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DNA barcoding of catfishes (Order: Siluriformes) with description of two new records from eastern region of Nepal

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Abstract

Altogether 99 specimens catfishes collected from the Eastern Region of Nepal (ERN), representing 29 species belonging to 21 genera and eight families were successfully barcoded employing mitochondrial COI gene sequence analysis showing 98.5-100% identity with both GeneBank and BOLD databases. Of these, two species *i.e. Amblyceps, arunachalense* and *Erethistoides sicula* were recorded for the first time from Nepal. Out of the 29 species, one species was found to be Endangered, two species under Near Threatened, one species under Vulnerable, one species under Data Deficient and 24 species under Least Concern category. The intra-specific genetic distances within species ranged from 0 to 5%. The highest intraspecific K2P and p-distances were found among the two species *viz., E. sicula* (4%) and *Glyptothorax telchita* (5%), indicating the presence of putative species. The inter-specific genetic distances ranged from 0 to 17%. GC content at 1st, 2nd and 3rd codon was found to be 42.4, 49.3 and 43.8%, respectively. The findings of the study will definitely support the upcoming initiatives on fisheries resource management and conservation in the region. It also forms an important input towards the DNA barcode library of catfishes of the eastern region of Nepal.

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Introduction

Catfishes (Order: Siluriformes) represent a diverse group of ray finned fishes (Nelson *et al.,* 2016), comprising approximately forty families, 500 genera and about 4300 valid species (Fricke *et al.,* 2023). They are distributed across all continents (Grande and Eastman, 1986; Diogo, 2004), except Australia (Malabarba, 1998). Of these, some speices such as *Heteropneustes fossilis* and *Plotosus lineatus* are poisonous or venomous, and can inflict severe wounds by injecting toxins through their spines. Catfishes are widely valued as a food source, as popular game fishes and as tropical aquarium fishes in many regions (Nelson *et al.,* 2016). While most of the catfish species inhabit freshwater, families like Ariidae and Plotosidae, also include representatives found in brackishwater and marine environments (Malabarba, 1998). Catfishes are characterised by the absence of scales, the presence of spines on the dorsal and pectoral fins, and unique skeletal features such as the fusion of the third and fourth neural crests to form a complex centrum and the fusion of the parietal and supraoccipital bones, resulting in a robust neurocranial structure (Ballen and De Pinna, 2022). They typically have tiny eyes and prominent barbels extending from each side of the upper jaw (Shrestha, 2019). Further distinguishing features include the absence of the symplectic, sub-opercular, basihyal and intermuscular bones, which highlight their unique anatomical adaptations.

Accurate species identification is crucial for fisheries resources management and conservation, but morphology-based species identification can be challenging, especially for non-specialists (Pandey *et al.,* 2020). Traditional taxonomic methods, relying on morphometric and meristic characterisations (Rosso *et al.,* 2012), often result in misidentification due to phenotypic and genotypic variations, cryptic species or different life stages (Chen *et al.,* 2015). Considering the shortage of taxonomic experts, there is a growing need for reliable, efficient, and reproducible molecular tools for species identification (Steinke *et al*., 2009; de Carvalho et *al*., 2011).

DNA barcoding, using the mitochondrial cytochrome c oxidase I (COI) gene, has become a widely used and reliable method for the rapid and accurate identification of fish species across diverse aquatic systems worldwide (Hebert *et al.,* 2003; Chen *et al.,* 2015; Kundu *et al.,* 2019). This technique has demonstrated a high success rate, with mean intraspecific COI diversity levels in fish species ranging from 3-4% (Ward, 2009). In addition to accurate species identification, DNA barcoding plays a crucial role in detecting the probable existence of morphologically cryptic species (Chen *et al.,* 2015), further enhancing its utility in fish taxonomy and biodiversity studies.

The eastern region of Nepal (ERN) encompasses three distinct ecological zones *viz.,* the Terai, mid-hills and the mountains, ranging in altitude from 60 to 8,848.86 m asl. The key aquatic resources in the region include, the Koshi river system (comprising Tamor, Arun, Tamakoshi, Dudkoshi, Bhotekoshi, Sunkoshi and Indrawati), the Kankai river system (including Deumai, Puwamai, Jogmai and Mai) and the Mechi River. These water bodies provide critical habitats for a diverse range of fish species, from small species like *Puntius phutunio* to large sized fishes like *Bagarius bagarius*. Studies on fish taxonomy and systematics in Nepal remian in the early stages. Morphology-based ichthyofaunal surveys in the ERN (Subba *et al.,* 2017; Limbu *et al.*, 2018, 2021a, b, 2023; Adhikari *et al.,* 2021; Rajbanshi *et al.,* 2021; Tumbahangfe *et al.,* 2021), have faced challenges, including potential errors and the inability to identify cryptic species. DNA barcoding is yet to be employed for fish species identification in Nepal, leaving scope for the discovery of cryptic and potentially new fish species. Herein, based on extensive sample collection in the ER, DNA barcoding technology was used to evaluate the fish diversity order Siluriformes. During an ichthyofaunal surveys in ERN undertaken during July 2021 to August 2023, we collected several individuals of *Erethistoides* spp. and *Amblyceps* spp., which could not be identified to the species level. Subsequent morphometric, meristic and mitochondrial COI analyses revealed that these two genera had two species new to Nepal. This study marks the first application of mitochondrial COI-based DNA barcoding for fish species delineation in Nepal, focusing on Siluriformes catfishes collected from the ERN.

