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RESEARCH PAPER

Characterizing the Dark Mahseer, *Naziritor chelynoides* (McClelland, 1839): A Morphological, Osteological, and Molecular Approach

Neetu Shahi[1,*], Debajit Sarma[1], Bhupendra Singh[1], Sumanta Kumar Mallik[1], Deepjyoti Baruah[2], Ravindra Posti[1], Radheshyam Haldar[1], Irengbam Linthoingambi[3]

[1]ICAR-Directorate of Coldwater Fisheries Research, Bhimtal, 263 136, Nainital, Uttarakhand, India.
[2]ICAR-Indian Agricultural Research Institute, Dirpai Chapori, Gogamukh, 787 035, Dhemaji, Assam, India.
[3]Dhanamanjuri University, Thangmeiband, Imphal, 795 001, Manipur, India.

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Corresponding Author
Tel.: +917409148538
E-mail: neetuicar@gmail.com

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Abstract

Dark mahseer, *Naziritor chelynoides* is a vulnerable stream fish in several Asian countries, and developing an effective conservation strategy is impeded by incomplete information about its taxonomy. To address this research gap, in the current study dark mahseer collected from Himalayan stream in India (29°21'99.5" N and 79°33'23.9" E) were examined for their morphometric, meristic, osteological, and molecular characteristics. This study discovered unique features of dark mahseer, including its thin non-hypertrophied lower lip without median lobe, complete post-labial groove, 33 to 34 central perforated lateral line scales, a dark elliptical blotch at the angle of the operculum, and numerous fine black dots on a crescent pattern on scales. This species has 9 to 11 slender gill rakers (595.93±148.71 µm in length), 40 to 41 total vertebrae, 38 to 39 neural spines, 14 to 15 hemal spines, and fused first to fourth abdominal vertebrae. Molecular analysis of cytochrome c oxidase subunit 1 gene (*cox1*), cytochrome b (*cytb*), and ATPase subunit 6&8 (*ATPase 6&8*) genes revealed the genetic distance of 0.08 to 0.10, 0.12 to 0.13, and 0.01 to 0.11 respectively from other *Tor* species. Our study enhances knowledge of dark mahseer's taxonomy, supporting conservation strategies for this valuable fish species.

Introduction

Mahseers are important freshwater fish species in Southeast Asian countries such as India, Nepal, Bhutan, Thailand, Bangladesh, Pakistan, Indonesia and Myanmar (Sen and Jayaram, 1982; Talwar and Jhingran, 1991; Shahi *et al.,* 2014; Jaafar *et al.,* 2021). They belong to the Cyprinidae family, and consist of three genera: *Tor*, *Neolissochilus*, and *Naziritor* (Khare *et al.,* 2014; Sarkar *et al.,* 2015). Among these three genera, *Tor* is recognized as the true mahseer owing to distinctive characteristics such as the presence of a median lobe in the lower lip, large body size, prominent scales, and 22 to 28 central perforated lateral line scales (Sen and Jayaram, 1982; Jaafar *et al.,* 2021).

The genus of mahseer, *Naziritor* is found in India, Nepal, and Pakistan. It includes two species: *N. zhobensis* (zhobi mahseer; Mirza, 1967) and *N. chelynoides* (dark mahseer; McClelland, 1839). *N. zhobensis* is endemic to the western region of Pakistan (Mirza, 1967), and *N. chelynoides* is found in shallow, fast-flowing mountain streams in India, Nepal, and Pakistan (Talwar and Jhingran, 1991).

Currently the population of dark mahseer is in decline, and this species is considered vulnerable by the International Union for Conservation of Nature (IUCN)

red list (IUCN, 2021). The primary threat to dark mahseer in its natural habitat is destructive fishing methods, hydroelectric dam construction, habitat degradation and climate change (Kattel *et al.,* 2022). Despite its decline and vulnerable status, dark mahseer conservation efforts have yet to be initiated so far due to its smaller size, taxonomic ambiguity, and sporadic distribution. Additionally, the lack of holotype specimens and osteological details, incomplete morphometric and meristic information in the original description have posed severe constraints to its taxonomy.

Therefore, the objective of this research was to analyze the morphological, osteological, and molecular taxonomic characteristics of dark mahseer collected from the central Himalayan region of northern India. The taxonomic classification of this species was determined using a combination of morphometric and meristic characters, alongside osteological analysis of gill rakers, pharyngeal bones, and other body bones.

Molecular taxonomy was also carried out, utilizing mitochondrial genes, including cytochrome c oxidase subunit 1 (*cox1*), cytochrome b (*cytb*) and ATPase subunit 6 and 8 (*ATP6 & ATP8*). These mitochondrial gene serve as common molecular markers for identifying fish species. Their selection for DNA barcoding studies is due to their conserved nature across diverse fish species, ensuring reliable identification and comparison. The *cox1* gene, acts as a prevalent DNA barcode marker in fish and other organisms. It possesses conserved regions for reliable species identification, alongside variable sequences. Similarily, the *cytb* gene serves as another frequently used mitochondrial marker, offering valuable insights into species identification and phylogenetic studies due to its moderate evolutionary rate and inter-species variation. Moreover, the *ATPase 6&8* gene, constituting the mitochondrial ATP synthase complex, are employed in combination as molecular markers to examine genetic diversity and phylogenetic relationships among fish species. Analyzing these three gene sequences allows to compare them with established database, enabling species identification and genetic exploration.

Materials & Methods

Fish Sampling and Voucher Specimen Preservation

Live *N. chelynoides* specimens (15.32±3.10 g; n=42) were collected using cast net by fishermen engaged in mahseer fishing. The fish sampling was carried out at Kalsa stream (Figure 1) (29°21’99.5” N; 79°33’23.9” E; altitude 1,506 meters above mean sea level), Uttarakhand, India, in-between February 2021, and June 2022. The live fish were transported to the wet laboratory of ICAR-DCFR, Bhimtal, India within 1 to 2 h of collection. Prior to the measurement of morphometric and meristic variables, preservation of voucher specimens, and collection of fin clippings for molecular identification, all the sampled fish were euthanized with 180 mg/L of tricaine methane sulphonate (MS-222, HiMedia, India).

Among 42 *N. chelynoides* specimens, 5 fish were randomly selected for voucher specimen preservation. These 5 specimens were identified and labelled before being fixed in 10% formalin for a period of 6 days, followed by long-term storage in 5% formalin in a sealed glass jar. The voucher specimens were deposited at the repository of ICAR-DCFR, Bhimtal, India museum for future reference and study.

Taxonomic Description of Morphometric and Meristic Variables

All the morphometric measurements and meristic counts of *N. chelynoides* were determined by following the previously described procedures (Britz *et al.,* 2019). In brief, 27 morphometric measurements were taken by an electronic digital calliper (Fisher Scientific, USA) to the nearest of 0.1 mm, and 23 meristic counts were obtained from the collected specimens under transmitted light with the aid of a Nikon SMZ18 stereozoom microscope (Tokyo, Japan). All morphometric measurements and meristic counts were recorded at least three times independently for the 42 specimens. Other recorded fish characteristics are pigmentation and coloration on the body and fins, presence of spots on the body, mouth and body shape, presence or absence of tubercles, and size and position of the eyes.

Statistical analysis tool Paleontological Statistic Software Package (PAST) version 4.04 was used for morphometric and meristic data analysis (Hammer *et al.,* 2001), and to perform the Principal Component Analysis (PCA). The morphometric and meristic data of other mahseer species used in PCA was collected by us in our previous study (unpublished data). Data of morphometric measurements are expressed as the proportion of the standard length (SL), and the subunit of the head is expressed as the proportion of head length (HL).

Molecular Characterization of Fish

Isolation of Genomic DNA

Approximately 30 to 40 mg pelvic fin clippings from each *N. chelynoides* specimen (n=42) were collected and stored individually in 75% ethanol. From these fin clippings, genomic DNA was extracted using Wizard® genomic DNA purification kit (Promega, Madison, USA), following the manufacturer’s protocol. The quantity and quality of the isolated genomic DNA were checked by Qubit 2.0 Fluorometer (Invitrogen, Oregon, USA), and 0.8% agarose gel electrophoresis, respectively. The isolated genomic DNA was stored at –20°C till further use.

