Integration of DNA barcodes with truss-network morphology for taxonomic validation and stock differentiation of *Heteropneustes fossilis*

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Abstract

The stinging catfish *Heteropneustes fossilis* is a commercially and ecologically important freshwater species, yet information on its taxonomic status, population structure, and genetic diversity remains limited. This study conducted an integrated assessment combining morphometric, meristic, truss-network, and molecular analyses to evaluate the population quality of three geographically distinct stocks collected from Head Trimmu, Head Taunsa, and Chashma Barrage. Twenty-seven morphometric, six meristic and twenty truss variables were measured. Total length of *H. fossilis* varied from 126.10-283.06 mm. Among six meristic characters, only PecFS showed no variation, while the remaining five traits exhibited significant differences in population. Significant morphometric traits included TL, SL, HBD, LBD, HL, HD, PrOL, PsOL, MXBL, MNBL, PrDL, PsDL, PrPevL, PrAnL, HPec, HPev, HAF, LAFB, CFL, and IO ($p< 0.05$), whereas SnL, ED, PrPecL, HDF, LDFB, LPevFB, and LPecFB were non-significant ($p>0.05$). Truss variables data showed significant difference ($p<0.05$). Principal component analysis of morphometric data revealed that the first four components (PC1–PC4) explained 77.7%, 8.7%, 3.7%, and 2.9% of total variance, respectively, while PCA of meristic traits accounted for 92.6% of variation. In truss analysis of *H. fossilis*, the loading proportion of the initial four elements from principal components; PC1, PC2, PC3 and PC4 defined 20.8%, 4.9%, 4.1% and 1.0% of the change, respectively. DNA barcoding was conducted on five representative samples. Sequence analysis revealed moderate AT-bias (A+T=58%) and highest GC content (42%) at the first codon position. Inter-population K2-P distances ranged from 0.0000 to 0.0102, indicating minor divergence among populations likely due to geographical and environmental influences. BLAST analysis confirmed 100% similarity with reference sequences, validating species-level identification. Phylogenetic analyses using Maximum Likelihood trees showed all *H. fossilis* sequences clustering into a cohesive clade with low divergence, confirming the reliability of *CO1* as a DNA barcode. Overall, this study provides an integrated morphological and molecular characterization of *H. fossilis* populations in Pakistan, validates DNA barcodes for the species, and establishes a foundational framework for future studies on biodiversity assessment, stock discrimination, and conservation management of threatened freshwater catfishes in the region.

Keywords: *Heteropneustes fossilis,* Taxonomic Identification, Genetic Analysis, Truss Variables, Principal Components Analysis, Conservation, Management

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Introduction

In Pakistan, natural freshwater resources are existing in the form of natural lakes, dams, streams, and rivers which have great value and potential for aquaculture and fisheries practices. Most importantly, freshwater fish fauna is considered the most valuable and important food source across the globe (Khan *et al*., 2021; Zafar *et al*., 2024). There are now 33,000 fish species identified across the world. In Pakistan, a total of 531 fish species have been known, out of which 233 are freshwater and 298 are marine fish species, 78 out of 233 freshwater fish species are commercially valuable (Ghouri *et al*., 2020; Khan *et al*., 2021). Their habitat ranges from the glacial water to hot springs and they can also withstand a broad range of salinity. That is why within the animal taxa, they are regarded as the vital element of biodiversity (Ali *et al*., 2020).

The Order Siluriformes contains 4,100 species, accounting for 6.3% of all vertebrates and 12% of all fish species. Since of their evolutionary location catfish have long been important in biological studies because there are more closely related to a common fishing ancestor unlike bony fish (infraclass Teleostei). Catfish is a significant source of essential protein that is well-known around the world (Hasan *et al*., 2021). *Heteropneustes fossilis*, is a stinging catfish that belongs to a family Heteropneustidae and order Siluriformes (Chew *et al*., 2020).

H. fossilis (Bloch, 1974), an Asian stinging catfish is a commercially significant fish species. In Pakistan common name of *H. fossilis* are Lahoord, Nullie and Lohar, in Bangladesh its native names are stinging catfish, singhee or shingi, in India it is known as Bitchu and in Sri Lanka its name is Hunga. It is found in Pakistan, Srilanka, India, Nepal, Bangladesh, Myanmar, Loas and Thailand among other Southeast and South Asian countries. Iran and Iraq also have Asian stinging catfish. This catfish is valuable as a food fish because it is high in protein, calcium and iron (Islam *et al*., 2021).

H. fossilis has an elongated body, a depressed head covered with osseous plates and four pairs of barbels. Along both sides of a vertebral column, pair of auxiliary respiratory organs in the shape of air sacs stretch backward from the gill chamber. The dorsal fin is tiny and lacks bony spines; its pectoral spine is sharp and serrated internally and caudal fin is round (Plamoottil *et al*., 2022). The pectoral fin spine of *H. fossilis* is very highly serrated and attached to a toxic gland, making it dangerous to humans. Because of its pectoral spines, *H. fossilis* is a venomous fish. It is traumatogenic (Ali *et al*., 2016).

Ponds, marshes, ditches, muddy rivers and swamps are among its preferred environments. It can withstand lengthy durations of submersion and somewhat high levels of ammonia in the environment. In the dry season, *H. fossilis* live in semi dry mud and semi liquid water even after the mud has dried it moves its body over the bottom of crevices and cracks because it has an air sac as an auxiliary respiratory organ. Due to many negative impacts, the output of *H. fossilis* in natural water bodies is steadily decreasing. If this continues for several years, it will eventually become endangered and go

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extinct. However, it is hoped that numerous environmental management projects (fish sanctuary, fishing laws etc.) as well as *H. fossilis* breeding and culture techniques would be effective in reviving natural populations across the country. *H. fossilis* has a faster growth rate, is easier to cultivate and has a higher market price when using additional foods making it popular among fish farmers (Chew *et al*., 2020).

All of this has drawn attention to the current situation of *H. fossilis* in our nation, which will be the subject of future study and growth. Information of a species' biology and structure of population is required for formulating protection and management plans, and it also used to investigate short-term and environmentally caused changes (Rahman *et al*., 2014). Natural populations of the vulnerable stinging catfish *H. fossilis* have decreased due to overfishing, habitat degradation and pollution make the surviving rare, wild populations of *H. fossilis* in Asian nations of critical conservation concern. However, this is a tough fish that can live in a variety of water conditions. It is categorized a least concern specie of Bangladesh (Bangladesh IUCN, 2015) as well as globally (IUCN, 2017) (Islam *et al*., 2021).

One of the most important duties of taxonomy is to identify fish species. The apparent morphology of freshwater fish species is widely used to identify them and several morphological keys are used for identification of freshwater fishes (Zafar *et al*., 2024). Morphometric is a term that refers to a collection of major statistical processes that are used to assess differences in the size and form of organisms. The accurate and simplest way of identifying a specimen was morphological systematic, which included measurements of morphometric features and meristic account. Meristic characteristics are countable characters, whereas morphometry is the outer measurement of an organism's body parts (Tripathy, 2020; Durrani *et al*., 2023).

