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DNA BARCODING OF SMILIOGASTRINAE (TELEOSTEI: CYPRINIFORMES) OF BANGLADESH BASED ON CYTOCHROME C OXIDASE SUBUNIT I (COI) SEQUENCES

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Abstract. This study aims to molecularly characterize the phylogenetic relationship of small barbs under the Subfamily Smiliogastrinae (Cypriniformes: Cyprinidae) in Bangladesh using a fragment of the mitochondrial gene cytochrome c oxidase subunit I (COI). Samples were collected from rivers, haors (a seasonal wetland), baors (an oxbow lake), beels (perennial waterbody), and floodplains. A total of eleven species under five genera were confirmed based on both morphological and molecular approaches. The average Kimura two-parameter (K2P) distances for intraspecies and interspecies were 0.0058 and 0.1538, respectively. The mean GC content was markedly low (44.03%) in the COI sequences of the smiliogastrin species compared to the mean AT content (55.97%). In addition to the barcode-based species identification, phylogenetic relationships among the species were also explored. Phylogenetic (neighbor-joining, parsimony, and maximum likelihood) as well as species delimitation (ASAP and mPTP) analyses of all the eleven species revealed distinct clusters in concurrence with the taxonomic status of the species.

INTRODUCTION

The open inland waters of Bangladesh are rich in faunal diversity consisting of 253 species of fish (IUCN Bangladesh 2015). A group of small freshwater fish species of the subfamily Cyprininae *sensu lato* (Cypriniformes: Cyprinidae) are called Barbs (*puntimachh* in Bengali). These small fishes (length < 25cm) are an important source of animal protein, fatty acids, essential vitamins and minerals, and are mainly consumed by local people in rural areas (Roos et al. 2007). According to estimates, these barb species constitute about 16–19% of the total annual catch depending on water bodies (Haroon et al. 2002; Hossain et al. 2008). Some of the barb species *viz., Systomus sarana* (Hamilton, 1822), *Puntius sophore* (Hamilton, 1822) are used as potential aquaculture species (IUCN Bangladesh 2015).

These barbs are characterized by a moderately to deeply compressed body, varying from silvery to greenish silvery or reddish-brown in color (Talwar and Jhingran 1991; Banglapedia 2015). The presence of spots, blotches, bands on the body, and the presence of 4 or 2 barbels (or their absence) are the key identifying characters (Talwar and Jhingran 1991; Banglapedia 2015). However, for non-experts, it is difficult to taxonomically identify the species as some other species exhibit very similar morphological characteristics. The body length of the adults varies from 5 cm to about 20 cm (Talwar and Jhingran 1991) and they inhabit all types of freshwater habitats including rivers, streams, haors (seasonal wetland), baors (oxbow lake), beels (perennial waterbody), and floodplains throughout the country (Rahman 2005; Siddiqui et al. 2007; Hossain et al. 2008; Mian et al. 2013; Ahmed et al. 2019, 2020). At least ten species of barbs under two genera *Puntius* (9 species) and *Oreichthys* (1 species) are described from Bangladesh (Siddiqui et al. 2007). The latest taxonomy classifies them under five genera: *Pethia* (5 species), *Puntius* (3 species), *Systomus* (1 species), *Oreichthys* (1 species) and *Osteobrama*. When revising the classification of cyprinine fishes, Yang et al. (2015) proposed to place small-sized barbs of Asia (*Puntius* and allies), including *Osteobrama,* in the Tribe Smiliogastrini under the subfamily Cyprininae. Later, based on the phylogenetic classification of the extant genera of fishes of the order Cypriniformes, Tan and Armbruster (2018) placed *Puntius* and allies in the subfamily Smiliogastrinae. In Bangladesh, there have been several studies done on the abundance and distribution of barbs in rivers, floodplains, and mountain streams in different regions (Haroon et al. 2002; Mian et al. 2013; Mohsin et al. 2013). Correct identification of barbs and regular monitoring for adulteration are highly important as these fish have both nutritional and ornamental value.

It is now well established that a specific fragment of the mitochondrial cytochrome c oxidase subunit I (COI) sequence (DNA barcodes) can be used as an alternative to the traditional species identification based on morpho-

logical characters (Hebert et al. 2003; Frézal and Leblois 2008; Leray and Knowlton 2015). Besides, the reconstruction of phylogenetic trees based only on morphology is controversial due to the complex evolutionary changes in morphological and physiological characters. Analysis of molecular characters provides more insights into the specific patterns of differentiation among the isolated fish samples. However, the use of short-length single-locus markers such as COI has a shortcoming because these sequences represent the whole phylogenetic history only partially. More sophisticated species delimitation systems using multiple loci should be employed for a more accurate inference (Brower 2006; Rubinoff et al. 2006; Dasmahapatra et al. 2010; Dupuis et al. 2012; Fujita et al. 2012; Collins and Cruickshank 2013).

Despite their great significance, few studies have been done on the taxonomy, biology, and biodiversity of barbs in Bangladesh (Hossain et al. 2008; Mian et al. 2013; Mohsin et al. 2013; Ahmed et al. 2019). Hence, the present study was carried out to identify smiliogastin species based on morphometric and molecular approaches and to infer phylogenetic relationships among them.

MATERIALS AND METHODS

Collection of samples

The target species were collected from the Tanguar Haor, Sunamganj (25.1503 N 91.0603 E); Hakaluki haor, Moulvibazar (24.6767 N 92.0469 E); Sirajdikhan, Munshiganj (23.65 N 90.38 E); Padma, Rajshahi (24.35 N 88.65 E); Kuakata, Patuakhali (21.85 N 90.10 E); Dacope, Khulna (22.57 N 89.49 E); Sonargaon, Narayanganj (23.64 N 90.62 E); Boiddar Bazar Ghat, Sonargaon, Narayanganj (23.65 N 90.63 E); and Chalan Beel, Singra, Natore (24.5134 N 89.0539 E) during fishing expeditions from July 2015 to June 2018 (Figure 1). The samples in frozen condition were immediately transported to the DNA Barcoding Lab, Department of Zoology, University of Dhaka. At least 3–5 individuals per taxon were examined for the morphometric and meristic study except for *Oreichthys cosuatis* (Hamilton, 1822) and *Pethia guganio* (Hamilton, 1822), in which case, two individuals were analyzed to assess variability within the species. In this study, we used two-letter abbreviations, *Pu.* and *Pe.* for the genera *Puntius* and *Pethia*, and *Or.* and *Os.* for the genera *Oreichthys* and *Osteobrama*, respectively to avoid any ambiguity for the readers.

