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DNA Barcoding depicts cryptic diversity within Barilius bendelisis (Cypriniformes: Cyprinidae) from India

Saha ABHISEK[1], Singh MAHENDER[2], Das DEBASHIS[3], De Chandra GOBINDA[4], Vyas VIPIN[5], Gurjar PARUL[6], Singha DEVKANT[7]

[1,3]Tufanganj College, Coochbehar, West Bengal, 736160 India
[2]National Bureau of Fish Genetic Resources, Lucknow, Uttar Pradesh, 226 002 India
[4]Coochbehar Panchanan Barma University, Coochbehar, West Bengal, 736101 India
[5]Department of Bioscience, Barkatullah University, Bhopal, Madhya Pradesh, 462026
[6]Department of Environmental Science, Barkatullah University, Bhopal, Madhya Pradesh, 462026
[7]Centre for DNA Taxonomy, MSD, Zoological Survey of India, Kolkata, West Bengal, 700053 India
https://orcid.org/0000-0002-0670-520X

E-mail: devkant144@gmail.com

Abstract: Members of the genus Barilius are known for their economic values. However, due to human activities and natural calamities, Barilius bendelisis faces massive population loss, especially in eastern India. We applied both morphology and molecular approaches to identify Barilius bendelisis from transboundary river Torsa, Raidak-1 and Mansai (Jaldhaka), West Bengal, India. Further, we compared genetic divergence of Barilius bendelisis from other members of the genus Barilius available in India. The Bayesian (BA) phylogeny clearly distinguishes all the studied species with reciprocal monophyletic criteria and represents multiple clades within Barilius bendelisis, indicating cryptic diversity and probable occurrence of allopatric speciation within India.

Keywords: Barilius bendelisis, DNA barcoding, cryptic diversity, allopatric speciation, India.

1. INTRODUCTION:

India comprises of diverse Ichthyofauna with 868 freshwater fishes, of which 192 species are enlisted as endemic and 327 species as threatened by IUCN. The Genus *Barilius* are small to moderate-sized fishes commonly known as Bariline fishes, inhabits in medium to fast-flowing torrential mountain streams of Asia (Dishmaand & Vishwanath 2012). Members of the genus Barilius are characterized by compressed body, blue-black bars or spots on the body and dorsal fin inserted behind the middle of the body (Hamilton 1822). So far, 36 species of bariliine fishes are reported globally, and 24 have been enlisted in India (Fricke et al. 2019, Qin et al. 2019).The conservation status of this *Barilius*species are marked as 'lower risk near threatened' (LRnt) according to the CAMP (Conservation Assessment and Management Plan) report for freshwater fishes of India (Molur & Walker

1988). *Barilius bendelisis* locally known as 'Boroli', is a tropical freshwater species with economic significance due to ornamental value and potential food fish (Mishra et al. 2012).However, despite economic and ecological importance, *Barilius bendelisis* is facing a rapid reduction in India, transboundary river Torsa, Raidak-1 and Mansai (Jaldhaka), West Bengal, India. due to overfishing, habitat loss, hydrologic modification and water pollution (Mishra et al. 2012). For example, in recent years, *Barilius bendelisis* has become infrequent in the sub-Himalayan transboundary river i.e., Torsa, Raidak-1 and Mansai (Jaldhaka) River, West Bengal, India (Sah et al. 2011). Moreover, due to the high degree of phenotypic plasticity, sexual dimorphism, and lack of available identification keys for immature stages, the identification *of Barilius species,* including *B. bendelisis* has been a major issue to be addressed (Mishra et al. 2012). The transboundary river Torsa, Raidak-1 and Mansai (Jaldhaka) originates from the Sikkim, Darjeeling, Bhutan and Tibetan Himalayas and enters Bangladesh through Jalpaiguri district, West Bengal, India.

For the last one and half decades, molecular tools, especially DNA barcoding, have successfully demonstrated their efficacy in determining fish diversity worldwide, including in India (Khedkar et al. 2014). Besides species identification, DNA barcoding also evidenced as an effective tool for resolving several taxonomic quagmires of fishes such as species complexes and cryptic diversity (Laskar et al. 2013, 2018). However, most of the integrated studies has been limited to identification of Indian ichthyo faunal diversity from different riverine ecosystem. The present study aimed to fill the research gap in *Barilius* species, with special reference to *Barilius bendelisis* by inferring phylogenetic studies and genetic divergence from generated and available COI sequences from India.