Truss values created with landmark points, as well as morphometric and meristic features measurements, are important tools that may be applied for stock identification, explaining population dynamics, and differentiating morphologically similar species from other species (Zafar *et al*., 2024). The body is divided into units based on a network of vertical, horizontal, and diagonal distances between sites selected based on regional morphological characteristics. Compared to conventional morphometric character sets, which typically consist of length, width, and depth data, this method has advantages (Tripathy, 2020).

The accurate identification of each species is the first step in protecting and managing the threatened fishes. Identification based on physical traits sometimes seems inadequate because of genetic diversity, phenotypic plasticity, morphologically cryptic taxa, various stages of life cycle, and the requirement of high level taxonomic expertise. Short sequence of universal DNA can be used by DNA barcoding for species identification and appropriately classify an unknown specimen to a previously recognized species in order to resolve this conundrum (Chen *et al.*, 2021).

In DNA Barcoding, small section of mDNA, almost 650bp of COI gene from

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the 5/ were used as a standard marker for the purpose of precise identification. *COI* gene is the bio-identical indicator as it has best distinction ability for about entire animal phyla because of the following vital features: it is quiet easy to separate, free of re-assimilation, great copy number, deletion and insertion is rare, having negligible intra-species distinctions, it appears to have arrangement rareness that is sufficient to isolate strongly associated species and may be restored from despoiled along with small samples (Ghouri *et al*., 2020). DNA barcoding method offers an innovative view in systematic and fish ecology (Hubert *et al*., 2008).

The present study aimed to evaluate the genetic and morphological identification of species within the family *Heteropneustidae*. In Pakistan, only a single species from this family, *Heteropneustes fossilis* is reported. This species is commercially important due to its high market value, strong consumer preference, tolerance to low oxygen environments and its increasing potential in inland aquaculture. However, despite its significance, the taxonomic status, updated classification and phylogenetic relationships of *H. fossilis* populations cultured and harvested in Pakistan remain poorly understood. Accurate species identification is critical, because misidentification can cause errors in stock management, biodiversity reporting, conservation planning and fisheries policy. Therefore, this study not only examined morphological identification using both traditional morphometric techniques and the Truss Network approach, but also incorporated molecular genetic analysis through DNA barcoding to validate species identity and assess genetic variation in *H. fossilis* population from Punjab, Pakistan.

Materials and methods

Sampling site

The current research was carried out in three different locations of Punjab Pakistan. These three locations are Head Trimmu in Jhang situated at (31°8'41.71" N, 72°8'45.7" E), Taunsa Barage in Kot Adu (30º42´N 070º50´E) and Chashma Barage in Mianwali (32.43389°N, 71.37889°E) (Latif *et al*., 2016) (Fig. 1).

Sample collection

Fish sample of *H. fossilis* were collected with the help of fisherman by using commercial fish gears such as gill net, baskets and trammel nets. The fresh fish samples were carefully cleaned and place in ice and delivered to the laboratory in polythene bags as soon as possible. Fish samples was transferred from sampling location to the research laboratory of Government College University Faisalabad for further analysis. Morphometric analysis was done by using a key to the fishes of the Punjab by Mirza, 1996. After morphometric analysis specimen were preserved in 70% ethanol for genetic analysis (Fig. 2).

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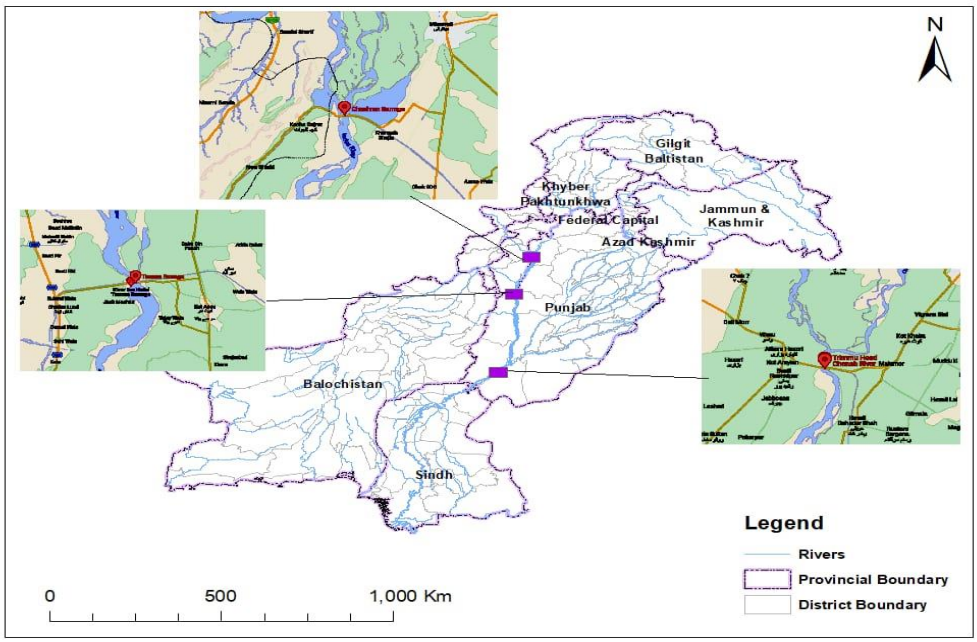

Figure 1: Map of three sampling site in Punjab, Pakistan.

Figure 2: Measurement on the basis of morphometric traits.

Morphological Identification

Morphological identification was based on meristic and morphometric characters of fish. Each specimen's measurements were taken from left to right. The morphometric parameter such as Total length (TL), standard length (SL), pre dorsal length (PrDL), post dorsal length (PsDL), pre pectoral length (PrPecL), pre pelvic length (PrPevL), pre anal length (PrAL), snout length (SnL), highest body depth (HBD), lowest body depth (LBD), head length (HL), head depth (HD), eye diameter (ED), inter orbital (IO), pre orbital length (PrOL), post orbital length (PsOL), maximum barbell length (MXBL), minimum barbell length (MNBL), anal fin base (AFB), height of anal fin (HAF), dorsal fin base (DFB), height of dorsal fin (HDF), height of pelvic fin (HPevF), height of pectoral fin (HPecF), pelvic fin base (LPevFB), pectoral fin base (LPecFB), Length of anal fin base (LAFB), caudal fin length (CFL), upper jaw length (UJL) and lower jaw length (LJL) were measured with the help of vernier caliper. A vernier caliper with a precision of 0.01 mm was used to perform traditional measurement (Table 1; Fig. 3).

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Table 1: Morphological parameters of *H. fossilis*.