Morphometric and meristic study

All the collected specimens were fresh, having visually distinguishing characteristics (Table 1) and were identified in accordance with the reference materials (Rahman 2005; Talwar and Jhingran 1991). Morphometric characters were measured using Digital Callipers with an accuracy of 0.01 mm, and the specimens were identified according to the previous taxonomic description following Rahman (2005) and Talwar and Jhingran (1991). Meristic characters were counted using standard methods. Body color, shape, spot, barbels, lateral line scales, fin formula, etc. were examined.

DNA Extraction

20 mg of muscle tissue was removed aseptically from each fish sample, just above the caudal fin. Total genomic DNA was obtained by addition of 500 µl of TES buffer (0.05 M Tris-HCl, pH 8.0, 0.025 M EDTA, and 0.15 M NaCl) and digestion with 15 µl of proteinase-K (20 mg/ml). Incubation was carried out at 56°C for 12–18 hours until the tissue was totally dissolved. Further steps in isolating genomic DNA were performed following the standard phenol-chloroform-isoamyl alcohol method described by Sambrook et al. (1989).

Visualization and quantification of extracted DNA

The quality of the isolated DNA was visualized in 1% agarose gel using fluorescence of ethidium bromide under UV light, by direct comparison with a

Figure 1. A map of the study area depicting the sampling location.

Table 1. Morphometric and meristic characteristics of barb species along with their Red list status (Rahman 2005; Talwar and Jhingran 1991).

Species	Lateral line and no. of scales	No. of barbel	Dorsal spine and rays	Spots on body	IUCN red list status (Bangladesh 2015)
Putius sophore	Complete 24–26	No barbel	Last unbranched dorsal ray ossificated and smooth	Two black blotches; at base of dorsal rays and base of caudal fin	LC
P. chola	Complete 24–26	2	Last unbranched dorsal ray ossificated, strong and smooth	Two conspicuous dark blotches; at the base of 2nd-5th dorsal rays and near base of caudal fin	LC
P. terio	Incomplete 22–23	No barbel	Last unbranched dorsal ray ossificated, strong and smooth	A black blotch over anal fin, from which a fine dark line runs back to base of caudal fin; a reddish orange spot on operculum	LC
Pethia conchonius	Incomplete 24–28	No barbel	Last unbranched dorsal ray ossificated and strongly denticulated along the posterior edge	Large black spot over posterior portion of anal fin, A band of black marks along the middle dorsal rays during breeding season	LC
P. ticto	Incomplete 23–26	No barbel	Last unbranched dorsal ray ossificated, fairly strong and serrated at its posterior edge	A block spot at commencement of lateral line, another at sides of the tail above anal fin.	VU
P. gelius	Incomplete 22–23	No barbel	Last unbranched dorsal ray ossificated, strong and serrated	A dark band over tail. Anterior base of dorsal, pelvic and anal with black mark	NT
P. phutunio	Incomplete 21–23	No barbel	Last unbranched dorsal ray ossificated, strong and serrated	Five steel-blue transverse bars which fade into three black blotches, 1st behind operculum, 2nd above anal fin and 3rd on caudal peduncle	LC
P. guganio	Incomplete 36	No barbel	Last unbranched dorsal ray ossificated, strongly denticulated along the posterior edge	A light greenish silvery lateral band	LC
Systomus sarana	Complete 32–34	4	Last unbranched dorsal ray strongly ossificated and finely serrated along its posterior edge	No spot, body silvery, darker on the back	NT
Oreichthys cosuatis	Interrupted 22–23	No barbel	Last unbranched dorsal ray weak, non-ossificated and smooth	Absence of a spot on the caudal-fin base, a bright-yellow dorsal fin with a large black mark covering the distal margin of the dorsal fin, and red ventral fins, and a black spot in the anal fin	EN
Osteobrama cotio	Complete 60–65	No barbel	Dorsal with weak and serrated ossificated ray	Scales on upper half of the body with minute black dots. A black spot at commencement of dorsal, another over nape. Silvery, fins yellowish.	NT

CR – Critically Endangered; EN – Endangered; VU – Vulnerable; NT – Near Threatened; LC – Least Concern; and DD – Data Deficient.

standard marker (GeneRuler 1 kb Plus DNA ladder, 0.1 µg/µL, Catalogue number: SM1333, Thermo Scientific). The fluorescence was documented using AlphaImager® gel documentation systems (ProteinSimple, USA). For the quantification of the extracted DNA, UV-spectrophotometry (NanoDrop 2000) was used to infer the concentration of DNA (ng/µL) at the absorption of 260 nm (OD). Additionally, the quality of the isolated DNA was assessed using OD_{260}/OD_{280} (Down and Wilfinger 1983).

PCR amplification

For PCR amplification of the mitochondrial COI gene, we used primers FishF1 (5′-TCAACCAACCACAAAGACATTGGCAC-3′) and FishR1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) (Ward et al. 2005). The final volume of each PCR was 25 µl and consisted of 12.5 µl of GoTaq® G2 Hot Start Colorless Master mix (Promega, Madison, WI USA), 1 µl of each primer (0.01 mM), 8.5 µl of nuclease-free water, and 2 µl of DNA template. Amplifications were performed using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Inc.). The thermocycling profile consisted of initial denaturation at 95°C for 2 min, 30 cycles of denaturation at 95°C for 30s, annealing at 52–54°C for 30s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products were visualized on a 1% agarose gel using a standard mini-horizontal Agarose Gel Electrophoresis System (Catalogue No. MGU-602T, CBS Scientific, Inc.).

Sequencing

Bidirectional sequencing was performed using BigDye® Terminator version 3.1 cycle sequencing kit chemistry (Applied Biosystems Inc., USA) on an ABI 3730XL capillary sequencer by First BASE Laboratories Sdn Bhd, Malaysia. Sequences of both strands were assembled using Sequence Assembly Program CAP3 (Huang and Madan 1999). The bidirectional sequences were subsequently aligned using the MUSCLE 3.8.31 (Edgar 2010). The generated sequences were deposited with the NCBI GenBank (GB) and were accordingly assigned GB Accession Numbers (KX455895-96, KX455909, KY124379-80, KT353106, KT364771-73, KT762359-60, MH087036, MK988520, MK988542, MN013419, MN083131, MN171353-54, MN171373, MN200455, MN200463-65, MN200473).

Species delimitation and Molecular phylogenetic analysis

Pairwise sequence comparisons of the set of COI gene sequences were performed using the K2P model (Kimura 1980). The amount of the nucleotide variation was determined after alignment and trimming gaps from both ends using the software MEGA version X (Kumar et al. 2018). For evaluation of the maximum-likelihood (ML) estimate of Transition/Transversion Bias, the substitution pattern and rates were estimated according to the K2P model (+G + I). Then, to test whether the genes had reached the substitution saturation plateau, the COI gene sequence substitution saturation was investigated by plotting the number of transitions and transversions against pairwise genetic divergence using DAMBE (Data Analysis in Molecular Biology and Evolution) version 6.0 (Xia, 2017).

