# 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%), Č (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 а 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 environmental of 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 distinguishing within groups and between species similar of shape Anumudu, (Mojekwu and 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 meristic characteristics for stock identification or discrimination research has recently been less familiar. Interbreeding resulted from long-term isolation. geographic 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 fish fillet and 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 time-consuming; and yet, species documentations typically need a substantial amount of taxonomic data. Additionally, morphological identification is limited to specific life

Additionally, a variety of phases. taxonomists possess varving 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 low number of taxonomists the (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 Ν. notopterus especially is 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 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.

# 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).	

Genus	Species	Sample No	Max Length (mm)	Location
Notopterus	N. notopterus	15	290	TB, TH, CB
Chitala	C. chitala	15	383	ТВ, ТН, СВ
	<b>Genus</b> Notopterus Chitala	GenusSpeciesNotopterusN. notopterusChitalaC. chitala	GenusSpeciesSample NoNotopterusN. notopterus15ChitalaC. chitala15	GenusSpeciesSample NoMax Length (mm)NotopterusN. notopterus15290ChitalaC. chitala15383

# 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).

Table 2: Description of morphometric characters used in this study.					
Characters	Abbreviations	Description			
Total Length	ТІ	The distance between the snout tip and the longest caudal			
Total Length	IL	fin ray			
Standard Length	SI	The distance between the tip of snout and the end of			
Standard Dength	SE	vertebral column			
Snout Length	SnL	The distance between the tip of mouth and anterior edge of			
Shour Dongu	SIL	eye			
Head Length	HL	The distance between the tip of snout and posterior edge of			
	UD	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 shout to anterior part of the eye			
Post-Orbital Length	PsOL	operculum			
Inter-Orbital	IO	The distance of eyes from each other			
Upper Jaw Length	Ш	The distance between the two end points along the upper			
Opper Jaw Length	CJE	jaw margin			
Lower Jaw Length	LIL	The distance between two endpoints along the lower jaw			
Lower van Lengu		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	I he distance between tip of the shout and base of the first			
		The distance between tip of the spout and base of the first			
Pre-Pectoral Length	PrPecL	ne distance between up of the shout and base of the first			
		The distance between tip of the spout and base of the first			
Pre-Pelvic Length	PrPevL	nelvic fin ray			
Length Dorsal Fin		The distance between dorsal fin base's most anterior and			
Base	LDFB	posterior points			
Total anal caudal		Length from the anterior edge of anal fin to the tip of caudal			
base	TACB	fin along fin base			
Length of Pectoral		The distance between pectoral fin base's most anterior and			
Fin Base	LPecFB	posterior points			
Length of Pelvic Fin	I DavED	The distance between pelvic fin base's most anterior and			
Base	LPevFB	posterior points			
Height of Dorsel Fin	LIDE	The distance from the base of the dorsal fin origin to the end			
Height of Dorsai Fill	IIDI	of the longest fin ray			
Height of Anal Fin	НАБ	The distance from the base of the anal fin origin to the end			
Height of Anal Phi	IIAI	of the longest fin ray			
Height of Pectoral	HPecF	The distance from the base of the pectoral fin origin to the			
Fin	111 001	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 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/)

Landmark No	Landmark position
1	The anterior tip of the snout of
1	mouth
2	The most backward part of the
2	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
	The prior attachment of the
6	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
11	ventral side

Table 4: Description of truss networkcharacters used in the study.

The PCR reaction mixture was prepared with a final volume of 25  $\mu$ l, containing 1X reaction buffer, 1 µl of template DNA, 2.5 mM dNTPs, 2.5 mM MgCl<sub>2</sub>, 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 followed by minutes. 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 comparison of mean the 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

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 razorshaped 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 С. 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 morphometric analysis traditional 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 showed significant result (HPecF) (p < 0.05). While other, Snout Length (SnL), Head Length (HL), Pre-Orbital Length (PrOL), Pre-Dorsal Length (PrDL), Post-Dorsal Length (PsDL),

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	c Overall CB TH TB ANG				ANOV	'A test			
characters	Mean±SD	Mean±SD	Mean±SD	Mean±SD	F	<i>p</i> -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	$12.80 \pm 1.30$	12.35±1.21	12.70±1.35	$13.35 \pm 1.40$	.750	.493			
HL	51.05±10.28	44.26±9.79	49.19±8.80	59.69±6.41	4.345	.038			
HD	$28.23 \pm 5.65$	24.47±5.96	$27.54 \pm 4.40$	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	$14.82 \pm 1.18$	17.49±1.63	6.646	.011			
ED	$8.72 \pm .66$	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	$6.70 \pm .45$	6.47±0.29	6.40±0.14	7.22±0.32	15.129	.001			
UJL	$16.08 \pm 1.20$	$15.17 \pm 1.40$	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	$125.78 \pm 7.91$	2.496	.124			
PrAL	69.51±13.02	60.71±13.87	68.20±11.56	79.61±6.39	3.707	.056			
PrPecL	$45.64 \pm 8.24$	$40.09 \pm 8.05$	43.93±6.92	52.91±4.07	5.030	.026			
PrPevL	65.67±12.1	59.25±15.77	63.92±10.20	$73.83 \pm 4.70$	2.221	.151			
LDFB	$6.36 \pm .46$	$6.07 \pm 0.50$	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	$6.30 \pm .97$	5.57±1.29	6.36±0.50	6.99±0.36	3.689	.056			
LPevFB	$3.52 \pm .84$	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	$28.03 \pm 4.08$	29.20±2.04	32.83±0.32	4.502	.035			
HPevF	5.71±.93	5.29±0.56	$5.29 \pm 0.94$	$6.54 \pm 0.72$	4.538	.034			
CFL	19.46±2.12	18.61±2.51	$19.37 \pm 1.43$	20.41±2.33	.883	.439			
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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.