Materials and methods

Sample collection

Sample collection for this study was undertaken in the eastern region of Nepal (ERN) from forty five sampling stations in the different rivers *viz.,* Koshi, Kankai, Lohandra, Mechi, Ratuwa, Singhiya and Tamor (Fig. 1). A total of 700 specimens were collected during July 2019 to August 2023. Fishes were captured using cast net, gillnet and local fishing tools like Dhadiya, Ghorlang, and Mosquito net. The captured fish specimens were photographed in the field to accurately capture their live colouration. For molecular study, the muscle tissue or caudal fin of 2-6 individuals of each species (except for *Ailia coila* and *Mystus bleekeri*) were preserved in 95% ethanol in the field and then transferred to 75% ethanol. Voucher specimens were identified using following Talwar and Jhingran (1991); Jayaram (2010) and Shrestha (2019) and were deposited at the museum of Central Department of Zoology, Tribhuvan University, Kirtipur Kathamandu,

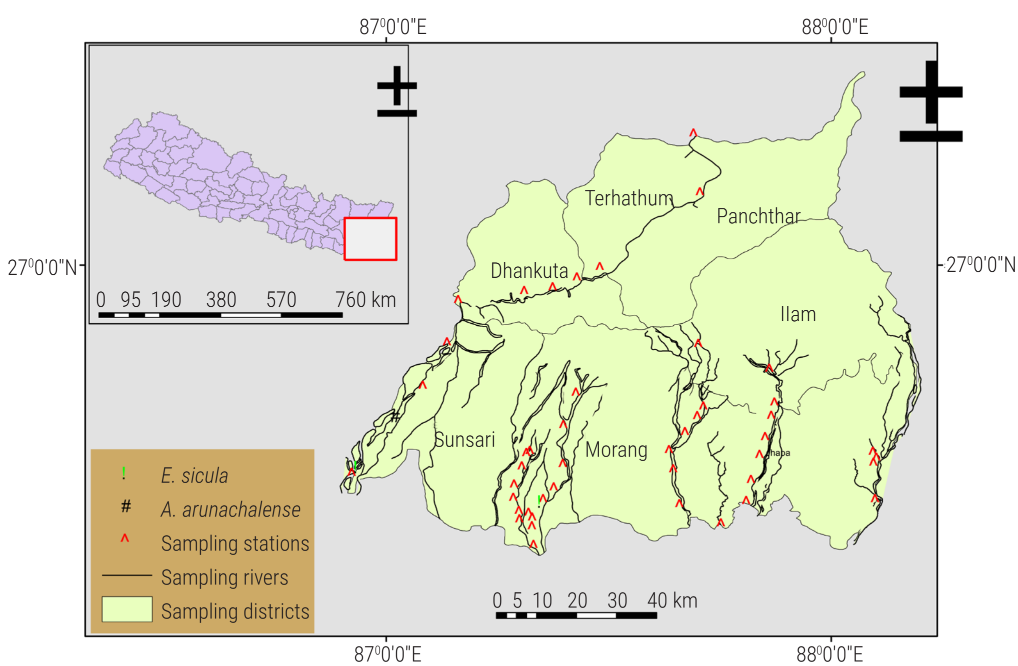

Fig. 1. Map of study area showing sampling stations

Nepal. We followed FishBase (www.fishbase.org) and Fricke *et al*. (2023) for confirming the recent valid names of species, genus and family. Subsequently, we checked the International Union for the Conservation of Nature (IUCN) (https://www.iucnredlist.org/) status of all the species studied.