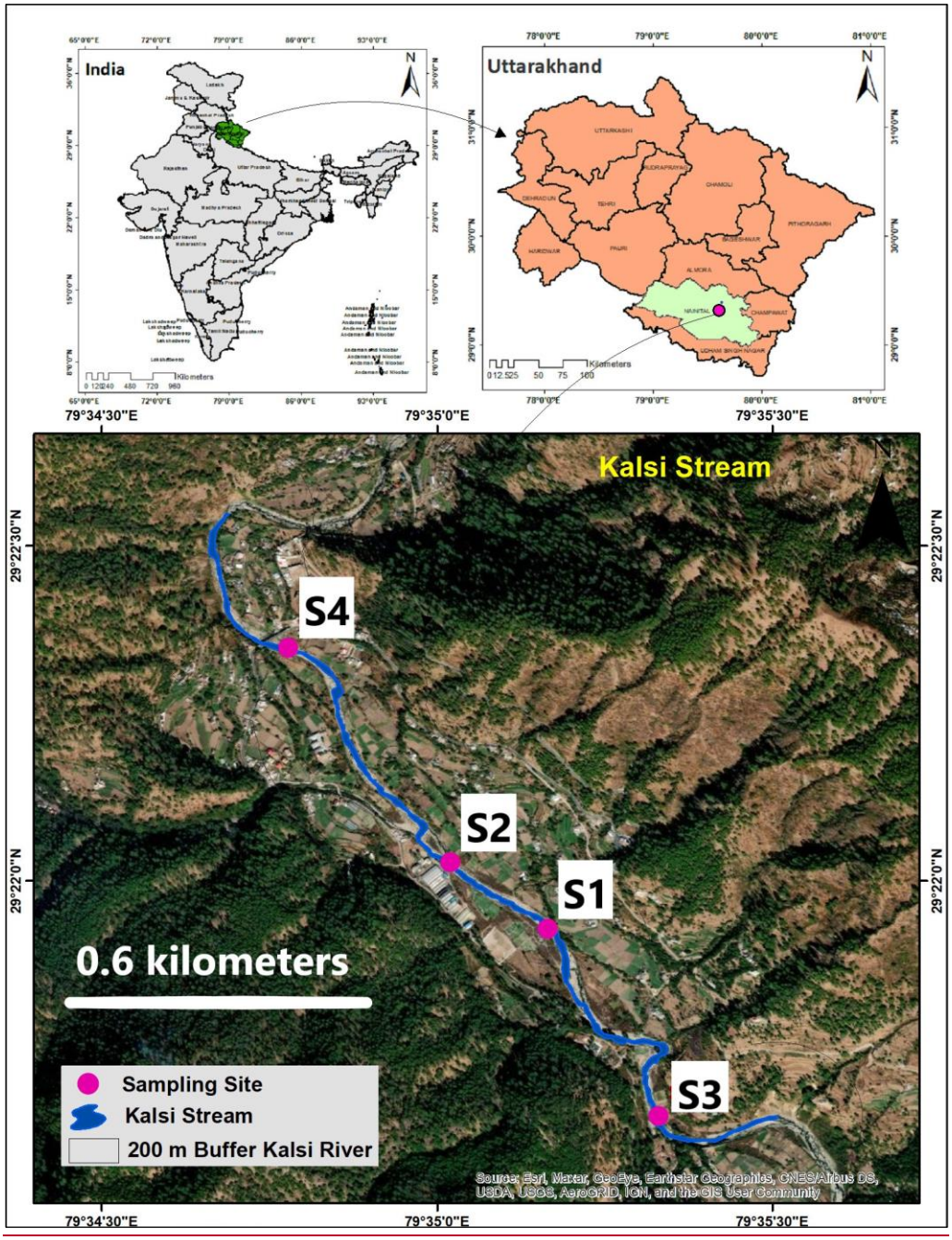

Figure. 1. Sampling site of dark mahseer, *N. chelynoides* at Kalsa stream of Himalayan region of northern India. The dots along the blue line indicate the sampling site located in the stream.

Mitochondrial Gene Amplification

All the chemicals used for mitochondrial gene amplification were purchased from Fermentas (Massachusetts, USA), unless otherwise mentioned.

Lyophilized, desalted primers used for mitochondrial gene amplification were purchased from Integrated DNA Technologies (IDT, Iowa, USA). Lyophilized Primers were resuspended (40 pmol/µL) in DNA rehydration solution (Promega, Madison, USA), and stored as 50 µL aliquots at –20°C. PCR amplification of the *cox1* gene was carried out with the set of previously published FishF1 and FishR1 primers (Ward *et al.,* 2005). The *cytb* gene was PCR amplified with L14724 and H15915 primers (Xiao *et al.,* 2001), whereas *ATPase 6&8* genes were amplified with ATP8.2 L8331 and COIII.2H9236 primers (Sivasundar *et al.,* 2001). Nucleotide sequence of the primers used in this study and the profile of the amplification cycle of each mitochondrial gene is given in Supplementary Table S1 and Supplementary Table S2, respectively.

PCR amplification of mitochondrial genes was performed using 96 well veriti thermal cycler (Applied Biosystems, Massachusetts, USA) in 50 µL reaction volume, consisting of 1x *Taq* buffer with KCl (-$MgCl_2$), 2.5 mM $MgCl_2$, 40 pmol of forward and reverse primers each, 0.25 mM of each dNTPs, 0.25 U *Taq* DNA Polymerase (recombinant), and 40 to 50 ng of genomic DNA. The PCR amplified fragments of 655 bp (*cox1*), 1121 bp (*cytb*) and 842 bp (*ATPase* 6&8)

(Supplementary Figure S1) were gel purified by MinElute gel extraction kit (Qiagen, Hilden, Germany), following the manufacturer's instruction. Gel purified fragments were bi-directionally Sanger sequenced at AgriGenom Laboratories Private Limited, Kochi, India.

Bioinformatic Analysis of Nucleotide Sequence

Forward and reverse nucleotide sequences of *cox1*, *cytb*, and *ATPase6&8* were assembled using QIAGEN CLC genomic workbench version 11.01 (https://digitalinsights.qiagen.com/), and alignment was done using the MUSCLE algorithm with Maximum Likelihood Tree with 500 replicates. Nucleotide sequences were Basic Local Alignment Search Tool (BLAST) searched at National Centre for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov) for sequence similarity. Assembled sequences were submitted to GenBank of NCBI, and accession number was obtained. The accession number of *N. chelynoides* is given in Supplementary Table S3.

For conducting statistical analysis of molecular evolution using mitochondrial genes and constructing the phylogenetic tree, the Molecular Evolutionary Genetics Analysis (MEGAX) tool was used (Kumar *et al.,* 2018). Phylogenetic analysis was carried out by the maximum likelihood method, and the kimura-2-parameter with 500 bootstrap replications using MEGAX. The analysis and phylogenetic tree were made individually for each independent *cox1*, *cytb* and *ATPase 6&8* dataset. For phylogenetic tree the nucleotide sequences of three genes (*cox1*, *cytb* and *ATPase* 6&8) of other mahseer species were obtained from GenBank of NCBI.

Genetic distance between *N. chelynoides*, *Tor* spp, and other fish taxa was determined using assembled *cox1*, *cytb*, and *ATPase 6&8* nucleotide sequences. These nucleotide sequences were analyzed in MEGAX tool (Kumar *et al.,* 2018). Genetic distance was computed by estimating the number of nucleotide substitution per site between two sequences of each gene, and analysis were conducted by the Maximum Composite Likelihood model. The nucleotide composition of this species was determined by the MEGAX tool.

Osteological Examination

Gill Arch

After removing the operculum of *N. chelynoides* (n=6), the first-gill arch from the left side of each randomly chosen fish was dissected out intact and washed immediately in distilled water to remove the blood and other adhered tissue. Subsequently, the gill was immersed in 70% ethanol + 3% Alizarin red for 2 to 3 h, and then stained gill was re-washed in 1% KOH for 20 min. Gill arch and gill rakers were examined under transmitted light of Nikon SMZ18 stereozoom microscope (Tokyo, Japan) for the number of gill rakers in an anterior and posterior row, inter-raker space, length of the gill raker, shape of gill arch and rakers, and other gill arch characteristics. Photographs of the gill were taken by the camera mounted on the microscope and by using the imaging software NIS-Elements D version 5.01 (NIKON, Japan).

Pharyngeal Bone

After removing the operculum, the pharyngeal bones were carefully dissected out from the same 6 fish, from which gill arch was collected. Adhered tissue and other materials were removed carefully, and the pharyngeal bones were immersed in 70% ethanol + 3% Alizarin red for 24 h. After staining, the pharyngeal bone was washed in 1% KOH for 2 h. The number of the pharyngeal teeth, morphology, and the size of the teeth were recorded for each examined specimen. Pharyngeal bones were observed under Nikon SMZ18 stereozoom microscope (Tokyo, Japan) and the photographs were taken using the camera mounted on the microscope, and the imaging software NIS-Elements D version 5.01 (NIKON, Japan).

Vertebral and Skull Bone

The *N. chelynoides* (n=06) were fixed in 10% neutral buffered formalin for 24 h. From fixed fish, viscera were removed, and the fish was dipped in 1% KOH solution for 1 week. Subsequently, 1% KOH was replaced with fresh stock of 1% KOH, after every 3rd day. The muscle was taken out gently to avoid damage to bones and ribs. Buffer solution containing 35 mL of saturated sodium borate, 65 mL of distilled water, and 1% of trypsin was used to remove the fish tissue by incubating the fish at 37^{o}C. Fish was incubated in this solution till the tissue was completely digested. Bones were stained with 1% Alizarin red for 3 days, and the stained fish skeleton was stored in 100% glycerol with thymol. Vertebral count in this study includes the 1st four vertebrae from the Weberian apparatus, and vertebrae of the abdominal region were counted from the 1st four vertebrae of the Weberian apparatus to the last vertebrae having pleural rib. Caudal vertebrae are counted from the vertebrae immediately after the anal fin pterygiophore.

Results

Voucher Specimen

Voucher specimens of *N. chelynoides* were deposited at the museum of the ICAR-DCFR. The details are as follows: ICAR-DCFR museum identification number; DCFRMU 0129, 89.32 mm SL, Male, India, Uttarakhand, Nainital district, Kalsa stream, Chafi village, N 29^{o}21.995' and E 79^{o}33.239'; 1,506 m above mean sea level. Collected by Neetu Shahi, Bhupendra

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DCFRMU 0130, 108.4 mm SL, Male; DCFRMU 0131, 78.4 mm SL, Male; DCFRMU 0132, 112.1 mm SL, Female, and DCFRMU 0133, 89.2 mm SL, Male, India, Uttarakhand, Nainital district, Kalsa stream near Chafi village, N 29°21.985' and E 79°33.249'; 1,503 m above mean sea level. Collected by Neetu Shahi, Bhupendra Singh, Debajit Sarma, Krishna Kala, and Sumanta Kumar Mallik. Date of collection 01/06/2021.

Distinct Taxonomic Characteristics

The Figure 2A to Figure 2C depicts the overall external appearance of freshly euthanized, unfixed *N. chelynoides* specimen (DCFRMU 0129, 89.32 mm SL), while Figure 2D to F portrays the external appearance of *N. chelynoides* (DCFRMU 0133, 89.20 mm SL) fixed in formalin.

N. chelynoides exhibits distinguishing features, such as a smaller lateral line scale ranging from 33 to 34, the absence of a median lobe in the lower lip, and thin upper and lower lips that are not hypertrophied. In contrast, the *Tor* species possess larger lateral line scales, varying lengths of median lobes, and hypertrophied lower lips, which was not observed in *N. chelynoides*.

