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Arpita Dey
Research Scholar, Aquaculture and Limnology Research Unit, Department of Zoology, University of North Bengal, Darjeeling, Siliguri -734013, West Bengal, India.

Rashmi Verma
Molecular Biology and Biotechnology Division, National Bureau of Fish Genetic Resources, Canal Ring Road, Dilkusha, Lucknow-226002, Uttar Pradesh, India.

Mahender Singh
Molecular Biology and Biotechnology Division, National Bureau of Fish Genetic Resources, Canal Ring Road, Dilkusha, Lucknow-226002, Uttar Pradesh, India.

Sudip Barat
Aquaculture and Limnology Research Unit, Department of Zoology, University of North Bengal, Darjeeling, Siliguri -734013, West Bengal, India.

Correspondence
Arpita Dey
Research Scholar, Aquaculture and Limnology Research Unit, Department of Zoology, University of North Bengal, Darjeeling, Siliguri -734013, West Bengal, India.

Evolutionary and taxonomic relationships in loach (Genus: *Botia*) through molecular characterization in a river of Terai region of West Bengal, India

Arpita Dey, Rashmi Verma, Mahender Singh, Sudip Barat

Abstract
Loaches which are quite near to extinction are high demanding fish species having both ornamental and edible food value. Their identification is difficult due to morphological variation especially amongst *Botia lohachata* and *Botia almorhae*. Genetic diversity and molecular phylogeny among six loaches belonging to genus *Botia* were studied. Cytochrome Oxidase I (COI) gene (655 bp) was amplified using PCR and sequenced. The pairwise genetic distances among *Botia* species ranged from 0.004 to 0.200. The interspecies Kimura's 2- parameter pair-wise distance was highest (0.200) between *B. modesta* and *B. lohachata* and lowest (0.004) for *B. almorhae* and *B. lohachata.* The overall transition/transversion bias is R = 3.084. The phylogenetic tree showed that *B. almorhae* and *B. lohachata* formed a monophyletic group (supported by 100% bootstrap value) and then constituted one clade with *B. kubotai*. Other Asian species *B. rostrata, B. striata, B. dario B. modesta* and *B. macracanthus* also contributed to this clade but were distant to native *Botia* species. It was deduced that loaches need attention towards conservation due to its endangerment and vulnerability status.

Keywords: Genus *Botia*, Cytochrome Oxidase subunit I gene, Barcoding, Phylogeny, Terai region of West Bengal.

Introduction
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. They lead a nocturnal life and remain buried in the sand or silt for most of the time. The fishes are very colourful with bright bands, peaceful nature, lesser scales and barbles. Occasionally, they make a loud cracking sound which is produced by the grinding of their pharyngeal teeth. Loaches are omnivores, and usually prefer *Daphnia*, earthworms, bloodworms, snails and animal proteins. Nelson [1] estimated a total of 85 genera and 770 species of loaches in the world. Recently, Kottelat [2] has reviewed and reported 1043 species under 111genera of loach in the world, Acharjee and Barat [3] reported 20 loach species which are available in Terai region of West Bengal. Among the huge biodiversity, loaches belonging to the family Cobitidae contribute to a major share of the world ornamental fish trade market due to their beautiful colouration.

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 [4] *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 [4] and distributed widely in North-East India and Bangladesh. *Botia macracanthus or Chromobotia macracanthus* (Bleeker), commonly known as "Clown loach" is an endemic and not evaluated species [4] of Indonesia. *Botia modesta or Yasuhikotakia modesta* (Bleeker), popularly known as "Blue loach", is a threatened species [4] distributed in Vietnam, Cambodia, Laos and Thailand. These two exotic loaches are also very dominant over the Indian ornamental fish.