Sr.	Acronym	Morphometric Trait	Description
1.	TL	Total Length	Distance from the snout tip to the end of the caudal fin
2.	SL	Standard Length	Distance from the snout tip to the posterior edge of vertebral column
3.	PrDL	Pre Dorsal Length	Distance from the snout tip to the insertion of dorsal fin
4.	PsDL	Post dorsal length	Distance from the end of the dorsal fin to the end of caudal fin ray
5.	PrPecL	Pre Pectoral length	Distance from the anterior margin of upper lip to the starting point of pectoral fin
6.	PrPevL	Pre Pelvic length	Distance from the anterior margin of upper lip to the starting point of pelvic fin
7.	PrAL	Pre anal length	Distance from the anterior margin of upper lip to the starting point of anal fin
8.	SnL	Snout Length	The length from the tip of the snout to the anterior edge of eye margin
9.	HBD	Highest Body Depth	Length from the dorsal's fin anterior part to the ventral portion of the body
10.	LBD	Lowest Body Depth	Length at the end of vertebral column
11.	HL	Head Length	Length from the tip of the snout to the posterior tip of the operculum
12.	HD	Head Depth	Vertical measurement just posterior to the eye orbit
13.	ED	Eye Diameter	Distance between anterior and posterior eye margin
14	IO	Inter Orbital	Length between the eye orbits
15.	PrOL	Pre Orbital length	Length from the anterior part of the body to the front margin of the orbit
16.	PsOL	Post Orbital Length	Distance from the posterior edge of orbit to the end of operculum
17.	MXBL	Barbel Length Maximum	Largest barbell length
18.	MNBL	Barbel Length minimum	Smallest barbell length
19.	LAFB	Length of anal Fin Base	Distance from the anterior origin of anal fin to the posterior end of anal fin
20.	HAF	Height of Anal Fin Base	Measurement from anal fin base to the tip of the largest fin ray
21.	DFB	Dorsal Fin Base	Distance from the anterior origin of dorsal fin to the posterior end of dorsal fin
22.	HDF	Height of Dorsal Fin	Measurement from dorsal fin base to the tip of the largest fin ray
23.	HPevF	Height of Pelvic Fin	Measurement from pelvic fin base to the tip of the largest fin ray
24.	HPecF	Height of Pectoral Fin	Measurement from pectoral fin base to the tip of the largest fin ray
25.	LPevFB	Length of Pelvic Fin Base	Distance from the anterior origin of pelvic fin to the posterior end of pelvic fin
26.	LPecFB	Length of Pectoral Fin Base	Distance from the anterior origin of pectoral fin to the posterior end of pectoral fin
27.	CFL	Caudal Fin Length	Distance from anal fin base to the caudal fin base

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Figure 3: Morphometric measurement of *H. fossilis.*

Meristic Count

A magnifying lens was used to count the total number of fin rays. Six meristic features were investigated, dorsal fin rays (DFR), pectoral fin rays (PFR), ventral fin rays (VFR), anal fin rays (AFR) and caudal fin rays (CFR). With the use of a needle and little pins all of the meristic counts were put up against the incoming light direction in the room for simple counting (Table 2).

Table 2: Meristic characters of *H. fossilis.*

Sr.	Acronym	Characters	Description
1.	DFR	Dorsal fin rays	Total number of Dorsal fin rays
2.	PecFR	Pectoral fin rays	Total number of Pectoral fin rays
3.	AFR	Anal fin rays	Total number of Anal fin rays
4.	PevFR	Pelvic fin rays	Total number of Pelvic fin rays
5.	CFR	Caudal fin rays	Total number of Caudal fin rays
6.	PecFS	Pectoral fin spine	Total number of Pectoral fin spine

Truss Analysis

The "Truss network system" was used to collect morphometric data. A little piece of fabric was used to handle the fish and blotting paper was used to absorb the water. Around the fish data points were organized in "trusses," a pattern that maximized the number of observations and increased the accuracy of the analysis. On the body, 10 landmarks designating 20 distances were measured (Fig. 4). Each landmark was gathered by positioning a fish on the graph paper and using colored markers to detect the landmarks allowing for precise and repeatable measurements. Finally, Vernier calipers were used to measure the lengths on the graph paper (Fig. 5; Table 3).

DNA extraction and quantification

The high quality DNA was extracted from fins of collected samples by following the Kit method “QIAamp DNA Mini Kit”. Genomic DNA was photographed on the 1% agarose gel and kept at–20ºC. Purity and quantification of extracted DNA was determined through Nano Drop.

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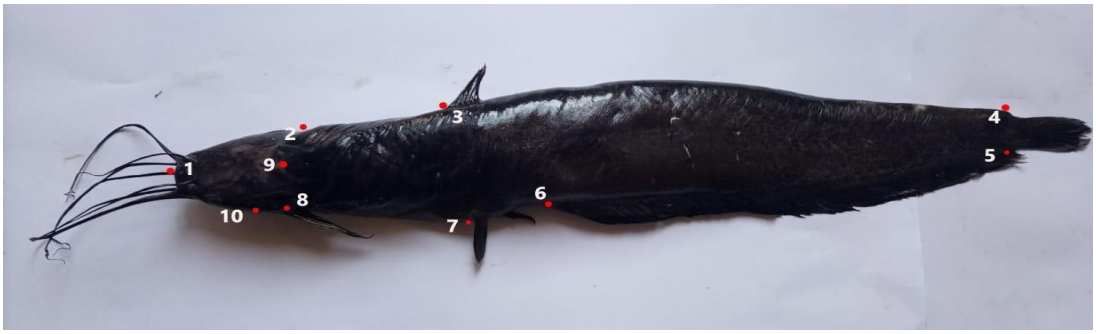

Figure 4: *H. fossilis* indicated the location of 10 landmarks for truss analysis.

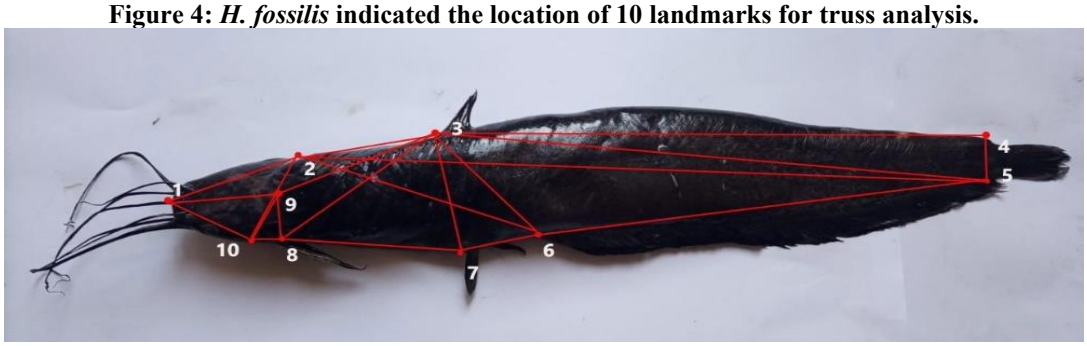

Figure 5: *H. fossilis* indicated the 10 landmarks distance used for morphological analysis.

Table 3: Description of truss landmarks used in this study.