Compared to other species within the genus *Neolissochilus*, *N. chelynoides* is characterized by a set of morphological traits that distinguish it from its

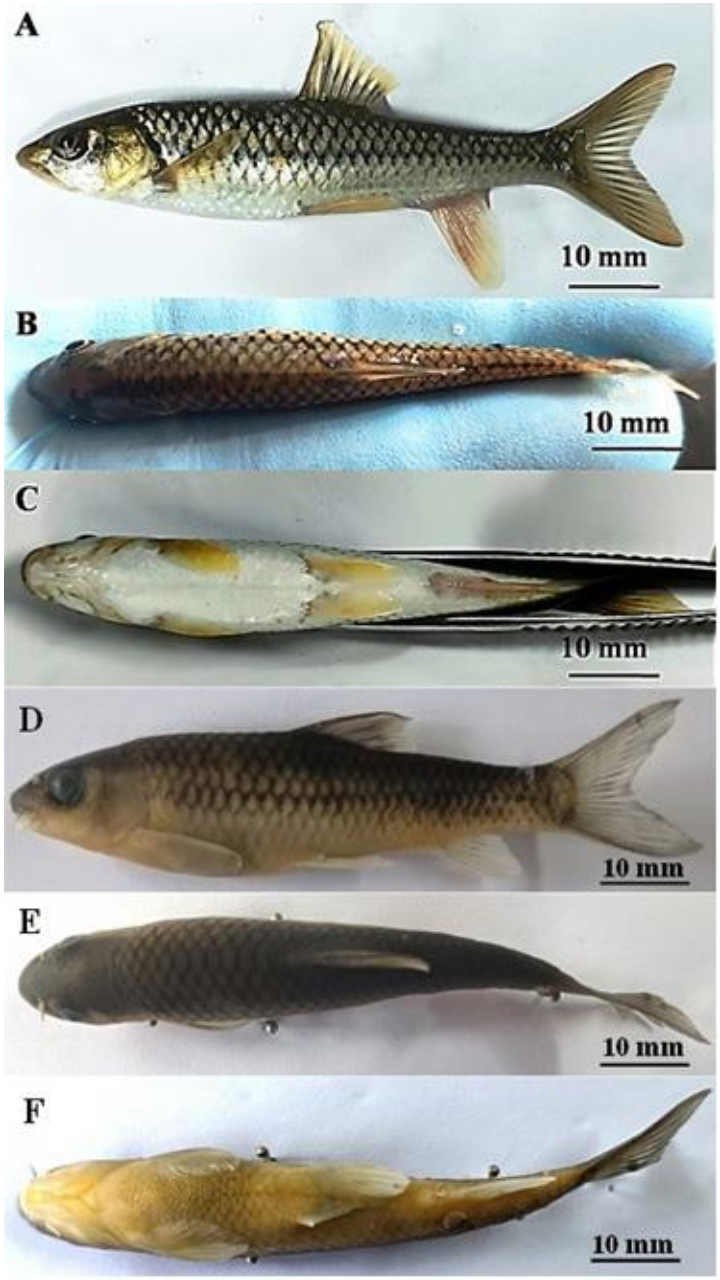

Figure. 2. Image of the samples of dark mahseer, *N. chelynoides* collected from Kalsa stream of Uttarakhand, India. (A) Lateral (B) dorsal and (C) ventral view of the live specimen of *N. chelynoides* (DCFRMU 0129, 89.32 mm SL). (D) Lateral, (E) dorsal and (F) ventral view of the formalin fixed *N. chelynoides* specimen (DCFRMU 0133, 89.2 mm SL). Scale bar is shown at the bottom right side of the images.

congeners. Specifically, *N. chelynoides* exhibits a smaller, pointed head in contrast to the broad head with a blunt snout typically observed in *Neolissochilus* species. Additionally, *N. chelynoides* possesses narrower infraorbital bones and lacks the numerous tubercles found on the cheeks of *Neolissochilus* species. The lips of *N. chelynoides* are thin and non-hypertrophied, and lack the rostral flap on the upper lip, often present in *Tor* and *Neolissochilus*. Furthermore, *N. chelynoides* has smaller and lighter scales compared to other species with large and heavy scales. Lastly, *N. chelynoides* is distinguished by a unique lateral line scale count of 33 to 34, which differs from the count of 20 to 29 seen in other mahseer species.

Description of *N. chelynoides*

The body of the *N. chelynoides* is elongated and subcylindrical, exhibiting equally convex dorsal and ventral profiles. The dorsal fin base slopes slightly at the posterior part of the ventral side, and the standard length is 4.2 to 4.6 times the body depth and 3.3 to 3.6 times the head length. The profile from the posterior end of the dorsal fin base to the caudal fin base slopes slightly posteriorly. The ventral profile of the body is convex from the tip of the snout to the pelvic fin origin and slightly compressed ventrally from the pelvic fin to the origin of the caudal peduncle. The head is moderately large and slightly depressed near the snout at the interorbital space. The snout is rounded dorsally and ventrally and appears pointed in lateral view. Neither snout nor nostrils exhibit any tubercles, rostral cap, or rostral lobe. Head length is slightly greater than or equal to body depth. The eyes are dorsally placed, and visible from the dorsal view (Figure 3A) and marginally visible from the underside of the head (Figure. 3B).

In addition to the body morphology, the study also reports the presence of two pairs of barbels, with the rostral barbel located anterolaterally and dark grey in colour, and the maxillary barbel located at the corner of the mouth and pale cream in colour. The buccal cavity

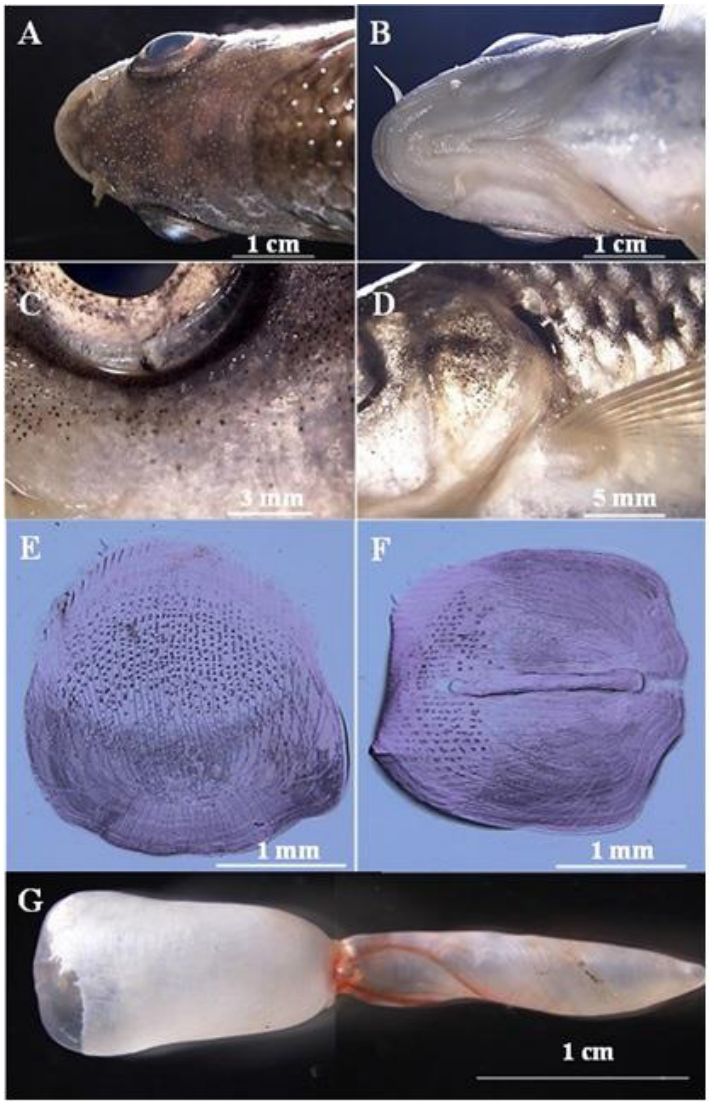

Figure. 3. Image of the various body parts of the dark mahseer, *N. chelynoides* collected from Kalsa stream of Uttarakhand, India (DCFRMU 0129, 89.32 mm SL). (A) Dorsal and (B) ventral view of the head of the freshly collected *N. chelynoides*. (C) Tubercles around the eye in male. (D) Black spot at the edge of the operculum in fish. (E) Scale from dorsal region of fish and (F) scale from lateral line region of the fish. (G) Swim bladder of *N. chelynoides* has two chambers. Scales were stained with 2% alizarin red, before examining under the stereo zoom microscope at 1x. Scale bar is shown at the bottom right side of the images.

lacks teeth, and the mouth is non-protractile. The eyes are placed slightly above on the upper region of the head, with red/orange spots in the cornea, and males have 9 to 12 pointed soft tubercles encircling the eyes (Figure 3C). The lips are thin, fleshy, and continuous at the angle of the mouth. A single black blotch is present at the angle of the operculum (Figure 3D). The edge of the scales has numerous fine black dots arranged in a crescent-shaped pattern (Figure 3E). The lateral line scale is centrally perforated, and the perforation covers more than 2/3 of the length of the scale (Figure 3F). A dark grey colour band is present all along the lateral line. The swim bladder lies deep into the body and has two chambers (Figure 3G), with the anterior chamber being bigger and broader, and the posterior chamber being tube-like with its end pointed.

Furthermore, the species showed slightly arched and complete lateral lines, relatively smaller scales except on the head, and a post-labial groove that was continuous until the angle of the mouth. The pelvic and pectoral fins were lightly reddish, with the first ray/spine of the pectoral, anal, and pelvic fin showing a light red coloration. The ventral region of the body appeared silvery, while the dorsal part of the body was dark grey.

Table 1 and Table 2 give morphometric measurements and meristic counts, respectively, of *N. chelynoides*. The dorsal fin displays 3 (42) simple rays and 8 (5) or 9 (37) branched rays, with the last branched ray equal in length to the pre-orbital length. The pectoral fin exhibits 1 (42) simple ray and 15 (5) or 16 (37) branched rays, while the pelvic fin displays 1 (42) simple ray and 8 (5) or 9 (37) branched rays. The anal fin is equal in length to the pelvic fin and has 2 (42) simple rays and 6 (5) or 7 (37) branched rays. The caudal fin is emarginated, with pointed lobes of equal length, and the 9th ray is the shortest. The 2nd unbranched ray of the dorsal fin is smooth and longest, and 3 times the length of the 1st unbranched ray. The distal margin of the dorsal fin is concave, with the origin between the snout tip and caudal fin base, and the dorsal fin is inserted just above the pelvic fin, with the 1st branched ray being the longest. The pectoral fin extends beyond the midway to the pelvic fin origin, and is shorter than the head length, while the pelvic fin does not reach the origin of the anal fin and has its first branched ray as the longest. The anal fin extends up to the base of the caudal fin, with the first branched ray being the longest and non-serrated. The first simple ray of the anal fin is half the length of the 2nd simple ray, and the posterior margin of the fin is straight, with the anal fin starting immediately after the anus.