DNA extraction and PCR

Total genomic DNA was extracted from the muscle tissue or caudal fin by using Tiangen Genomic DNA purification kit (Tiangen Biotech, Beijing, China). Partial sequences (~661 bp) of the COI gene was amplified using the primer pair, CGCTGATTCTTCTCTACCAAYCAYAAAGA and ACTTCTGGGTGGCCGAAGAAYCARAA, from 2~6 individuals of each species using a volume of 20 µl, containing 10 µl, Taq Master Mix (Vazyme), 6 µl ultrapure water, 2 µl DNA template and 1 µl, of each forward (F) and reverse (R) primers. The cycling conditions for PCR compised 35 cycles with initialisation step at 98˚C for 2 min, denaturation at 95˚C for 30 s, annealing at 55˚C for 45 s, elongation at 72˚C for 45 s and final elongation at 72˚C for 5 min. The amplified products were checked on 1% agarose gel before sending for sequencing.

Data analysis

All the resulting sequences were assembled using Geneious Prime 9.0.2 software and aligned and analysed using MEGA11 (Tamura *et al.,* 2021). The noisy sequences of both ends were trimmed before subsequent sequence analysis (Chen *et al.,* 2015). The 99 sequences obtained and analysed in the present study were submitted to NCBI GenBank database and the NCBI assigned accession numbers are given in Table 1. The phylogenetic trees of the newly recorded species *i.e. Amblyceps arunachalense* and *Erethistoides sicula* were constructed with their respective congeners using MEGA11 software (Tamura *et al.,* 2021), applying the maximum likelihood method (Felsenstein, 1981), with Kimura's two parameter evolutionary model (Kimura, 1980) using the bootstrap method (Kimura *et al.,* 2021), set to 1000 replication for evaluating the tree. The resulting trees were visualised in Fig Tree v. 1.4.4 (Rambaut, 2018). For the big data set, phylogenetic analysis was performed using maximum likelihood (ML) methods through IQ-TREE v1.6.12 (Nguyem *et al.,* 2015; Trifinopoulos *et al.,* 2016), using the bootstrap method (Kimura *et al.,* 2021), set to 1000 replication for evaluating the tree. We used the evolutionary model TIM2+F+I+G4 as the best-fit model, which was selected by Model Finder (Kalyaanamoorthy *et al.,* 2017) applying the Bayesian information criterion. Subsequently, using MEGA 11 software (Tamura *et al.,* 2021), we calculated sequence nucleotide composition and the mean genetic distances of various taxonomic levels, within and between species, genus and family, using K2P and p-distance model and set to bootstrap 1000 replicates. Moreover, the genetic diversity, nucleotide and haplotype diversity were analysed using DnaSP6 software. To detect whether the DNA barcode gaps existed, we calculated intraspecific and interspecific of K2P and p-distances including minimum, maximum and mean values. Tajima's D, Fu and Li's D test statistics analysis were done using DnaSP 6.12.03 (Librado and Rozas, 2009). Additionally, we applied online version (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) of Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.,* 2012), with both pairwise distance analysis using the default values to generate histograms and distance ranks.

Results

Identification

The proper and authentic taxonomic delineation and nomenclature of the collected fish species were done following the standard taxonomic references (Talwar and Jhinghran, 1991; Jayaram, 2010). The morphological and molecular categorisation affirmed that all 99 sequences belong to 29 species, 21 genera and eight families. Of these, two species *viz*., *Amblyceps arunachalense* and *Erethistoides sicula* were recorded for the first time from Nepal (Fig. 2, 3). On the basis of International Union for the Conservation of Nature (IUCN, 2022), one species was found to be Endangered (EN), two species under Near Threatened (NT), one species under Vulnerable (VU), one species under Data Deficient (DD) and 24 species under Least Concern (LC) category (Table 1).

(a)

(b)

Fig. 2. (a) *A. arunachalense*, KR-130; (b) *A. mangois* LR-129

Fig. 3. *E. sicula*, KR120

Table 1. Fish species collected from ERN with their IUCN status and GeneBank accession numbers