Landmark points	Description
1	Anterior tip of snout on the upper jaw
2	The posterior margin of the supra-occipital bone
3	Origin of the dorsal fin base
4	Origin of the caudal fin
5	Posterior edge of the anal fin base
6	Anterior edge of the anal fin base
7	Insertion of the pelvic fin base
8	Point of insertion of the pectoral fin
9	Posterior margin of the operculum
10	The ventral edge of operculum and ventral side of the body

PCR Amplification and Sequencing

PCR was done by using the Thermocycler T100 BioRad. Barcode region was amplified by using standard protocol of PCR. For the amplification of short segment of mitochondrial DNA, universal forward and reversed primer was used. For the amplification, reaction mixture of 20µL was made in the PCR tubes. Amplification by a polymerase chain reaction, the pair of primers was used to amplify the *CO1* gene found in the mitochondrial genome.

FishF1: 5` TCAACCAACCACAAAGACATTGGCAC

FishR1: 5` TAGACTTCTGGGTGGCCAAAGAATCA

Composition of the reaction mixture for PCR amplification is Master mix (10µL), Deionized water (3µL), Forward primer (2µL), Reverse primer (2µL) and DNA template (3µL). Tubes were then placed in thermo-cycler which was programmed with the suitable PCR standard protocol. The thermal regime followed by the 35 cycles at 95˚C for 0.5 minute, annealing at 58˚C for 1 minute and the extension at 72˚Cfor 1 minutes. Final extension for 10 minutes at 72˚C

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was observed. The amplified products of PCR sized and visualized on the 1% agarose gel. The amplified products of PCR were purified before Sanger sequencing by using FavorPrepPCRClean-UpMiniKit (Cat. #FAPCK001-1). Sanger sequencing completed uni-directionally for perception of the freshwater species. Entire nucleotide sequences were deposited in GenBank.

Statistical Analysis

Descriptive statistics, Univariate analysis of variance (ANOVA) and principal component analysis (PCA) were implemented for morphometric features to estimate the significant change with significance level of 5% was measured to check the significance of truss measurements, meristic, and morphological using Minitab 17 software. The *COI* sequence of each specimen was found by aligning the sequences generated by the forward and reverse primers. The sequences were aligned and scrutinized using BioEdit sequence alignment version 7.0.5.3. For validation and certain analyses, additional sequences were acquired from GenBank (http://www.ncbi.nlm.nih.gov/) and BOLD (Barcode of Life Data Systems, www.boldsystems.org). The Kimura 2-P method (K2P) was used to compute pairwise genetic divergence between species, across genus and family members, and within species. With the MEGA 11 and K2P model, maximum likelihood (ML) trees at 1000 pseudo replicates and genetic distance were created. Additionally, GenBank was provided with accession numbers for all five *COI* sequences of *H. fossilis*.

Results

Taxonomic identification

Morphometric features define the traits of body shape. Examples of morphological characteristics include shape and size. The mean SL of *H. fossilis* for 30 samples was 171.2087 mm and ranged 107.86-260.01 mm. The mean values of 27 morphometric characters were considerably different in the specific fish from all three locations. Chashma Barrage population comparatively, showed high range of body shape and size as compare to trimmu head and Taunsa Barrage. Trimmu head population, showed high range of body shape and size except three characters HAF, LDFB and LPevFB. These three characters showed higher mean value in the samples collected from Taunsa Barrage (Table 4).

Meristic counts

The meristic counts among three populations were done with equivalent descriptive statistical factors (mean±SD). The maximum and minimum values for each meristic character ranged from 5-7 for DFR, 64-67 for AFR, 13-16 for CFR, 1-1 for PecFS, 5-7 for PecFR and 5-7 for PevFR among three population tested by descriptive statistical parameters (mean±SD). Among six meristic characters, only one trait (PecFS) showed no variation (Table 5).

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Remaining five has significant variation. As Chashma Barrage has greater mean values among three populations, results showed there was no influence of SL on meristic counting. In case of DFR the mean value of Trimmu head was greater than the other two locations. In case of AFR Taunsa Barrage showed high value as compare to Trimmu Head and Chashma Barage. The mean value of PecFR is highest in Trimmu Head. In case of PevFR there was no variation between Trimmu Head and Chashma Barage but observed higher at taunsa barrage.

Table 4: Comparision of the mean and standard deviation of morphometric trait of *H. fossilis* in three populations.

Morphometric Characteristics	Overall			Chashma Barrage	Trimmu Head	Taunsa Barrage
	Min (mm)	Max (mm)	Mean±SD	Mean±SD	Mean±SD	Mean±SD
TL	126.10	283.06	197.37±45.70	230.41±43..76	182.80±30.42	178.90±45.39
SL	107.86	260.01	171.21±41.45	202.95±44.48	162.72±28.84	147.96±30.43
SnL	7.99	17.56	12.28±2.73	13.94±2.55	11.60±2.24	11.30±2.79
HBD	15.96	45.63	28.18±7.39	33.93±8.68	26.30±2.90	24.29±5.87
LBD	9.36	17.87	12.81±2.24	14.75±2.43	12.15±1.35	11.54±1.40
HL	18.67	58.04	34.73±9.52	40.34±11.64	34.15±6.15	29.70±7.41
HD	6.32	20.17	12.64±3.67	15.13±4.05	12.35±2.56	10.44±2.84
ED	2.37	4.92	3.56±0.53	3.73±0.42	3.55±0.50	3.38±0.65
PrOL	6.71	14.03	9.97±1.99	11.60±2.40	9.53±0.65	8.78±1.36
PsOL	6.04	23.62	15.86±2.98	17.05±2.97	15.41±3.31	15.13±2.53
MXBL	23.46	72.59	37.05±15.64	48.80±19.16	33.48±12.52	28.86±4.76
MMBL	9.92	49.78	26.49±11.62	35.11±13.20	23.07±9.45	21.28±6.78
PrDL	34.20	81.13	55.99±13.93	66.03±13.35	52.05±11.15	49.88±12.34
PsDL	67.92	169.08	110.41±25.33	129.94 ±27.57	106.51±17.55	94.78±16.96
PrPecL	19.93	37.42	27.75±4.82	30.14±4.31	26.56±3.03	26.55±6.12
PrPevL	40.79	85.96	58.18±12.31	67.83±13.19	56.19±8.71	50.55±8.11
PrAL	47.18	106.94	70.71±16.54	83.83±17.50	67.95±12.21	60.35±10.32
HPecF	9.63	25.02	18.28±4.42	21.04±4.02	17.24±2.89	16.57±5.05
HPevF	8.95	20.87	13.57±3.17	15.46±3.80	13.22±2.19	12.02±2.50
HDF	12.24	25.78	18.35±3.44	20.43±3.95	17.37±1.05	17.25±3.76
HAF	4.33	19.30	7.88±3.24	9.94±4.69	6.76±1.01	6.95±1.94
LDFB	4.42	8.97	6.94±1.27	7.20±1.183	6.65±0.81	6.97±1.71
LPecFB	3.04	7.89	5.94±1.40	6.41±1.25	5.81±0.74	5.59±1.94
LPevFB	2.74	6.96	4.69±1.34	4.91±1.20	4.38±0.92	4.79±1.83
LAFB	64.12	154.98	101.83±24.52	120.84±25.46	96.18±17.71	88.45±18.42
CFL	12.66	22.46	17.61±3.07	19.35 ± 3.19	17.60±2.21	15.89±2.93
IO	9.08	19.45	13.23±2.80	15.38±3.25	12.58±1.30	11.73±2.20

Min=Minimum; Max=Maximum; SD=Standard deviation

Table 5: Comparison of the mean and standard deviation of *Heteropneustes fossilis* for meristic characteristics in three populations.