Different molecular markers, such as allozymes, mitochondrial DNA, RAPD have been used to observe genetic variation and evolutionary relationship amongst the different taxa.DNA barcoding is species level identification system based on mitochondrial DNA. Mitochondrial DNA was used to examine the evolutionary and taxonomic relationships amongst taxa. The DNA barcoding is based on a small sequence of about 655bp of mitochondrial gene Cytochrome oxidase subunit I (COI) with universal primers [5]. An international interest in fisheries sparked to launch the "Barcode of Life Project (iBOL)" [6] which determined that

mtDNA cytochrome oxidase subunit 1 (CO1) was a suitable gene marker for fish species identification due to the fast evolution of the mtDNA, its maternal inheritance and haploid condition [7]. The use of COI gene for barcoding is a suitable marker for discriminating between closely related species of fishes [8, 9, 10, 11, 12, 13, 14].

The present study was, therefore, focussed to establish the evolutionary and taxonomic relationships amongst the 6 species of genus *Botia* using mtDNA and to show the genetic distance between them. These species were contributed by different Continents under Gondwana Land. No other literature is available either on behaviour, breeding or conservation aspects of loaches. These lacunae instigated the present investigation on molecular identification and phylogenetic relationship of the above six mentioned loach species. The study may thus contribute to some extent to the information database and conservation approach of the fish diversity in natural resources.

Materials and Methods

Sampling site

The sampling sites located at Bhelakopa, Dwitia Khanda of Cooch Behar lie at $26^0$18' North latitude and $89^0$34'East 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 [15] and [16].

Genomic DNA Isolation

DNA was isolated from approximately 50 mg of pectoral or pelvic fins and muscle tissue following standard phenol/chloroform method [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.

Fig 1: *Botia dario*

Fig 2: *Botia rostrata*

Fig 3: *Botia almorhae*

Fig 4: *Botia lohachata*

Fig 5: *Botia macracanthus*

Fig 6: *Botia modesta*

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',

(Ward *et al.*) [9]. 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 ^{0}C 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 [18] using ABI Prism Big Dye Terminator v3.1 Cycle Sequencing kit, and sequenced following Applied Biosystems, USA.

Sequencing analysis

The raw DNA sequences were edited using BioEdit sequence alignment editor [19], aligned using CLUSTALW [20], referred against electropherogram and submitted to GenBank (Table-1). To analyze the evolutionary isolation of six 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 [21] under Gama distribution estimated in MEGA 5.1(Molecular Evolutionary Genetics Analysis) software [22].

Results

DNA sequence variation analysis

Mitochondrial DNA 655bp Cytochrome Oxidase Subunit I (COI) gene were successfully amplified from individuals of *Botia dario, Botia rostrata, Botia almorhae, Botia lohachata, Botia macracanthus* and *Botia modesta* 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. The average base composition [Thymine/Uracil (T/U); Cytosine (C); Adenine (A) and Guanine (G)] over all the three codon positions is 31.5, 25.7, 25.8 and 17.0, respectively. The transition/transversion rate ratios are k1 =4.836 (purines) and k2 = 6.772 (pyrimidines). The overall transition/transversion bias is R = 3.084(Table 2).

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

Sl. No.	Species	Genbank Accession number	Authors
1	*Botia almorhae*	KF738184	NCBI
2	*Botia almorhae*	KF738185	NCBI
3	*Botia almorhae*	KF738183	NCBI
4	*Botia almorhae*	KT781504	Present Study
5	*Botia lohachata*	KT781505	Present Study
6	*Botia lohachata*	KF742423	NCBI
7	*Botia kubotai*	KF738178	NCBI
8	*Botia kubotai*	KF738179	NCBI
9	*Botia kubotai*	KF738180	NCBI
10	*Botia kubotai*	KF738181	NCBI
11	*Botia rostrata*	KT781497	Present Study
12	*Botia rostrata*	KT781498	Present Study
13	*Botia rostrata*	KT781499	Present Study
14	*Botia rostrata*	KF738189	NCBI
15	*Botia rostrata*	KF738190	NCBI
16	*Botia rostrata*	KF738191	NCBI
17	*Botia rostrata*	KT781500	Present Study
18	*Botia rostrata*	KF738192	NCBI
19	*Botia striata*	KF738186	NCBI
20	*Botia striata*	KF738187	NCBI
21	*Botia striata*	KF738188	NCBI
22	*Botia dario*	KT781502	Present Study
23	*Botia dario*	KT781503	Present Study
24	*Botia dario*	JX105475	NCBI
25	*Botia dario*	KF511556	NCBI
26	*Botia dario*	JX105468	NCBI
27	*Botia dario*	JX105477	NCBI
28	*Botia dario*	JX105478	NCBI
29	*Botia macracanthus*	KT781506	Present Study
30	*Chromobotia macracanthus*	KF738204	NCBI
31	*Chromobotia macracanthus*	KF738207	NCBI
32	*Chromobotia macracanthus*	KF738205	NCBI
33	*Chromobotia macracanthus*	KF738206	NCBI
34	*Botia modesta*	KT781501	Present Study
35	*Yasuhikotakia modesta*	JQ346170	NCBI
36	*Glyptothorax brevipinnis*	EU637829	NCBI

