Unveiling intraspecific variations in *Notopterus* notopterus and *Chitala chitala* through landmark-based morphometry and mitochondrial DNA barcoding

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Abstract

This study assessed the biodiversity of Notopteridae family by involving *Notopterus notopterus* and *Chitala chitala* because of their contribution to the ecological balance of freshwater habitats, food security, economic importance and aquaculture. Both fish species are under pressure and facing threats in their natural habitats and especially *Chitala chitala* is considered near threatened. Fish were sampled from the natural riverine population of the Punjab, Pakistan including Taunsa Barrage, Trimmu Head and Chashma Barrage. Fish samples were recognized morphologically, and the *COI* gene sequence was used to prepare them for genetic study. Maximum Likelihood (ML) trees were constructed using MEGA 11 and the K2P model. A total 30 individual of *N. notopterus* and *C. chitala* (15 of each) were identified by using morphometric and truss analysis. Twenty-five morphological, five meristic and 27 landmarks were measured. In this study, all meristic characters of *N. notopterus* and *C. chitala* species showed non-significant (p>0.05) results and 14 out of 25 morphological characters of *N. notopterus* showed significant (p<0.05) results while *C. chitala* showed non-significant results in all morphological characters. Clustered analysis of *N. notopterus* and *C. chitala* showed higher mean values of the external morphological traits in Taunsa barrage and Chashma barrage, respectively. In truss study PC1 and PC2 revealed 74.9% and 11.2% in *N. notopterus* and 85.6% and 11.5% in *C. chitala*, respectively. The overall mean of base composition of family Notopteridae was observed as T (29.45%), C (26.17%), A (27.75%), G (16.63%). Generally, GC content (42.8%) was less than AT content (57.2%), which revealed a distinct pattern of anti-G bias. The genetic distance within two genus of family Notopteridae species were found to be 0 while between species, the mean distance varied from 0.002 to 0.149. Phylogenetic tree revealed the identity in the range of 99% for sequences of *N. notopterus* and *C. chitala* that clustered in two clads due to two distinct genera but having same family. In this study, 12 barcodes of family Notopteridae with an e-value of 0.0 and a maximum similarity of 99–100% with a maximum length of 600 bp were found. The current work developed the idea for the establishment of the Pakistani fish gene bank, which is still needed, and supported the effectiveness of the *COI* gene for the species identification in the riverine population. Information of population structure and genetic variation obtained from current study would be helpful for planning appropriate strategies for the rehabilitation, conservation and efficient management for these two species.

Keywords: *N. notopterus, C. chitala*, Morphological, Meristic, Truss analysis, DNA barcoding, Phylogeny, Conservation

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Introduction

Pakistan has one of the world's greatest freshwater resources and a correspondingly huge variety of freshwater animals, among which fishes are particularly noteworthy (Rehman *et al*., 2015). Freshwater fish also provide important ecological services in terms of economics, nutrition, science, history, and culture. Freshwater fishes have a variety of responses to anthropogenic stressors, making them useful markers for monitoring the biological and ecological integrity of freshwaters. Freshwater fish are frequently used to assess the functioning of freshwater ecosystems and habitat quality (Radinger *et al*., 2019).

The taxonomy aims to recognize the fish species. Fish industry benefit, evaluation of environmental consequences, ceasing illegal trafficking demonstrating protective areas and structuring of fishery resources are vital for the recognition of fishes. Fish species are commonly identified based on their visible morphology, with various morphological keys employed for this purpose. Fish have a wide range of morphological traits as they progress through ontogenetic metamorphism and their morphometric characteristics vary as a result of this. In recognition procedure, comparably many challenges are forced by convergent and divergent modifications (Prasad and jogi, 2020).

Misidentification of species can endanger not just the species, but also the environment, due to inaccuracies in monitoring, inefficient resource allocation for preservation efforts and an unnoticed drop in fish stocks. For fisheries to be sustainable and productive, accurate stock determination is vital (Wariaghli *et al*., 2021).

Fish descriptive qualities; morphometric and meristic methods can both be used for fish identification. Morphometric is a term that refers to a collection of complex statistical processes that are used to assess differences in the size and form of organs and organisms. Morphological systematic was defined as the measurement of morphometric features and the meristic account, which was regarded as the most reliable and straightforward means of identifying a specimen. Morphometric analysis is a crucial technique for understanding organism growth and development, as well as the systematics, changes and structure of population features. This tool can be used to investigate the interaction of environment, selection and heredity on a species' body shape and size (Tripathy, 2020).

Morphometric analysis, like image analysis provides more efficient and powerful tools for finding variations within groups and distinguishing between species of similar shape (Mojekwu *and Anumudu*, 2015). Meristic characteristics are countable characters. Meristic involves counting the number of fin rays and other fish body parts. Observing fish characteristics, such as tail fin shape, color patterns, coloring and others are descriptive characteristic. Due to a lack of taxonomic skill, the traditional strategy of using fish morphometry and

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meristic characteristics for stock identification or discrimination research has recently been less familiar. Interbreeding resulted from long-term geographic isolation, morphometric characteristics are crucial for studying stock structure (Ramya *et al*., 2021).

The standard morphometric approaches have been questioned since they have been found to have some limitations in defining fish shape. As a result, the "truss network technique," a powerful landmark-supported statistical analysis tool, is utilized to classify species. Many workers have employed this technique extensively to discriminate between species. In order to create a regular pattern of connected quadrilaterals or cells across the body, a series of distances between landmarks are computed to create a truss network. In order to learn more about an organism's morphology, landmarks from digital photographs can be analyzed using geometric morphometrics (Gupta *et al*., 2018).

The truss network system encompasses the entire fish in a consistent network, increasing the potential for extracting differences between specimens. Truss network helps to overcome size dependent variation and also takes into account the form variation, whereas size-related morphometric measurements are mostly age dependent and as a result fail to disclose the actual variance both within and between the populations. The development of truss analysis has made it a valuable taxonomic tool for stock identification as well as for separating morphologically similar species. Because it can analyse a large number of samples in a short amount of time, the Truss Network System is employed as a fisheries management tool (Mallik *et al*., 2020).

DNA barcoding is extensively acknowledged as the leading genetic method for species identification and has played a crucial role in the discovery of new species across different organismal groups (Ude *et al*., 2020; Tsoupas *et al*., 2022). This method, which employs a small mitochondrial DNA fragment for species identification, was originally pioneered by Hebert and colleagues at the University of Guelph, Canada (Rahman *et al*., 2019).

Taxonomic uncertainty is prevalent among many fish species, making accurate identification essential for effective management and trade. Additionally, issues such as species mislabeling and fish fillet misrepresentation are widespread in global fish markets. To address these challenges, Hebert *et al*. (2003) introduced the concept of DNA barcoding, proposing a novel method for species identification. DNA barcoding provides several benefits over traditional taxonomic approaches (Tsoupas *et al*., 2022).

Furthermore, based on physical characteristics, classification and taxonomic identification can be difficult and time-consuming; yet, species documentations typically need a substantial amount of taxonomic data. Additionally, morphological identification is limited to specific life

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phases. Additionally, a variety of taxonomists possess varying identification skills and abilities, which can lead to contradictory identifications of the same specimen when comparing and summarizing data (Hulley *et al*., 2019). Therefore, precise species-level specimen identification is essential for environmental research and protection. Genetic approaches are necessary for the identification of fish species due to the limitations of traditional taxonomy and the low number of taxonomists (Sheraliev and Peng, 2021).