A PCA was conducted on a set of 23 meristic counts that were plotted on a two-dimensional graph. Four distinct clusters were observed that corresponded to different taxa, with *N. chelynoides* forming a separate cluster (Figure 4).

Table 1. Morphometric measurements of *Naziritor chelynoides* (n = 42) collected from Kalsa stream of Uttarakhand, India.

Morphometric characters	***Naziritor chelynoides***			
	Paratype (≤ 80.0 mm in SL, n=36)		Paratype (≥ 80.0 mm in SL, n=6)	
	Average ± S. D*	Range	Average ± S. D	Range
Standard length (SL, mm)	67.70 ± 7.74	79 – 49	85.5 ± 3.06	89 – 82
Head length (HL, mm)	17.70 ± 2.78	23 – 12	23 ± 1.20	24 – 21
% SL				
Head length	26.07 ± 2.13	30.13 – 21.31	26.90 ± 1.38	28.91 – 25.28
Body depth	23.75 ± 2.16	28.76 – 17.72	22.69 ± 2.56	26.86 – 19.1
Pre – dorsal length	50.33 ± 2.27	54.09 – 43.66	49.70 ± 1.22	51.8 – 48.27
Dorsal fin length	20.69 ± 2.26	25 – 16.66	17.17 ± 1.20	18.29 – 14.94
Anal fin length	18.03 ± 1.76	21.56 – 14.47	16.87 ± 1.38	18.42 – 14.94
Pectoral fin length	18.08 ± 1.63	20.4 – 14.75	18.47 ± 1.33	20.48 – 16.62
Ventral fin length	17.39 ± 1.24	19.7 – 15.38	15.90 ± 1.23	17.07 – 13.48
Dorsal – fin base length	10.77 ± 1.17	11.94 – 8.16	14.68 ± 5.57	26.5 – 11.23
Anal–fin base length	4.68 ± 1.11	18.13 – 1.66	6.82 ± 1.27	8.55 – 4.87
Pectoral – fin length	18.13 ± 1.66	20.4 – 14.75	18.55 ± 1.26	20.48 – 16.62
Ventral fin base	3.79 ± 0.72	4.47 – 2.81	4.24 ± 1.24	6.74 – 3.37
Caudal – peduncle length	27.57 ± 4.24	38.15 – 22.85	32.94 ± 8.56	46.38 – 20.73
Eye diameter	6.39 ± 0.69	7.84 – 5.00	5.66 ± 0.22	6.09 – 5.42
Preorbital length	7.96 ± 1.58	11.39 – 5.63	8.59 ± 2.41	13.25 – 5.74
Postorbital length	11.49 ± 1.65	14.75 – 7.59	12.48 ± 0.97	13.48 – 11.23
Interorbital width	7.94 ± 1.30	10.95 – 5.17	9.44 ± 1.05	11.23 – 8.43
In % of head length				
Eye diameter	24.65 ± 3.01	31.57 – 20	21.04 ± 1.26	22.72 – 18.75
Preorbital length	30.44 ± 4.89	42.85 – 22.22	31.74 ± 7.67	45.83 – 22.72
Postorbital length	43.72 ± 6.46	53.84 – 28.57	46.39 ± 2.70	47.82 – 41.66
Interorbital width	30.37 ± 3.49	36.36 – 21.42	35.19 ± 4.99	41.66 – 29.16
Ratios				
SL/HL	3.85 ± 0.32	4.69 – 3.31	3.71 ± 0.19	3.95 – 3.45
SL/body depth	4.23 ± 0.41	5.64 – 3.47	4.32 ± 0.61	5.23 – 3.56

*S.D is standard deviation.

Table 2. Meristic counts of *Naziritor chelynoides* (n = 42) collected from Kalsa stream of Uttarakhand, India. When viewed from the lateral orientation "½ scale" indicates that the visible scale is half of the full scale.

Meristic characters	**DCFRMU voucher specimens (n = 05)**	**Other samples (n = 37)**
Unbranched dorsal – fin rays	3	3
Branched dorsal – fin rays	8	9
Unbranched pectoral – fin rays	1	1
Branched pectoral – fin rays	15	16
Unbranched Pelvic – fin rays	1	1
Branched Pelvic – fin rays	8	9
Unbranched anal – fin rays	2	2
Branched anal – fin rays	6	7
Caudal – fin rays (procurrent + principal)	30 – 31	30 – 32
Caudal – fin upper lobe	15 – 18	15 – 18
Caudal – fin lower lobe	13 – 14	13 – 15
Caudal – fin rays (procurrent)	13 – 15	11 – 17
Caudal fin rays (principal)	16 – 17	16 – 19
Circumpeduncular scales	12	12
Lateral line scales	30 ½ – 32 ½	31 ½ – 32 ½
Pre – dorsal scales	13 ½ – 14 ½	13 ½ – 14 ½
Post – dorsal scales	16 – 18	15 – 18
Pre – pelvic scales	9 ½ – 10 ½	8 ½ – 10 ½
Post – pelvic scales	21 – 23	21 – 23
Pre – anal scales	17 – 18	16 – 18
Post – anal scales	10 – 11	10 – 12
Scales above the lateral line	5 ½	5 ½
Scales below the lateral line	3 ½	3 ½

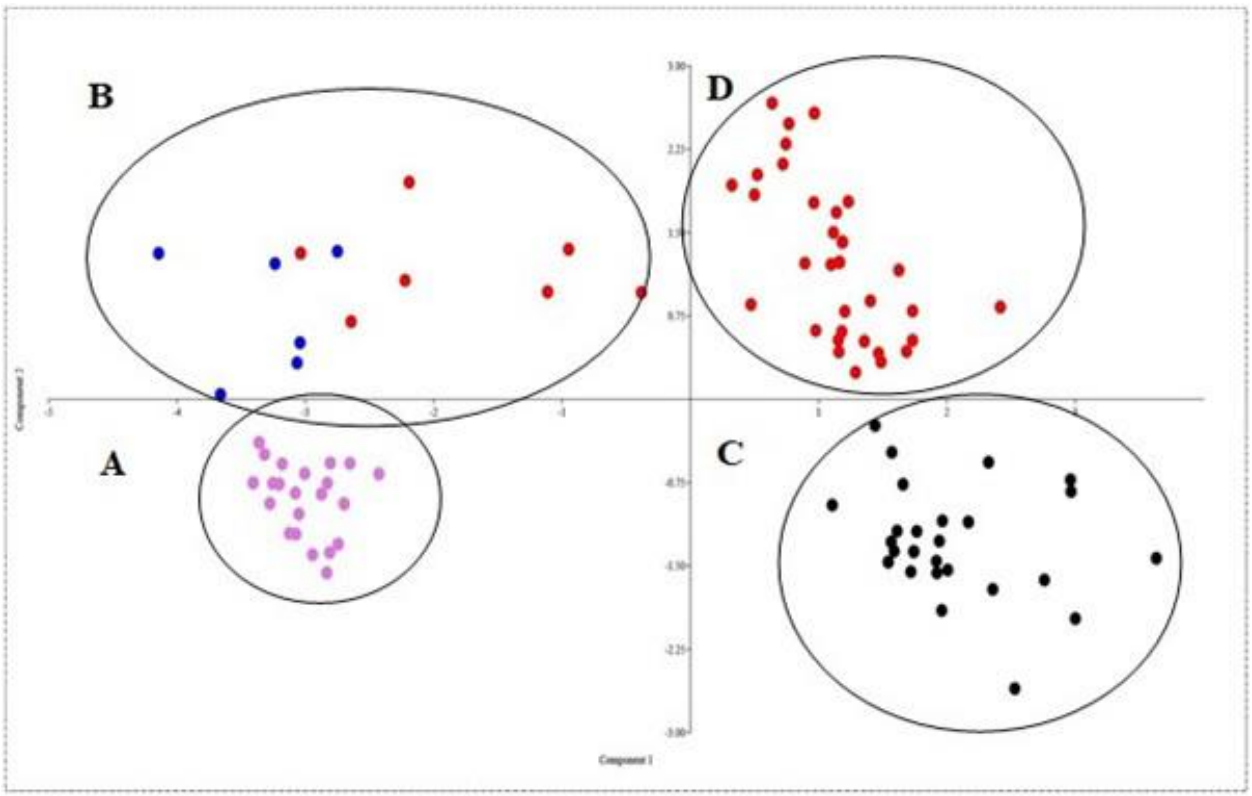

Figure. 4. Principal component analysis (PCA) of 23 meristic parameters of *N. chelynoides* samples (n = 42). In the image the cluster (A) is *N. chelynoides*, (B) is *Tor spp.*, (C) is *Tor putitora*, and (D) is *Tor tor* characterized in this study from central Himalayan region of northern India. Component 1 and component 2 were loaded on the axis 1 and axis 2, respectively for PCA analysis.

Body Coloration

Live *N. chelynoides* specimens exhibit a dark grey dorsal coloration with a silvery white ventral region and a metallic sheen. Examined specimens displayed pale red fins that turned pale yellow in the pectoral, pelvic, and anal fins a few hours post-mortem, while the dorsal and caudal fins remained light grey. Formalin-fixed specimens (Figure 2D, E and F) displayed a medium grey colour on the dorsal and lateral regions above the lateral line with a dark grey band marking the lateral line, and a pale beige ventral part below the lateral line. The head was medium grey on the dorsal and lateral sides and had a beige shade on the ventral side. The eyes were dark grey, and the caudal and dorsal fins were grey with a beige line. The anal, ventral, and pectoral fins were light grey with a light beige line, and both barbels were pale cream.