S. No.	Order	Family	Species	IUCN status (2022)	GeneBank Accession No.
1	Siluriformes	Amblycipitidae	*Amblyceps mangois* (Hamilton, 1822)	LC	OR793874-OR793877
2			**Amblyceps arunachalense* Nath & Dey, 1989	EN	OR801071-OR801073
3		Ailiidae	*Ailia coila* (Hamilton, 1822)	NT	OR793880
4			*Clupisoma garua* (Hamilton, 1822)	LC	OR726087, OR799676
5			*Eutropiichthys vacha* (Hamilton, 1822)	LC	OR733549, OR799677
6			*Eutropiichthys murius* (Hamilton, 1822)	LC	OR794001-OR794003,
7		Bagridae	*Batasio batasio* (Hamilton, 1822)	LC	OR793868-OR793871
8			*Mystus bleekeri* (Day, 1877)	LC	OR726099
9			*Mystus vittatus* (Bloch, 1794)	LC	OR801519-OR801522
10			*Mystus cavasius* (Hamilton, 1822)	LC	OR793864-OR793867
11			*Rita rita* (Hamilton, 1822)	LC	OR801529-OR801531
12			*Sperata aor* (Hamilton, 1822)	LC	OR801372-OR801374
13			*Sperata seenghala* (Sykes, 1839)	LC	OR801376-OR801381
14			*Olyra longicaudata* McClelland, 1842	LC	OR801375, OR801515
15		Siluridae	*Ompok bimaculatus* (Bloch, 1794)	NT	OR801463-OR801468
16		Sisoridae	*Bagarius bagarius* (Hamilton, 1822)	VU	OR801523-OR801526
17			*Erethistes pusillus* Muller &Troschel, 1849	LC	OR801436-OR801437
18			**Erethistoides sicula* Ng, 2005	DD	OR801443-OR801454
19			*Gagata cenia* (Hamilton, 1822)	LC	OR801457-OR801459
20			*Gogangra viridescens* (Hamilton, 1822)	LC	OR801461-OR801462
21			*Nangra assamensis* Sen & Biswas, 1994	LC	OR801527, OR801553
22			Glyptothorax telchitta (Hamilton, 1822)	LC	OR801537-OR801541
23			*Glyptothorax cavia* (Hamilton, 1822)	LC	OR801516-OR801517
24			*Glyptothorax trilineatus* Blyth, 1860	LC	OR801440-OR801441
25			*Glyptothorax indicus* Talwar, 1991	LC	OR801455-OR801456
26			*Pseudecheneis sulcata* (McClelland, 1842)	LC	OR801199-OR801203
27		Heteropneustidae	*Heteropneustes fossilis* (Bloch, 1794)	LC	OR801151-OR801155
28		Horabagridae	*Pachypterus atherinoides* (Bloch, 1794)	LC	OR801194-OR801198
29		Chacidae	*Chaca chaca* (Hamilton, 1822)	LC	OR801438-OR801439

**= New records for Nepal*

Amblyceps arunachalense Nath & Dey 1989

Material examined

KR130, 130b, 130c, 130d, 130e; 60.81-95.5mm SL; Koshi River; Nepal 50 km from Biratnagar Sub-metropolitan City, 26.65861°N and 87.02277°E, 96 m asl; Limbu and Rajbanshi, 12 January 2021.

Diagnosis: *A. arunachalense* has a slim and posteriorly compressed body (Fig. 2). Head is short and nearly rounded. Mouth is terminal having double folds lips with lower jaw longer than upper jaw. Teeth in the lower jaw have a bowed band which is divided in the central portion, whereas teeth in the upper jaw have a thin band with hind extensions on both edges isolated by an extremely narrow aperture. Eyes are small and subcutaneous. It has somewhat convex dorsal body contour, while the profile from the pectoral fin to the base of the pelvic fin is convex, the ventral body profile from the pelvic fin to the base of the anal fin is slightly concave. There are four pairs of barbels which are compressed throughout their length. Nasal and inner mandibular barbels are nearly equal with head length. Maxillary barbel extends just beyond the central part of the pectoral fin base, whereas outer mandibular barbel extends just beyond the posterior end of the pectoral fin base. Dorsal fin vertically located almost mid-part of the pectoral and pelvic fins, containing I, 7 rays. The spine is weak and concealed in the small bulging skin. Adipose fin located at vertical level of the anal fin origin, with its posterior part rounded and far from the caudal fin. Pectoral fin has I, 7 rays, comparatively smooth and stronger spine than dorsal spine. It extends at the back of dorsal fin base. Pelvic fin contains I, 5 rays, while the anal fin with V, 8 rays. Caudal fin deeply forked having upper lobe longer than lower lobe bearing 6 + 9 rays. Vertebrae 40. Lateral line absent.

Colouration: Live colouration is darkish brown; after preservation in 10% formaldehyde solution, colour changes to grey on lateral side and white creamy on ventral side.

Distribution: Sunsari and Saptari Districts and commonly available in Koshi River.

Remarks: Collected from slowly moving water near Koshi Barrage.

Economic importance: Species is locally popular as food fish.

Phylogenetic analysis: For phylogenetic analysis, fifteen COI sequences of *Amblyceps* were employed (Fig. 4). For phylogenetic tree reconstruction, *Danio rerio* (Hamilton, 1822) was used as the out-group. Using a bootstrap value >95%, the phylogenetic analysis revealed that the recently discovered Nepalese *Amblyceps* species is congruent with an Indian *Amblyceps* species known as *A. arunachalense*. Nepalese *A. arunachalense* had a genetic distance of 8.8% with *A. mangois*, 1.7% with Indian *A. arunachalense* (retrieved from NCBI), 13.1% with *A. apangi,* 10.5% with *A. laticeps*, 10.1% with *A. hmolaii*, 11.8% with *A. mucronatum* and 11.1% with *A. serratum*. The overall mean genetic distance of tested *Amblyceps* species was found to be 0.11±0.01.