Table 2: Molecular characterization information content of the mtDNA COI region of analyzed *Botia*

Number of bases analyzed	Nucleotide composition				Invariable Sites	Polymorphic informative Sites	Parsimony informative Sites	Estimated tv/ts bias (R)
	%A	%G	%T	%C				
655	25.8	17	31.5	25.7	196	164	172	3.084

Table 3: Evolutionary divergence between intra-species of Genus *Botia.*

	Botia dario	***Botia lohachata***	***Botia almorhae***	***Botia macracanthus***	***Botia modesta***	***Botia rostrata***	***Botia kubotai***	***Botia striata***
Botia dario		0.015	0.015	0.017	0.018	0.013	0.015	0.014
Botia lohachata	0.112		0.002	0.017	0.020	0.009	0.009	0.014
Botia almorhae	0.107	0.004		0.017	0.020	0.008	0.009	0.014
Botia macracanthus	0.169	0.137	0.140		0.020	0.018	0.018	0.018
Botia modesta	0.186	0.200	0.199	0.198		0.019	0.019	0.019
Botia rostrata	0.094	0.045	0.040	0.151	0.193		0.008	0.012
Botia kubotai	0.112	0.047	0.049	0.158	0.192	0.035		0.013
Botia striata	0.109	0.093	0.097	0.155	0.199	0.074	0.088	

Evolutionary distances

Intra-species pair wise distances of *Botia* genus is highlighted in Table 3. The COI sequence pair of *Botia* evolutionary distances ranged from 0.004 to 0.200. The interspecies Kimura's 2- parameter pair-wise distance was highest (0.200) between *B. modesta* and *B. lohachata* and lowest (0.004) for *B.almorhae* and *B. lohachata* (Table 3). 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*. 500 bootstrap re-sampling strategy was used to assess the reliability of a phylogenetic tree. All the populations of *Botia* were clearly separated from each other in phylogenetic tree (Figure 7).

Phylogenetic analysis

The nucleotide sequences of COI gene were aligned in order to determine the phylogenetic relationship among 6 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) and then constituted one clade with *B. kubotai*. Other Asian species *B. rostrata, B. striata, B. dario B. modesta and B. macrocanthus* also contributed to this clade but are distant to native *Botia* species.