Molecular Analysis

The partial nucleotide sequences of *cox1*, *cytb*, and *ATPase 6&8* genes of *N. chelynoides* in this study formed a distinct clade from the other fish taxa (Figure 5A, B, and C). The nucleotide sequence similarities among the *N. chelynoides* samples ranged in-between 99.1 to 100% for these three genes. Separate clusters were observed for *Puntius* species, *Tor tor*, *T. putitora*, *T. barake*, *T. remadevii*, *T. khudree*, and *T. mosal* on the phylogenetic trees, with *Puntius* spp. forming a distinct clade.

The resulting genetic distances between *N. chelynoides* and other *Tor* species, based on partial gene sequences of *cox1*, *cytb*, and *ATPase 6&8*, ranged in-between 0.08 to 0.10, 0.12 to 0.13, and 0.01 to 0.11, respectively (Table 3). The genetic distance between *N. chelynoides* and *Puntius* spp. based on *cox1*, *cytb*, and *ATPase 6&8* partial gene sequences ranged from 0.17 to 0.23, 0.13 to 0.31, and 0.19 to 0.26, respectively. The average AT frequency of *N. chelynoides* for *cox1*, *cytb*, and *ATPase 6&8* genes was 55.23±0.16%, 56.46±0.62%, and 58.60±0.83%, respectively. The average GC frequency of *N. chelynoides* for *cox1*, *cytb*, and *ATPase 6&8* genes was 44.80±0.07%, 43.43±0.56%, and 41.49±0.62%, respectively. The comparison of A, T, G and C composition of this fish with other taxa is given in Supplementary Figure S2.

Osteological Observations

Gill Rakers

The gill arch morphology of *N. chelynoides* exhibits a distinctive bow shape comprising an upper and a lower limb. The anterior row of gill rakers on the upper limb comprises 3 to 4 elongated rakers (Figure 6A) (mean length: 595.7±169.6 µm,), while the lower limb bears 7 to 8 shorter rakers (mean length: 324.2±47.5 µm). The posterior row consists of 14 to 15 pointed gill rakers (Figure 6B) (mean length: 292.3±39.45 µm) without melanin pigment. The base of both anterior and posterior gill rakers assumes a broad and triangular shape. The inter-row distance of gill rakers in this species is 307.05±29.3 µm and 323.6±27.3 µm for the anterior and posterior rows, respectively.

Pharyngeal Bones

The 5th ceratobranchial arch, which forms the inferior pharyngeal bone in *N. chelynoides*, exhibits a three-dimensional arched shape that is flattened and concave (Figure 6C). Positioned ventrolaterally to the pharyngobranchial, a toothed bony plate is evident, while the inner surface of the bone displays three rows of 9 robust teeth. The pharyngeal teeth in *N. chelynoides* are stout, with a concave apex and broader base, and exhibit a slight curvature at the tip. The lower row comprises 5 large teeth, the middle row has 3 teeth, and the upper row possesses 2 small teeth.

Vertebral Bones

N. chelynoides exhibits a total vertebrae count ranging from 40 to 41, comprising 38 to 39 neural spines and 14 to 15 hemal spines (Figure 7). The post-anal, neural, and hemal spines are uniformly curved towards the caudal peduncle, and exhibit nearly equal lengths. This fish had 23 abdominal and 17 caudal vertebrae. The eyes of *N. chelynoides* are encompassed by 4 suborbital bones and 1 lacrimal bone, interconnected via the infraorbital sensory canal (Figure 7A).

The dorsal and ventral structures of the *N. chelynoides* skull (excluding premaxillaries, maxillaries, and suborbital series) are described (Figure 7B and C). The pre-vomer is positioned on the ventral surface, immediately ahead of the anterior end of the para-shepnoid, and beneath the ethmoid and supraethmoid. The posterior region of the skull consists of 3 bones in the dorsal part, comprising 2 lateral epiotic bones, and a middle supraoccipital bone that exhibits distinct supraoccipital and epiotic spines. The ventral part of this posterior region transitions into the basioccipital region, which includes the pharyngeal plate.

The 1st four abdominal vertebrae of *N. chelynoides* are fused to form the Weberian chain, with the 1st centrum being flat in the anterior region. The 2nd and 3rd vertebrae are fused to form visually larger amphicoelous centrum complexes. Above these complexes are 3 neural arches with the fused 2nd and 3rd neural spines. The 4th fully developed vertebra is visible after the fused centrum complex, comprising proper neural and pleural spines at dorsal and ventral positions, respectively. Additionally, an interneural bone was observed between the neural spines (Figure 7D).

In *N. chelynoides*, the final centrum of the caudal bone is fused with preural 1 and ural 1, resulting in a compound centrum that has a concave shape towards the anterior (Figure 7E). A rudimentary neural arch extends dorsally from the compound centrum to more

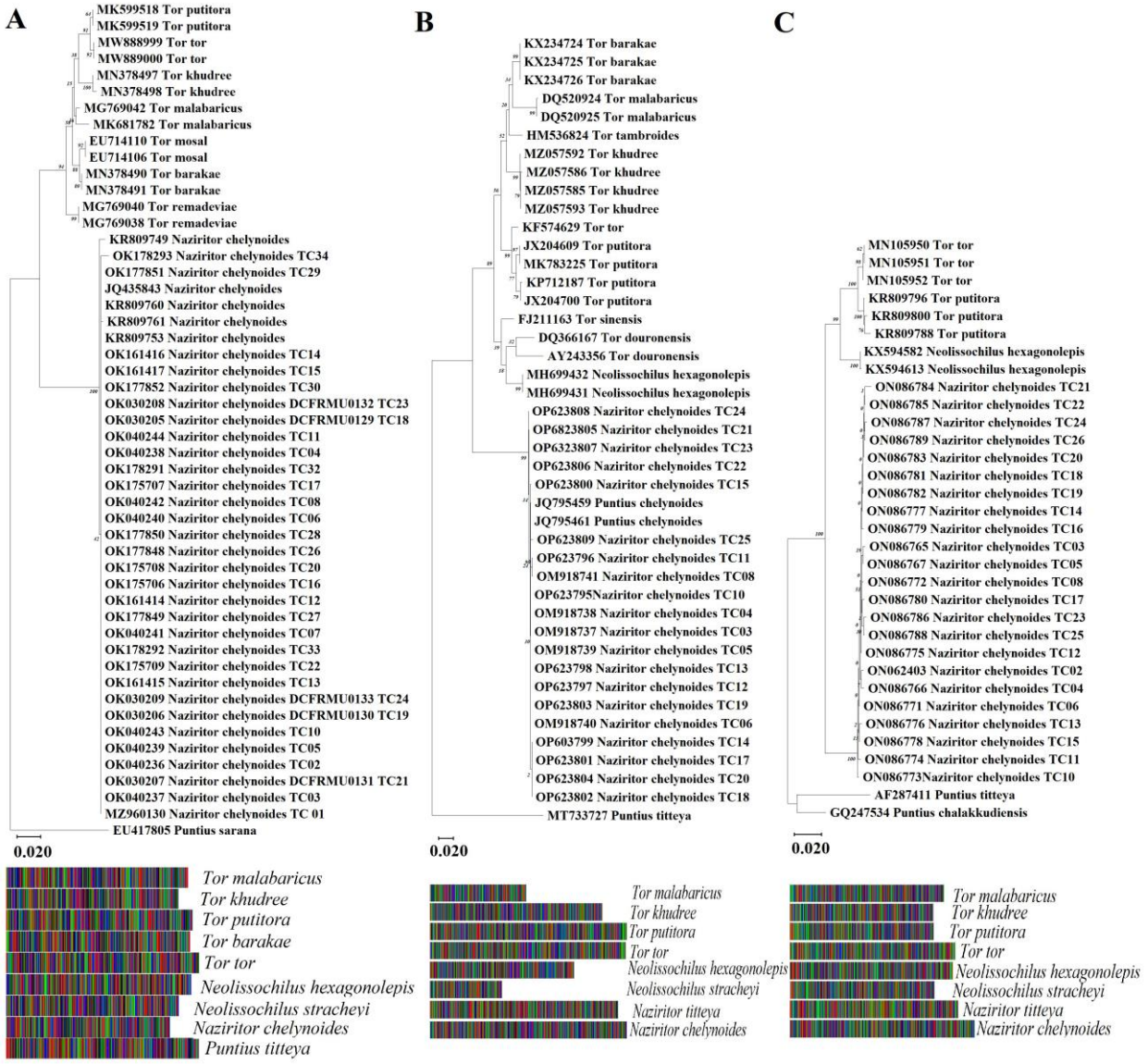

Figure. 5. The evolutionary history based on nucleotide sequence of *cox1* (A), *cytb* (B) and *ATPase 6&8* (C) was inferred using the Maximum Likelihood method, and Kimura 2 parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. DNA barcode for each gene is shown below the phylogenetic tree. These DNA barcodes are generated from the sequence data of nucleotides of the *cox1*, *cytb*, and *ATPase 6&8* genes. Each line of barcode represents a particular base pair: green = adenine, blue = cytosine, black = guanine, red = thymine. The arrangement of these bases is unique to its species and genus level.

Table 3. Genetic distance between *Naziritor chelynoides* and other fish taxa based on *cox1, cytb* and *ATPase 6&8* partial gene sequence. The number of base substitutions per site from between sequences are shown. Analysis was conducted using the Maximum Composite Likelihood model. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

***cox1* gene**	***N. chelynoides***	***cytb* gene**	***N. chelynoides***	***ATPase 6*&8 gene**	***N. chelynoides***
Naziritor chelynoides	0.000	*Naziritor chelynoides*	0.000	*Naziritor chelynoides*	0.000
Tor barakae	0.103	*Tor putitora*	0.121	*Neolissochilus hexagonolepis*	0.112
Tor khudree	0.101	*Tor tor*	0.125	*Tor tor*	0.115
Tor malabaricus	0.094	*Tor sinensis*	0.129	*Tor putitora*	0.157
Tor mosal	0.102	*Tor khudree*	0.134	*Tor khudree*	0.116
Tor remadeviae	0.087	*Tor barakae*	0.139	*Tor douronensis*	0.131
Tor tor	0.093	*Tor tambroides*	0.127	*Puntius titteya*	0.266
Tor putitora	0.087	*Neolissochilus hexagonolepis*	0.134	*Puntius chalakkudiensis*	0.198
Puntius sarana	0.174			*Puntius conchonius*	0.219
Puntius titteya	0.23				
Puntius fraseri	0.213				
Puntius terio	0.191				
Puntius sophore	0.209				
Puntius schanicus	0.172				

than half the length of the adjacent neural spine of the preural 2 centrum. The compound centrum is semi-radially differentiated at the lateral position into pleurostyle, uroneural, and hypural plate (5 to 1) in a clockwise position, with a distinct gap on either side of the origin of the hyplural plate bone 1, known as the hypural foramen and parhypural foramen. Osteological details for the vertebral bone, complex vertebrae, mesethmoid, pre-ethmoid, vomer, and caudal fin bone are presented in Table 4.

