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ORIGINAL ARTICLE

Morphometric and molecular characterization of *Channa marulius* from Riverine system of Punjab, Pakistan

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Abstract

Objective *Channidae* family, are major freshwater fish species amongst the local aquatic fauna of Pakistan, while, there is limited availability of local data on their molecular identification and phylogenetic analysis.

Methods *Channa* species were collected from four different geographical sites in the tertiary of Punjab province on the Indus and Chenab rivers of Pakistan. Morphometric records and molecular techniques were used to determine the intraspecific variations among populations of *Channa marulius*. Mitochondrial DNA was extracted from the flesh of *C. marulius*, while, COI gene was used for molecular identification and variation levels were estimated by using Principal Component Analysis.

Results Data recorded on the basis of morphometric parameters clearly divided the *C. marulius* of different locations into two distinct categories, which accounted for a cumulative variability of 97.6%. Non-significance ($P < 0.05$) among the *C. marulius* showed that it contains a unique control haplotype localized within the sub-population. The intra-species distance ranged from 0.000 to 0.001 for four different populations, in contrast, the sequences retrieved from the NCBI database exhibited a range span of 0.000–0.003, while, sequence diversity ranged from 0.000 to 0.006 for this intra-specific comparison. The cladogram was also constructed for *C. marulius* of different geographical locations for observation of phylogenetic relationship. The conclusion drawn from the phylogenetic analysis of *C. marulius* populations used in this study, contributes significantly to the understanding of genetic variations within populations of this species. The findings provide valuable insight to devise conservation strategies in fisheries management programs in Pakistan.

Keywords *Channa marulius* · Morphometric parameters · COI gene · Phylogeny · Principal component analysis

Introduction

Channidae family have of genera, *Parachanna* and *Channa*, former is present across various Asian countries including Pakistan, Iran, Korea, China, Sri Lanka, Vietnam, India, Thailand, Indonesia and Malaysia [1]. The freshwater ecosystem in Pakistan showcases considerable diversity, accommodating numerous species of warm-water fish. Within Pakistan, *C. marulius* is found throughout the riverine courses in the warm water region and is commonly referred as snakehead fish. *C. marulius* holds significant position in the freshwater food fish and is widely distributed throughout Asiatic region [2] and has attained notable attention due to its extensive distribution, natural abundance, and high market value [3].

In recent decades, the populations of *C. marulius* have witnessed a decrease, attributed to factors such as habitat loss, overexploitation, and introduction of exotic species, disease outbreaks, and the pollution [4]. IUCN recorded the

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distributions of *C. marulius* as native and near to vulnerable [3]. Unfortunately, limited attention has been paid to stocks and populations using morphometric or molecular approaches in Pakistan even in advance technological era [5].

Examining morphometric parameters is a prevailing approach for evaluating differences among various stocks and other animals in both aquatic and terrestrial ecosystems [6]. Although Pakistan owns a guide for freshwater fish identification based on morphological traits [7], but identifying snakehead species presents considerable difficulties [8] due to the lack of updated data. In recent years, molecular techniques have gained popularity as a dependable and accurate method for systematic, particularly through the use of DNA barcoding, which aids in species identification and discovery [9]. This approach has proven to be effective and relatively reliable for both vertebrates and invertebrates, as evident by numerous successful studies [10, 11]. The utilization of DNA barcoding has added noteworthy discoveries within the realm of fish species, particularly focusing on freshwater fish populations [7]. DNA barcoding plays an important role in the identification of fish species and delineation [12–14]. Specifically, the COI gene (Cytochrome oxidase I gene), a fragment of mtDNA, is commonly used for this purpose. COI serves as a robust marker due to its favorable characteristics, including easy isolation [15]; rarity of recombination, insertion, and deletion events, minimal intra-species variations, and sufficient sequence uniqueness for distinguishing closely related species [12]. Furthermore, COI can be recovered from small samples, enhancing its utility in DNA barcoding [16]. It is crucial to address the need for an in-depth investigation and updated taxonomic understanding of snakehead fishes in Pakistan, as their diversity remains poorly understood. The main objective of this study was to observe the morphometric parameters, genetic diversity and phylogenetic trends of *C. marulius* species from different geographical parts of riverine system of Punjab, Pakistan using the COI gene. The findings can contribute to devise efficient management and conservation strategies for *Channa* species and will help in understanding the impact of environmental fluctuations on the physical characteristics of fish populations.