Ancestral teleost lineages represented by the Order Osteoglossiformes contain fossil records that date to the late Jurassic to early Cretaceous epoch. Ten species from four genera make up the family Notopteridae, which are found in freshwaters in South Asia and Africa (Dutta *et al.,* 2020). The bronze featherback, *Notopterus notopterus* (Osteoglossiformes), belongs to a family Notopteridae of fishes known as "knife fishes," which are extensively spread in South and Southeast Asia, Africa, and other parts of the world. The vital food fish *N. notopterus* can be found in rivers, reservoirs, lakes, and ponds. The food fish *N. notopterus* is especially significant and has a high market value. For instance, this species has the highest export value of any fish in Cambodia. The fish is also prized in the aquarium industry as a species for decorations (Borkhanuddin *et al*., 2020).

One of the earliest fundamental teleost lineages is the humped featherback, *Chitala chitala* (Hamilton, 1822), which is a member of the Superorder Osteoglossomorpha, order Osteoglossiformes, and family Notopteridae. Due to its scarcity and delicate nature, *Chitala* is regarded as one of the most valuable and expensive fish for food, sport, and aquarium reasons. In the natural, this species plays a very important part in controlling the number of common carp, minnows, and insects (Mitra *et al*., 2018). As a commercially significant species, *C. chitala* has been given priority as a potential species for aquaculture. However, overexploitation and the degradation of its natural habitat and breeding grounds have led to a fall in its natural abundance (Chandran *et al*., 2020).

In this study, morphometric variations among fishes of the family *Notopteridae* were examined. The research was based on both traditional and truss network analyses**,** aimed at providing useful insights into the morphometric characters responsible for shape and size variations between two selected species, *Chitala chitala* and *Notopterus notopterus*. Although some morphological work had been conducted on these species in other countries, no such comprehensive study had been reported from Pakistan. Therefore, efforts were made to collect morphological data from Chashma Barrage**,** Trimmu Head**,** and Taunsa Barrage. In addition to morphological analysis, the study also employed DNA barcoding to identify the selected species at the genetic level**,** allowing for more accurate species delineation and detection of cryptic diversity. The

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integration of morphological and molecular approaches enhanced the accuracy of species identification and provided a more robust understanding of intra- and interspecific variation within Notopteridae. The data obtained in this research can be utilized by future researchers and will contribute to the management and conservation of these threatened species by highlighting population-level distinctions and supporting strategies for their sustainable preservation.

Materials and methods

Study area

The present research was conducted in three location of the Punjab, Pakistan including Trimmu Head, Chashma Barrage and Taunsa Barrage. In the Punjab province, the Chashma Barrage is situated southwest of Mianwali on the Dera Ismail Khan Road. Chashma barrage covers around 34,099 hectares and is located at a height of approximately 225 meters (Shelly *et al*., 2011). Trimmu Head is situated on the Bhakkar road, 21 kilometers far from District Jhang, close to Athara hazari, where river Jhelum falls down into the river Chenab. Trimmu Head covers 3,680,43 acres in the head pond. The Taunsa Barrage offers a vast and diversified macro-habitat. It is situated at a height of 137 meters in the Muzaffargarh area of southern Punjab Pakistan (Bibi and Ali, 2013) (Fig. 1).

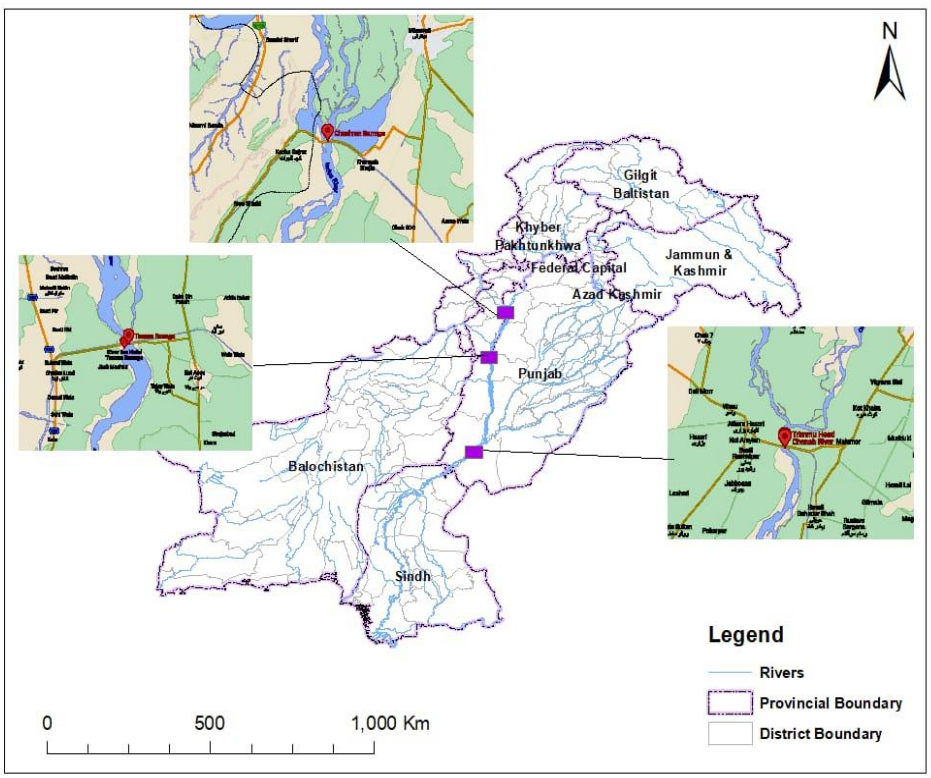

Figure 1: Map showing three different sampling location of Punjab, Pakistan.

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Sample collection

The fish samples were collected from three sites by fishing net device with the help of fisherman. The fish were morphologically recognized through visual examination. After collection, the sample were labeled, packed in polyethylene bag and carried back to the laboratory at Government College University Faisalabad for further analysis. Samples were recognized by using identification key "A Key to Fishes of Punjab" provided by Mirza. Samples were preserved in 70 percent ethanol after taking picture and for genetic analysis (Fig. 2 and Table 1).

(a) (b)

Figure 2: Fish specimen of (a) *Notopterus notopterus* and (b) *Chitala chitala.*

Table 1: Sampling details of *N. notopterus* and *C. chitala* collected from selected locations of Taunsa Barrage (TB), Trimmu Head (TH) and Chashma Barrage (CB).

Family	Genus	Species	Sample No	Max Length (mm)	Location
Notopteridae	Notopterus	*N. notopterus*	15	290	TB, TH, CB
	Chitala	*C. chitala*	15	383	TB, TH, CB

Morphological identification

Morphometric characters were measured with the help of digital vernier calliper. The following 27 morphometric characters were measured on each specimen. Descriptions of measured morphometric characters are presented in Table 2. Meristic features such as Anal fin rays (AFR), Dorsal fin rays (DFR), Caudal fin rays (CFR), Pelvic fin rays (PvFR), Pectoral fin rays (PcFR), of each sample were counted (Table 3). The main fin rays were manually counted. The fin rays were counted with the use of a magnifying glass.

Truss analysis

A total of 11 anatomical landmarks were chosen for the investigation, and the box-truss network, which represents a truss network of 27 lines, was created by interconnecting these landmarks. Manual procedures were used to measure each landmark line, which involved piercing the paper with a needle (Figs. 3 and 4).

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Table 2: Description of morphometric characters used in this study.