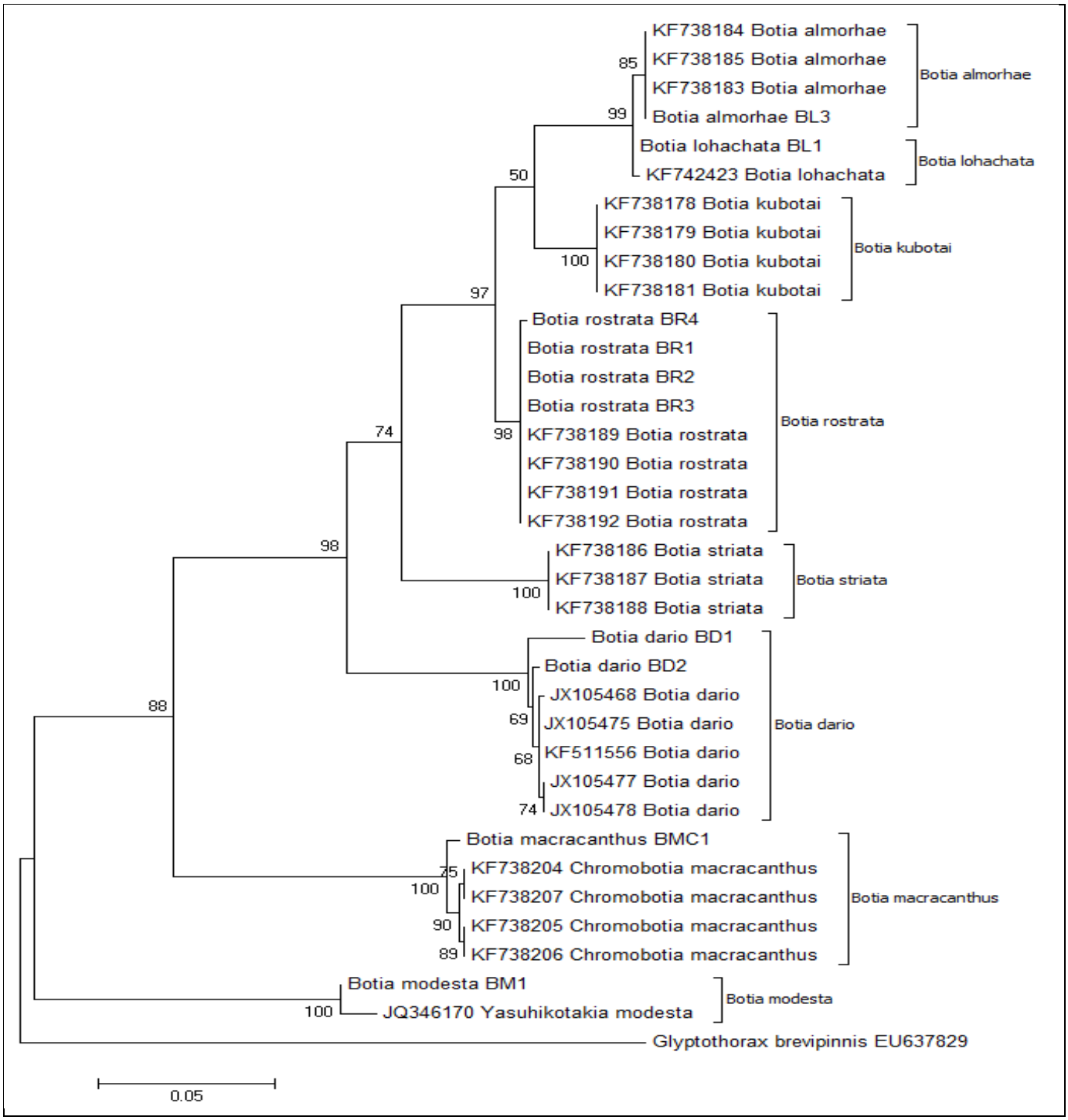

Fig 7: Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model [23]. The tree with the highest log likelihood (-2520.3362) is shown in Figure 8. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.0010% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 36 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 592 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [22].