Habitat Mapping

N. chelynoides occupies the pristine, shallow (0.5 to 2.5 feet), fast-flowing perennial streams situated in the mid-altitude Himalayan region of central India. These streams also have intermittent pools with depths ranging from 0.75 to 8.9 feet, and their substrate comprises bedrock, pebbles, sand, silt, and boulders (Figure 8A). The stream banks are characterized by sandy areas with rocks and gravels. The mapping of *N. chelynoides* habitat within a 2 km buffer zone indicated that the Kalsa stream is surrounded by vegetation, including forest cover and agricultural land (Figure 8B). Oxygen-rich, slightly alkaline water, and vegetation-covered areas are preferred by *N. chelynoides*. Other fish species that cohabit with *N. chelynoides* in this stream include *Schizothorax richardsonii* (10% of total catch), *Schizothorax* spp. (14% of total catch), *Barilius bendelisis* (11% of total catch), *Barilius* spp. (17% of total catch), *Garra* sp. (13% of total catch), and *Schistura* spp. (8% of total catch).

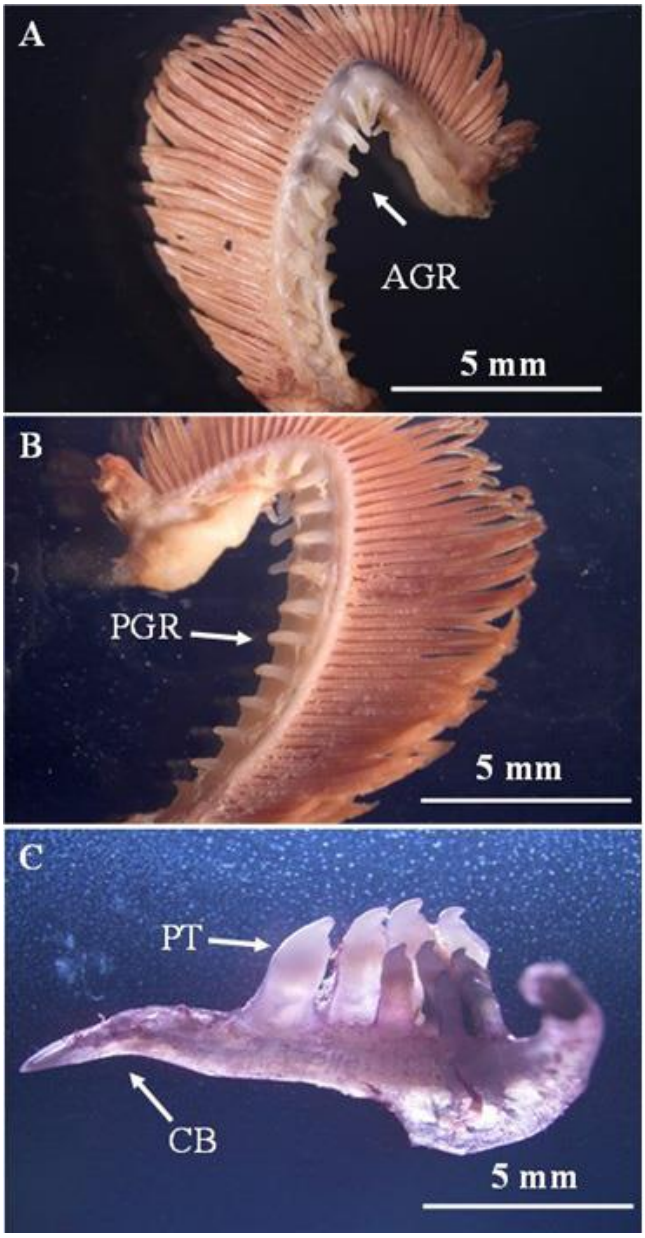

Figure. 6. Photomicrograph of the gill and the pharyngeal bone of the dark mahseer, *N. chelynoides*. (A) Anterior gill rakers (AGR) in the first gill arch of fish. (B) Posterior gill rakers (PGR) in the first gill arch of fish. (C) Posterior view of the unstained pharyngeal bone teeth of the fish. Gill rakers and pharyngeal bone were fixed in 70% ethanol, and pictures were taken under bright field of the stereozoom microscope. Scale bar is shown at bottom right side of each image.

Discussion

The *Naziritor* population in India is under the vulnerable category due to anthropogenic activities, including the construction of dams and barrages leading to habitat modification or destruction, unregulated and destructive fishing methods such as bleaching powder and electric fishing, and unpredictable changes in aquatic habitats caused by climate change. The taxonomy of *Naziritor*, despite its ecological significance, is frequently conflated with the genera *Tor* and *Puntius*. Consequently, formulating a conservation strategy for *Naziritor* is considerably hampered by inadequate information concerning its taxonomy, distribution patterns, and habitat preferences.

To date, two species have been reported within the genus *Naziritor*. *Naziritor zhobensis*, also known as the Zhob mahseer, is an endemic species found in the western region of Pakistan, with the river Zhob as its original reported location (Yousaf *et al.,* 2021). This species was initially identified as *Tor zhobensis* from the Zhob River by Mirza (1967), and was later reclassified under the new genus *Naziritor*. Another species, *N. chelynoides*, also known as the dark mahseer, is an endemic species found in the Ganges River basin of northern India (Khare *et al.,* 2014). This species holds cultural and local significance in several regions of the northern central Himalayas of India (Dahanukar, 2010; Froese and Pauly, 2013). However, *N. chelynoides* is currently recognized as *Puntius chelynoides* (Dahanukar, 2010; Froese and Pauly, 2013). Nonetheless, some researchers, such as Tilak and Baloni (1984), considered *N. chelynoides* as a valid mahseer species.

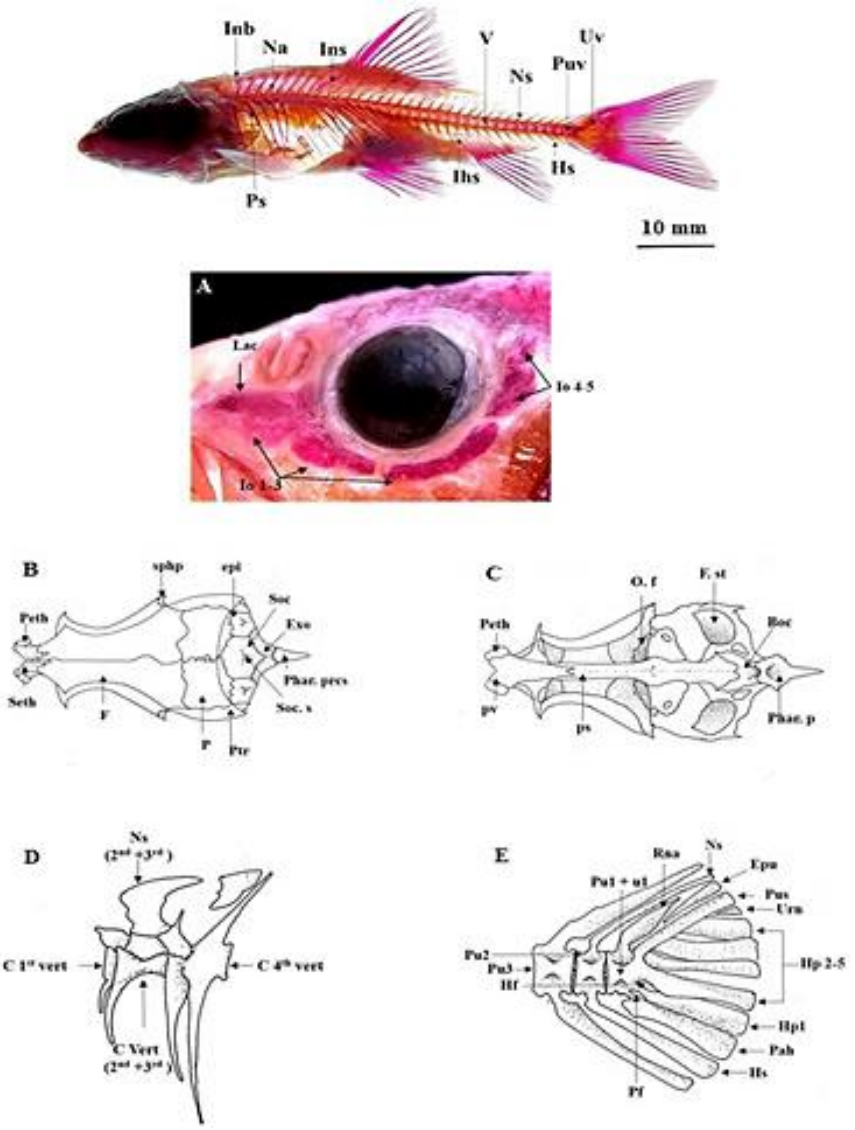

Figure. 7. Osteological details of dark mahseer, *N. chelynoides*. Lateral view of the cleared and alizarin red stained skeleton of *N. chelynoides*. (A) Lateral aspects of the suborbital bones of fish. The bone was examined after staining the fish. (B) Skull, dorsal view (line diagram). (C) Skull, ventral view (line diagram). (D) Weberian apparatus, lateral view (line diagram). (E) Caudal skeletan, lateral view (line diagram). Abbrevations: Inb-inter neural bone; Na-Neural arch; Ins-inter neural spine; V-Vertebrae; Ns-neural spine; Puv- pre ural vertebrae; Uv-ural vertebrae;; Ihs- Inter heamal spine; Ps-pleural spine; Lac-lachrymal; Io- Infra orbital bone; Peth-Pre ethmoid; Seth-Supra ethmoid; F-Frontal; Sphp-Sphenotic; P-Parietal; Ptr-Pterotic; Soc.s- Supraoccipital spine; Soc-Supraoccipital; Exo-Exoccipatal; Epi-Epiotic; Phar. Prcs-Pharayngeal process; Pv-Pre vomer; Ps-Parasphenoid; O. f-Optic foreman; F.St-subtemporal foramen; Boc-Basioccipital;and Phar. P-Pharyngeal plate; C-Centrum; Int n b-Interneural bone; Vert-Vertebrae; Ns-Neural spine; Na-Neural arch; and Ps-Pleural spine; Epu-epural; Hf-hypural foramen; Hp (1 – 5)-hypural plates (1 – 5); Hs-hemal spine; Ns-neural spine; Pah-parhypural; Pf-parhypural foramen; Pu-preurals; Pu1 + u1- compound centrum; Pus-pleurostyle; Rna-rudimentary neural arch; and Urn-uroneural.