Characters	Abbreviations	Description
Total Length	TL	The distance between the snout tip and the longest caudal fin ray
Standard Length	SL	The distance between the tip of snout and the end of vertebral column
Snout Length	SnL	The distance between the tip of mouth and anterior edge of eye
Head Length	HL	The distance between the tip of snout and posterior edge of operculum
Head Depth	HD	The space between the head's two broadest points
Higher Body Depth	HBD	Greatest vertical length of the body
Lower Body Depth	LBD	Smallest vertical length of the body
Eye Diameter	ED	The distance from anterior to posterior edge of the eye
Pre-Orbital Length	PrOL	The distance from tip of snout to anterior part of the eye
Post-Orbital Length	PsOL	The distance from posterior part of the eye to the edge of operculum
Inter-Orbital	IO	The distance of eyes from each other
Upper Jaw Length	UJL	The distance between the two end points along the upper jaw margin
Lower Jaw Length	LJL	The distance between two endpoints along the lower jaw margin
Pre-Dorsal Length	PrDL	The distance between tip of the snout and base of the first dorsal fin ray
Post-Dorsal Length	PsDL	The distance between origin of dorsal fin and base of caudal
Pre-Anal Length	PrAL	The distance between tip of the snout and base of the first anal fin ray
Pre-Pectoral Length	PrPecL	The distance between tip of the snout and base of the first pectoral fin ray
Pre-Pelvic Length	PrPevL	The distance between tip of the snout and base of the first pelvic fin ray
Length Dorsal Fin Base	LDFB	The distance between dorsal fin base's most anterior and posterior points
Total anal caudal base	TACB	Length from the anterior edge of anal fin to the tip of caudal fin along fin base
Length of Pectoral Fin Base	LPecFB	The distance between pectoral fin base's most anterior and posterior points
Length of Pelvic Fin Base	LPevFB	The distance between pelvic fin base's most anterior and posterior points
Height of Dorsal Fin	HDF	The distance from the base of the dorsal fin origin to the end of the longest fin ray
Height of Anal Fin	HAF	The distance from the base of the anal fin origin to the end of the longest fin ray
Height of Pectoral Fin	HPecF	The distance from the base of the pectoral fin origin to the end of the longest fin ray
Height of Pelvic Fin	HPevF	The distance from the base of the pelvic fin origin to the end of the longest fin ray
Caudal Fin Length	CFL	The distance from the base of the caudal fin origin to the end of the longest fin ray

Table 4 represents the description of 11 landmarks.

DNA extraction and PCR amplification

A small piece of ethanol-preserved tissue was excised for DNA extraction using the QIAamp DNA Mini Kit, according to the manufacturer's protocol. A conserved region of the *COI* gene's 5' end was then amplified with specific primers. The Cytochrome c oxidase subunit 1 (*COI*) gene was

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amplified using the universal primers Fish F1 and Fish R1, which were synthesized by MACROGEN Inc., Seoul, Korea. These primers have been previously described in studies by Ward *et al*. (2005).

Table 3: Description of meristic characters used in this study.

Characters	Abbreviation	Description
Dorsal fin rays	DFR	Total number of dorsal fin rays
Anal fin rays +Caudal fin rays	AFR+CFR	Total number of anal and caudal fin rays
Pectoral fin rays	PecFR	Total number of pectoral fin rays
Pelvic fin rays	PevFR	Total number of pelvic fin rays

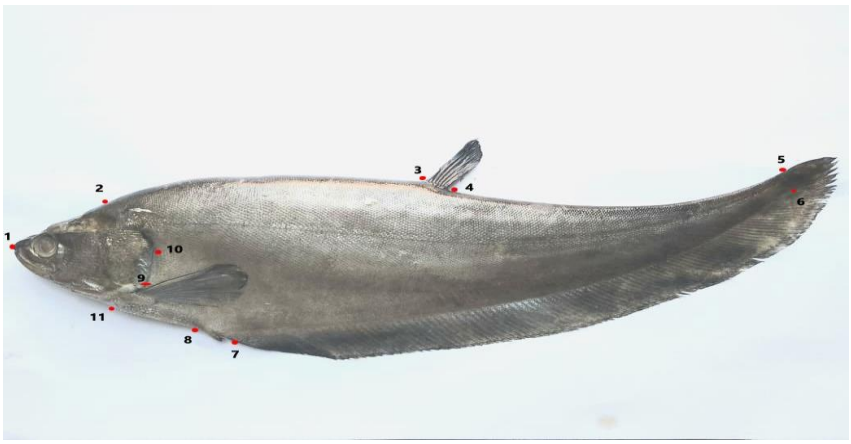

Figure 3: Points of 11 landmarks on fish body.

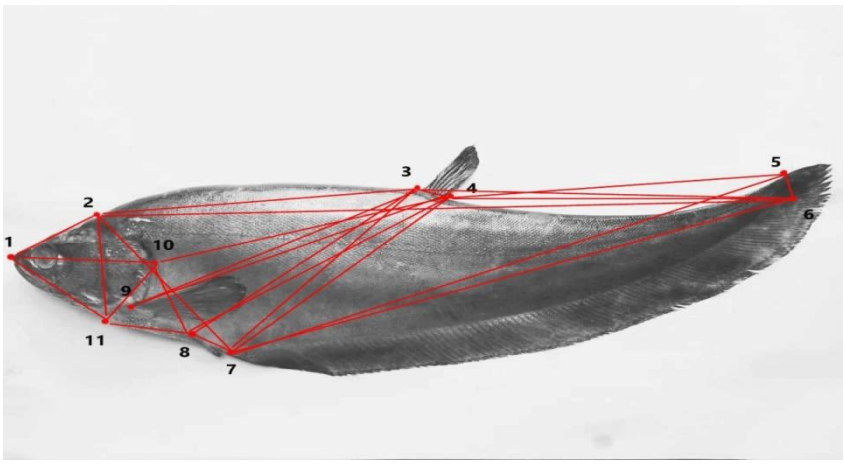

Figure 4: Points of landmarks representing truss network on fish body.

Primer sequences were:

FishF1: (5/ TCAACCAACCACAAAGACATTGGCAC 3/)
FishR1: (5/ TAGACTTCTGGGTGGCCAAAGAATCA 3/)

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Table 4: Description of truss network characters used in the study.

Landmark No	Landmark position
1	The anterior tip of the snout of mouth
2	The most backward part of the neuro-cranium
3	The origin of the dorsal fin
4	The end of the dorsal fin
5	The prior attachment of the dorsal membrane from the caudal fin
6	The prior attachment of the ventral membrane from the caudal fin
7	The origins of the anal fin
8	The insertion of the pelvic fin
9	The origin of the pelvic fin
10	Posterior edge of operculum
11	The gill line insertion points on ventral side

The PCR reaction mixture was prepared with a final volume of 25 µl, containing 1X reaction buffer, 1 µl of template DNA, 2.5 mM dNTPs, 2.5 mM $MgCl_2$, 0.2 U Taq DNA polymerase, and 0.5 µl of each primer. Amplification was performed using the DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD). The PCR conditions were as follows: an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at an optimized temperature for 30 seconds, extension at 72°C for 40 seconds, and a final extension at 72°C for 7 minutes. The PCR products were visualized via 1% agarose gel electrophoresis, with SYBR Green staining.

PCR amplification and sequencing were performed using the Thermocycler T100 from BioRad. Prior to Sanger sequencing, the amplified PCR products underwent purification using the FavorPrepPCRCleanUpMiniKit (Cat. # FAPCK001-1). Sanger sequencing was carried out uni-directionally to accurately characterize the freshwater species. The complete nucleotide sequences obtained from sequencing were deposited into GenBank for reference and further analysis.