Meristic Characteristics	Overall			Chashma Barrage	Trimmu Head	Taunsa Barrage
	Min (mm)	Max (mm)	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
DFR	5	7	6.37 ± 0.62	6.30 ± 0.67	6.60 ± 0.52	6.20 ± 0.63
AFR	64	67	65.30 ± 1.15	64.90 ±1.10	65.20 ±1.23	65.80 ±1.03
CFR	13	16	14.63 ± 0.72	14.90± 0.74	14.60 ±0.70	14.40 ±0.67
PecFS	1	1	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
PecFR	5	7	6.27 ± 0.64	6.00 ± 0.82	6.50 ± 0.53	6.30 ± 0.48
PevFR	5	7	6.17 ± 0.75	6.00 ± 0.82	6.00 ± 0.82	6.50 ± 0.53

Min=Minimum; Max=Maximum; SD=Standard deviation

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Image based truss-box analysis

Truss network analysis is the systematic way to depict the entire shape of the fish in the study of morphometrics. *Heteropneustes fossilis* of Trimmu Head population showed greater mean values of truss morphometry. The mean values of all variables of Chashma Barrage were observed higher than the Taunsa Barrage (Fig. 6).

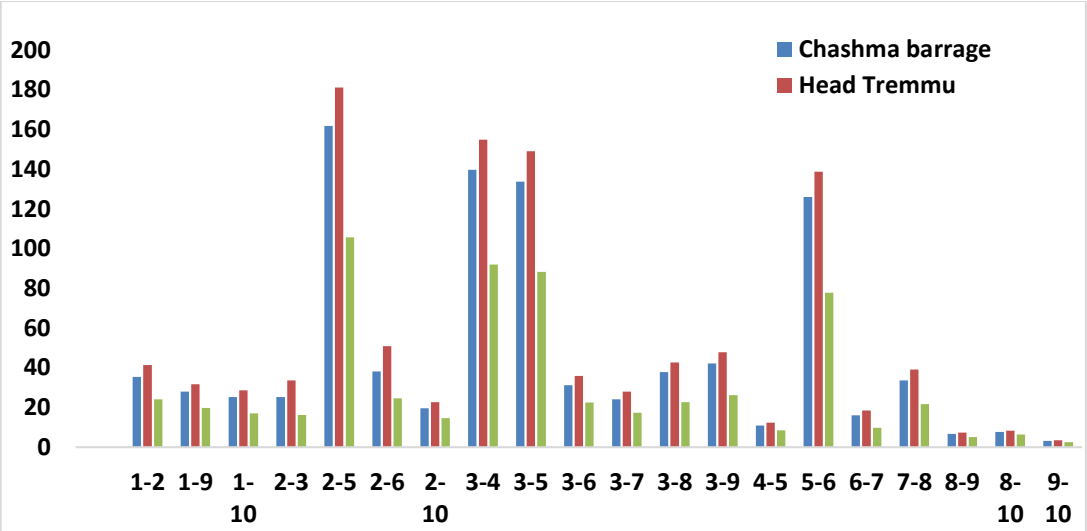

Figure 6: Showing Truss Box analysis of *Heteropneustes fossilis* samples collected from three different locations, illustrating the morphological variation among populations.

Principal Component Analysis

Principal component analysis was performed for multivariate analysis and to identify similar number of uncorrelated variables, principal component from our data set. PCA explained the maximum amount of variance with fewest number of Principle components. In *H. fossilis* the proportion loadings of the initial four elements from principal components (PC1, PC2, PC3 and PC4 respectively) defined 77.7%, 8.7%, 3.7% and 2.9% of the change respectively. PC1 was the axis which continually considered for the highest amount of difference. For intra-components relation, PC1 positively correlated with all variables (Table 6).

Table 6: Eigen analysis of the Correlation Matrix of *H. fossilis*.

Components	Eigen value	Proportion	Cumulative
PC1	20.988	0.777	0.777
PC2	2.340	0.087	0.864
PC3	0.991	0.037	0.901
PC4	0.793	0.029	0.930

In morphmeristic counts, results showed that TL, SL, HBD, LBD, HL, HD, PrOL, PsOL, MXBL, MMBL, PrDL, PsDL, PrPevL, PrAnL, HPec, HPev, HAF, LAFB, CFL, SnL, ED, PrPecL, HDF, LDFB, LPevFB and LPecFB and IO have large positive on first component. There was no negative loading measured, as shown in Figure 7. In meristic counts, loading plot showed the

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results for first four components. Our results represented that CFR, DFR, PevFR, PecFR, and AFR have large positive on first component. No negative loading was observed, as shown in Figure 8.

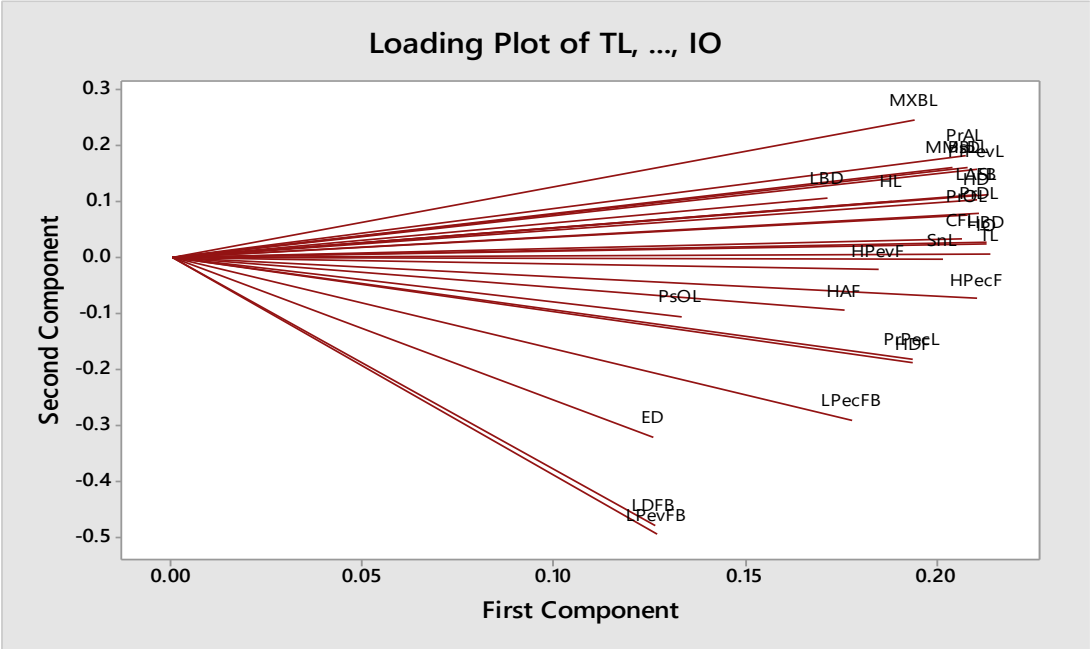

Figure 7: Loading Plot of morphometric parameters of *H. fossilis*.