Materials and methods

Samples collection and transportation

A total of 200 specimen of *C. marulius* were collected from four distinct geographical sites in Punjab at River Indus (Taunsa Barrage and Chashma Barrage) and River Chenab (Trimmu Barrage and Head Panjnad) amongst the Indus riverine system of Pakistan (Fig. 1). The collected samples were transported in an icebox to the Department of Fisheries and Aquaculture at the University of Veterinary and Animal Sciences in Lahore for subsequent analysis.

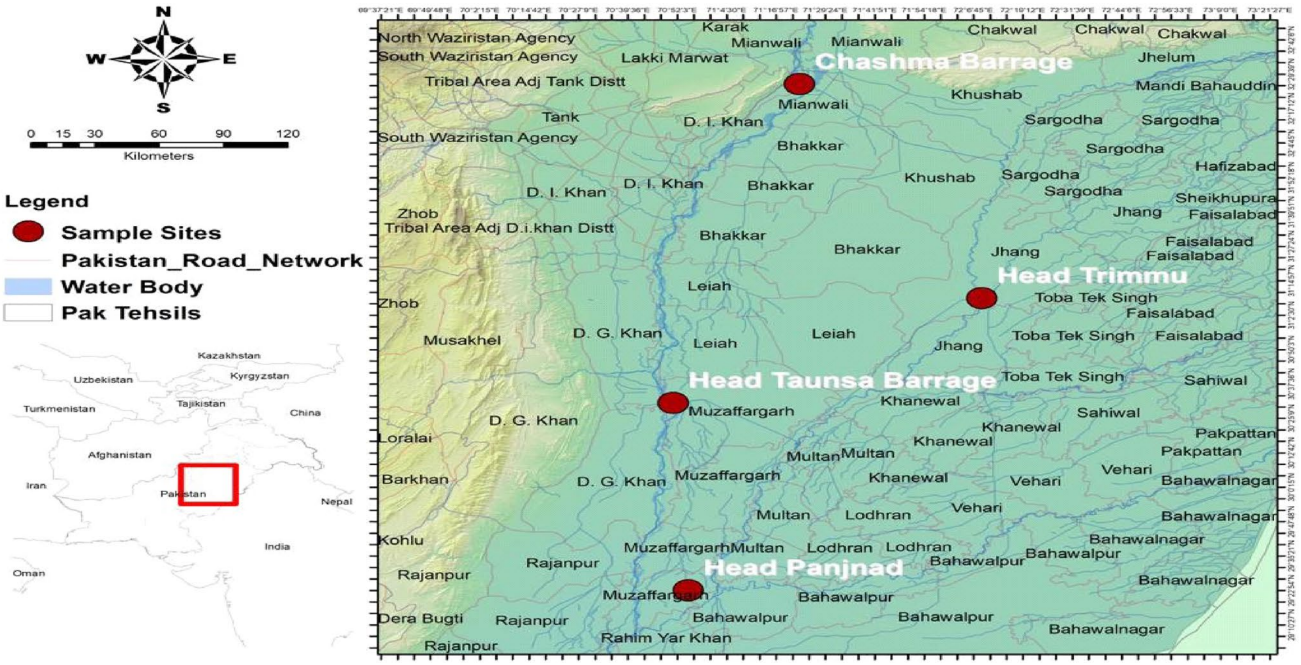

Fig. 1 Sampling location and geographical coordinates of sampled fish

Morphometric analysis

Morphometric parameters including body weight, total length, fork length, head length, paired pectoral, pelvic, and caudal fin length were documented for each individual sample obtained from various locations for comparison amongst the populations of different sites [17].