2. MATERIALS AND METHODS

Morphological investigation

A total of nine specimens of *Barilius bendelisis* were collected from the transboundary river Torsa (26°17'13"N, 89°27'33"E), Raidak-1 (26°18'39"N, 89°40'14"E), and Mansai (Jaldhaka) (26°19'10"N, 89°14'23"E), West Bengal, India [Figure 1(A)]. The specimens were collected using a cast net and identified by the available taxonomic keys (Talwar & Jhingran 1991, Srivastava 1992, Menon 1999, Jayaram 2010). After morphological and molecular analysis, the specimens were preserved in 10% formalin. A stereo-zoom light microscope was used to calculate fin rays (Kottelat 1990, Kottelat 2001). The voucher specimens are deposited in the laboratory of the Molecular Biology and Biotechnology Division, the National Bureau of Fish Genetic Resources, Lucknow, Uttar Pradesh, India.

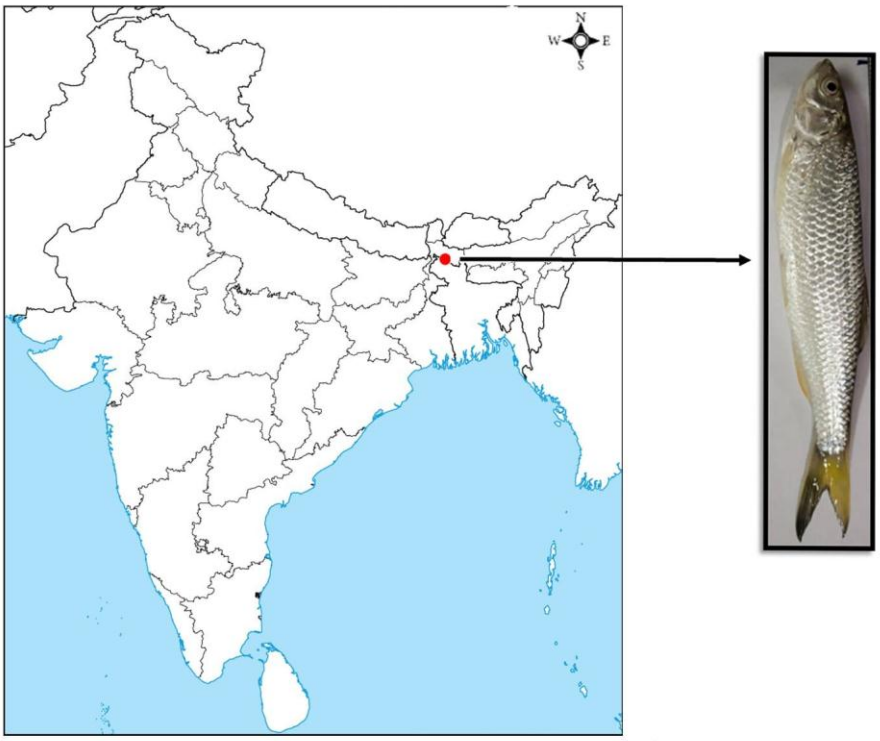

Figure 1. (A) Map showing the collection localities of *Barilius bendelisis*.

Molecular investigation

A required amount of muscle tissue was collected aseptically from specimen for molecular study and stored at the Laboratory of Molecular Biology & Biotechnology Division, National Bureau of Fish Genetic Resources(NBFGR), Lucknow-226002 India for future reference. The total genomic DNA was extracted by using the NucleoSpin® Tissue XS Kit (Macherey-Nagel, Germany). The Polymerase Chain Reaction (PCR) was performed by using published primer pairs: COIF1 (5'TCAACCAACCACAAAGACATTGGCAC- 3') and COIR1 (5'-TAGACTTCTGGGTGGC CAAAGAATCA-3') (24) in VeritiVR Thermal Cycler (Applied Biosystems, Foster City, CA). The PCR products were purified and sequenced by using the published protocol (Laskar et al. 2013). The generated bi-directional chromatograms were checked through MEGA6 (Tamura et al. 2013) to trim the noisy part and made the consensus sequences. The online tools, BLAST (https://blast.ncbi.nlm.nih.gov) and ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) were used to check the indels (insertion/deletions) and stop codons. The generated sequences were further checked through the similarity search in National Centre for Biological Information (NCBI) and Barcode of Life Data System (BOLD) databases. The final dataset was prepared with 66 COI sequences of *Barilius* including the generated sequences of *B. bendelisis* (accession no. MN810961-MN810965, MN994439-MN994442). Tor tor (JX983505) was taken as an out-group in the present study. The dataset was aligned using ClustalX software (Thompson et al. 1997). The genetic divergence was calculated in MEGA6 using the Kimura-2-Parameter (K2P) model.