Statistical analysis

Univariate analysis of variance (ANOVA) using post hoc Tukey test for the comparison of mean for morphometric and meristic characters of collected samples among different locations and multivariate (principal component analysis) were used for morphometric features to estimate the significant change with significance level of 5% ($p<0.05$) was measured to check the significance of truss and morphometric measurement. All data were analysed using Microsoft office excel and Statistical Packages for Social Sciences (SPSS) software. The sequencing data were converted into FASTA format, and BLAST searches of the *COI* sequences were conducted using the NCBI database to determine the closest homologous matches. The MEGA 11 program was used to perform evolutionary analysis on the aligned sequences. Kimura's two-parameter distance model was used to calculate evolutionary divergence.

Results

Body color and shape

N. notopterus specimens can be distinguished from all other Oriental freshwater fishes because of the tapering tail and the corners of the mouth below

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the eye. According to reports, the standard length of this species can reach up to 60 cm. For this species, a wealth of information about its cytogenetic (Barby *et al*., 2019), phylogeography (Yanwirsal *et al*., 2017), and reproductive behaviour is accessible (Lavoue *et al*., 2020).

Chitala's body is a coppery brown tint. The tiny, yellowish-gray dorsal fin projects posteriorly from the body's midline. The pelvic fins are basic, while the pectoral fins are substantially extended to the anal fin. The caudal fin, which has the appearance of a feather, is long and continuous and has a razor-shaped anal fin (100-130 fin rays). Near the tail, a variety of large dark dots can be seen. Between the thorax and the base of the ventral fin, the abdomen margin has around 51 serrations. On the left side, there may be 0 to 16 distinct black spots, and on the right, there may be 1 to 19 spots. The presence of spots and marks is influenced by the environment, genetics, and dietary composition (Mitra *et al*., 2018).

The maxilla only reaches the center of the eye in *N. notopterus* and well beyond the posterior margin in *C. chitala*. It was also noted that the dorsal fin of *C. chitala* was placed close to the base of the caudal fin, whereas the dorsal fin of *N. notopterus* was inserted more toward the tip of the snout. On the basis of the fish's craniodorsal profile, which was sharply concave in *C. chitala* and nearly straight in *N. notopterus* distinguished these two taxa. Furthermore, these species differ because, in contrast to *C. chitala*, which has jaws that never stop growing during its lifetime and reach past the back of its eyes (Rawal *et al*., 2020).

Morphological analysis

In this study, a total of 30 samples of *N. notopterus* and *C. chitala* were collected from three different locations i.e., Trimmu Head (TH), Chashma Barrage (CB) and Taunsa Barrage (TB). Population of *N. notopterus* and *C. chitala* showed higher values of the external morphological traits in TB and CB, respectively (Tables 5 and 6). The mean value of TL of *N. notopterus* and *C. chitala* was 247.17mm and 373.12mm, respectively. Mean value at CB was greater than TB and TH in *C. chitala* specimen. The finding of traditional morphometric analysis represents that there was significant variation in morphological character. One-way ANOVA followed by post-hoc Tukey test with 0.05 significance value (p<0.05). In *N. notopterus* morphometric characters like Total length (TL), Standard Length (SL), Head Length (HL), Higher Body Depth (HBD), Lower Body Depth (LBD), Eye Diameter (ED), Post-Orbital Length (PsOL), Inter-Orbital (IO), Upper Jaw Length (UJL), Lower Jaw Length (LJL), Pre-Pectoral Length (PrPecL) Length of Dorsal Fin Base (LDFB), Total Anal Caudal Base (TACB), Length of Pelvic Fin Base (LPevFB), Height of Pectoral Fin (HPecF) and Height of Pelvic Fin (HPecF) showed significant result (p<0.05). While other, Snout Length (SnL), Head Length (HL), Pre-Orbital Length (PrOL), Pre-Dorsal Length (PrDL), Post-Dorsal Length (PsDL),

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Pre-Anal Length(PrAL), Pre-Pelvic Length (PrPevL), Length of Pectoral Fin Base (LPecFB), Height of Dorsal Fin (HDF), Height of Anal Fin (HAF) and Caudal Fin Length (CFL) showed non-significant results (p>0.05) (Table 5). In *C. chitala* all parameters TL, SL, SnL, HL, HD, HBD, LBD, ED, PrOL, PsOL, IO, UJL, LJL, PrDL, PsDL, PrAL, PrPecL, PrPevL, LDFB, TACB, LPecFB, LPevFB, HDF, HAF, HPecF, HPevF and CFL showed non-significant results (Table 6).

Table 5: Mean values (mm) of morphometric traits of *N. notopterus.*

Morphometric characters	Overall	CB	TH	TB	ANOVA test	
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	F	*p*-value
TL	247.17±37.63	217.86±31.50	233.47±13.70	290.17±12.51	16.248	.000
SL	227.74±36.49	198.72±29.35	214.78±12.99	269.74±12.77	17.434	.000
SnL	12.80±1.30	12.35±1.21	12.70±1.35	13.35±1.40	.750	.493
HL	51.05±10.28	44.26±9.79	49.19±8.80	59.69±6.41	4.345	.038
HD	28.23±5.65	24.47±5.96	27.54±4.40	32.67±3.75	3.727	.055
HBD	66.35±6.26	61.39±7.71	66.04±2.97	71.61±1.87	5.463	.021
LBD	15.31±2.32	13.60±2.21	14.82±1.18	17.49±1.63	6.646	.011
ED	8.72±.66	8.26±0.72	8.54±0.30	9.38±0.24	7.702	.007
PrOL	8.45±1.13	8.32±0.97	7.86±0.57	9.18±1.46	1.980	.181
PsOL	33.80±8.65	28.77±9.58	33.52±8.47	39.10±5.62	2.051	.171
IO	6.70±.45	6.47±0.29	6.40±0.14	7.22±0.32	15.129	.001
UJL	16.08±1.20	15.17±1.40	15.94±0.67	17.14±0.36	5.862	.017
LJL	15.07±1.24	14.16±1.29	14.64±0.30	16.40±0.39	10.879	.002
PrDL	134.88±22.39	119.27±27.13	134.29±18.27	151.09±7.17	3.390	.068
PsDL	112.75±19.14	101.42±23.03	111.07±17.67	125.78±7.91	2.496	.124
PrAL	69.51±13.02	60.71±13.87	68.20±11.56	79.61±6.39	3.707	.056
PrPecL	45.64± 8.24	40.09±8.05	43.93±6.92	52.91±4.07	5.030	.026
PrPevL	65.67±12.1	59.25±15.77	63.92±10.20	73.83±4.70	2.221	.151
LDFB	6.36±.46	6.07±0.50	6.22±0.19	6.79±0.31	5.620	.019
TACB	169.64±24.52	150.21±25.84	165.56±13.67	193.15±8.11	7.717	.007
LPecFB	6.30±.97	5.57±1.29	6.36±0.50	6.99±0.36	3.689	.056
LPevFB	3.52±.84	3.10±0.73	3.06±0.70	4.38±0.22	7.931	.006
HDF	25.67±2.81	24.03±3.75	24.97±1.18	28.01±1.20	3.853	.051
HAF	20.68±1.94	20.45±2.87	19.78±0.28	21.81±1.47	1.540	.254
HPecF	30.02±3.23	28.03±4.08	29.20±2.04	32.83±0.32	4.502	.035
HPevF	5.71±.93	5.29±0.56	5.29±0.94	6.54±0.72	4.538	.034
CFL	19.46±2.12	18.61±2.51	19.37±1.43	20.41±2.33	.883	.439

CB= Chashma Barrage, TH = Trimmu Head, TB=Taunsa Barrage, SD = Standard deviation

Meristic analysis

From the descriptive statistic of meristic traits of *N. notopterus*, Dorsal Fin Rays (DFR) showed variation at Chashma Barrage. In case of Pectoral Fin Rays (PecFR) and Peclvic Fin Rays (PevFR), there were no variation between Chashma Barrage and Taunsa Barrage population, while Anal Fin Rays+Caudal Fin Rays (AFR+CFR) at Trimmu Head specimens showed greater average value. All parameters showed non-significant results (p>0.05) (Table 7). In *C. chitala*, DFR and PecFR showed no variation between Trimmu Head and Taunsa Barrage. AFR+CFR showed slight difference in mean values of population of Trimmu Head. Among three locations, variation had been observed in PecFR.

