this result corresponds to the DNA barcoding principle that interspecific divergence sufficiently outscores intraspecific divergence. In addition, the difference was greater than the 13.9-fold difference observed in the marine fishes commonly encountered in the Canadian Atlantic [31].

Figure 21 displays the whole NJ tree produced by the current study. The NJ tree showed that the majority of species were grouped into monophyletic groupings, which proves that DNA barcoding is highly effective at identifying different species. Misidentification based on morphology has the potential to alter the NJ tree's conclusion. The evolutionary relationships among the species were identical, according to the NJ tree. The phylogenetic relationships between the species were clearly established. Nodes with high bootstrap values (90–100%) supported the clustering of similar species under the same node. While, nodes with lower bootstrap values supported the clustering of different species under separate nodes. Although barcode research solely aims to identify species boundaries, COI sequencing data certainly contained some evolutionary information. In most instances, familial species also congregated with congeneric species. By using the divergences in COI sequence to identify a second species of Asian sea bass (Lates calcerifer), the authors [21] produced an intriguing discovery. In addition to species identification, DNA barcoding has been utilized to identify processed fish products [32].

"DNA contamination and incomplete knowledge of the taxonomic literature can contribute to ambiguous barcoding results" [33]. "On the other hand, a huge number of specimens, including eggs, larvae, and adults, are needed for the reference library of barcodes and species identification, because numerous morphometric traits vary throughout various developmental stages. Because of this, there will inevitably be some instances of misidentification, and this example shows how DNA barcoding may identify these instances of morphological misidentification. A prerequisite for creating a molecular database is the combining of morphological and molecular properties. It is possible to classify and broadly identify species using a successful reference barcode library. Our findings show that the great majority of fish species might be identified using DNA barcoding. Identification aided by DNA barcoding may be used to manage fisheries, assess fish biodiversity, and monitor fish conservation" [34].

Future research on fish species that need to be barcoded should follow the guidelines provided by this study. Fish barcoding has a wide range of scientific and practical advantages, once a fish DNA barcode database has been built. All fish species may be identified using DNA barcoding which can also identify their eggs, larvae, and corpse fragments. The findings provide fisheries managers and ecologists with more information about fish variety, as they develop plans for the protection and sustainable exploitation of fishing resources.

4.1. Conclusion

The effectiveness of COI in recognizing fish species has been clearly validated by this study. For the DNA barcoding method to be successful, DNA sequences within species must be more alike to one another than to sequences in other species. The findings imply that COI barcoding is a feasible method to identify the fish fauna of Head Panjnad, with requisition in its management and conservation.

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