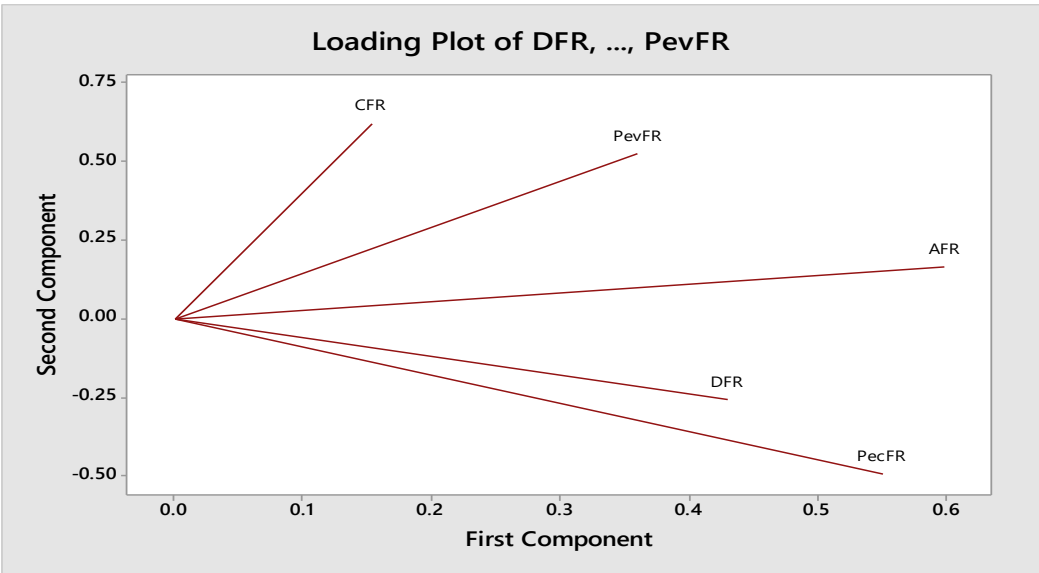

Figure 8: Loading Plot of meristic characters of *H. fossilis*.

Molecular Identification

Nucleotide base composition analysis

In this study, 5 sequences of *COI* gene were successfully analyzed, amplified and submitted to GenBank and the BOLD databases. The mitochondrial *COI* gene sequence of studied specimens were aligned that contained 586/622 conserved sites, 6/622 variable sites, and 6/622 singleton sites. No deletions, stop

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codon, or insertions were found in any of the aligned sequences. Moreover, results of our morphological identification also matched at least 100% similarity with the BLASTN annotations of BOLD and NCBI database. Average nucleotide pair frequencies for all studied sequenced were T (30.8%), C (25.2%), A (27.2%), and G (16.8%). The average GC content (42%) was lower than the AT content (58%) in the nucleotide base composition analysis of the COI sequences, which revealed that the average AT content (58%) was highest and the GC content (42%) was lowest (Table 7). With a R (si/sv) ratio of 1.89 for the dataset, it was found that transitional pairs (si=64.62) were more prevalent than transversional pairs (sv=35.36). In the final dataset, 662 locations altogether were located.

Table 7: Average base composition of *Heteropneustes fossilis* from selected river populations in Pakistan.

Accession No.	T	C	A	G	Total
Heteropneustes fossilis OQ318486	30.9	25.2	27.2	16.7	592
Heteropneustes fossilis OQ318850	30.9	25.2	27.2	16.7	592
Heteropneustes fossilis OQ318851	30.9	25.2	27.2	16.7	592
Heteropneustes fossilis OQ326839	30.4	25.4	27.2	17.0	622
Heteropneustes fossilis OQ318536	30.9	25.1	27.3	16.7	586
Average	30.8	25.2	27.2	16.8	597

Pairwise genetic analysis

In the present study, the Kimura-2-Parameter (K2-P) model was applied to calculate the pairwise genetic distances among *Heteropneustes fossilis* sequences obtained from different sampling locations. The results revealed zero genetic distance among individuals within each population, indicating high genetic similarity or genetic stability within the species. However, slight variation was detected among sequences from different localities, with inter-population K2-P distances ranging from 0.0000 to 0.0102. These values show that although individuals of *H. fossilis* are genetically very close, minor divergence exists between populations, which may reflect geographical influence or localized environmental differences (Table 8).

Table 8: Genetic distance using KP2 Model within *Heteropneustes fossilis*.

	OQ318486	OQ318850	OQ318851	OQ326839	OQ318536
OQ318486					
OQ318850	0.0000				
OQ318851	0.0000	0.0000			
OQ326839	0.0102	0.0102	0.0102		
OQ318536	0.0000	0.0000	0.0000	0.0086	

Phylogenetic divergence

The phylogenetic tree generated from the COI sequences showed that all *Heteropneustes fossilis* samples from this study grouped together with the authenticated reference sequences

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retrieved from GenBank (PP217346.1 and GQ466395.1), forming a single monophyletic cluster. This cluster was strongly supported with a bootstrap value of 100%, clearly indicating very high confidence in the grouping and confirming that all analysed samples belong to *H. fossilis*. The tight clustering pattern also reflects very low genetic variation within the species. The outgroup species *Notopterus notopterus* formed a separate branch, confirming the distinctness of *H. fossilis* and validating the use of COI sequences for species-level identification in this dataset (Fig. 9).