DNA extraction

DNA extraction was conducted using the phenol–chloroform protocol [29]. Genomic DNA was extracted and appropriately diluted to achieve a 100 ng/ml. COI gene for the *C. marulius* fish samples was accomplished by employing a pair of forward and reverse primers.

FishF1:$^{5'}$TCAACCAACCACAAAGACATTGGCAC$^{3'}$, FishR1:$^{5'}$TAGACTTCTGGGTGGCCAAAGAATCA$^{3'}$.

PCR amplification

The PCR reaction was devised with total volume of 25 µl with following constituents; 5 µl of genomic DNA, 2 µl each of forward and reverse primers, 12 µl of DreamTaq Green PCR Master Mix (2X) (Catalog number: K1081, ThermoFisher Scientific, USA), and 4 µl of deionized water. Amplification of the COI barcode region, spanning 658 bp, was performed using a thermal cycler (Bio-Rad, USA, Model 1861096) under specific thermal conditions: 95 °C initial denaturation for 5 min, followed by 30 cycles, each cycle involving denaturation at 94 °C for 35 s, annealing at 54 °C for 40 s, extension 72 °C for 1 min, and a final extension at 72 °C for 10 mins [18].

Gel electrophoresis and sequencing

The 1% agarose gel (Invitrogen) containing 5 µl ethidium bromide stain was visualized using a Gel Documentation System (Bio-Rad, USA). The COI gene was identified and subsequently subjected to Sanger sequencing, which was conducted by BGI Hong Kong Co. Ltd., China.

Morphometric and molecular data analysis

Regression analysis was used to estimate the length–weight relationship, while the analysis of variance (ANOVA) was performed to compare four populations from sampling sites on the basis of morphometric parameters through XLSTAT version 21.1.1. [17].

Alignment of the sequenced data was performed with sequences from NCBI using Clustal W [19]. The sequence was submitted to NCBI to acquire accession numbers. Additionally, the variations among species were assessed by averaging pairwise comparisons, and the count of haplotypes was evaluated using DnaSp 5.10 [20]. Utilizing the Kimura 2-parameter (K2P) model, and Neighbors Joining (NJ) trees were constructed with the assistance of MEGA 11 [21, 22].

Results

Morphometric parameters

The Principal Component Analysis (PCA) clearly demonstrated a correlation between the number of factors and the decrease in eigenvalues. This trend reached its peak at the second factor, as indicated in Table 1. The analysis involving transformation revealed that the first two factors (F1 and F2) significantly contribute to the variability, as specified by the bold values in Table 1. Utilizing the Kaiser criterion, which was based on eigenvalues greater than one, the PCA for morphometric character analysis indicated that, although the four populations of *C. marulius* did not exhibit high distinctiveness, 97.6% of the fish were accurately classified into their respective species clusters.

The first set of parameters, denoted as factor one (F1), includes fish body weight, total, dorsal fin, caudal fin, anal fin, average paired pectoral fins, and average paired pelvic fins length. This set accounted for 94.6% of the cumulative variability. The second distinct group, represented by the fork length of the fish body and referred to as factor two (F2), contributed 3% of the cumulative variability, as detailed in Table 1.

Positive correlations between fish body weight and fork, total, head, anal fin, and caudal fin length were recorded, as shown in Table 2.

The graphs generated using the PCA-processed data illustrate relationships among variables, observations, and the biplot, which represents both variables and observations. These graphs gave insights into the extent of correlation among individuals from various populations across four distinct geographical locations. The application of PCA for data transformation revealed that the graph based on the F1 and F2 factors effectively contributed up to 97.6% to the overall variability.