Erethistoides sicula Ng 2005

Material examined

KR120, 120a, 120b; 27-40.5 mm SL; Koshi River; Nepal, 50 km from Biratnagar Sub-metropolitan City, 26.65861°N and 87.02277°E, 96 m asl; Limbu and Rajbanshi, 12 January 2021. LR120, 120b; 32-47 mm SL; Lohandra River, Nepal, 6 km from Biratnagar Sub-metropolitan City, 26.54611°N and 86.9383°E, 70 masl; Limbu and Rajbanshi, 12 January 2021.

Diagnosis: E. sicula has broad head which is strongly depressed (Fig. 3). The body is broad and compressed with more compression in the caudal peduncle. Dorsal profile is slightly convex while the ventral profile nearly straight. Eyes are ovoid in shape having longest axis on horizontal side. Gill openings narrow; mouth small having upper jaw projected on the far side of lower jaw with papillated lips. Four pairs of barbels present with very short and slim nasal barbels extending to the posterior margin of posterior nares. Maxillary barbel slim, extending to the base of pectoral fin base whereas outer mandibular barbel extending on the far side of the base of posterior-most pectoral fin ray. Similarly, inner mandibular barbel is short and nearly reach the base of pectoral spine. On the flanks, along with skin numerous elongate and flattened plaque like tubercles are present in the longitudinal rows. Lateral line is complete and located in the midlateral position. Vertebrae 29-31. Fin numbers found same as described by the original author. Dorsal fin with 5(12) rays with its margin straight. Furthermore, it has robust, straight and compressed spine, having smooth spine on its anterior region and posterior region with 24 small serrations. Pectoral fin with 5(5) rays, anal fin with 10-11 rays and caudal fin forked having tips of both lobes pointed with lower lobe slightly longer than upper lobe.

Colouration: Dorso-lateral side of the body and head are light chocolate brown as described by Ng (2005). Both paired and unpaired fins are hyaline. Dorsal fin has transverse faint brown bands in the direction of base and in the centre portion. Sometimes, dark brown colour is present in the pelvic, pectoral and anal fins. All barbels are creamy white but sometimes maxillary barbel with brown rings.

Distribution: Known from the Koshi River which is the largest river of Nepal and also known from the Lohandra River of Morang District, 6 km away from Biratnagar Sub-metropolitant City.

Remarks: E. sicula was collected from Koshi and Lohandra rivers, from the fast flowing areas with gravelly bottom (Fig. 1).

Economic importance: The speices is a locally popular food fish.

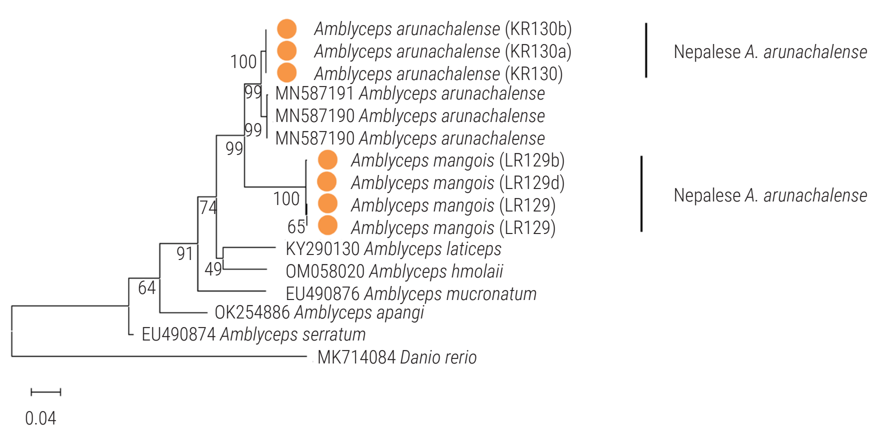

Fig. 4. Maximum-likelihood (ML) tree of newly recorded *A. arunachalense* and other species of the Amblicipitidae, based on sequences (~661 bp) covering the partial barcoding region of COI gene

Phylogenetic analysis: For the phylogenetic analysis of *E. sicula*, we employed five newly generated sequences and five from NCBI database (Fig. 5). There were no mitochondrial COI sequence available for other *Erethistoide*s spp. So we used other closely related species for tree construction. Newly generated sequence data was submitted to both NCBI and BOLD databases, which showed 100% match with *E. sicula* from other countries. The K2P genetic distance of Nepalese *E. sicula* with those of retrieved data from NCBI was found to be 0%, indicating the same species.