Phylogenetic and molecular evolutionary analysis was conducted using MEGA version X (Kumar et al. 2018). Best-fitting substitution models were selected according to the Bayesian information criterion (BIC). For molecular phylogenetic analysis by ML reconstruction, evolutionary relationships were inferred based on the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) with 1000 bootstrap replicates. A total of 26 sequences belonging to six genera and 11 species were randomly included in the study as ingroups from the GenBank (GB accession nos. and Country of origin listed in Supplementary Table 2). As outgroups, *Esomus danricus* from the tribe Cyprinini of the same subfamily and *Psilorhynchus sucatio* from the family Psilorhynchidae of the same order were selected. In addition, a neighbor-joining (NJ) tree of K2P-distances (Tamura et al. 2004) and a maximum-parsimony (MP) tree were created using the Subtree-Pruning-Regrafting algorithm (Nei and Kumar 2000) with 1000 replications for bootstrap analysis to assess the phylogenetic relationships among species.

Species delimitation was performed using the Kimura K80 (ts/tv 2.0) substitution model of the software Assemble Species by Automatic Partitioning (ASAP) (https://bioinfo.mnhn.fr/abi/public/asap/) with the default parameters (i.e., recursive split probability of 0.01), since it rapidly offers a full graphical exploratory interface relevant species hypothesis (Puillandre et al. 2020). We also conducted a multi-rate Poisson Tree Processes (mPTP) analysis (https://mcmc-mptp.h-its.org/mcmc/) using the ML-generated tree, keeping all the parameters default (Kapli et al. 2017).

RESULTS AND DISCUSSION

Species diversity and status

A total of eleven species of freshwater barbs belonging to five genera; *Pethia* (5 species), *Puntius* (3 species), *Systomus* (1 species), *Oreichthys* (1 species) and *Osteobrama* (1 species) under the subfamily Smiliogastrinae were confirmed based on morphometric and meristic

characteristics. Among them, *Pe. ticto* and *Pu. terio* were confusing, as their descriptions and photographs provided in the published books were contradictory (Rahman 2005; Siddiqui et al. 2007). As such, we considered the description provided by Talwar and Jhingran (1991). According to IUCN Red Book (IUCN Bangladesh 2015), out of these eleven smiliogastrins, six species were ranked as least-concern (LC), three near threatened (NT), one as vulnerable (VU), and one as endangered (EN) (Table 1).

Molecular characterization, compositional bias, and saturation

A total of 24 COI sequences belonging to six genera and 11 species were generated. The BLAST analysis of each of our sequences (around 636 nucleotides, coding for 212 amino acids in length) revealed that they have 97–100% similarity with the pre-existing sequences of barb species in the NCBI database. Among the 557 nucleotide sites that we included in the final sequence alignment, 186 (33.39%) were variable, 371 (66.61%) were conserved, and 172 (30.88%) were parsimony-informative. The mean base composition in COI sequences of the Smiliogastrinae species showed a markedly low G content (17.46 ± 1.12%) compared to that of A, T, and C (27.04 ± 1.01%, 28.93 ± 0.55%, and 26.57 ± 0.66%, respectively) (Supplementary Table 1). Thus, the mean base composition in COI sequences of the species showed a lower average GC content (44.03 ± 1.50%) than the average AT content (55.97 ± 1.50%). To be specific, the average GC content of the three codon positions was 57.46 ± 1.08%, 42.77 ± 0.47%, and 31.90 ± 3.77%, respectively (Figure 2), indicating a significant decrease in the second and third codon positions compared to that of the first codon position, which reflects the pattern of AT and GC content observed in small indigenous fish species (SIS) of Bangladesh (Ahmed et al. 2019).

While working on cytochrome b gene of barbs, Tsigenopoulos and Berrebi (2000) reported that G was under-represented in the second and, particularly, in the third codon position (13% and 6.1%, respectively), but there was no such bias found in the first codon position. In our analysis, significant compositional biases were found to exist in the second and third codon positions of the COI gene, where the G content (14.22% and 7.00%, respectively) was noticeably low compared to 31.20% in the first codon position. However, the composition of the rest of the bases exhibited heterogeneity in all codon positions throughout the species in our study. For example, there was an underrepresentation of T (18.41%) in the first codon position, whereas T over-representation (41.61%) occurred in the second codon position. Such reverse representation was also observed for A in the second (15.62%) and third (41.34%) positions of the codons.

The Transition/Transversion bias (R) was estimated to be 3.58. Next, the substitution saturation analysis using DAMBE clearly depicted the linear increase of both transitions (s) and transversions (v) along with the no. of transitions exceeding the no. of transversions (Figure 3). This indicates that the COI gene sequences were well under the saturation threshold level, and therefore, the data still retained an ample phylogenetic signal for the estimation of true genetic distances.

In our study, all the species exhibited unique barcodes distinguishable from each other, and all individuals within a species and genus could be discriminated. The number of base substitutions per site from averaging over all sequence pairs between groups is shown in Table 2. The interspecies mean genetic divergence was 0.1538, while the mean intraspecies divergence was only 0.0058, indicating a high DNA barcoding gap. Therefore, there is no overlap in the range of intra- and interspecific COI sequence divergence of smiliogastrins. However, the presence of only a single sequence for 4 out of the 11 analyzed species is a limiting factor for obtaining any information about intraspecific variability.

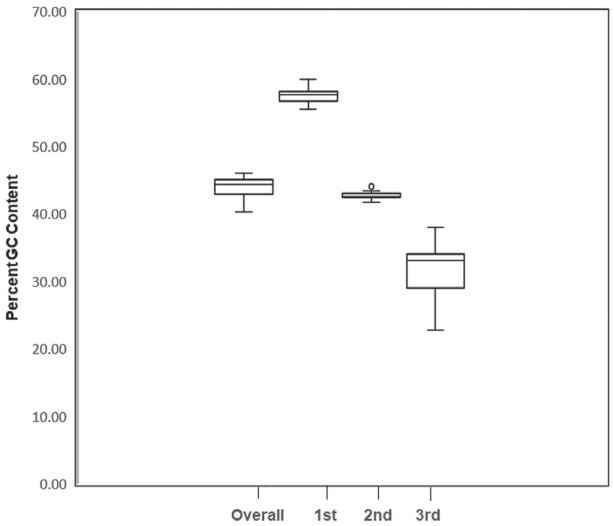

Figure 2. GC content (%) in different codon positions in the sequenced COI region of Smiliogastrinae fishes.