To test the reciprocal monophyletic criteria for species delimitation, Bayesian analysis (BA) was implemented. The best-fit model candidate model was estimated to be GTR+I+G using PartitionFinder version 1.1.1 with the lowest Bayesian Information Criterion score. The BA was performed in MrBayes 3.1 (Ronquist & Huelsenbeck 2003) with the Markov Chain Monte Carlo (MCMC) run for 10,000,000 generations with trees sampled every 100 generations (the first 1000 trees were disposed as 'burn in'). The MCMC analysis was steady when the determined standard deviation of split frequencies reached below 0.01, and the potential scale reduction factor (PSRF) approached 1.0. The generated BA tree was visualized using the web based tool Interactive Tree Of Life (iTOL) (Letunic & Bork 2007).

3. RESULT:

Morphological identification and Morphometric analysis:
The diagnostic characteristics of the examined *Barilius bendelisis* were compared with the earlier studies, which agree sufficiently with the description given by the researcher (Jayaram & Singh 1977, Rahman 1977, Rahman 1989, Kundu 2000). The studied species were identified and morphometric analyses were conducted with the prominent characters (Table 1) of the shiny body with dark greyish bands towards the horizontal line.

Table-1: Morphometric measurements of *Barilius bendelisis*.

Morphometric Measurements	***Barilius bendelisis***
Standard length/ Head length	3.63-4.12. (M 3.87)
Standard length/ Pre pelvic length	1.94-2.01. (M 1.97)
Standard length/ Body depth	3.32-3.80. (M 3.56)
Standard length/ Predorsal length	1.58-1.65. (M 1.61)
Head length/ Eye diameter	4.70-4.79. (M 4.74)
Standard length/Pre anal length	1.40-1.46. (M 1.44)
Head length/Head height	1.30-1.42. (M 1.34)
Interorbital width/Snout length	1.00-1.36. (M 1.18)
Standard length/Height of anal fin	6.18-7.31. (M 6.74)
Standard length/Pectoral fin length	4.80-5.01. (M 4.90)
Interorbital width/Eye diameter	1.58-1.76. (M 1.67)
Standard length/Pelvic fin length	6.80-7.30. (M 7.05)
Head length/Snout length	2.60-2.68. (M 2.64)
Length of caudal peduncle/width of caudal peduncle	1.30-1.42. (M 1.36)
Head length/Head width	1.70-1.88. (M 1.79)
Standard length/Height of dorsal fin	4.90-5.42. (M 5.16)
Standard length/Length of caudal peduncle	5.00-5.90. (M 5.45)
Caudal fin rays	18
Anal fin rays	ii-iii,8-9
Dorsal fin rays	ii,7
Pectoral fin rays	I,11-12
Pelvicfinrays	i,8
Lateral line scales(L.I)	40-43
Lateral line scales(L.tr)	10-13

Predorsal scales	19-22
Circumpeduncular scales	11
Number of barbels	Two pairs, well-developed
Number. of blotch/bars	Scales bears a black spot at the base

M=Mean, All length are measured in mm scale.