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Table 6: Mean values (mm) of morphometric traits of *C. chitala*.

Morphometric Characters	Overall	CB	TH	TB	ANOVA test	
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	F	*p*-value
TL	373.12±45.96	382.68±57.48	355.29±48.52	381.40±33.74	.527	.603
SL	346.89±43.77	356.68±55.20	329.28±44.71	354.70±32.82	.572	.579
SnL	16.43±1.29	17.08±1.14	16.07±1.78	16.14±0.75	.942	.417
HL	78.82±10.18	78.26±14.20	75.91±10.88	82.28±4.12	.462	.641
HD	31.75±1.65	32.37±1.58	30.89±1.68	31.99±1.65	1.103	.363
HBD	93.20±11.95	98.93±13.69	90.23±11.46	90.44±10.92	.844	.454
LBD	21.07±2.67	21.23±3.49	20.65±2.76	21.33±2.23	.082	.922
ED	9.56±0.60	9.81±0.49	9.40±0.93	9.46±0.18	.639	.545
PrOL	10.25±1.56	10.80±1.66	10.06±2.12	9.89±0.85	.437	.656
PsOL	57.53±10.57	57.73±14.26	54.03±11.72	60.84±4.62	.481	.629
IO	10.66±1.75	11.36±2.09	10.51±2.05	10.12±1.05	.627	.551
UJL	21.28±2.24	22.34±2.48	21.32±1.73	20.18±2.34	1.201	.335
LJL	20.01±2.15	20.77±2.56	19.99±1.48	19.29±2.48	.549	.591
PrDL	186.38±25.80	194.82±34.50	178.06±23.60	186.25±20.15	.490	.625
PsDL	180.92±30.47	183.24±40.89	169.94±33.37	189.57±14.58	.503	.617
PrAL	93.22±14.69	96.45±22.39	89.95±10.68	93.27±10.68	.217	.808
PrPecL	69.45±10.10	70.73±13.33	66.00±11.14	71.62±5.70	.409	.673
PrPevL	90.71±10.73	92.74±12.84	86.58±11.69	92.81±8.35	.517	.609
LDFB	9.13±1.44	9.81±1.73	8.69±1.23	8.88±1.37	.842	.455
TACB	272.24±45.21	281.66±54.26	254.26±49.88	280.78±27.97	.555	.588
LPecFB	8.52±0.76	8.46±0.79	8.12±0.93	8.97±0.21	1.825	.203
LPevFB	3.46±1.32	4.19±1.47	3.43±1.59	2.75±0.30	1.617	.239
HDF	30.30±2.37	30.80±3.16	30.59±1.84	29.50±2.24	.398	.680
HAF	27.63±4.52	29.70±4.87	27.90±3.79	25.29±4.59	1.249	.322
HPecF	40.61±2.86	41.38±3.87	40.49±1.92	39.94±2.94	.292	.752
HPevF	8.72±9.56	6.71±1.40	13.90±16.30	5.55±1.04	1.141	.352
CFL	25.69±2.84	26.43±3.73	25.65±1.87	24.99±3.10	.290	.753

CB= Chashma Barrage, TH = Trimmu Head, TB=Taunsa Barrage, SD = Standard deviation

Table 7: Mean values (number) of meristic characters of *N. notopterus*.

Morphometric characters	Overall	CB	TH	TB	ANOVA test	
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	F	*p*-value
DFR	7.40±0.507	7.00±0.00	7.60±0.55	7.60±0.55	3.000	0.088
AFR+CFR	116.93±3.99	114.00±3.81	118.80±1.64	118.00±4.70	2.531	0.121
PecFR	14.93±0.799	15.00±0.71	14.80±0.84	15.00±1.00	0.091	0.914
PevFR	4.87±0.743	4.80±0.84	5.00±0.71	4.80±0.84	0.105	0.901

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All character of *C. chitala* showed non-significant results (p<0.05) (Table 8).

Table 8: Mean values (number) of meristic characters of *C. chitala.*

Morphometric Characters	Overall	CB	TH	TB	ANOVA test	
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	F	*p*-value
DFR	8.87±.743	8.60±0.89	9.00±0.71	9.00±0.71	0.444	0.651
AFR+CFR	114.73±1.668	114.60±1.67	115.00±2.00	114.60±1.67	0.083	0.921
PecFR	16.00±1.604	15.40±1.95	16.40±1.34	16.20±1.64	0.506	0.615
PevFR	5.73±.704	5.60±0.55	5.80±0.84	5.80±0.84	0.118	0.890

Truss analysis

In this study, total of 25 landmarks were recorded for the examination analysis of body shape. In *N. notopterus*, PC1 contributes the highest range of variation (74.9%) among selected for components. PC2, PC3 and PC4 were led by the PC1. However, variation showed by PC2, PC3 and PC4 components as followed 11.2%, 5% and 3%, respectively (Table 9).

Table 9: Eigen values, percentage of proportion and cumulative variance for 4 PC in *N. notopterus* and *C. chitala* truss measurements.

Components	*N. notopterus*			*C. chitala*		
	Eigen value	Proportion	Cumulative	Eigen value	Proportion	Cumulative
PC1	18.717	0.749	0.749	21.402	0.856	0.856
PC2	2.795	0.112	0.860	2.876	0.115	0.971
PC3	1.260	0.050	0.911	0.561	0.022	0.994
PC4	0.742	0.030	0.941	0.089	0.004	0.997

In case of intra-component analysis, PC1 expressed positive relation among all variables except two traits; 1-10 and 8-10 while, PC2 show positive relation among 1-2, 1-10, 2-3,2-11,3-7,3-8,4-5,4-7,4-8,4-9,5-6,7-8,8-11 and 10.11 and remaining showed negative relation (Fig. 5). PC3 and PC4 expressed randomly both positive and negative correlations. In *C. chitala*, PC1 contributes the highest range of variation (85.6%) among selected components. However, variation showed by PC2, PC3 and PC4 components as 11.5%, 2.2% and 0.4%, respectively (Table 9). In case of intra-component analysis, PC1 expressed positive relation among all variables while in PC2 following variable 1-2,1-10,1-11,2-3,2-6,2-11,3-4,5-6,7-8 and 8-10 showed positive while other were negatively correlated (Fig. 6). PC3 and PC4 expressed randomly both positive and negative correlations.

Molecular identification

Nucleotide base composition analysis

The nucleotide frequency analysis was conducted on 12 sequences, including all codon positions (1^{st}, 2^{nd}, 3^{rd}) and non-coding regions. Ambiguous positions in each dataset were removed using pairwise deletion. An average of all nucleotide bases for all studied fishes were T (29.45%), C (26.17%), A (27.75%), G (16.63%).