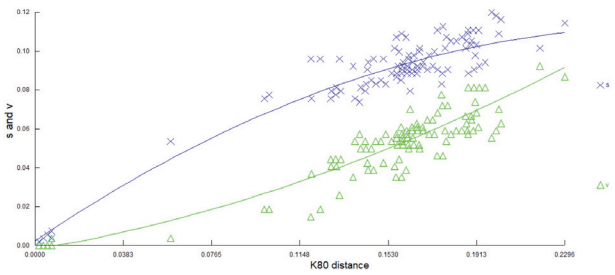

Figure 3. Transition (S) and transversion (V) values plotted against the K80 distance for COI gene of smiliogastrins.

Table 2. Estimates of evolutionary divergence over sequence pairs between groups using the K2P model.

	Pe. conchonius	*Pe. phutunio*	*Pe. gelius*	*Pu. sophore*	*Pu. chola*	*Pu. terio*	*Or. cosuatis*	*S. sarana*	*Pe. guganio*	*Pe. ticto*	*Os. cotio*
Pe. conchonius											
Pe. phutunio	0.127										
Pe. gelius	0.156	0.176									
Pu. sophore	0.184	0.164	0.158								
Pu. chola	0.166	0.189	0.159	0.116							
Pu. terio	0.188	0.200	0.192	0.118	0.124						
Or. cosuatis	0.201	0.219	0.195	0.181	0.202	0.202					
S. sarana	0.164	0.174	0.157	0.144	0.164	0.152	0.178				
Pe. guganio	0.172	0.175	0.150	0.181	0.171	0.172	0.227	0.157			
Pe. ticto	0.101	0.118	0.161	0.161	0.166	0.169	0.175	0.140	0.146		
Os. cotio	0.151	0.186	0.181	0.144	0.151	0.161	0.188	0.151	0.187	0.162	

All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 675 positions in the final dataset.

Species delimitation and molecular phylogenetic analysis

When we subjected the sequences to ASAP to build species partitions from single-locus sequence alignments, we generally found a good agreement between molecular operational taxonomic units (MOTUs) and morphological species, except for two taxa (*Os. cotio* and *Or. cosuatis*), which split into two well-differentiated clades (Figure 4). Thus, among the ten best ASAP partitions, 13 groups were identified based on the two lowest ASAP scores (partitions ranked first and second). For the partition with the "best" ASAP-score (2.50), the Proba (probability that the partition at step n is different from the partition at step n-1) and the barcode gap width were found to be 1.389759e-03 and 7.93e-03, respectively.

Although mPTP has been suggested to underperform consistently when there is a small number of species, and quite well when the number of species exceeds or is equal to 50 (Puillandre et al. 2020), our mPTP analysis was in congruence with the ASAP analysis, identifying 13 groups (Figure 4). Although *Os. cotio* specimens reported in our study were morphologically indistinguishable when we first identified them after their collection in 2015, both species delimitation analyses implied that one of them (Accession no. KT762359.1), placed as a distinct lineage separated from all the other *Os. cotio*, can be *Os. cotio* sensu lato.

The phylogenetic analyses of smiliogastrins using ML, NJ, and MP methods revealed similar clades in concordance with the taxonomic status of the eleven species under five genera (Figure 4). The ML tree distinctly formed seven lineages for the five genera: *Puntius, Systomus, Oreichthys*, *Osteobrama*, *Pe. gelius, Pe. guganio,* and the remaining *Pethia* spp. Considering the nodes supported by more than 70% of bootstrap replicates as robust in accordance with previous reports (Hillis and Bull 1993; Lecointre et al. 1994; Zharkikh and Li 1992), in our study, the three *Puntius* spp. form a well-supported clade, but with unclear relationships among its three species. However, this might change if other species were included in the analysis. As indicated by the low support in branching, *Pethia* spp. are weakly supported as monophyletic. Even though the relationship between the species *Pe. conchonius* and *Pe. ticto* was weakly supported by 54% bootstrap value, our tree indicated them to be sister taxa. Although no support exists, *S. sarana* and *Os. cotio* could be in a sister position to *Or. cosuatis* in the tree.

Based on the presented data, *Or. cosuatis* have diverged separately at an early stage of evolution from one in-group *Oreichthys* sp. reported from India (Figure 4). BLAST results showed 100% sequence similarity with the whole mitochondrial genome of *Or. crenuchoides* (GB AP012064.1) sequenced from Japan and 90.98% similarity with *Or. parvus* (GB JF915631.1) sequenced from Singapore, rather than *Or. cosuatis*. Therefore, we presume that this entry (GB FJ459528) might be a cryptic species and needs taxonomic revision. Otherwise, to confirm that the two distinct haplotypes have occurred due to the adaptation to different geographical distribution, a more comprehensive study, involving a larger sample size, has to be carried out.

Similarly, it is evident from our phylogenetic tree that the *Os. cotio* collected from Tanguar Haor is genetically divergent (pairwise *p*-distances 0.056) from the other

