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DNA Barcoding of Four Ornamental Fishes of Genus *Botia* from Eastern Himalaya

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Abstract: ***Botia loaches are high demanding species having both ornamental and economical important food value and contribute to a major share of the world market for beautiful coloured indigenous ornamental fish. Their identification is difficult due to morphological variation. Genetic variation amongst the four species namely Botia alomorhae, Botia dario, Botia lohachata and Botia rostrata using mtDNA were studied. Cytochrome Oxidase I (COI) gene (655 bp) was amplified using PCR and sequenced. The pairwise genetic distances among Botia species ranged from 0.002 to 0.112. The interspecies Kimura's 2- parameter pair-wise distance was highest (0.112) between Botia dario and B. lohachata and lowest (0.002) for B.almorhae and B. lohachata. The phylogenetic tree showed that B. almorhae and B. lohachata formed a monophyletic group (supported by 100% bootstrap value). The present study is helpful for identification of the Endangered and Vulnerable Botia species from Eastern Himalayan region and population studies, management and conservation programs.***

Keywords: Genus *Botia*, Cytochrome Oxidase subunit I gene, DNA Barcoding

1. Introduction

Ornamental fishes are called 'living jewels' for their beautiful colour and playful behaviour and are typically small sized; attractive and bizarre shaped in appearance [4]. The tropical ornamental fishes from North Eastern and Southern provinces of India are in great demand in the hobbyists market like loaches, barbs, badis, zebra fishes, catfishes and glass fishes. Terai and Dooars regions of Eastern Himalaya are considered as "hot spot" for fresh water fish biodiversity [3]. A great number of species have been reported from Cooch Behar district on fish biodiversity and 10 species of loaches are available in Cooch Behar district [3]. The fishes of the family Cobitidae are popularly known as 'Loach'. The loaches are high demanding species having both ornamental and economical important food value and contribute to a major share of the world market for beautiful coloured indigenous ornamental fish. Among the loaches, *Botia dario* (Hamilton-Buchanan) commonly known as "Queen loach*"* or "Rani Mach*"*, *Botia rostrata* (Gunther), commonly known as "Ladder loach", are vulnerable fishes [9] whereas, *Botia almorhae* (Grey), commonly known as "Almorha loach*"* and *Botia lohachata* (Chaudhuri), popularly known as "Y-loach*"* or "Tiger loach*"* or "Lohachata*"*, are endangered species [9] are distributed widely in North-East India and Bangladesh. These loaches are considered as Endangered. The Endangered status of the loaches are mainly because of the deterioration of the environment particularly, water quality which may be due to agricultural run-offs or pesticidal effect of tea gardens in the Terai and Dooars regions, and big-water bodies being fragmented into small water bodies; thus drying up the water. Their identification is difficult due to morphological variation especially amongst *Botia almorhae*, *Botia lohachata* and *Botia rostrata.*

DNA barcode is a new tool for taxon recognition and classification of biological organisms based on sequence of a fragment of mitochondrial gene, Cytochrome Oxidase I (COI). In view of the growing importance of the fish, DNA barcoding for species identification, molecular taxonomy and fish diversity conservation is essential [14]. The DNA barcoding is based on a small sequence of about 655bp of mitochondrial gene Cytochrome oxidase subunit I (COI) with universal primers [7]. The present study was, therefore, focussed to establish the genetic variation amongst the four species namely *Botia alomorhae*, *Botia dario*, *Botia lohachata* and *Botia rostrata* using mtDNA and to show the genetic distance between them. No other literature is available on DNA barcoding of the four *Botia* species. These lacunae instigated the present investigation on molecular identification of the above four mentioned *Botia* loach species.

2. Materials and Methods

Sampling site

River Kaljani situated in Cooch Behar district covers a stretch of about 9 Km upto the lower reaches of the river, that is, from Amlaguri in the north to Chhat Bhelakopa in the south. The sampling areas which were divided into four sites and having a distance of 3 km between them included Amlaguri (26^0 34' N latitude and 89^0 58' E longitude), Chhatoa (26^0 32' N latitude and 89^0 58' E longitude), Jaigir Chilakhana (26^0 31' N latitude and 89^0 58' E longitude) and Chhat Bhelakopa (26^0 29' N latitude and 89^0 58' E longitude). Live fishes were sampled from different sampling sites of Kaljani River. The fishes were identified following their general body form, morphometric and meristic characteristics according to Talwar and Jhingran [19] and Jayaram [10].


Figure 1: *Botia almorhae*


Figure 2: *Botia dario*


Figure 3: *Botia lohachata*


Figure 4: *Botia rostrata*

Genomic DNA Isolation

DNA was isolated from approximately 50 mg of pectoral or pelvic fins and muscle tissue following standard phenol/chloroform method Sambrook *et al.* [17]. Precipitated DNA was resuspended in TE buffer (10mM tris –HCl, 0.1 mM EDTA, pH 8) with a final concentration of 100 ng⁄ µl using Nanodrop 2000 (Thermo Scientific, USA), for all samples.