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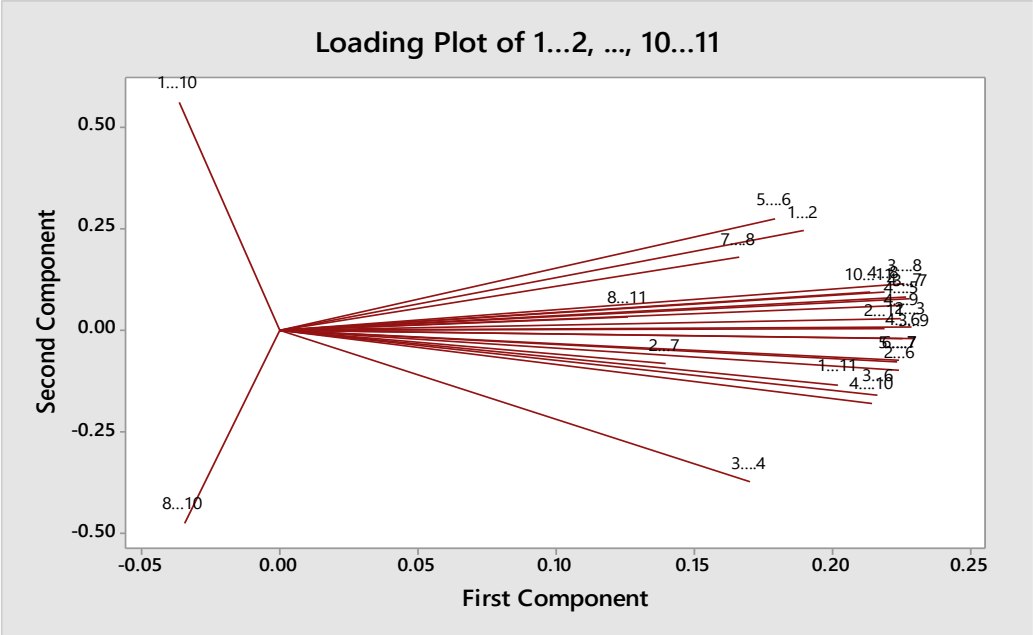

Figure 5: Loading Plot of truss analysis of *N. notopterus.*

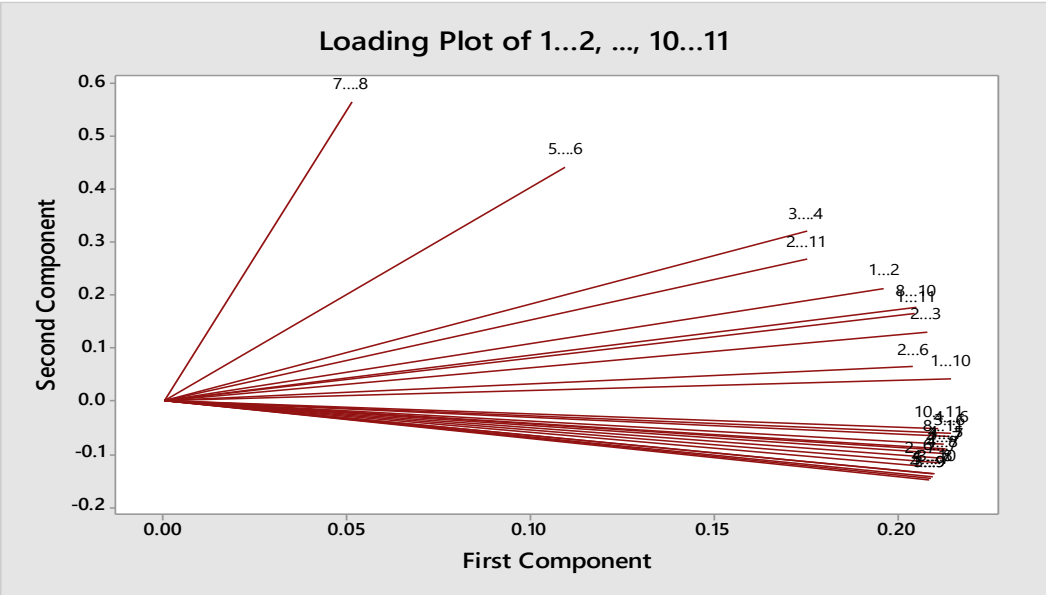

Figure 6: Loading plot of truss analysis of *C. chitala.*

The average AT (57.2%) was higher than GC (42.8%) content of nucleotide base composition analysis of *COI* gene sequences (Table 10). After editing, all barcode sequences exhibited a consensus length of 655 bp, with no observed insertions, deletions, or stop codons. Each analyzed sequence exceeded 600 bp in length, which supports the exclusion of nuclear mitochondrial DNA segments (NUMTs). Mitochondrial DNA sequences shorter than 600 bp were not included in this study.

Nucleotide pair frequency analysis

Out of 661 nucleotide positions analyzed in the barcode sequences (Fig. 7), 545

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sites were conserved (Cs), 95 were variable (Vs), 72 were parsimony-informative (Pis), and 22 were singleton variable sites (Sv). The estimated transition/transversion bias (R) was 1.48. All evolutionary analyses were performed using MEGA11 software.

Table 10: Base composition (%) of all samples of family Notopteridae from the selected locations.

Accession No.	T	C	A	G	Total
Notopterus notopterus OQ339202	29.56	26.01	27.53	16.89	592
Notopterus notopterus OQ338349	28.99	25.90	27.20	17.92	614
Notopterus notopterus OQ338351	29.90	25.84	27.36	16.89	592
Notopterus notopterus OQ338347	29.52	26.20	27.20	17.08	603
Notopterus notopterus OQ338348	29.56	26.01	27.53	16.89	592
Notopterus notopterus OR018531	29.77	26.15	27.14	16.94	608
Chitala chitala OQ338197	28.78	26.39	28.30	16.53	629
Chitala chitala OQ332354	29.62	26.37	28.08	15.92	584
Chitala chitala OQ338199	29.46	26.09	28.28	16.16	594
Chitala chitala OQ338196	28.74	26.44	28.08	16.75	609
Chitala chitala OQ338198	29.44	26.48	28.29	15.79	608
Chitala chitala OQ933370	30.15	26.13	27.97	15.75	597
Average	29.45	26.17	27.75	16.63	602

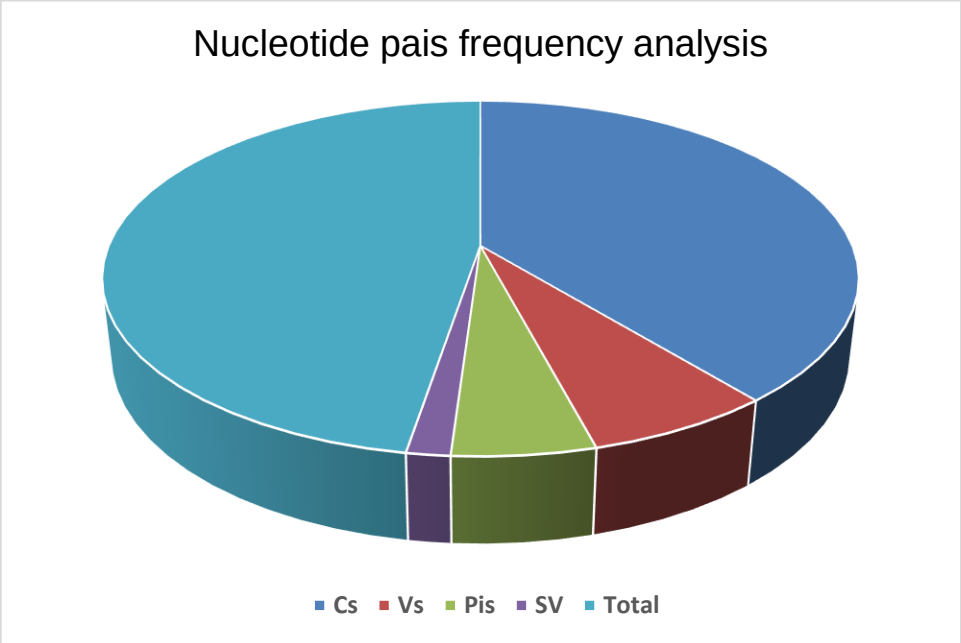

Figure 7: Nucleotide pair frequency analysis of Notopteridae fish from Selected rivers.