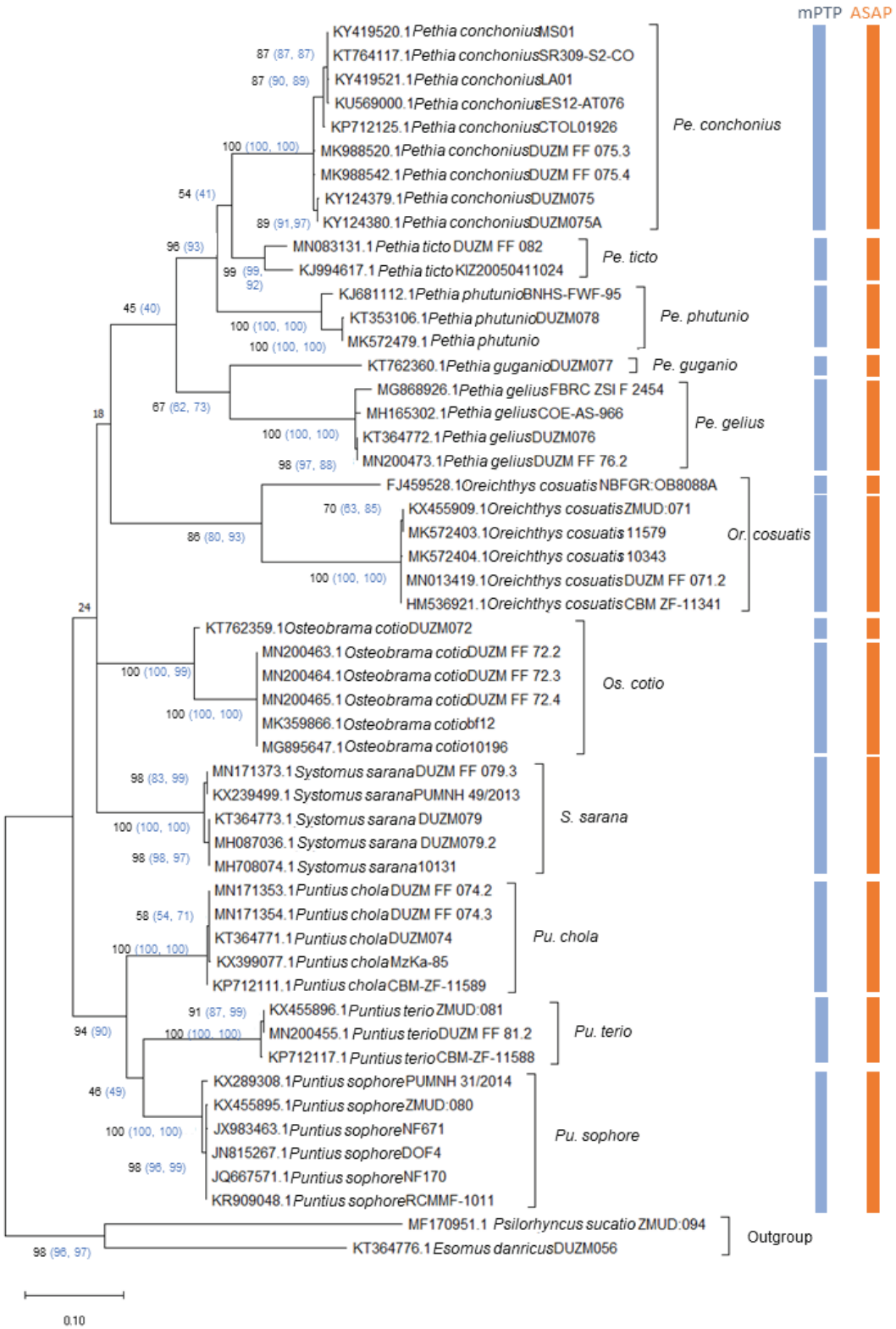

Figure 4. Summary of results obtained through different analyses of eleven smiliogastrin fishes. On the left: Molecular phylogenetic analysis generated from COI gene sequences employing the maximum likelihood method. The evolutionary analyses were conducted with 1000 bootstrap replicates (values are indicated next to the branches). The values in blue color inside brackets are the bootstrap values from the neighbor-joining and maximum parsimony methods. Sequences of the present study are designated as DUZM and ZMUD. On the right: Species delimitation analysis using mPTP and ASAP. The unique partition predicted by mPTP (blue vertical bars) and the partition with the "best" ASAP-score (2.50) using the K80 substitution model (orange vertical bars) are shown. Proba (probability that the partition at step n is different from the partition at step n–1) = 1.39e-03, barcode gap width = 7.93e-03. nb (number of species as identified by ASAP in the corresponding partition) groups = 13.

three specimens of *Os. cotio* amassed from Boiddar Bazar Ghat. However, not unlike the previous findings (Rahman et al. 2018) on specimens collected from different rivers of India and Bangladesh, we observed no distinct morphological difference among the *Osteobrama* species allowing their separation at sub-species level or their representation as different species, which contradicts the proposition put forward by Singh et al. (2018). Furthermore, an in-depth study with a larger sample size can solve the conundrum of this cryptic species.

In conclusion, the results of morphometric, meristic, and molecular analyses of Smiliogastrinae species of Bangladesh that were carried out in the present study were consistent, thus validating the COI barcode's effectiveness in accurately discriminating the species. Nevertheless, the low bootstrap value or the weakly supported nodes can be explained by the small sample size, which could be resolved if there were more in-groups and samples from closely related genera of the same family. The resulting phylogenetic trees from the analyses were largely congruent and revealed the same pattern of clustering for the studied populations with slightly different tree topologies, which differ only at weakly supported internal nodes (Figure 4). However, similarly to the previous studies (Tan and Armbruster 2018), we would like to stress the need for the further taxonomic revision of the Cyprinidae family so as to solve the conundrum of the cryptic species. More importantly, the frightening decline of barbs, especially the disappearance of *Pu. puntio*, *Pe. gelius, Pe. guganio* and *Pe. ticto* from natural habitats zeroes in on the need to introduce conservation measures for these species immediately, before they become locally extinct.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The sequence data have been submitted to the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers KX455895-96, KX455909, KY124379-80, KT353106, KT364771-73, KT762359-60, MH087036, MK988520, MK988542, MN013419, MN083131, MN171353-54, MN171373, MN200455, MN200463-65, MN200473 which have open public access.

REFERENCES

Ahmed, M. S., M. M. K. Chowdhury, and L. Nahar. 2019. Molecular characterization of small indigenous fish species (SIS) of Bangladesh through DNA barcodes. *Gene* 684: 53–57. https://doi.org/10.1016/j.gene.2018.10.048

Ahmed, M. S., S. K. Datta, and A. A. Zhilik. 2020. Molecular diversity of freshwater fishes of Bangladesh assessed by DNA barcoding. *Bangladesh Journal of Zoology* 48 (1): 1–19.

Banglapedia 2015. *National Encyclopaedia of Bangladesh*. http://en.banglapedia.org/index.php/Barb

Brower, A. V. Z. 2006. Problems with DNA barcodes for species delimitation: 'ten species' of *Astraptes fulgerator* reassessed (Lepidoptera: Hesperiidae). *Systematics and Biodiversity* 4: 127–132.

Collins, R. A., and R. H. Cruickshank. 2013. The seven deadly sins of DNA barcoding. *Molecular Ecology Resources* 13 (6): 969–75. doi: 10.1111/1755-0998.12046. Epub 2012 Dec 27. PMID: 23280099.

Dasmahapatra, K. K., M. Elias, R. I. Hill, J. I. Hoffman, and J. Mallet. 2010. Mitochondrial DNA barcoding detects some species that are real, and some that are not. *Molecular Ecology Resources* 10: 264–273.

Down, T. R., and W. W. Wilfinger. 1983. Fluorometric Quantification of DNA in Cells and Tissue. *Analytical Biochemistry* 131: 538–547.

Dupuis, J. R., A. D. Roe, and F. A. H. Sperling. 2012. Multilocus species delimitation in closely related animals and fungi: one marker is not enough. *Molecular Ecology* 21: 4422–4436.

Edgar, R. C. 2010. Quality measures for protein alignment benchmarks. *Nucleic Acids Research* 38 (7): 2145–2153. https://doi.org/10.1093/nar/gkp1196

Frézal, L., and R. Leblois. 2008. Four years of DNA barcoding: current advances and prospects. *Infection, Genetics and Evolution, Elsevier* 8 (5): 727–736. https://doi.org/10.1016/j.meegid.2008.05.005ï

Fujita, M. K., A. D. Leache, F. T. Burbrink, J. A. McGuire, and C. Moritz. 2012. Coalescent-based species delimitation in an integrative taxonomy. *Trends in Ecology and Evolution* 27: 480–488.