DNA barcoding of catfishes of the ERN

To validate the DNA barcode, at least 2-6 specimens from each species were barcoded (except *A. coila* and *M. bleekeri*). We created barcodes for 99 specimens belonging to 29 species, representing 21 genera and eight families from forty-five locations in the ERN (Fig. 1; Table 1). The created sequences were submitted to NCBI and BOLD public databases, which displayed a similarity of 98.5-100% and matched with the obtained morphological data except for one species (*Glyptothorax indicus*). The sequences generated in this study have been deposited in the GeneBank database (Table 1).

The nucleotide base composition analysis, as shown in Table 4, portrayed that out of 648, invariable sites were 318 (49.07%), variable sites were 247 (38.11%), singleton sites were 15 (2.31%) and parsimony informative sites were 232 (35.8%). The average nucleotide composition showed that the composition of T was the highest (29.0%), while that of G was the lowest (18.0%). The base composition of C and A were 27.2 and 25.8% respectively. It was also found that AT content (54.8%) was higher than that of GC content (45.2%). GC content at 1st, 2nd, ad 3rd codon was 42.4, 49.3 and 43.8%, respectively.

Phylogenetic analysis

The maximum likelihood (ML) tree (Fig. 6) was constructed for the 99 sequences belonging to 29 species, 21 genera and eight families. The family Sisoridae represented 11 species, Bagridae 9 species, Ailiidae 5 species, Amblycipitidae 2 species and Siluridae, Horabagridae, Heteropneustidae and Chacidae represented single species each. In the phylogenetic tree, COI barcode sequences discriminated all the species and clustered the similar species with significant bootstrap values of 99-100% under the same nodes (Fig. 6). The ML tree formed two separate clades. The Sisoridae family formed a separate monophyletic clade with interspecific separation, whereas the family Bagridae formed a separate paraphyletic clade with interspecific separation. *Batasio batasio* distantly separated from the other members of the Bagridae family. The members of the family Ailiidae also clustered into one clade with the strong support of 100% bootstrap value for each genus. The fish belonging to genus *Amblyceps i.e. A. arunachalense* and *A. mangois* formed a distinct monophyletic clade. The species belonging to the genus *Glyptothorax* i.e. *G. indicus, G. cavia, G. trilineatus* and *G. telchitta* formed a strong monophyletic clade. Similarly, species belonging to the genus *Mystus i.e. M. vittatus, M. bleekeri* and *M. cavasius* formed a strong monophyletic clade. In addition to this, fish of the genus *Sperata i.e. S. aor*, and *S. seenghala* also formed a strong monophyletic clade. The fish belonging to the genera *Ompok, Batasio, Rita* and *Chaca* formed a paraphyletic clade.

Genetic diversity and mean genetic divergence analysis

DNA sequence polymorphism 6.12.03 analysis divulged the nucleotide diversity (p) and haplotype diversity (Hd) (Table 2). The highest haplotype diversity was found to be 1.00 for *A. mangois, C. garua, E. pussilus* and *C. chaca* while the lowest was found to be 0.167 for *E. sicula*. Moreover, species of *M. bleekeri, E. murius* and *H. follilis* were also found with high haplotype genetic diversity *i.e.* 0.99, 0.833 and 0.800 respectively. The highest nucleotide diversity was found to be 0.27795 for *E. murius* and the lowest (0.00101) in the case of *M. cavasius*. But *A. arunachalense, A. coila, E. vacha, S. aor, O. caudate, O. bimaculatus, B. bagarius, G. viridescens* and *G. trilineatus* did not show any polymorphism in the analysed

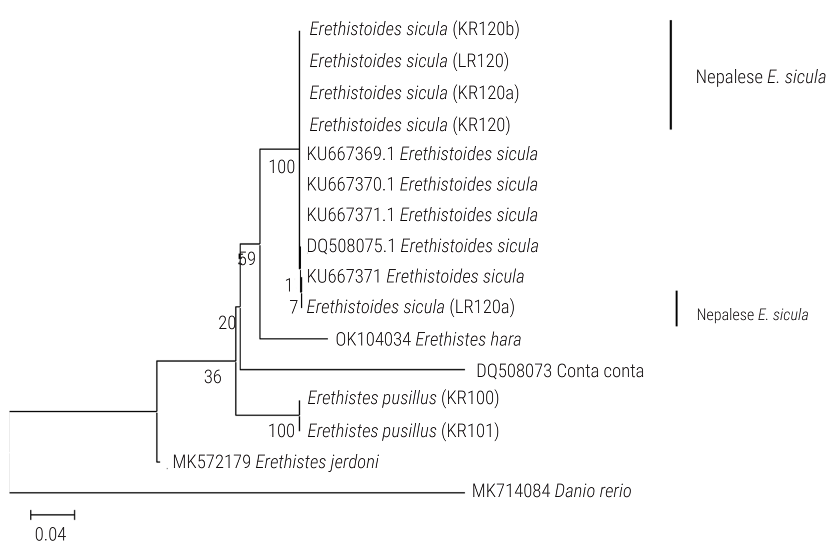

Fig. 5. Maximum-likelihood (ML) tree of newly recorded *E. sicula* and other species of the Sisoridae, based on sequences (~661 bp) covering the partial barcoding region of COI gene