Table 1 Eigen values for *Channa marulius*

	F1	F2	F3	F4	F5	F6	F7	F8
Eigen value	7.5647	0.2401	0.0965	0.0439	0.0367	0.0159	0.0022	0.0000
Variability %	0.946	0.030	0.012	0.005	0.005	0.002	0.000	0.000
Comulative %	0.946	0.976	0.988	0.993	0.998	1.000	1.000	1.000

Table 2 Correlation matrix of morphometric parameters for *Channa marulius*

Variables	WT	FL	TL	HL	DFL	PFL	AFL	CFL
WT								
FL	0.979							
TL	0.996	0.974						
HL	0.980	0.958	0.983					
DFL	0.976	0.962	0.977	0.965				
PFL	0.970	0.941	0.956	0.951	0.929			
AFL	0.976	0.962	0.977	0.965	1.000	0.929		
CFL	0.857	0.827	0.851	0.840	0.850	0.844	0.850	

Specifically, F1 contributed to 94.6% of this variability, while F2 to 3.0%.

Upon examining the variable plot, it became evident that all populations fall within a similar range of values (ranging from 0 to + 1.0), displaying minimal differences depicted in Fig. 2. Similarly, the observation plot, which was also generated from the influence of these two factors, portrayed the same proportion of variability. In constructing this plot, a selection of 50 samples was made from each geographical location.

The observation plot highlighted that nearly 90% of the randomly chosen representative samples from all population clusters within a shared region, with values ranging from 0 to + 5. The remaining individuals were situated within a comparable range, extending from 0 to −4.5 (Supplementary Fig. 1). Consequently, based on PCA analysis, all geographical representative samples were categorized into two major groups, with their degrees of relatedness ranging from 0 to + 5 for the first group and from 0 to −4.5 for the second group. A similar trend was observed in the bi-plot, which was generated using these factors (Supplementary Fig. 2).

In the scree plot graph, the X-axis represented factor components such as fork length, total length, head length, dorsal fin length, pectoral fin length, and anal fin length. The Y-axis displayed eigenvalues, totaling 7.56 in Supplementary Fig. 3. Regression analysis of body weight with the total length of *C. marulius* from all sites yielded an R2 value of 0.992 Fig. 3.

Genetic diversity and population dynamic of *Channa marulius*

Total of four sequences were acquired and subsequently submitted to the GenBank database, where they have been assigned unique accession numbers OP169690, OP169691, OP169692 and OP169693. Furthermore, 5 haplotypes (h) on the basis of the COI gene of *C. marulius* were recognized in the present study. In general, haplotype diversity (Hd) was 0.42647, average number of nucleotide differences (Kt) 1.01471 and nucleotide diversity (PiT) was recorded 0.00182. *C. marulius* contains a unique control haplotype localized within the sub-population the non-significant *P*-value was documented. Table 3 presents the computed K2P genetic distance values based on COI sequences. These calculations were performed using MEGA X. The Transition/Transversion bias (R) was estimated to be 3.81. To determine the substitution pattern and rates, the K2P model was employed. The frequencies of nucleotides are documented as follows: A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. The intraspecies (*C. marulius*) from four