Haroon, A., G. Halder, S. Rahman, M. Razzaque, M. Alam, and S. Amin. 2002. *Sylhet-Mymensingh basin fish stock assessment. Final Report* 81. Chandpur, Bangladesh: Bangladesh Fisheries Research Institute, Riverine Station.

Hasegawa, M., H. Kishino, and T.-A. Yano. 1985. Dating of the Human-Ape splitting by a Molecular Clock of Mitochondrial DNA. *Molecular Evolution* 22: 160–174.

Hebert, P. D. N., A. Cywinska, S. L. Ball, and J. R. DeWaard. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences* 270 (1512): 313–321. https://doi.org/10.1098/rspb.2002.2218

Hillis, D. M., and J. J. Bull. 1993. Empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* 42 (2): 182–192. https://academic.oup.com/sysbio/article abstract/42/2/182/1730933?redirectedFrom=fulltext

Hossain, M. E., M. M. Chowdhury, and K. F. Iqbal. 2008. A Survey on the faunal diversity of Savar Upazilla, Dhaka, Bangladesh. *Pakistan Journal of Biological Sciences* 11 (3): 373–379.

Huang, X., and A. Madan. 1999. CAP3: A DNA sequence assembly program. *Genome Research* 9: 868–877. www.genome.orgIUCN

IUCN Bangladesh. 2015. *Red list of Bangladesh: Freshwater Fishes* (Vol. 5). International Union for Conservation of Nature and Natural Resources, IUCN Bangladesh Country Office.

Kapli, P., S. Lutteropp, J. Zhang, K. Kobert, P. Pavlidis, A. Stamatakis, and T. Flouri. 2017. Multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. *Bioinformatics* 33: 1630–1638.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Molecular Evolution* 16: 111–120.

Kumar, S., G. Stecher, M. Li, C. Knyaz, and K. Tamura. 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

Lecointre, G., H. Philippe, H. L. Vân Lê, and H. le Guyader. 1994. How many nucleotides are required to resolve a phylogenetic problem? The use of a new statistical method applicable to available sequences. *Molecular Phylogenetics and Evolution* 3 (4): 292–309. https://doi.org/10.1006/mpev.1994.1037

Leray, M., and N. Knowlton. 2015. DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. *PNAS* 112 (7): 2076–2081. https://doi.org/10.5883/DS-ARMS

Mian, S., M. J. Ferdous, M. Y. Sarker, M. J. Islam, A. M. Reza, M. M. Iqbal, and M. A. R. Hossain. 2013. Status of biodiversity and conservation of freshwater barbs in Bangladesh. *World Journal of Fish and Marine Sciences* 5 (6): 701–708. https://doi.org/10.5829/idosi.wjfms.2013.05.06.7665

Mohsin, A. B. M., S. M. M. Haque, S. M. Galib, M. F. H. Fahad, N. Chaki, M. N. Islam, and M. M. Rahman. 2013. Seasonal abundance of fin fishes in the Padma river at Rajshahi district, Bangladesh. *World Journal of Fish and Marine Sciences* 5 (6): 680–685. https://doi.org/10.5829/idosi.wjfms.2013.05.06.75109

Nei, M., and S. Kumar. 2000. *Molecular Evolution and Phylogenetics*. Oxford university press.

Puillandre, N., S. Brouillet, and G. Achaz. 2020. ASAP: Assemble Species by Automatic Partitioning. *Molecular Ecology Resources* 21 (7). DOI: 10.1111/1755-0998.13281.

Rahman, A. K. A. 2005. *Freshwater fishes of Bangladesh* (2nd ed.). Dhaka 1000: Zoological Society of Bangladesh, Department of Zoology, University of Dhaka.

Rahman, M. M., M. Norén, A. R. Mollah, and S. Kullander. 2018. The identity of *Osteobrama cotio*, and the status of "*Osteobrama serrata*" (Teleostei: Cyprinidae: Cyprininae). *Zootaxa* 4504 (1): 105–118. https://doi.org/10.11646/zootaxa.4504.1.5

Roos, N., M. A. Wahab, M. A. R. Hossain, and S. H. Thilsted. 2007. Linking human nutrition and fisheries: Incorporating micronutrient-dense, small indigenous fish species in carp polyculture production in Bangladesh. *Food and Nutrition Bulletin* 28 (2 Suppl.), S280–S293. https://doi.org/10.1177/15648265070282s207

Rubinoff, D., S. Cameron, and K. Will. 2006. A genomic perspective on the shortcomings of mitochondrial DNA for 'barcoding' identification. *Journal of Heredity* 97: 581–594.

Sambrook, J. F., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual* (2nd ed., Issue Ed. 2). Cold Spring Harbor, USA: Cold Spring Harbor Laboratory Press.

Siddiqui, K. U., M. A. Islam, S. M. H. Kabir, M. Ahmad, A. T. A. Ahmed, A. K. A. Rahman, E. U. Haque, Z. U. Ahmed, Z. N. T. Begum, M. A. Hasan, M. Khondker, and M. M. Rahman. 2007. *Encyclopedia of Flora and Fauna of Bangladesh* (Vol. 23). Asiatic Society of Bangladesh.

Singh, M., R. Verma, R. Yumnam, and W. Vishwanath. 2018. Molecular phylogenetic analysis of genus *Osteobrama* Heckel, 1843 and discovery of *Osteobrama serrata* sp. nov. from North East India. *Mitochondrial DNA Part A: DNA Mapping, Sequencing, and Analysis* 29 (3): 361–366. https://doi.org/10.1080/24701394.2017.1285289

Talwar, P. K., and A. G. Jhingran. 1991. *Inland Fishes of India and Adjacent Countries* (Vols. 1–2). Oxford-IBH Publishing Co. Pvt. Ltd.