Table 6: Mean values (mm) of morphometric traits of C. chitala.							
Morphometric	Overall	СВ	ТН	ТВ	ANOV	A test	
Characters	Mean±SD	Mean±SD	Mean±SD	Mean±SD	F	<i>p-</i> value	
TL	373.12±45.96	$382.68 \pm 57.48$	355.29±48.52	381.40±33.74	.527	.603	
SL	346.89±43.77	$356.68 \pm 55.20$	329.28±44.71	354.70±32.82	.572	.579	
SnL	16.43±1.29	$17.08 \pm 1.14$	$16.07 \pm 1.78$	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	$20.65 \pm 2.76$	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	$10.80 \pm 1.66$	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	
ΙΟ	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	$186.38 \pm 25.80$	194.82±34.50	178.06±23.60	186.25±20.15	.490	.625	
PsDL	$180.92 \pm 30.47$	$183.24 \pm 40.89$	169.94±33.37	$189.57{\pm}14.58$	.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	$280.78 \pm 27.97$	.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	

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	,	0					1		

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

|--|

Morphometric - characters	Overall	СВ	TH	ТВ	ANOV	'A test
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	F	<i>p</i> - value
DFR	$7.40\pm0.507$	$7.00 \pm 0.00$	$7.60 \pm 0.55$	7.60±0.55	3.000	0.088
AFR+CFR	116.93±3.99	$114.00 \pm 3.81$	$118.80{\pm}1.64$	$118.00 \pm 4.70$	2.531	0.121
PecFR	14.93±0.799	$15.00 \pm 0.71$	$14.80 \pm 0.84$	$15.00 \pm 1.00$	0.091	0.914
PevFR	4.87±0.743	$4.80 \pm 0.84$	$5.00 \pm 0.71$	$4.80\pm0.84$	0.105	0.901

Table 8: Mean values (number) of meristic characters of C. chitala.							
Monnhomotrio	Overall	СВ	TH	ТВ	ANOV	/A test	
Characters	Mean±SD	Mean±SD	Mean±SD	Mean±SD	F	<i>p</i> - value	
DFR	8.87±.743	$8.60 \pm 0.89$	9.00±0.71	9.00±0.71	0.444	0.651	
AFR+CFR	114.73±1.668	$114.60 \pm 1.67$	$115.00 \pm 2.00$	$114.60 \pm 1.67$	0.083	0.921	
PecFR	$16.00 \pm 1.604$	$15.40 \pm 1.95$	$16.40 \pm 1.34$	$16.20 \pm 1.64$	0.506	0.615	
PevFR	$5.73 \pm .704$	$5.60 \pm 0.55$	$5.80 \pm 0.84$	$5.80 \pm 0.84$	0.118	0.890	

All character of C. chitala showed non-

significant results (p < 0.05) (Table 8).

### 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.

		N. notopterus		C. chitala		
Components	Eigen value	Proportion	Cumulativ e	Eigen value	Proportion	Cumulativ e
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 noncoding 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%).



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 sites were conserved (Cs), 95 were variable (Vs), 72 were parsimonyinformative (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: B	ase composition (	%) of all samples of fai	milv Notopteridae fron	n the selected locations.
I GOIC IV. D	abe composition (	( ) of an sumples of fa	my rocopteridate iron	i ine serected rocarions.

Accession No.	Т	С	Α	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*.

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 С. 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 morphometric and meristic and 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

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 morphometric 27 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 response local in to 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

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 **Barbodes** in 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. terms particularly in of water and temperature dissolved oxygen concentrations (Haque et al., 2019).

The Truss network method relies on specific anatomical landmarks for that is very effective analysis 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 of species identification. means 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 nuclear or mitochondrial pseudogenes (NUMTS) were found, with an average consensus length observed more than 600 bp in sequences. The barcode 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

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

### Conclusion

The successfully present study distinguished N. notopterus and C. chitala from three different locations in Punjab using meristic counts. conventional morphometrics, and landmark-based truss network analysis. revealed The findings significant morphological variations between the species, highlighting two the effectiveness of modern morphometric techniques in identifying stock structure understanding and species-level complement differences. To 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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