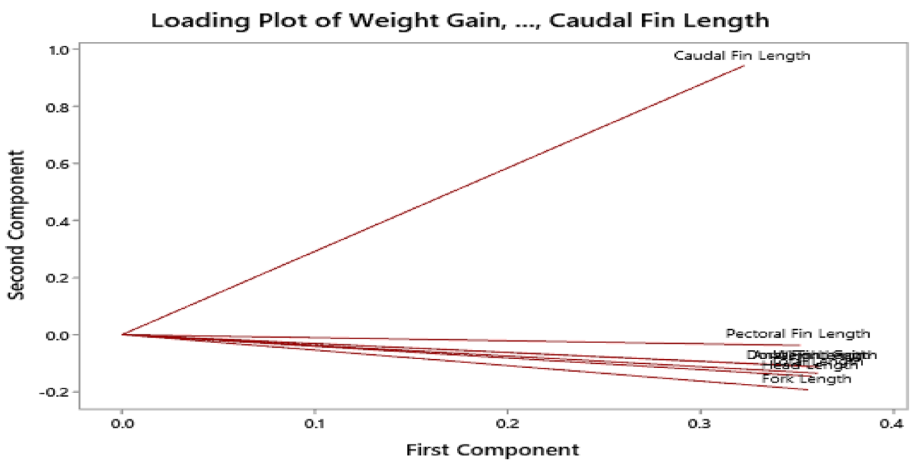

Fig. 2 Morphometric parameters (variables) plot

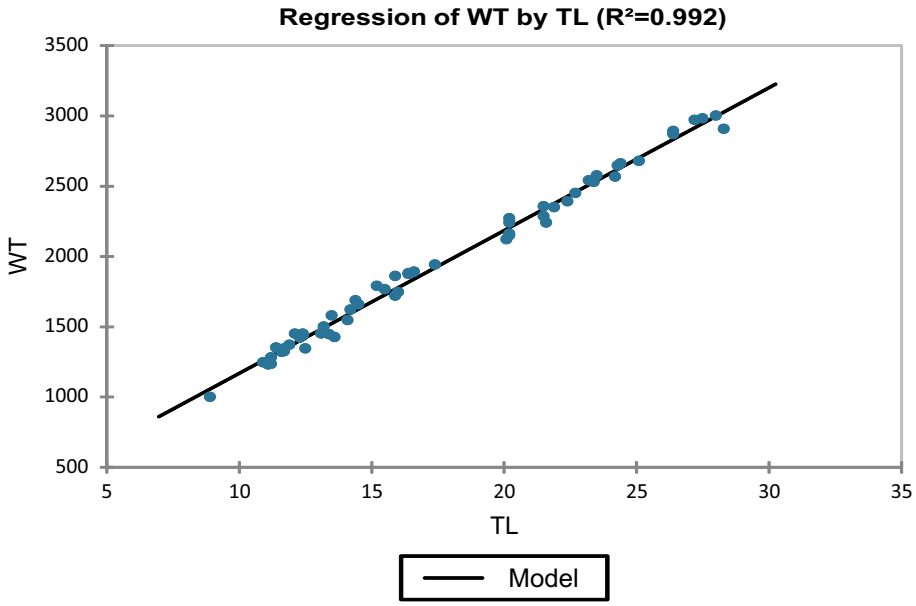

Fig. 3 Regression analysis WT by TL of *C. marulius*

different geographic location represented the distance ranged from 0.000 to 0.001, while with the sequences from database (NCBI) showed the distance ranged from 0.000 to 0.003. A pairwise sequence divergence is presented in Table 4. The range of intra-species sequence diversity varied from 0.006 to 0.000. Utilizing the K2P model, the NJ tree was created using both the *C. marulius* gene sequences from this study and additional sequences obtained from the database. This NJ tree construction is shown in Fig. 4.

Discussion

The morphometric analysis in the present study align with the work of [23] in the context of morphometric analysis of *Channa striata*. This study employed PCA Gen8 software to investigate the overall shape variability among specimens collected from three localities. The examination of the *C. striata* population unveiled morphometric variation among specimens from different areas within Laguna de Bay. Particularly, the morphology of *C. striata* from the Binangonan and Tanay regions displayed the least similarity, with the most pronounced variation in the cranial region. Similarly, specimens from Calamba exhibited the highest morphometric values in terms of length and weight measurements.