Tamura, K., M. Nei, and S. Kumar. 2004. Prospects for infer-

ring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America* 101 (30): 11030–11035. https://doi.org/10.1073/pnas.0404206101

Tan, M., and J. W. Armbruster. 2018. Phylogenetic classification of extant genera of fishes of the order Cypriniformes (Teleostei: Ostariophysi). *Zootaxa* 4476 (1): 6–39. https://doi.org/10.11646/zootaxa.4476.1.4

Tsigenopoulos, C. S., and P. Berrebi. 2000. Molecular phylogeny of north Mediterranean freshwater barbs (genus *Barbus*: Cyprinidae) inferred from cytochrome b sequences: Biogeographic and systematic implications. *Molecular Phylogenetics and Evolution* 14 (2): 165–179. https://doi.org/10.1006/mpev.1999.0702

Ward, R. D., T. S. Zemlak, B. H. Innes, P. R. Last, and P. D. N. Hebert. 2005. DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B: Biological Sciences* 360 (1462): 1847–1857. https://doi.org/10.1098/rstb.2005.1716Xia, X. 2017. DAMBE6: New tools for microbial genomics, phylogenetics, and molecular evolution. *Journal of Heredity* 108 (4): 431–437. https://doi.org/10.1093/JHERED/ESX033

Yang, L., T. Sado, M. Vincent Hirt, E. Pasco-Viel, M. Arunachalam, J. Li, X. Wang, J. Freyhof, K. Saitoh, A. M. Simons, M. Miya, S. He, and R. L. Mayden. 2015. Phylogeny and polyploidy: Resolving the classification of cyprinine fishes (Teleostei: Cypriniformes). *Molecular Phylogenetics and Evolution* 85: 97–116. https://doi.org/10.1016/j.ympev.2015.01.014

Zharkikh, A., and W. Li. 1992. Statistical properties of bootstrap estimation of phylogenetic variability from nucleotide sequences. I. Four taxa with a molecular clock. *Molecular Biology and Evolution* 9 (6). https://doi.org/10.1093/oxfordjournals.molbev.a040782 GenBank https://www.ncbi.nlm.nih.gov/genbank/

Supplementary Table 1. Analysis of nucleotide compositions for the obtained mitochondrial COI gene length of the species explored in this study.

Species	Accession no.	Length of obtained sequence (bp)	Base composition (%)					
			T	C	A	G	AT	GC
Pu. sophore	KX455895.1	675	29.78	25.63	27.56	17.04	57.33	42.67
Pu. chola	KT364771.1	668	27.40	27.54	26.65	18.41	54.04	45.96
	MN171353.1	614	27.36	28.18	26.55	17.92	53.91	46.09
	MN171354.1	617	27.23	28.04	26.74	17.99	53.97	46.03
Pu. terio	KX455896.1	671	29.96	25.04	27.12	17.88	57.08	42.92
	MN200455.1	604	30.79	24.67	27.32	17.22	58.11	41.89
Pe. conchonius	KY124379.1	634	28.71	26.34	26.34	18.61	55.05	44.95
	KY124380.1	643	29.08	26.59	25.82	18.51	54.90	45.10
	MK988520.1	620	28.87	27.10	26.45	17.58	55.32	44.68
	MK988542.1	622	28.78	27.01	26.21	18.01	54.98	45.02
Pe. gelius	KT364772.1	668	29.34	26.50	25.60	18.56	54.94	45.06
	MN200473.1	607	29.65	26.69	25.86	17.79	55.52	44.48
Pe. phutunio	KT353106.1	669	28.85	26.16	25.86	19.13	54.71	45.29
Pe. guganio	KT762360.1	669	29.00	26.31	26.16	18.54	56.44	43.56
Pe. ticto	MN083131.1	566	30.74	24.56	28.98	15.72	59.72	40.28
S. sarana	KT364773.1	668	28.29	26.50	28.14	17.07	56.49	43.51
	MH087036.1	655	28.55	26.56	27.94	16.95	56.86	43.14
	MN171373.1	612	28.27	26.80	28.59	16.34	55.16	44.84
O. cosuatis	KX455909.1	675	30.96	26.22	26.96	15.85	57.93	42.07
	MN013419.1	624	31.41	26.28	26.92	15.38	58.33	41.67
O. cotio	KT762359.1	669	27.65	27.20	27.95	17.19	55.61	44.39
	MN200463.1	605	27.93	27.27	27.77	17.02	55.70	44.30
	MN200464.1	607	27.84	27.18	28.01	16.97	55.85	44.15
	MN200465.1	605	27.93	27.27	27.77	17.02	55.70	44.30
Average ± SD		636	28.93 ± 0.55	26.57 ± 0.66	27.04 ± 1.01	17.46 ± 1.12	55.97 ± 1.50	44.03 ± 1.50

Supplementary Table 2. Information about ingroup and outgroup sequences utilized for the phylogenetic analysis in the study.

Species	GenBank Accession No.	Country of Origin	Author/ Reference
Pe. conchonius	KP712125.1	India: Market between Kolkata and Basirhat, North 24 Parganas, West Bengal	Yang, L., T. Sado, M. Vincent Hirt, E. Pasco-Viel, M. Arunachalam, J. Li, X. Wang, J. Freyhof, K. Saitoh, A. M. Simons, M. Miya, S. He, and R. L. Mayden. 2015. Phylogeny and polyploidy: Resolving the classification of cyprinine fishes (Teleostei: Cypriniformes). *Molecular Phylogenetics and Evolution* 85: 97–116. https:doi.org/10.1016/j.ympev.2015.01.014
	KY419520.1	India: Tamilnadu	Direct Submission by Rajasekaran, N., S. Chandrasekar, and R. Sivakumar, Molecular identification of *Pethia conchonius* from South Indian rivers (Unpublished)
	KY419521.1		
	KT764117.1	India: Tamilnadu	Direct Submission by Sabaridasan, A., and R. Soranam (Unpublished)
	KU569000.1	South Africa: Tyger, Cape Town, Western Cape	van der Walt, K. A., T. Mäkinen, E. R. Swartz, and O. Weyl. 2017. DNA barcoding of South Africa's ornamental freshwater fish-are the names reliable? *African Journal of Aquatic Science* 42 (2). https://doi.org/10.2989/16085914.2017.1343178
Pe. phutunio	KJ681112.1	India: Sambalpur, Odisha	Katwate, U., C. Katwate, R. Raghavan, M. S. Paingankar, and N. Dahanukar. 2014. *Pethia lutea*, a new species of barb (Teleostei: Cyprinidae) and new records of *P. punctata* from northern Western Ghats of India. *Journal of Threatened Taxa* 6 (6): 5797–5818. https://doi.org/10.11609/jott.o3929.5797-818
	MK572479.1	Bangladesh: Hakaluki Haor in Borolek, Moulvibazar, Sylhet	Rahman, M. M., M. Norén, A. R., Mollah, and S. O. Kullander. 2019. Building a DNA barcode library for the freshwater fishes of Bangladesh. *Scientific Reports* 9 (1). https://doi.org/10.1038/s41598-019-45379-6
Pe. gelius	MH165302.1	India: Agartala, Tripura	Direct Submission by Parhi, J., N. Chaoba Devi, H. Priyadarshi, P. Biswas, and P. K. Pandey.
	MG868926.1	N/A	Direct Submission by Laskar, B. A., A. Harikumar, S. Mandal, S. Rehanuma, and A. D. Narahari. J. DNA Barcoding of Eastern Ghats Fauna (Unpublished)
Pe. ticto	KJ994617.1	China	Zheng, L. P., J. X. Yang, and X. Y. Chen. 2016. Molecular phylogeny and systematics of the Barbinae (Teleostei: Cyprinidae) in China inferred from mitochondrial DNA sequences. *Biochemical Systematics and Ecology* 68: 250–259. https://doi.org/10.1016/j.bse.2016.07.012
Pu. sophore	JQ667571.1	India: Anchinal River, Harda, Madhya Pradesh	Khedkar, G. D., R. A. Jamdade, R. H. Hanner, and P. D. N. Hebert. DNA Barcoding can Help Ornamental Fish Trading in Changing Regime of Indian Biodiversity Act (Unpublished)
	JX983463.1	India: Narmada ghat, Narmada River, Hoshangabad, Madhya Pradesh	Khedkar, G. D., R. Jamdade, S. Naik, L. David, and D. Haymer. 2014. DNA barcodes for the Fishes of the Narmada, India's longest rivers. *PLoS ONE* 9 (7). https://doi.org/10.1371/journal.pone.0101460
	JN815267.1	India	Dhar, B., and S. Ghosh. 2015. Genetic assessment of ornamental fish species from North East India. *Gene* 555 (2): 382–392. https://doi.org/10.1016/j.gene.2014.11.037
	KR909048.1	India: Manipur	Direct Submission by D. N. Sobita, and D. C. Basudha. Taxonomic relationship of some cyprinid fishes of Manipur, India (Unpublished)
	KX289308.1	India	Raja, M., and P. Peruma. 2017. DNA barcoding and phylogenetic relationships of selected South Indian freshwater fishes based on mtDNA COI sequences. *Journal of Phylogenetics & Evolutionary Biology* 5 (184). https://doi.org/10.4172/2329-9002.1000184
Pu. chola	KX399077.1	India	Barman, A. S., M. Singh, and P. K. Pandey. 2018. DNA barcoding and genetic diversity analyses of fishes of Kaladan River of Indo-Myanmar biodiversity hotspot. *Mitochondrial DNA Part A: DNA Mapping, Sequencing, and Analysis* 29 (3): 367–378. doi:10.1080/24701394.2017.1285290