Body type is elongated and compacted. The ventral shape is more convex than that of the dorsal. Size accomplishes 138 mm approx, in total length. Snout bears tubercles. The posterior extremity of the maxilla reaches below the first third of the orbit. Eye diameter shows a mean value of 4.74 (4.70-4.79) in head length. Head length 3.87 (3.63-4.12) and body depth 3.56 (3.32-3.80) in standard length. Barbels two pairs (Hora 1921, Tilak 1967) shorter than eye diameter. Height of anal 6.74 (6.18-7.31), dorsal 5.16 (4.90-5.42), pelvic length 7.05 (6.80-7.30), pectoral length 4.90 (4.80-5.01) in the standard length. Pectoral may or may not attain the pelvic region and highly robust and inflamed in adult male specimens. Lateral sides of immature specimens with 7-9 bars and slowly disappear in the adult specimens. The extent of the postorbital part of the head is less than twice the snout length. The minimum elevation of the caudal peduncle is 1.36 (1.30 - 1.42) in its length, Height of head 1.34 (1.30 - 1.32), the width of head 1.79 (1.70 - 1.88) in the head length. Pre pelvic distance 1.97 (1.94 - 2.01), pre dorsal distance, 1.61 (1.58 - 1.65), Snout length 2.64 (2.60 - 2.68) in the head length, 1.18 (1.00 - 1.36) in inter orbital width. Caudal peduncle length was noted 5.49 (5.10-5.88) in the standard length. Least height of the caudal peduncle 1.35 (1.30-1.41) in its length. The pre anal distance was calculated at 1.43 (1.40 - 1.47). Length and width measurements were done on an mm scale. All the studied Specimens belonging to *Barilius bendelisis* showed black dots at the base of all scales with double spots on the lateral line. Fins are whitish tinged with colorful orange. The boundary of dorsal and caudal fins is grayish. The fins are yellow-tinted with black edges. Observed fin structure noted as Dorsal rays ii, 7; anal rays ii-iii, 8-9; pectoral rays i, 11-12; pelvic rays i, 8 and caudal rays 18. The dorsal fin was commencing closer to the base of the caudal fin than the snout and higher than its base is long which never extends to over anal fin. The caudal fin divided and the lower lobe is a little bit longer than the upper part. Brilliant yellowish operculum, greenish snout, orange-hued lower jaw, little tubercles on the two jaws, orange edges on the paired and anal fins, and with yellowish yet dark margined caudal fin. Each scale in the adult is with a dark spot at its base. Predorsal scale counts 19-22 and circumpeduncular scale counts 11. Lateral line scale counts noted with a total of 40-43 scales. Lateral line and base of pelvic fin scales covered with 11-13 scales (Negi et al. 2002).

Molecular Investigation:

Integrated study of classical taxonomy and DNA barcoding has been adopted globally for illuminating species diversity, cryptic diversity, species complexes and route of invasion of invasive species (Hebert et al. 2003, Tyagi et al. 2017, Singha et al. 2018).A total of nine COI sequences of *Barilius bandelisis* were generated in the present study collected from the

Torsa, Raidak-1 and Mansai (Jaldhaka) River, West Bengal, India. The final dataset of ~648 bp were prepared with 67 nucleotide sequences representing 10 species of *Barilius* (*B. ardens*, *B. bakeri*, *B. barna*, *B. bendelisis*, *B. gatensis*, *B. malabaricus*, *B. ngawa*, *B. tileo*, *B. vagra*), *Tor tor* (JX983505) was taken as an out-group. The BA phylogeny illustrated cohesive clustering of the generated sequences with the representative database sequences [Figure 1(B)].