Genetic divergence

The current study used the K2-P model to evaluate the genetic distance between fish species of *N. notopterus* and *C. chitala*. All fishes have zero genetic distance within their species. The interspecific mean genetic distance ranged from 0.002 to 0.149, indicating that genetic variation among species was substantially higher than the intraspecific variation, which remained below 1%. The mean distance between fish species *N. notopterus* and *C. chitala* was calculated as 0.07 (Table 11).

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Table 11: Genetic distance using KP2 Model.

**	1	1	1	1	1	1	2	2	2	2	2	2
1												
1	0.005											
1	0.005	0.011										
1	0.000	0.007	0.005									
1	0.000	0.005	0.005	0.000								
1	0.000	0.005	0.005	0.000	0.000							
2	0.118	0.117	0.125	0.118	0.118	0.118						
2	0.136	0.120	0.139	0.136	0.136	0.142	0.016					
2	0.138	0.121	0.145	0.138	0.138	0.142	0.019	0.003				
2	0.116	0.120	0.123	0.118	0.116	0.116	0.005	0.004	0.007			
2	0.138	0.119	0.145	0.137	0.138	0.149*	0.022	0.007	0.007	0.009		
2	0.137	0.119	0.140	0.137	0.137	0.149*	0.019	0.002	0.005	0.002*	0.005	

* Evaluates the highest and lowest genetic distance. ** Number of respective species, 1 = *Notopterus notopterus*, 2 = *Chitala chitala*

Evolutionary analysis

The phylogenetic analysis of the *COI* sequences from the fish species of *N. notopterus* and *C. chitala* resulted in a well-supported tree revealing distinct evolutionary lineages within the family Notopteridae. The Maximum Likelihood (ML) tree was constructed among 20 sequences (12 studied sequences and 6 reference sequences from NCBI, 2 outgroups) by using Mega 11 software (Fig. 8).

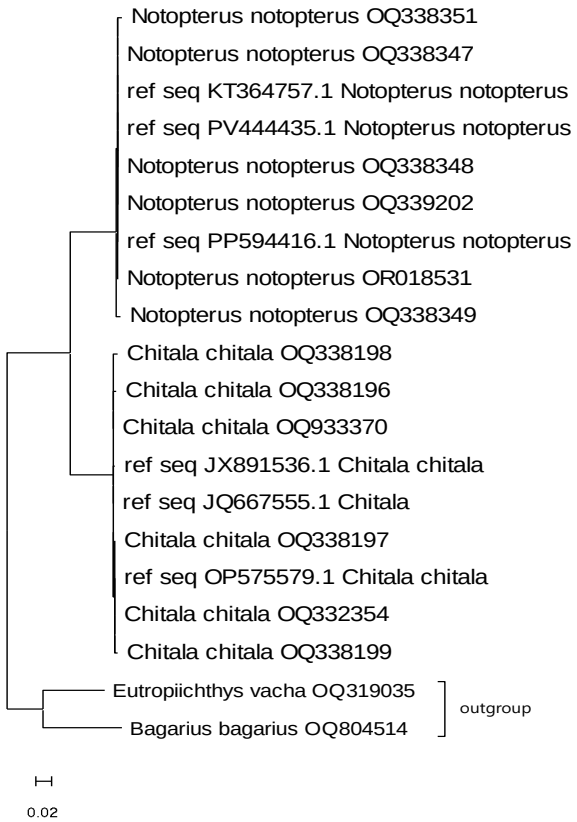

Fig. 8. Maximum likelihood tree representing genetic relationship among *N. notopterus* and *C. Chitala*.

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The branch length of tree was 0.01 with 1000 bootstrap replicates with as an out group. This analysis clearly divided the fish species of family Notopteridae into two major clades on the basis of relationship among fishes.

Individuals of the same species grouped within the same node, while distinct species were positioned on separate nodes. All specimens of *N. notopterus* (Family Notopteridae) included reference sequences were present in one clade and *C. chitala* (Family Notopteridae) in separate clade with 99% bootstrap value. An outgroup of *Bagarius bagarius* (Family Sisoridae) and *Eutropiichthys vacha* (Family Schilbeidae) showed a total separate clade and these findings revealed the significant insights into the evolutionary history and genetic relationships among these species, as determined by *COI* sequence analysis.

Discussion

Osteoglossiformes is a monophyletic order of freshwater teleosts that is currently limited to tropical area of South America, Africa, Asia, and Australia. *N. notopterus* and *C. chitala* were identified on the basis of morphometric, meristic and truss network system, they belong to the family Notopteridae.

Stock differentiation of *N. notopterus* and *C. chitala* was carried out using a truss network based on anatomical landmarks, along with conventional morphometric and meristic trait analyses. Shape analysis enables more accurate and direct comparison of the long-term morphological evolution of stocks (Rawat *et al*., 2017). Fish have a very high level of phenotypic plasticity and morphometric and meristic investigations yield useful information for identifying fish stocks (Das *et al*., 2020). These species are considered threatened, with catch rates having significantly decreased as a result of overfishing and ecological alterations in river systems driven by human activities (Chandran *et al*., 2020). Stock identification study was conducted in *N. notopterus* and *C. chitala* at Taunsa Barrage, Chashma Barrage and Trimmu Head. Analysis of the fish stock structure is a significant technique for controlling the wild population. In the current study, landmarks and morphology were used in multivariate analyses to distinguish between the stocks.

Fish morphological traits are those authoritative traits that offer a pertinent means of identification, taxonomic study, as well as greater comprehensions of common facts about fishes.

When conducting taxonomy and ecological research, the interpretation of morphological structure serves as a powerful instrument (Das *et al*., 2020; Zafar *et al*., 2024). It is acknowledged that morphometric variations between stocks of a species are crucial for assessing population structure and serving as a foundation for stock identification. The employment of sophisticated tools in morphometry has allowed for the testing and visualisation of shape differences, the separation of shape from size variation, and the identification of species stocks with

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distinctive morphological traits that will allow for better management of the species (Tripathy, 2020).

Analysis from morphometric study represented that *N. notopterus* showed significant differences among three locations. The mean value of TL in study varied among all selected locations noticed greater in Taunsa Barrage population (290.17 mm) and followed by Trimmu Head (233.47 mm) and Chashma Barrage (217.86 mm). The mean value of TL of *N. notopterus* was determined as 247.17 mm. The present study agrees with the finding of Chai *et al*. (2021) that showed TL mean value of *N. notopterus* collected from Malaysia was 303.8 mm. Moreover, the range of TL of *N. notopterus* was (184.2-300.69 mm). Same study was conducted in Malaysia by Isa *et al*. (2010) that showed range of TL was (154–264 mm). It showed that fish body length varies country to country which indicates that environmental factors have impact on body length and growth due to change in ecosystem.