Furthermore, our findings align with the research conducted by [24] concerning the morphometric variation of *C. punctatus*. This investigation revealed significant variations in multivariate morphometric analysis among subpopulations of the freshwater murrel, *C. punctatus*, residing in distinct habitats with varying environmental conditions. Linear correlations were identified between various body measurements and the overall length of the fish across both habitats. Among the seventeen morphometric parameters and five meristic parameters analyzed through univariate (one-way ANOVA) analysis, fourteen and three parameters, respectively, exhibited statistical significance. Subsequently, these significant parameters were subjected to multivariate analysis utilizing principal component and discriminant function analyses.

In the PCA, the first component accounted for 57% of the variance, while the second and third components explained 13 and 6% of the total variance, respectively. Collectively, these three components elucidated 76% of the total variance within the two sub-populations. Morphological distinctions documented by [25] among five *Channa* species, their PCA unveiled a cumulative contribution rate of 78.928% by the first five principal components.

In our study, *C. striata* was also identified and characterized at the molecular level using the COI gene. These findings were consistent with a prior study by [26], which employed the partial cytochrome C oxidase subunit I (COI) for *Channa* species identification. Notably, the interspecific genetic distance (K2P) surpassed the intraspecific distance. Among the various species, the lowest interspecific distance occurred between *Channa argus* and *Channa maculata* (0.091), whereas the highest distance was observed between *C. argus* and *C. striata* (0.219). Evident barcode gaps were noted between interspecific and intraspecific distances within each species.

Similarly, [27] conducted DNA barcoding and phylogenetic analysis on catfish. Their study reported intraspecific genetic distances within 14 species ranging from 0 to 0.0368, with an average of 0.0063. The most substantial intraspecific genetic distance was identified within *Pseudobagrus eupogon*. Interspecific genetic distances ranged from 0.0069 to

Table 3 Pairwise genetic distances (Kimura 2-parameter) for *Channa marulius* species based on COI sequences

	OP169690	OP169691	OP169692	OP169693	KX946609	KX946607	MF496846	OL658834	OP1696911
OP169690									
OP169691	0.001								
OP169692	0.000	0.001							
OP169693	0.000	0.001	0.000						
KX946609	0.000	0.001	0.000	0.000					
KX946607	0.000	0.001	0.000	0.000	0.000				
MF496846	0.001	0.003	0.001	0.001	0.001	0.001			
OL658834	0.001	0.000	0.001	0.001	0.001	0.001	0.003		
OP169691	0.001	0.000	0.001	0.001	0.001	0.001	0.003	0.000	
KY425551	0.000	0.001	0.000	0.000	0.000	0.000	0.001	0.001	0.001
KY425550	0.000	0.001	0.000	0.000	0.000	0.000	0.001	0.001	0.001
MF496849	0.006	0.005	0.006	0.006	0.006	0.006	0.008	0.005	0.005
MF496845	0.006	0.005	0.006	0.006	0.006	0.006	0.008	0.005	0.005
OK093345	0.000	0.001	0.000	0.000	0.000	0.000	0.001	0.001	0.001
OK093329	0.003	0.005	0.003	0.003	0.003	0.003	0.001	0.005	0.005
KX946608	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000
OL440718	0.001	0.000	0.001	0.001	0.001	0.001	0.003	0.000	0.000
OK093355	0.001	0.000	0.001	0.001	0.001	0.001	0.003	0.000	0.000
	KY425551	KY425550	MF496849	MF496845	OK093345	OK093329	KX946608	OL440718	OK093355
OP169690									
OP169691									
OP169692									
OP169693									
KX946609									
KX946607									
MF496846									
OL658834									
OP169691									
KY425551									
KY425550	0.000								
MF496849	0.006	0.006							
MF496845	0.006	0.006	0.003						
OK093345	0.000	0.000	0.006	0.006					
OK093329	0.003	0.003	0.010	0.010	0.003				
KX946608	0.000	0.000	0.005	0.005	0.000	0.003			
OL440718	0.001	0.001	0.005	0.005	0.001	0.005	0.000		
OK093355	0.001	0.001	0.005	0.005	0.001	0.005	0.000	0.000	

0.2088, with an average distance of 0.1170 between distinct bagridae species. The greatest interspecific genetic distance was observed between *Mystus wyckioides* and *Pelteobagrus intermedius* (0.2088).