Fig. 6. ML tree showing the phylogenetic relationship among the catfish species of the ERN

sequence data. In total, 41 haplotype diversity were found in the data set. The overall haplotype and nucleotide diversity were found to be 0.972 and 0.17160 in the whole data set, which indicated high genetic diversity in the rivers of ERN. The average p was found to be 0.447 in the whole data set. The values of both tests *i.e* Tajima's and Fu's F tests were found to be negative.

The K2P and p-distances were computed within the various taxonomic levels, including species, genus and family (Table 3). The mean genetic distances increased from within species to within family hierarchically. The overall mean distances of K2P and p-distance for the COI sequences were 22±0.02 (mean±SE) and 17±0.01 (mean±SE). A summary of genetic distances of different taxonomic levels are given in the Table 3. The intraspecific K2P genetic distances ranged from 0 to 5% [mean = 0.52, standard error [SE] = 0.0023] at the species level, whereas the intraspecific p-distances ranged from 0 to 4% [mean = 0.45, standard error [SE] = 0.0011]. The highest intraspecific K2P and p-distances were found among the two species *i.e. E. sicula* (4%) and *G. telchita* (5%), indicating the presence of putative species. The interspecific K2P distances ranged from 0 to 17% [mean = 2.6%, standard error [SE] = 0.0098] at the genus level, while the interspecific p-distances ranged from 0 to 15% [mean = 2.14%, standard error [SE] = 0.0073]. Some genus and family have only one species which could lead to the minimum interspecific K2P and p-distances of the genus and family level to 0%.

Table 2. Haplotype and nucleotide diversity of Siluriformes fishes collected from the ERN

Species	Haplotype diversity (Hd)	Nucleotide diversity (p)
Amblyceps mangois	1	0.00299
A. arunachalense	0.00	0.00
Ailia coila	0.00	0.00
Clupisoma garua	1	0.00351
Eutropiicthys vacha	0.00	0.00
E. murius	0.833	0.27795
Batasio batasio	0.667	0.00201
Mystus bleekeri	0.99	0.13404
M. cavasius	0.667	0.00101
M. vittatus	0.40	0.303
Rita rita	0.667	0.11161
Sperata aor	0.00	0.00
Sperata seeenghala	0.60	0.00092
Olyra longicaudata	0.00	0.00
Ompok bimaculatus	0.00	0.00
Bagarius bagarius	0.00	0.00
Erethistes pussilus	1.00	0.00309
Erethistoides sicula	0.167	0.03226
Gagata cenia	0.667	0.00906
Gogangra viridescens	0.00	0.00
Nangra assamensis	0.32	0.0140
Glyptothorax telchitta	0.446	0.00134
G. cavia	0.781	0.0435
G. trilineatus	0.00	0.00
Pseudecheneis sulcata	0.500	0.00151
Heteropneustes fossilis	0.800	0.00483
Chaca chaca	1.00	0.00150
Pachypterus atherinoides	0.700	0.00271

Table 3. Mean K2P and p-distances within species, genus and family of fish collected from ERN

	Taxonomic level	Min (%)	Mean (%)	Max (%)	SE
K2P distance					
	Within species	0.00	0.52	5.0	0.0023
	Within genus	0.00	2.6	17.0	0.0098
	Within family	0.00	8	21.0	0.0031
p-distance					
	Within species	0.00	0.45	4.0	0.0011
	Within genus	0.00	2.12	14.0	0.0073
	Within family	0.00	7.25	15.0	0.0014

Discussion

This study marks the first use of the COI gene for fish species identification in Nepal, advancing molecular research, which is still in its early stages. Despite Nepal's relatively small geographic area, it hosts remarkable species diversity. Traditionally, ichthyologists in Nepal have relied on morphology-based identification, which has limitations in resolving cryptic or morphologically similar species, suggesting the likelihood of undiscovered species. In this study, two new records for Nepal were identified, *i.e. A. arunachalense* and *E. sicula*. Nearly for two decades, *A. arunachalense* was treated as synonym of *A. mangois* in Nepal. Ng (2005) supported this synonymy, but Vishwanath and Linthoingambi (2007) later redescribed and resurreted it as a valid distinct species. Similarly, *E. sicula*, first described by Ng (2005) in West Bengal, India, is now recorded from south-eastern region of Nepal, almost after 18 years. Given the geographical connection between south-eastern part of Nepal and West Bengal, with rivers including Nepal's biggest Koshi River, flowing between them, the presence of *E. sicula* in Nepal is plausible. Both morphology and barcoding reiterate that these two species exists in Nepal. Both morphological analyses and DNA barcoding confirm the occurrence of these two species (*A. arunachalense* and *E. sicula*) in Nepal, contributing to the understanding of the country's fish biodiversity.