Species	GenBank Accession No.	Country of Origin	Author/ Reference
Pu. terio	KP712117.1	India: Jagatpur Market, Kolkata, West Bengal	Yang, L., T. Sado, M. Vincent Hirt, E. Pasco-Viel, M. Arunachalam, J. Li, X. Wang, J. Freyhof, K. Saitoh, A. M. Simons, M. Miya, S. He, and R. L. Mayden. 2015. Phylogeny and polyploidy: Resolving the classification of cyprinine fishes (Teleostei: Cypriniformes). *Molecular Phylogenetics and Evolution* 85: 97–116. https://doi.org/10.1016/j.ympev.2015.01.014.
S. sarana	KX239499.1	India	Raja, M., and P. Perumal. 2017. DNA barcoding and phylogenetic relationships of selected South Indian freshwater fishes based on mtDNA COI sequences. *Journal of Phylogenetics & Evolutionary Biology* 5 (184). https://doi.org/10.4172/2329-9002.1000184
	MH708074.1	Bangladesh: Pyiang River at Jaflong, about 60 km from Sylhet city	Rahman, M. M., M. Norén, A. R. Mollah, and S. Kullander. 2018. The identity of *Osteobrama cotio*, and the status of "*Osteobrama serrata*" (Teleostei: Cyprinidae: Cyprininae). *Zootaxa* 4504 (1): 105–118. https://doi.org/10.11646/zootaxa.4504.1.5
Or. cosuatis	HM536921.1	India	Yang, L., R. L. Mayden, T. Sado, S. He, K. Saitoh, and M. Miya. 2010. Molecular phylogeny of the fishes traditionally referred to Cyprinini *sensu stricto* (Teleostei: Cypriniformes). *Zoologica Scripta* 39 (6): 527–550. https://doi.org/10.1111/j.1463-6409.2010.00443.x
	MK572403.1	Bangladesh: Surma River left bank, at Kheaghat point, 1.5 km upstream from Golapganj, Sylhet	Rahman, M. M., M. Norén, A. R. Mollah, and S. O. Kullander. 2019. Building a DNA barcode library for the freshwater fishes of Bangladesh. *Scientific Reports* 9 (1). https://doi.org/10.1038/s41598-019-45379-6
	MK572404.1	Bangladesh: Padma River near Srinagar, Dhaka	Rahman, M. M., M. Norén, A. R. Mollah, and S. O. Kullander. 2019. Building a DNA barcode library for the freshwater fishes of Bangladesh. *Scientific Reports* 9 (1). https://doi.org/10.1038/s41598-019-45379-6
	FJ459528.1ǂ	India: Assam	Lakra, W. S., M. Singh, M. Goswami, A. Gopalakrishnan, K. K. Lal, V. Mohindra, U. K. Sarkar, P. P. Punia, K. v. Singh, J. P. Bhatt, and S. Ayyappan. 2016. DNA barcoding Indian freshwater fishes. *Mitochondrial DNA Part A: DNA Mapping, Sequencing, and Analysis* 27 (6): 4510– 4517. https://doi.org/10.3109/19401736.2015.1101540
Os. cotio	MK359866.1	Republic of Korea: Busan	Direct Submission by Alam, M. J., S. Andriyono, S. P. Sektiana, A. Eunus, and H. -W. Kim.
	MG895647.1	Bangladesh: Town fish market (Principally from Kaptai Lake), Rangamati, Chittagong	Rahman, M. M., M. Norén, A. R. Mollah, and S. Kullander. 2018. The identity of *Osteobrama cotio*, and the status of "*Osteobrama serrata*" (Teleostei: Cyprinidae: Cyprininae). *Zootaxa* 4504 (1): 105–118. https://doi.org/10.11646/zootaxa.4504.1.5
Psilorhynchus sucatio¶	MF170951.1	Bangladesh: Chittagong University	Ahmed, M. S., M. M. K. Chowdhury, and L. Nahar. 2019. Molecular characterization of small indigenous fish species (SIS) of Bangladesh through DNA barcodes. *Gene* 684: 53–57. https://doi.org/10.1016/j.gene.2018.10.048
Esomus danricus¶	KT364776.1	Bangladesh: Tanguar Haor, Sunamganj	Ahmed, M. S., M. M. K. Chowdhury, and L. Nahar. 2019. Molecular characterization of small indigenous fish species (SIS) of Bangladesh through DNA barcodes. *Gene* 684: 53–57. https://doi.org/10.1016/j.gene.2018.10.048

¶ = *Outgroup*, ǂ = Cryptic species.