Same results were postulated in study conducted by [28] for identification and to analyze *Channa* species from the riverine system of Pakistan, employing the COI gene as a DNA barcoding marker. Their outcomes, based on the K2P model, demonstrated that intraspecific divergence for three species ranged from 0.00 to 0.023, while interspecific divergence ranged from 0.182 to 0.195. The most significant genetic distance was noted between *C. striata* and *C. marulius*, whereas the lowest distance was observed between *C. punctata* and *C. marulius*. The Neighbor Joining (NJ) tree, constructed based on the K2P model for twenty-seven specimens within the Channidae family, exhibited three distinct clades, affirming the presence of separate species. These findings further corroborate a consistent phylogenetic relationship among the species under investigation.

Table 4 Pairwise sequence divergence in COI sequences derived from *Channa marulius*

	OP169690	OP169691	OP169692	OP169693	KX946609	KX946607	MF496846	OL658834	OP169691
OP169690									
OP169691	0.001								
OP169692	0.000	0.001							
OP169693	0.000	0.001	0.000						
KX946609	0.000	0.001	0.000	0.000					
KX946607	0.000	0.001	0.000	0.000	0.000				
MF496846	0.001	0.003	0.001	0.001	0.001	0.001			
OL658834	0.001	0.000	0.001	0.001	0.001	0.001	0.003		
OP169691	0.001	0.000	0.001	0.001	0.001	0.001	0.003	0.000	
KY425551	0.000	0.001	0.000	0.000	0.000	0.000	0.001	0.001	0.001
KY425550	0.000	0.001	0.000	0.000	0.000	0.000	0.001	0.001	0.001
MF496849	0.006	0.005	0.006	0.006	0.006	0.006	0.008	0.005	0.005
MF496845	0.006	0.005	0.006	0.006	0.006	0.006	0.008	0.005	0.005
OK093345	0.000	0.001	0.000	0.000	0.000	0.000	0.001	0.001	0.001
OK093329	0.003	0.005	0.003	0.003	0.003	0.003	0.001	0.005	0.005
KX946608	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000
OL440718	0.001	0.000	0.001	0.001	0.001	0.001	0.003	0.000	0.000
OK093355	0.001	0.0000	0.001	0.001	0.001	0.001	0.003	0.000	0.000

	KY425551	KY425550	MF496849	MF496845	OK093345	OK093329	KX946608	OL440718	OK093355
OP169690									
OP169691									
OP169692									
OP169693									
KX946609									
KX946607									
MF496846									
OL658834									
OP169691									
KY425551									
KY425550	0.000								
MF496849	0.006	0.006							
MF496845	0.006	0.006	0.003						
OK093345	0.000	0.000	0.006	0.006					
OK093329	0.003	0.003	0.010	0.010	0.003				
KX946608	0.000	0.000	0.005	0.005	0.000	0.003			
OL440718	0.001	0.001	0.005	0.005	0.001	0.005	0.000		
OK093355	0.001	0.001	0.005	0.005	0.001	0.005	0.000	0.000	