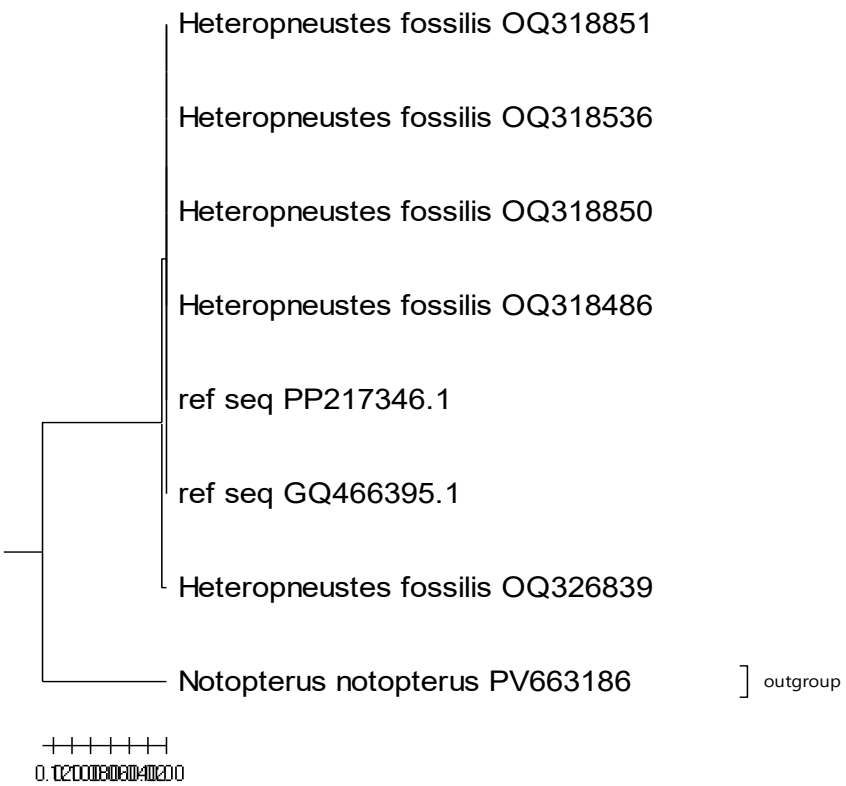

Figure 9: Phylogenetic tree showing the clustering of *Heteropneustes fossilis* sequences.

Discussion

Heteropneustes fossilis is a widely distributed freshwater catfish species in South Asia, including Pakistan, India, Bangladesh, Nepal, and Sri Lanka. It is characterized by an elongated body that is slightly compressed laterally and a depressed head with moderately sized lateral eyes and nostrils that are spaced widely apart, and a short, terminal, transverse mouth. Two cephalic fontanels, with the anterior one being longer than the latter. There are four pairs of barbels: the nasal, which extends one eye orbit diameter at the front of the dorsal fin base and the tip of the pectoral fin; the maxillary, which extends to the pelvic and dorsal fin bases; and the outer and inner mandibles, which extend to the pectoral fin tip. The pectoral fin has a

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strong spine, an inner edge that is serrated, and an outer edge that is granulated. The pectoral fin does not reach the pelvic fin, but it does fairly reach the anal fin. Although the anal and caudal fins are nearly identical in length, there is a little space between the two. Round caudal fin. Smooth skin with noticeable muscular bands. Color: vibrant colours Barbels are brown, eyes are blue, and the body and fins are light to dark brown (Plamoottil, 2022).

The *Heteropneustes fossilis* samples collected from Trimmu Head exhibited white spots on the body, whereas samples from other locations were entirely black and lacked spots. These differences likely reflect environmental and climatic variations among the sites. Factors such as water flow, substrate, vegetation, and genetic variation can induce morphological differences within a species (Secre *et al*., 2022). Water flow is considered a primary driver of morphological variation (Kelly *et al*., 2017; Shuai *et al*., 2018), and phenotypic diversity often increases with environmental heterogeneity (Malinich, 2019). Given geographical separation and potential ancestral differences, morphological variations between populations are expected. Fish can rapidly adjust morphometric traits in response to ecological factors, including food availability, demonstrating their sensitivity to environmental changes (Rahman *et al*., 2014).

Morphometric analysis has been widely used to assess variations in the shape and size of fish, evaluate population structure, and characterize stocks and species. It provides information on phenotypic stocks with similar growth patterns, reproductive potential, and mortality rates (Nama *et al*., 2022). To date, no detailed morphometric information on *Heteropneustes fossilis* in Pakistan had been reported. The present study described the morphometric characteristics of *H. fossilis* from three different locations in Punjab, Pakistan. The analysis included multiple length measurements, such as total length (TL), standard length (SL), pre-dorsal length (PrDL), post-dorsal length (PsDL), pre-pectoral length (PrPecL), pre-pelvic length (PrPevL), pre-anal length (PrAL), snout length (SnL), highest body depth (HBD), lowest body depth (LBD), head length (HL), head depth (HD), eye diameter (ED), interorbital distance (IO), pre-orbital length (PrOL), post-orbital length (PsOL), maximum barbel length (MXBL), minimum barbel length (MNBL), dorsal-fin base length (DFB), height of dorsal fin (HDF), length of anal-fin base (LAFB), height of anal fin (HAF), length of pectoral-fin base (LPecFB), height of pectoral fin (HPecF), length of pelvic-fin base (LPevFB), and height of pelvic fin (HPevF), as well as caudal fin length (CFL). These measurements provided a comprehensive overview of the morphological variation among *H. fossilis* populations in Punjab (Zafar *et al*., 2024).

Analysis from morphometric study represented that *H. fossilis* showed difference among three locations. The mean of TL in study varied among the

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location, noticed greater in Chashma Barrage location (230.41mm) and followed by Trimmu Head (182.80mm) and Taunsa Head (178.90mm). The variation in TL in *H. fossils* from different locations might be due to drastic change in aquatic parameters like water current, salinity and temperature. The maximum total length (TL) of *Heteropneustes fossilis* recorded in the present study was 283.06 mm, which was greater than the previously reported maximum TL of 268 mm in Bangladesh (Rahman *et al*., 2019), but lower than the maximum TL of 310 mm reported from the Ganga River, India (Khan *et al*., 2012). Information on the maximum length is essential for calculating population parameters, including the coefficient of growth and relative length, which are critical for the management and resource planning of fish populations (Hossain *et al*., 2019).

Moreover, in morphometric characters of *H. fossilis* TL, SL, HBD, LBD, HL, HD, PrOL, PsOL, MXBL, MMBL, PrDL, PsDL, PrPevL, PrAnL, HPec, HPev, HAF, LAFB, CFL and IO showed significant (p<0.05) results among 27 characters in ANOVA test While SnL, ED, PrPecL, HDF, LDFB, LPevFB and LPecFB were showed non-significant (p>0.05). In the meristic analysis, the mean ranges observed in the present study were as follows: dorsal-fin rays (DFR) 5–7, anal-fin rays (AFR) 64–67, caudal-fin rays (CFR) 13–16, pectoral-fin rays (PecFR) 5–7, pelvic-fin rays (PevFR) 5–7, and pectoral-fin spines (PecFS) 1. These results closely corresponded with the findings of Rahman *et al*. (2019), who reported DFR 6, PecFR 6–7, PecFS 1, PevFR 6, AFR 64–69, and CFR 16–18. Similar observations were also reported by Rahman *et al*. (2014). Both meristic and morphometric characteristics provided valuable information for the identification and differentiation of *Heteropneustes fossilis*. According to One-way ANOVA testing, among six meristic traits of *H. fossilis*, only one; DFS (p= 0.001) displayed significant (p<0.05) result. While all other parameters showed non-significant results. The stock's mean values of AFR, VFR and DFR were considerably different (p<0.05) (Rahman *et al*., 2019).