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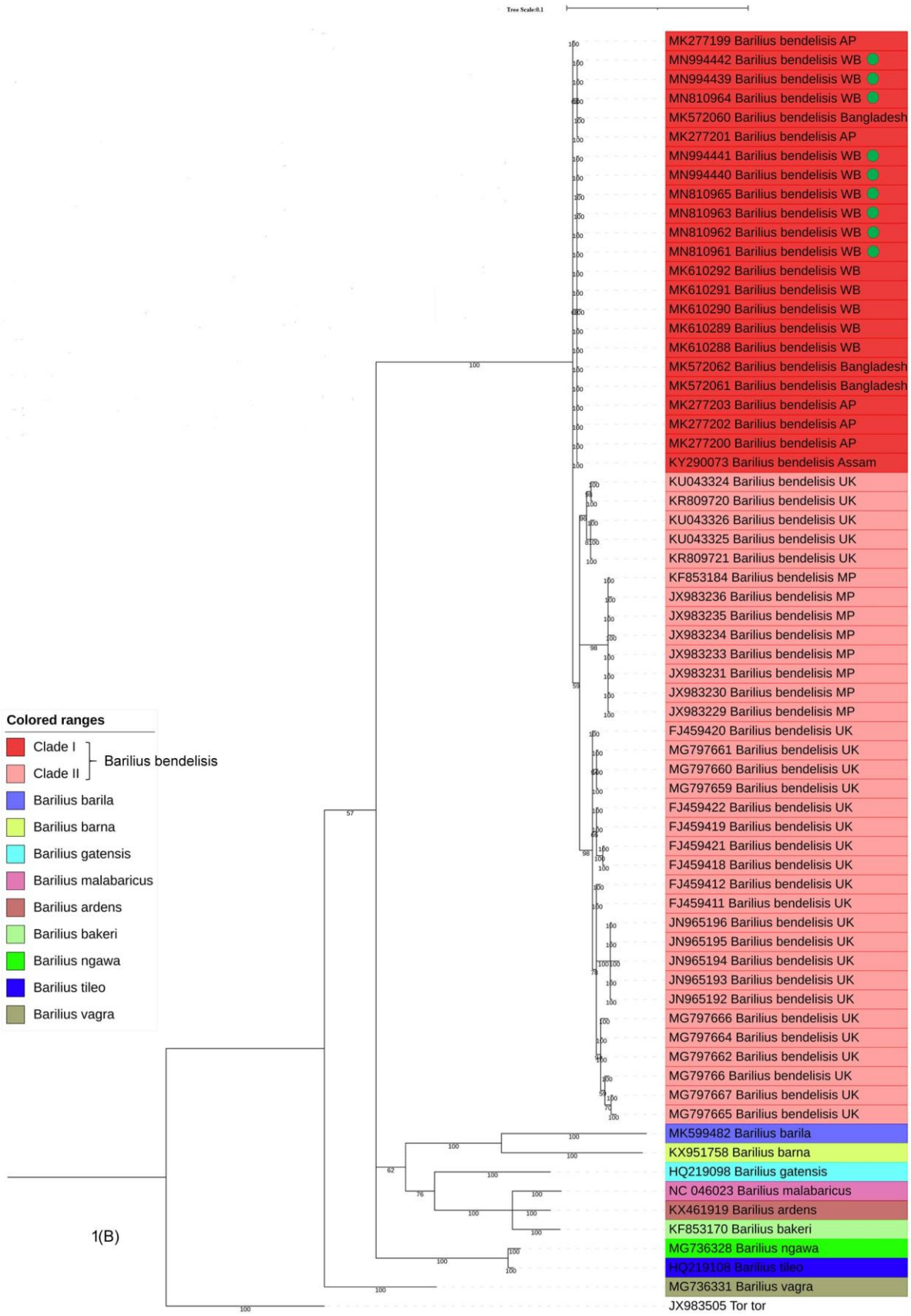

Figure 1.(B) Bayesian phylogeny with posterior probability showed the monophyletic clustering with possible cryptic diversity in *B. bendelisis*. (AP- Arunachal Pradesh; MP- Madhya Pradesh; UK- Uttarakhand; WB- West Bengal).

The phylogenetic tree displayed 11 distinct lineages of 10 morphospecies, with multiple clades within *Barilius bandelisis* (Clade I and Clade II). The phylogeny indicated *Barilius barila* as the closely related species of *Barilius bandelisis* and *Barilius vagra* as the distant species.

Table 2: The species level and Clades level K2P genetic divergence of the studied Barilius species.

Species level										Clade level	
Species	Mean inter-specific (%)									No. of estimated sub-clades	Inter-sub-clade (%)
Bariliusbendelisis										*B. bendelisis* I	
Bariliusardens	18.8									*B. bendelisis* II	2.3
Bariliusbakeri	17.8	5.90									
Bariliusbarila	19.2	17.4	17.2								
Bariliusbarna	21	18.3	17.8	16.1							
Bariliusgatensis	19.9	13.7	14.1	19.6	18						
Bariliusmalabaricus	19.1	5.7	6.2	19	18.3	13.9					
Bariliusngawa	19.7	16.3	18.2	19.6	19.9	15.7	15.7				
Bariliustileo	19.8	15.7	17.1	19.6	20.6	15.3	15	10			
Bariliusvagra	17.2	16.7	15.1	17.1	18.7	17.2	16.9	18	17.8		

The overall mean genetic divergence of the present dataset was estimated to be 6.1%. The highest mean intraspecific genetic divergence of 17.1% were observed within *Barilius bandelisis*. However, the mean highest interspecific genetic divergence (20.6%) was observed between *B. barna* and *B. tileo* (Table 2). The two clades of *B. bendelisis* depicted an average of 2.3% genetic divergence in the present dataset. Previous study suggested a barcode gap of ~2% for conspecific divergences in fishes. Multiple clades of *B. bendelisis* with supportive genetic divergence indicated cryptic diversity and probable occurrence of allopatric speciation of *B. bendelisis* within eastern, north-eastern, central and northern India.

However, in-depth taxonomic studies with multiple molecular markers needed for validating the cryptic diversity. Altogether, this study was a preliminary approach for estimation of genetic divergence of the genus *Barilius* from India.

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