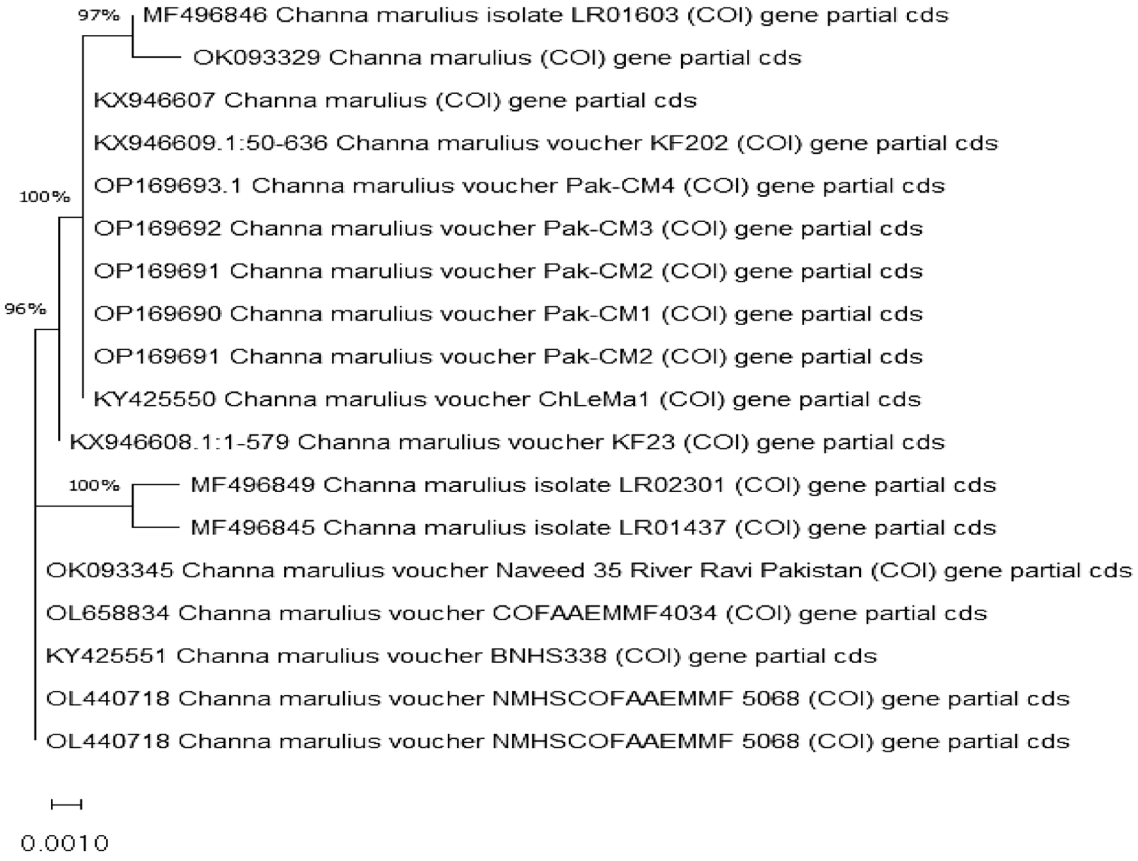

Fig. 4 NJ tree constructed of *C. marulius* from different geographical locations

Conclusion

This study concluded that the construction of a cladogram showcasing the evolutionary relationships of *C. marulius* from diverse geographical localities, contributes significantly to the understanding of genetic variations within populations this species. The findings underscore the importance of molecular techniques in elucidating population structures and genetic diversity, providing valuable insights for conservation efforts and fisheries management in Pakistan's freshwater ecosystems. Further research in this direction has the potential to uncover more intricate genetic patterns and evolutionary dynamics among *Channa* species, fostering a deeper comprehension of their ecological roles and adaptive strategies.

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Author's contribution GR and FR conceived and designed the study. GR executed the experiment and finalized the data. MF and MBBM helped in sampling. MF assisted in experimentation. GR wrote the manuscript. GR and FR contributed equally to this work. All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this article.

Declarations

Competing interest The authors declare that they have no known financial interests or personal relationship that could have appeared to influence the work reported in this paper.

Ethical approval Research Synopsis was first approved by ethical committee of the university and based on their approval, layout of the experimentation was approved/allowed by Advanced Studies Research Board (ASRB) of the University of Veterinary & Animal Sciences, Lahore-Pakistan (Approval/Memorandum Attached).

Consent to participate Not applicable.

Consent to publish Not applicable.

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