PCR amplification and Sequencing

The quality and quantity of the extracted DNA were estimated on 0.8% agarose gels stained with ethidium bromide (EtBr). Approximately 655 bp nucleotide was amplified from the 5' region of the COI gene from mtDNA using different combinations of two pairs of primers:

FishF1-5'TCAACCAACCACAAAGACATTGGCAC-3'

and

FishR1 -5'TAGACTTCTGGGTGGCCAAAGAATCA-3',

[22]. The amplifications were performed in 40 µl reactions containing in 4µl of 10X assay buffer, 0.8µl of MgCl2 (25mM), 0.2 µl of each dNTP, 0.4µl of each primer (10mM), 3U of *Taq* polymerase (0.4 µl) and 1.6 µl (50ng/ µl) of genomic DNA. To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. Thermocycler conditions were used as initial preheat at 94 ºC for 3 min, of denaturation 35 cycles at 94 ºC for 30 s, annealing 54 ºC for 30 s, extension 72 0C for 60s and final extension for 10 min at 72 ºC. The PCR products were visualized on 1.2% agarose gels and the most intense product were selected for sequencing. Nucleotide sequencing was performed by the dideoxy chain-termination method Sanger *et al.* [18] using ABI Prism Big Dye Terminator v3.1 Cycle Sequencing kit, and sequenced following Applied Biosy stems, USA.

Sequencing analysis

The raw DNA sequences were edited using BioEdit sequence alignment editor [5] aligned using CLUSTALW [23] referred against electropherogram and submitted to GenBank (Table-1). To analyze the evolutionary isolation of four species and the level of divergence within species, K2P distance was calculated by averaging pair wise comparisons of sequence difference across all individuals by the Kimura 2-Parameter method [16] under Gama distribution estimated in MEGA 5.1(Molecular Evolutionary Genetics Analysis) software [20].

3. Results

DNA sequence variation analysis

Mitochondrial DNA 655bp Cytochrome Oxidase Subunit I (COI) gene were successfully amplified from individuals of *Botia almorhae*, *Botia dario*, *Botia lohachata* and *Botia rostrata* and sequences were submitted to Genbank databases (Table 1). Simplicity and un-ambiguity were observed among all the sequences, and no insertions, deletions or stop codons were observed in any of the sequences. Some sequences were also derived from NCBI. Out of 655 positions in the COI gene sequences analyzed in 10 specimens, 196 positions were variable, and 172 were parsimoniously informative.

Intra-species pair wise distances of *Botia* genus is highlighted in Table 2. The COI sequence pair of *Botia* evolutionary distances ranged from 0.002 to 0.112. The interspecies Kimura's 2- parameter pair-wise distance was highest (0.112) between *Botia dario* and *B. lohachata* and lowest (0.002) for *B.almorhae* and *B. lohachata* (Table 2). Best fit models for COI dataset was Hasegawa-Kishino-Yano (HKY+ I) model for different population of *Botia* and closely related species such as *B.lohachata* and *B. almorhae*.

Table 1: The mitochondrial COI sequences of Genus *Botia* loaches with the accession number

Sl. No.	Species	Genbank Accession number	Authors
1	*Botia almorhae*	KT781504	PRESENT STUDY
2	*Botia lohachata*	KT781505	PRESENT STUDY
3	*Botia rostrata*	KT781497	PRESENT STUDY
4	*Botia rostrata*	KT781498	PRESENT STUDY
5	*Botia rostrata*	KT781499	PRESENT STUDY
6	*Botia rostrata*	KT781500	PRESENT STUDY
7	*Botia dario*	KT781502	PRESENT STUDY
8	*Botia dario*	KT781503	PRESENT STUDY

Table 2: Evolutionary divergence between Genus *Botia*

	Botia dario	*Botia lohachata*	*Botia almorhae*	*Botia rostrata*
Botia dario		0.015	0.015	0.013
Botia lohachata	0.112		0.002	0.009
Botia almorhae	0.107	0.004		0.008
Botia rostrata	0.094	0.045	0.040	

The nucleotide sequences of COI gene were aligned in order to determine the phylogenetic relationship among four species of *Botia*. The topology of ML and NJ tree estimated were identical. The phylogenetic tree showed that *B. almorhae* and *B. lohachata* formed a monophyletic group (supported by 100% bootstrap value).

4. Discussion

Hebert *et al.* [8] proposed a concept, a short nucleotide sequence of mitochondrial genome will act as a DNA barcode of species identification of eukaryotic in particular animals. The technology has proven to be a rapid tool for precise identification of biological specimens. DNA barcoding works under the principle that interspecies variations are greater than the intraspecies variations allowing one to distinguish the species using nucleotide sequences. Six hundred fifty nucleotide bases of 5

Cytochrome C oxidase sub – unit I gene (COI) have been accepted as universal barcode to delineate animal life in this planet. Identification of juveniles and immature stages of loach is very difficult using traditional taxonomic approach and molecular phylogenies help resolve taxonomic confusion of species. In Indian waters, similar types of findings were reported on barcoding by Lakra *et al.*, [13]; Krishna *et al.*, [12]; Chandra *et al.*, [2]; Rahman *et al.*, [15]; Vij *et al.*, [21]; Ambili *et al*, [1] and Kannan *et al.*, [11]. The present study thus highlighted the validity of DNA barcoding to differentiate the loaches at the species level and helped to understand the loaches in different reaches of rivers of Terai region of West Bengal.

5. Conclusion

The blast search analyses of sequences were also carried out for further strengthening of these sequenced data. The phenotypical identification of the present studied species of *Botia* showed 100% similarity with same species sequence in Genbank. From the present study it was concluded that *Botia almorhae* and *B. lohachata* were closely related species. The present study is helpful for identification of the Endangered and Vulnerable *Botia* species from Eastern Himalayan region and conservation programs.

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