The fish species collected from ERN were successfully identified with a 98.5-100% match in both NCBI and BOLD public databases, except for one *Glyptothorax* species. was unable to identify through both databases. Morphological identification suggested it as *G. indicus,* but database results showed a 100% match to the genus *Glylptothorax* and less than 92% similarity at the species level. Further analysis revealed that a previously submitted sequence for *G. indicus* was actually *G. telchitta*, highlighting potential errors in public databases (Wong *et al.,* 2011). This study enhanced the number of COI gene sequences for important fish species in the ERN, in both databases, contributing to improved identification and addressing previous misidentifications in Nepal. DNA barcoding supported morphological identification, achieving a nearly 100% success rate, consistent with previous reports (Lakra *et al.,* 2015; Shen *et al.,* 2016; Ude *et al.,* 2020). Intraspecific genetic divergence of *E. sicula* and *G. telchitta* exceeded 2%, indicating the presence of putative species. According to Ward (2009), genetic divergence above 2% with over 95% probability, indicates distinct species. Further, the GC content of the 99 sequences was found to be on the higher side (45.2%), aligning with earlier reports (Pandey *et al.,* 2020; Ude *et al.,* 2020).

The phylogenetic analysis of the fish of the order Siluriformes in the ERN, in the present study, disclosed that the species belonging to genus *Eutropiichthys* are closely related to the members of the family Ailiidae. The family Ailiidae includes three genera (*Ailia, Laides* and *Clupisoma*) and family Schilbeidae includes the genera *Eutropiichthys, Proeutropiichthys* and *Silonia.* Fricke *et al.* (2023) placed *Ailiichthys*, *Proeutropiichthys*, *Silonia* and *Eutropiichthys* in Ailiidae, where they included seven genera (*Ailia, Laides*, *Clupisoma,* and *Ailiichthys, Proeutropiichthys, Silonia* and *Eutropiichthys*), with 25 legitimate nominal species. Our study aligns with the placement in Eschmeyer Fish Catalog, which classifies species of the genus *Eutropiichthys* under the family Ailiidae. However this conclusion appears to rely on circular reasoning highlighting the need for further research to accurately determine the taxonomic positions of genera within this family. Karinthanyakit and Jondeung (2012) found that the genus *Eutropiichthys* is a sister group of the genus *Clupisoma* and also found *Clupisoma sinense* is more closely related to *Laides longibarbis* with 85.63% sequence identity. The mitochondrial genome studies conducted by Alam *et al.* (2019) suggested that *Ailia coila* is more closely related to *Eutropiichthys vacha*. Molecular analysis on Ailiids are meagre. Mitochondrial gene cannot alone be taken into consideration for phylogenetic studies and therefore it can be suggested that more studies are needed for both families *i.e.* Schilbeidae and Ailiidae using nuclear genes for confirmation. While the Bagridae family created a distinct paraphyletic clade with interspecific separation, the Sisoridae family formed a different monophyletic clade. Several families, including Chacidae, Horabagridae, Heteropneutidae and Siluridae, consists of only a single species. As a result, the minimum genetic distance between species, genera and families was found to be zero. Similarly, in the phylogenetic tree, the presence of a single species may lead to a genus or family forming a paraphyletic clade.

The results of haplotype diversity analysis suggested that the majority of the species in the current study exhibited high haplotype diversity, although a few species showed lower values. However nucleotide diversity was generally low across most species. The species with highest haplotype diversity (=1), included *A. mangois*, *Clupisoma garua, Erethistes pussilus,* and *Chaca chaca.* Additionally, species such as *M. bleekeri, E. murius, H. fossilis, B. batasio, M. cavasius, G. cavia* and *P. atherinoides* also displayed relatively high haplotype diversity (Table 2). On the other hand, most of the species studied exhibited low nucleotide diversity, indicating a significant divergence between them. According to Song *et al.* (2014) populations that show high haplotype diversity coupled with low nucleotide diversity, are likely to have recently diverged from one another. Additionally, the negative values observed in both neutrality tests, *i.e.* Tajima's D and Fu's F test statistics, suggest an excess of rare mutations within the current population, pointing to recent population expansion.

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