Analysis from morphometric study represented that *C. chitala* showed difference among three locations. The mean value of TL in study varied among all selected location, noticed greater in Chashma Barrage population (382.68 mm) and followed by Taunsa Barrage (381.40 mm) and Trimmu Head (355.29 mm). The mean value of TL of *C. chitala* was 373.12 mm. The present study contradicts with the finding of (Deka and Bura, 2015) that showed TL mean value of *C. chitala* collected from River Ravi was 582.2 mm. Moreover, the range of TL of *C. chitala* was (300.81-430.12 mm). Same study was conducted by Hussain *et al*. (2015) that showed range of TL was (506 mm – 684 mm). Variation in TL of *N. notopterus* and *C. chitala* from different locations might be due to drastic change in aquatic parameters like temperature, salinity and water current.

Among the three populations of *N. notopterus* and *C. chitala*, no significant difference was observed in case of meristic parameters. But in case of morphological characters' significant results were found in three selected locations. Results obtained from univariate analyses showed that out of 27 morphometric characters, 16 characters [(TL), (SL), (HL), (HBD), (LBD), (ED), (PsOL), (IO), (UJL), (LJL), (PrPecL) (LDFB), (TACB), (LPevFB), (HPecF) and (HPecF)] were significant (p<0.05) and 11 characters [(SnL), (HL), (PrOL), (PrDL), (PsDL), (PrAL), (PrPevL), (LPecFB), (HDF), (HAF) and (CFL)] were non-significant (p<0.05) (Table 5). Das *et al*. (2020) conducted their studies on morphological and meristic traits of *Tenualosa ilisha* and *Tenualosa toli* in Bangladesh. Although it might be challenging to pinpoint the exact reasons behind morphological variations among populations, it is generally accepted that these variations may have a hereditary component or be the result of phenotypic plasticity in response to local environmental conditions (Ethin *et al*., 2019). In *N. notopterus,* PC1 contributes the 72.3% of variation. However, PC2, PC3 and PC4 represent 16.1%, 4.4% and

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2.9% respectively. In *C. chitala,* PC1 contributes 58.4% variation. However, PC2, PC3 and PC4 represent 25%, 12.7% and 1.9%, respectively. Ramya *et al*. (2021) also found variations in morphological traits in *Barbodes carnaticus.* The morphological variations seen in this study may be explained by environmental influences, among other variables. Indeed, earlier research noted variations in these four rivers' water quality indicators, particularly in terms of water temperature and dissolved oxygen concentrations (Haque *et al*., 2019).

The Truss network method relies on specific anatomical landmarks for analysis that is very effective at gathering details on the shape of an organism. It has no restrictions on the directions of variation or the localization of shape changes (Malik *et al*., 2020; Zafar *et al*., 2024). In *N. notopterus,* PC1 and PC2 contribute the 74.9% and 11.2% of variation among selected components. PC1 expressed positive relation among all variables except two while, PC2 show positive relation among 14 variables and remaining 16 show negative relation. Truss analysis was also used by Muchlisin *et al*. (2013) and Biswal *et al*. (2018) in *Rasbora sp.* and *Systomus sarana,* respectively. In truss analysis of *C. chitala,* PC1 and PC2 contribute the 85.6% and 11.5% of variation among selected for components. All the variable of PC1 was positively correlated. Moreover, in PC2 10 variables showed positive while other 15 were negatively correlated. Nasren *et al*. (2019) conducted their study on truss parameters in *Hypselobarbus jerdoni.* The current findings differ significantly from those reported by Chandran *et al*. (2020), who recorded that the PC1 and PC2 contributed 32.90% and 23.56%, respectively in *Chitala chitala* in Indian river. As a result, there are significant variances in the habitat characteristics and environmental factors at different sites in terms of the rocks, minerals, terrain, water current, and water level. The current phenotypic variation may be brought on by the habitat's changing temperature, salinity, turbidity, and alkalinity levels (Biswal *et al*., 2018).

The utilization of the *COI* gene and DNA barcoding for species identification is widely recognized and extensively documented, especially in the fisheries sector. The *COI* gene is employed as a marker in DNA barcoding, a technology that has lately garnered interest as a very efficient means of species identification, particularly for fish (Knebelsberger *et al*., 2014). The choice of *COI* as a standard barcode gene is mainly based on its characteristic variation pattern, which exhibits distinct divergence and little overlap between genetic distances within species and between species (Hebert *et al*., 2003).

No stop codons or nuclear mitochondrial pseudogenes (NUMTS) were found, with an average consensus length observed more than 600 bp in barcode sequences. The average nucleotide base composition of the examined between *N. notopterus* and *C. chitala* species were GC (42.8%) and AT (57.2%). Overall, the average GC

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content was lower than the average AT content. This pattern was also observed in the species of *Clupisoma garua* (Saraswat *et al*., 2014). The similar average nucleotide base composition was also observed in *Wallago attu* and Labeo genus (Sajjad *et al*., 2023; Zafar *et al*., 2024). The base composition analysis of freshwater fishes from the Beas River found similar with the results of our study (Modeel *et al*., 2024). Transitional substitutions were higher than transversionsal with R (si/sv) ratio of 1.48 for the dataset. The identified transitional mutations were more abundant than the transversional ones, similar to the labeo genus (Zafar *et al*., 2024).

A nucleotide pair frequency analysis was performed with the help of Mega 11 software. Total 661 sites were observed. Out of 661 sites 545 conserved (cs) sites, 95 variable (vs) sites, 72 parismony informative (pis) sites and 22 singleton (sv) sites were analyzed (Fig. 7). Of the sites analyzed, 150 were constant, while, 470 showed variability, including 251 parismony informative sites and 219 singletons were observed within the fishes of the brackish water lake in South Asia (Agneeswaran *et al*., 2023).

Overall, mean of genetic distance between species was observed as 0.07. Average distance within two species of family Notopteridae was calculated as 0.002 to 0.149, and this distance increases with the increase of taxonomic level. Our findings match with the results of Indian freshwater fishes with the distance of 0.8 within species, 9.06 within genus and 15.35 within family (Modeel *et al*., 2024). Fish diversity from Batanghari River, Indonesia results showed K2P distance values 0.05, 0.12 and 0.16 for genera to families, also related with current findings (Marnis *et al*., 2024).

Evolutionary tree was constructed with the help of K2P to understand the evolutionary relationships among species which is fundamental to exploring their origins, diversification, and ecological interactions. The topology of the tree revealed two major clades with 99 bootstrap values that clearly identified that these two fish species belongs to different genus but similar family Notopteridae. An out group showed in different clad showed that it belongs to separate ancestor. Some earlier researches also explored the same results (Ude *et al*., 2020; Zafar *et al*., 2024).

Globally, fish diversity faces significant threats, exacerbated not only by harmful effects on the studied species but also by the worsening natural conditions for native species. The presence of toxic chemicals and other inorganic pollutants has led to genetic alterations in fish species (Tickner *et al*., 2020). The main objective of this study was to investigate the diversity of fish species and to develop an accurate identification method for two fish species *N. notopterus* and *C. chitala* present in the selected locations. This was achieved by establishing potential DNA barcodes to support conservation efforts for the examined fish species. Additionally, the study offers baseline data on these freshwater fish species,

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which can be utilized for fishery management, fish stock assessment in riverine population, and educational purposes at academic institutions and research centers.

Conclusion

The present study successfully distinguished *N. notopterus* and *C. chitala* from three different locations in Punjab using meristic counts, conventional morphometrics, and landmark-based truss network analysis. The findings revealed significant morphological variations between the two species, highlighting the effectiveness of modern morphometric techniques in identifying stock structure and understanding species-level differences. To complement the morphological data, DNA barcoding was also conducted on *N. notopterus* and *C. chitala*, confirming species identity at the genetic level and supporting the presence of distinct genetic lineages. The integration of morphometric and molecular data strengthened the reliability of stock discrimination and emphasized the need for combined approaches in fisheries biology.

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