Table 4. Comparative osteological descriptions of genus *Tor*, *Neolissochilus* and *Naziritor*.

Characters/Genus		***Tor***	***Neolissochilus***	***Naziritor***
Mesethmoid		Broad and longitudinal median depression on the dorsal surface from anterior frontal up to the tip of the ethmoid	Broad and flat dorsal surface	Narrow and longitudinal median depression on the dorsal surface from the anterior frontal up to the tip of the ethmoid.
Pre – ethmoid		Large, irregular hemispherical bone.	Small, irregular hemispherical bone.	Large, irregular hemispherical bone.
Vomer		Long, narrow, and posteriorly extends beyond the posterior edge of the lateral ethmoid.	Short, broad, and narrows towards the posterior end up to a little behind the lateral ethmoid.	Long, narrow, and posteriorly extends beyond the posterior edge of the lateral ethmoid.
Frontal	Length in % neurocranium length	67.61 %	60.07 %	70.00 %
	Breadth in % neurocranium breadth	53.08 %	50.51 %	82.00 %
Parietal	Length in % neurocranium length	11.91 %	27.10 %	16.60 %
	Breadth in % neurocranium breadth	77.70 %	73.70 %	82.30 %
Vertebral column		40 – 44	42 – 44	40(2) – 41(1)
Complex vertebrae		2nd and 3rd vertebrae fused Neural spine and neural arch of the 4th vertebra are fused with those of the 3rd vertebra.	2nd and 3rd vertebrae fused Neural spine of the 4th vertebra is not fused and well – developed First Interneural bone is present between the 4th neural spine and 5th neural spine	1st,2nd, 3rd, and 4th vertebrae fused but can be distinguished Neural spine of the 4th vertebra is not fused and well – developed. First Interneural bone is present between the 4th neural spine and the flattened neural complex

Characters/Genus		***Tor***	***Neolissochilus***	***Naziritor***
Caudal fin bones	PU3 & PU2 (Neural spine)	3	7	5
	Epural	4		
	Urostyle			2
	Hypural 6	1 (principal ray)	2 (includes 1 principal ray)	2 (includes 1 principal ray)
	Hypural 5	3	3	3
	Hypural 4	3	3	2
	Hypural 3	3	2	3
	Hypural 2	2	2	2
	Hypural 1	4	2	2
	Parhypural	3 (includes 1 principal ray)	4	3
	PU3& PU2 (haemal spine)	3 + 4	3 + 5 (includes 1 principal ray)	3 + 6 (includes 1 branched ray, 1 principal ray)
Procurrent rays	Dorsal	7 (4 segmented+ 3 unsegmented)	7 (3 segmented+ 4 unsegmented)	7(2 segmented + 5 unsegmented)
	Ventral	7 (4 segmented+ 3 unsegmented)	7 (3 segmented+ 4 unsegmented)	7 (2 segmented+ 5 unsegmented)

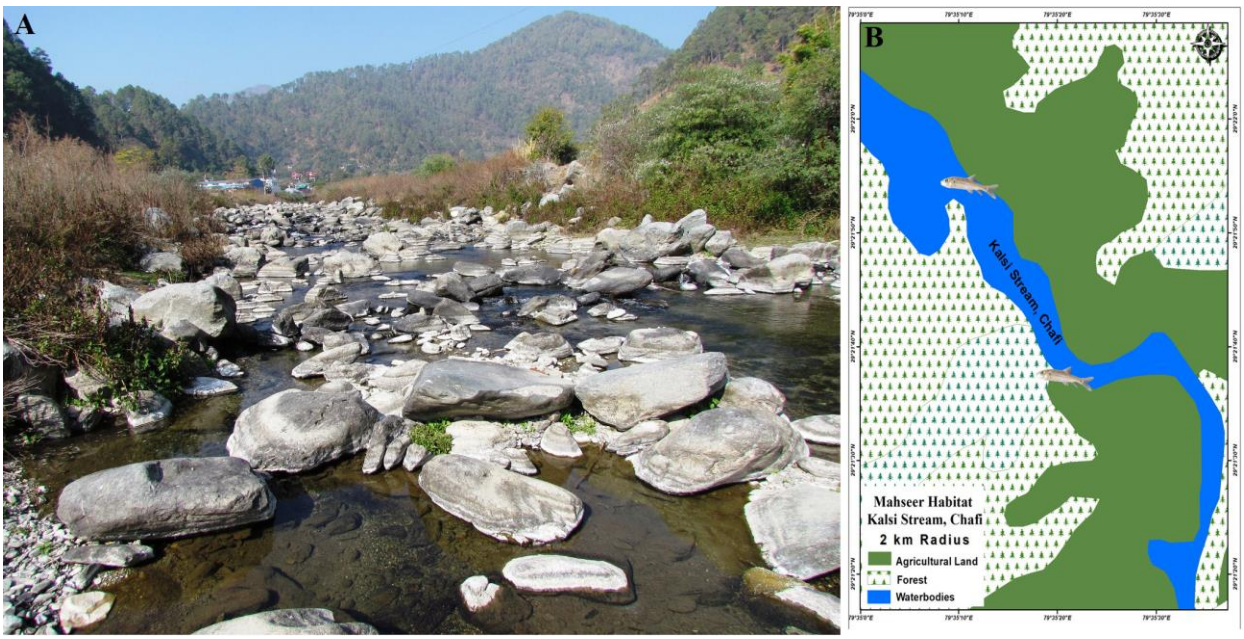

Figure. 8. Habitat of dark mahseer, *N. chelynoides.* (A) Type locality of fish at Kalsa stream, District Nainital, Uttarakhand, India. (B) Habitat mapping of fish at Kalsa stream covering 2 km buffer zone, which covers agricultural land, forest area and grass land.

The taxonomic delineation of the genus *Naziritor* is currently inadequate and lacks several pertinent details regarding osteology, classical, and molecular taxonomy. Thus, the present study aimed to address this issue by identifying and characterizing *N. chelynoides*, collected from one of its type localities, the Kalsa stream in Uttarakhand, India. The identification of *N. chelynoides* was based on its morphometric measurements, meristic counts, distinctive body features, osteological details, and mitochondrial barcoding genes: *cox1*, *cytb*, and *ATPase 6&8*.

While *N. chelynoides* and *N. zhobensis* belong to the same genus, and exhibit similarities in their morphometry in several aspects, they differ in the number of lateral line scales, with *N. chelynoides* having 33 to 34 scales compared to 32 to 37 scales in *N. zhobensis*. Moreover, *N. chelynoides* can be distinguished from *N. zhobensis* by having a greater number of caudal fin rays (including procurrent and principal rays) at 30 to 32 (compared to 19 to 21), a higher number of anal fin rays at 8 to 9 (compared to 7), 9 to 10 pelvic fin spines (compared to 9), 16 to 17 pectoral fin spines (compared to 15 to 18), and 11 to 12 dorsal fin spines (compared to 10), as well as pale red fins (compared to yellow fins). Additionally, *N. chelynoides* has a fusiform head with a pointed snout (compared to a broad, snake-like head), and its head length is equal to or greater than body depth (compared to always greater than body depth), while it has maxillary barbel is longer than the rostral barbel (compared to shorter).

A principal component analysis was performed on meristic counts of mahseer specimens collected from various water bodies in the central Himalayan region of northern India, including *N. chelynoides*. The resulting sample clusters from the PCA were identified based on meristic parameters, and other characteristics using taxonomic keys. *N. chelynoides* formed a distinct clade in the PCA analysis, which differed from those of *T. putitora*, *T. tor*, and other *Tor* species.

In the constructed phylogenetic tree based on nucleotide sequences of *cox1*, *cytb* and *ATPase 6&8*, *N. chelynoides* was observed to form a distinct cluster separated from *Tor* spp. and *Puntius* spp., confirming its separate taxonomic status. Previous studies by Khare *et al.* (2014) and Laskar *et al.* (2013) also demonstrated the differentiation of *Naziritor* genus from *Puntius*, and the validity of this species using mtDNA barcoding, respectively. Our study further supports the placement of *N. chelynoides* within the *Naziritor* genus (Talwar and Jhingran, 1991), along with *N. zhobensis*, and contradicts its classification under the genera *Tor* and *Puntius* (Dahanukar, 2010).

Over the past few years, DNA barcoding based on mitochondrial DNA has emerged as a valuable tool for accurate species-level identification, and validation of fish fauna (Laskar *et al.,* 2013; Pinder *et al.,* 2018; da Rocha *et al.,* 2021; Sudasinghe *et al.,* 2021). Our study further supports the usefulness of *cox1*, *cytb*, and *ATPase 6&8* genes as barcodes to accurately identify *N. chelynoides*. The lack of DNA barcode information for *N. chelynoides* in reference databases has hindered the development of a complete digital taxonomic guide for this species and its range distribution in India, Pakistan, Nepal, and neighbouring countries.