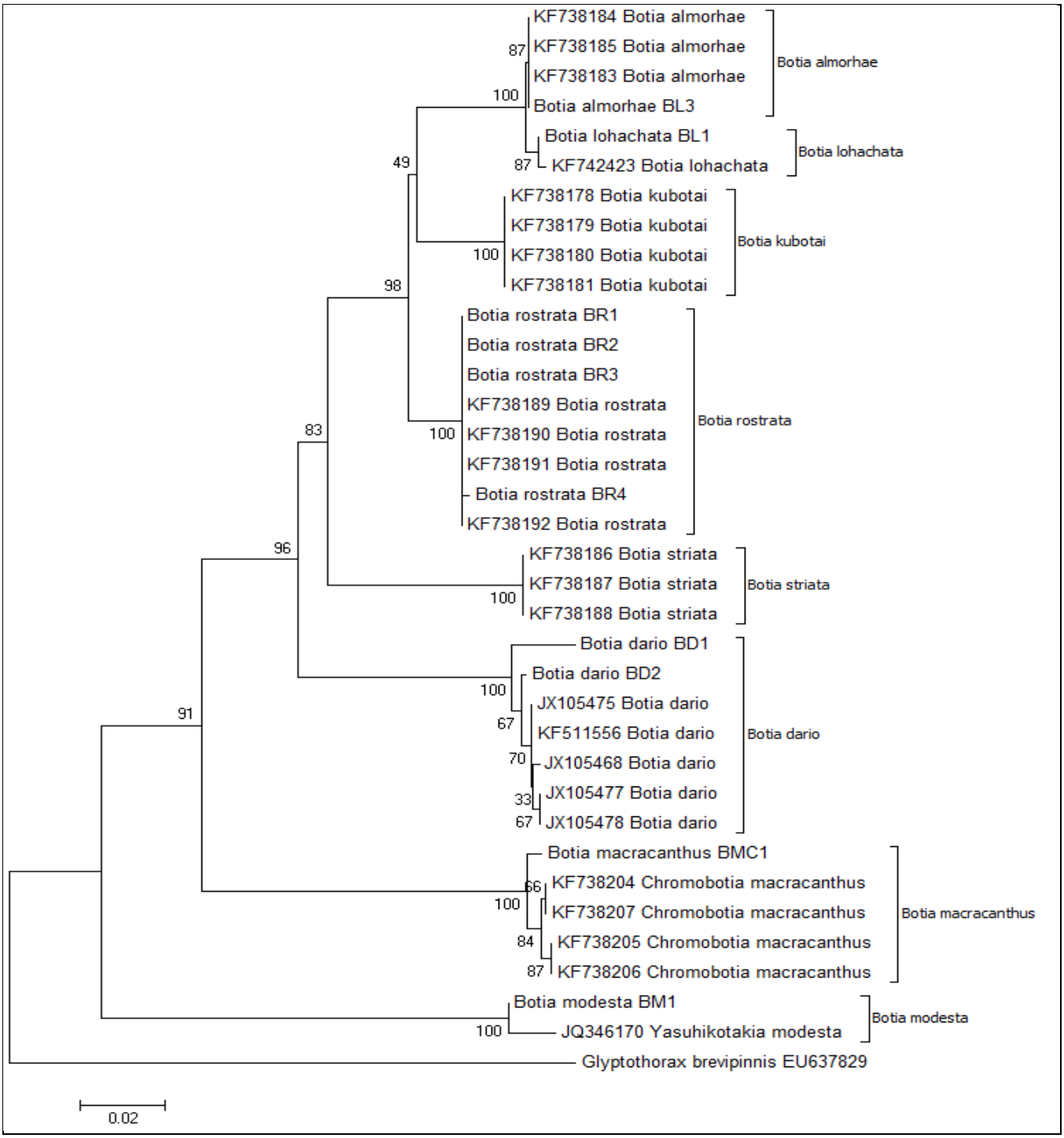

Fig 8: Evolutionary relationships of taxa by Neighbour-Joining method

The evolutionary history was inferred using the Neighbour-Joining method [21]. The optimal tree with the sum of branch length = 0.60289640 is shown in Figure 8. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [24]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [25] and are in the units of the number of base substitutions per site. The analysis involved 36 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 592 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [22].

Discussion

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 (Figure 7 and 8). Hebert *et al*., [26] 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. DNA barcoding of fishes in different parts of the globe gained momentum and it has been well established in Australia [9]. In Indian waters, similar type of findings were reported on barcoding by [27-32, 12-14]. 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.

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