In *Heteropneustes fossilis*, the proportion of variation explained by the first four principal components (PC1, PC2, PC3, and PC4) was 77.7%, 8.7%, 3.7%, and 2.9%, respectively. PC1 accounted for the highest proportion of variation and was positively correlated with all measured morphometric variables, indicating its dominant influence on the overall morphological differences among populations. Similar patterns of morphological variation have been reported in other fish species; for example, Ramya *et al*. (2021) observed variations in morphometric traits in *Barbodes carnaticus*. The morphological variations observed in this study may be attributed to environmental factors, among other influences. Previous research has documented differences in water quality parameters, such as temperature and dissolved oxygen, across the rivers studied, which could contribute to the

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observed variation (Haque *et al*., 2019). Many other authours used the technique of principal component analysis to described the truss variables morphometric and meristic characters such as (Su *et al*., 2019; Das *et al*., 2020; Biswal *et al*., 2020; Bal *et al*., 2021; Secer *et al*., 2022).

In this study the truss network analysis revealed that the Trimmu Head population of *H. fossilis* exhibited higher mean values of most truss distances compared with the other two populations, and the Chashma Barrage population showed greater mean values than the Taunsa Barrage population. These differences indicate that the shape of *H. fossilis* vary among the three sampled sites, consistent with the influence of spatially-variable environmental conditions and population history. The truss network approach is valuable because, unlike classical linear morphometrics alone, it captures the overall body geometry by linking anatomical landmarks across the body, thus providing a detailed shape "map" of the fish. For example, a prior study on *H. fossilis* in the Ganga, Yamuna and Gomti rivers of India used eleven truss landmarks and could successfully differentiate stocks based on shape (Khan *et al*., 2012).

To understand fish speciation, phylogenetic relationships were previously inferred primarily using morphological characteristics. Because of the similarity in external morphology, it is challenging to distinguish between the many species of freshwater catfish. Because of the complex evolutionary changes in either morphological or physiological characteristics, the rebuilt phylogenetic trees based on morphology were controversial. Recently, mtDNA genetic analysis has been done to address the controversial taxonomic issue (Erguden *et al*., 2010).

A total of five mitochonderial *COI* barcode sequences were effectively accuired and analyzed from fishes of genus Heteropneustes and amplified with two bidirectional primers in PCR. All barcode sequences were 650bp in length after manual edition, sequences had nodeletions, insertions and stop codons. In this study five specie of *Heteropneustes fossilis* were studied.

There were no stop codons found, no nuclear mitochondrial pseudogenes (NUMTs), and the average consensus length was observed 586 to 622 base pairs in all barcodes sequences. In general, vertebrate have smaller NUMTs than 600 bp (Ward *et al*., 2005). The biodiversity may be underestimated if NUMTs are present. Conserved primers should amplify mitochondrial DNA more frequently than NUMTs since there are more *COI* genes than pseudogenes, yet there was no evidence of NUMTs in the fish. A nucleotide pair frequency analysis showed 586/622 conserved sites, 6/622 variable sites, and 6/656 singleton sites. Transitional pairs (si=64.62) was more common than the transversional pairs (sv=35.36) with the R (si/sv) ratio of 1.89.The identified transitional mutations were more abundant than the transversional ones as earlier detected in *Channa*

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striata (Boonkusol and Tongbai, 2016) and Labeo genus (Zafar *et al*., 2024).

The average nucleotide base composition of examined catfish species was 58% AT and 42% GC. Overall, in studied freshwater catfishes, the average GC content was lower (42%) than AT content (58%). The current variations revealed a significant amount of heterogeneity within the fish species under this study. This position clearly displayed anti-G bias,and similar patterns have been seen in Unveiling intraspecific variations in *Notopterus notopterus* and *Chitala chitala* through landmark -based morphometry and mitochondrial DNA barcoding by Zafar *et al*. (2025). Similarly, nucleotide composition 56% AT and 43.96% GC was reported in the study of edible freshwater fish species of Pakistan through DNA barcoding (Ghouri *et al*., 2020). Similar finds were also observed by Sajjad *et al*. (2023) in *Wallago attu*.

The pairwise genetic distance analysis revealed very low divergence among the COI sequences, ranging from 0.0000 to 0.0102. Most sequences, including OQ318486, OQ318850, OQ318851, and OQ318536, were identical (0.0000), indicating extremely high genetic similarity within these populations. Sequence OQ326839 showed slightly higher divergence (0.0102) compared to the others, suggesting minor genetic variation among the sampled populations. Overall, these low genetic distances are typical of intraspecific variation in mitochondrial COI sequences and confirm that all sampled individuals belong to the same species, *H. fossilis*. The slight differences observed may reflect geographic separation, limited gene flow, or microevolutionary changes within populations. These results are consistent with previous studies on freshwater fish, where *COI* sequences typically show minimal divergence within species but sufficient variation to distinguish between species. Similar low genetic distance was observed among species of *wallago attu* by Sajjad *et al.* (2023) and labeo genus by Zafar *et al.* (2024).

The phylogenetic analysis based on *COI* sequences revealed that all *Heteropneustes fossilis* samples from this study clustered together with authenticated reference sequences from GenBank (PP217346.1 and GQ466395.1), forming a single, well-supported monophyletic group. The cluster received a high bootstrap support of 99%, indicating strong confidence in the grouping and confirming that all analyzed samples belong to *H. fossilis*. The tight clustering also reflects the low intraspecific genetic variation within the species, consistent with the low pairwise genetic distances observed. The outgroup species, *Notopterus notopterus*, formed a separate branch, clearly demonstrating the genetic distinctness of *H. fossilis* and validating the effectiveness of *COI* sequences for accurate species-level identification in this study. These results align with previous DNA barcoding studies in freshwater catfishes, where *COI*-based phylogenies reliably distinguish species while showing minimal variation within

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species (Ude *et al*., 2020; Sajjad *et al*., 2023; Zafar *et al*., 2024).

Conclusion

This study provides a thorough assessment of the population quality and stock structure of *Heteropneustes fossilis* from three geographically distinct locations in Pakistan through an integrated approach combining morphometric, meristic, truss-network, and molecular analyses. Morphometric and truss-network analyses revealed significant variation among certain traits, indicating subtle differences in body shape and size across populations, while some meristic traits were consistent, suggesting overall phenotypic stability. Principal component analyses further highlighted the key morphological variables contributing to population differentiation, providing a quantitative framework for distinguishing stocks. The genetic analysis using cox1-based DNA barcoding demonstrated high sequence similarity among individuals, confirming species-level identification and revealing minor inter-population divergence likely influenced by localized environmental conditions or geographic separation.

Overall, the results of this study establish baseline data on the morphological and genetic diversity of *H. fossilis* in Pakistan, which is critical for sustainable management and conservation planning. The integrated morpho-genetic approach not only validates the use of cox1 as a reliable barcode for this species but also offers a reference framework for future biodiversity assessments, stock discrimination, and aquaculture initiatives. These findings underscore the importance of monitoring wild populations to prevent over-exploitation and habitat degradation, and they provide a foundation for developing evidence-based conservation strategies, policy decisions, and regional management plans aimed at preserving the ecological and commercial value of *H. fossilis* in the Indo-Pak freshwater systems.

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