This study aimed to investigate the genetic divergence between *N. chelynoides* and other mahseers found in India, using mitochondrial DNA barcode markers (*cox1*, *cytb* and *ATPase 6&8*). The uncorrected pair-wise genetic divergence between *N. chelynoides* and other *Tor* species ranged from 0.08 to 0.10, 0.12 to 0.13 and 0.01 to 0.11, based on *cox1*, *cytb* and *ATPase 6&8* partial gene sequence, respectively. Similarly, the genetic distance between *N. chelynoides* and *Puntius* spp. ranged from 0.17 to 0.23, 0.13 to 0.31 and 0.19 to 0.26, based on *cox1*, *cytb* and *ATPase 6&8* partial gene sequence, respectively. Our findings confirm that *N. chelynoides* is genetically distant from other *Tor* and *Puntius* species. However, the genetic closeness between *N. chelynoides* and *N. zhobensis* was unestablished due to the non-availability of nucleotide sequences of *N. zhobensis* in public domain. Nevertheless, based on morphometry and meristic data, we consider *N. chelynoides* and *N. zhobensis* as different species under the same genus. Phenotypic plasticity and morphological dissimilarities in mahseer may occur due to environmental variations; therefore, genetic sequence information is crucial to accurately characterize any species.

The osteology of several mahseer species, including *N. chelynoides* remains poorly understood despite the morphological and taxonomic diversity within this group. To address this knowledge gap, we conducted a detailed study of the gill arch, pharyngeal teeth, and neurocranium in *N. chelynoides*. Our osteological investigation of the gill arch structure in *N. chelynoides* revealed that the number and arrangement of gill rakers were like other *Tor* and *Neolissochilus* species (Talwar and Jhingran, 1991). Cyprinid fishes, lacking true teeth in their buccal cavity, possess a prominent tooth on the 5th branchial arch, whose presence and arrangement are critical diagnostic features of the family Cyprinidae (Nakajima, 2018). The morphology of these teeth provides valuable information about the fish. Like other mahseer, *N. chelynoides* exhibits a stout tooth with 3 rows (5+3+2) of hooked teeth.

A detailed examination of vertebral bones revealed significant differences in osteological parameters among three genera: *Naziritor*, *Tor*, and *Neolissochilus*. Notably, *N. chelynoides* exhibits unique characteristics compared to *Tor* and *Neolissochilus*, where the 2nd and 3rd abdominal vertebrae are fused to form the Weberian chain. In contrast, the 1st four vertebrae of the abdominal region fuse to form the Weberian chain in *N. chelynoides*. Furthermore, *N. chelynoides* has a lower number of vertebral bones, ranging from 40 to 41, compared to 40 to 44 in the *Tor* genus and 42 to 44 in

Neolissochilus. The percentage of frontal neurocranium length and breadth in *N. chelynoides* is relatively greater than that of the *Tor* and *Neolissochilus* genera. Thus, an osteological investigation is a promising tool for accurate identification and differentiation of *N. chelynoides* from other closely related fish species.

The information generated in this study can be used for developing the conservation strategies for the preservation of *N. chelynoides*, including protecting their natural habitats, regulating fishing practices, raising awareness, implementing stocking programs and captive breeding, and establishing legal protection and enforcement. These stratergies aim to safeguard the species by preventing habitat destruction, overfishing, and illegal activities, while also promoting responsible fishing practices and community involvement. It is important to maintain the ecological balance of dark mahseer populations by combining habitat protection with scientific research, education, and legal measures.

Conclusion

In conclusion, using an integrative taxonomic approach, we describe dark mahseer, *N. chelynoides*, which is found in the shallow, fast-flowing streams of the northern part of the central Himalayan region of India. Our phylogenetic analysis, osteological study, morphological and statistical analysis corroborate the validity of *N. chelynoides* as a separate and well separated clad.

Ethical Statement

The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Indian Council for Agricultural Research-Directorate of Coldwater Fisheries Research (ICAR-DCFR), Bhimtal, India (DCFR/IACUC/12/06/2020/9), and conducted as per the guidelines of institutional regulations.

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References

Britz, R., Dahanukar, N., Anoop, V.K., Anvar, A. (2019). *Channa rara*, a new species of snakehead fish from the Western Ghats region of Maharashtra, India (Teleostei: Labyrinthici: Channidae). *Zootaxa* 4683, 589 – 600. https://doi.org/10.11646/zootaxa.4683.4.8.

Dahanukar, N. (2010). *Puntius chelynoides*. In IUCN 2013. *IUCN Red List of Threatened Species* version 2013: https://www.iucnredlist.org.

da Rocha, R.H., Fernandes, C.A., Bignotto, T.S., Margarido, V. P., Tencatt, L. F. C., Graça, W. J., Gubiani, É. A. (2021). Integrated analysis reveals a new species of *Corydoras Lacépède*, 1803 (Siluriformes: Callichthyidae) in the lower Iguassu River, Brazil. *Organisms Diversity and Evolution*. 22, 457–474. https://doi.org/10.1007/s13127-021-00534-8.

Froese, R., Pauly, D. (2013). FishBase. *World Wide Web electronic publication*. www.fishbase.org.

Hammer, Ø., Harper, D. A. T., Ryan, P. D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica*, 4, 1 – 9. http://palaeo electronica.org/2001_1/past/issue1_01.html.

IUCN. *The IUCN Red list of Threatened Species*. Version 2020 – 3. https://www.iucnredlist.org.

Jaafar, F., Na-Nakorn, U., Srisapoome, P., Amornsakun, T., Duong, T.Y., Gonzales-Plasus, M.M., Hoang, D.H., Parhar, I.S. (2021). A current update on the distribution, morphological features, and genetic identity of the Southeast Asian mahseers, *Tor* species. *Biology*. 10(4), 286 – 315. https://doi.org/10.3390/biology10040286.

Kattel, G.R. (2022). Climate warming in the Himalayas threatens biodiversity, ecosystem functioning and ecosystem services in the 21st century: is there a better solution? *Biodiversity and Conservation* 31, 2017–2044. https://doi.org/10.1007/s10531-022-02417-6.

Khare, P., Mohindra, V., Barman, A.S., Singh, R.K., Lal, K.K. (2014). Molecular evidence to reconcile taxonomic instability in mahseer species (Pisces: Cyprinidae) of India. *Organisms Diversity & Evolution,* 14, 307–326. https://doi.org/10.1007/s13127-014-0172-8.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35(6), 1547-1549. https://doi.org/10.1093/molbev/msy096/4990887.

Laskar, B.A., Bhattacharjee, M.J., Dhar, B., Mahadani, P., Kundu, S., Ghosh, S.K. (2013). The Species Dilemma of Northeast Indian Mahseer (Actinopterygii: Cyprinidae): DNA Barcoding in clarifying the Riddle. *PLoS ONE* 8(1), 1-11. https://doi.org/10.1371/journal.pone.0053704.

Mirza, M. R. (1967). *Tor zhobensis* sp. nov. a new mahseer from the River Zhob, West Pakistan. *Pakistan Journal of Scientific and Industrial Research* 19:54-56.

McClelland., 1839. *Barbus chelynoides*. *Asiatic researches* 19(2): 271, 340.

Nakajima, T. (2018). Comparative studies on the pharyngeal teeth of cyprinids. *Tokai University Press.* 166 pp.

Pinder, A.S., Manimekalan, A., Knight, J.D.M., Krishnankutty, P., Britton, J.R., Philip, S., Dahanukar, N., Raghavan, R. (2018). Resolving the taxonomic enigma of the iconic game fish, the hump-backed mahseer from the Western Ghats biodiversity hotspot, India. *PLoS ONE* 13(6), 1 – 17. https://doi.org/10.1371/journal.pone.0199328.

Sarkar, U., Mahapatra, B.K., Singh, A. (2015). Mahseer in India: An overview on Research Status and Future Priorities. *Journal of Ecophysiology and Occupational Health* 15(1 – 2): 45 – 52 DOI:10.15512/joeoh/2015/v15i1&2/91183.

Sen, T.K., Jayaram, K.C. (1982). The Mahseer fishes of India-a review. *The Records of the Zoological Survey of India.* Occasional Paper. No.39, 1 – 38. http://recordsofzsi.com/index.php/zsoi.

Shahi, N., Mallik, S.K., Sarma, D. (2014). Golden mahseer- *Tor putitora*-a possible candidate species for hill aquaculture. *Aquaculture Asia* Vol. 14, 23 – 28. https://enaca.org/?id=408&title=aquaculture-asia-magazine-april-june-2014.

Sivasundar, A., Bermingham, E., Ortì, G. (2001). Population structure and biogeography of migratory freshwater fishes (Prochilodus: Characiformes) in major South American rivers. *Molecular Ecology* 10, 407 – 417. doi: 10.1046/j.1365-294x.2001.01194. x.

Sudasinghe, H., Raghavan, R., Dahanukar, N., Pethiyagoda, R., Rüber, L., Meegaskumbura, M. (2021). Diversification and biogeography of Dawkinsia (Teleostei: Cyprinidae) in the Western Ghats-Sri Lanka Biodiversity Hotspot. *Organisms Diversity and Evolution* 21, 795 – 820. https://doi.org/10.1007/s13127-021-00515-x.

Talwar, P.K., Jhingran, A.G. (1991). Inland Fishes of India and Adjacent Countries. *Oxford-IBH Publishing Co. Pvt. Ltd.,* New Delhi, pp. 1158.

Tilak, R., Baloni, S.P. (1984). On the fish fauna of Tehri-Garhwal, UTTAR PRADESH. *The Records of the Zoological Survey of India*. 81 (3 – 4): 255 – 272. http://recordsofzsi.com/index.php/zsoi.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., Hebert, P.D.N. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B*, 360, 1847 – 1857. https://doi.org/10.1098/rstb.2005.1716.

Xiao, W., Zhang, Y., Liu, H. (2001). Molecular systematics of Xenocyprinae (Teleostei: Cyprinidae): Taxonomy, biogeography, and coevolution of a special group restricted in East Asia. *Molecular Phylogenetics and Evolution* 18, 163 – 173. https://doi.org/10.1006/mpev.2000.0879.

Yousaf, M., Hasan, Z., Zaidi, F., Rasheed, S.B. (2021). An overview of the taxonomic instability of endangered Golden and Zhobi mahseer in Pakistan. *Brazilian Journal of Biology*. 83., e243975. https://doi.org/10.1590/1519-6984.243975.