Environment and Ecology 37 (3A) : 873—876, July—September 2019
Website: environmentandecology.com ISSN 0970-0420

Molecular Characterization of Hill Stream Cyprinid Fish *Crossocheilus latius latius* Using the Mitochondrial DNA Control Region

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Received 25 February 2019; Accepted 29 March 2019; Published on 20 April 2019

Abstract *Crossocheilus latius latius* is a Hill Stream fish belonging to genus *Crossocheilus,* family Cyprinidae and subfamily Garrinae. Being a non-protein coding gene, the mitochondrial control region or D-loop plays an important role in the field of molecular taxonomy. Hitherto, there has been no reported study about the complete or partial sequence of this region of the *Crossocheilus latius latius*. For these reasons, the objective of this research was to assess the molecular characterization of this species using mitochondrial control region. The evolutionary history and phylogenetic tree of the sample species was inferred using the maximum likelihood method. MEGA 7 was used to infer all the phylogenetic studies.

Keywords *Crossocheilus latius latius,* Cyprinid, Mitochondrial control region, Molecular characterization.

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Introduction

Fishes of the family Cyprinidae comprise 16% of the world's fishes approximately 321 recognized genera and 3,268 species (Nelson 2006). Cyprinids range in size from 12 mm to 2.5M long and are egg layers that mostly spawn their eggs with no parental care. Many cyprinid species are important food fishes for humans, some have been farmed and fished for centuries. Some are biocontrol agents to remove pests such as mosquitoes, many are popular aquarium trade and as ornamental pets.

Crossocheilus latius latius is a hill stream fish of genus *Crossocheilus,* family Cyprinidae and subfamily Garrinae. It is herbivorus and bottom dweller. The molecular techniques in the field of biology has helped us to establish genetic relationships between the members of different taxa. DNA sequence analysis has been applied for 30 years to assist species identification (Ward et al.2005). The mtDNa is the best studied molecular marker applied in molecular characterization because of its justifying properties like high copy number, maternal inheritance. The MCR (MtDNA Control Region) plays a pivot role in the field of molecular taxonomy. The control region is the non-protien coding region of mitochondrial DNA, and it has been proven to be an ideal marker for assessing the genetic structure of recently diverged or closely related populations or species (Avise 1994, Bremer et al. 1996, Iguchi et al. 1999, Tabata and Taniguchi 2000, Ishikawa et al.2001, Liet al. 2012).

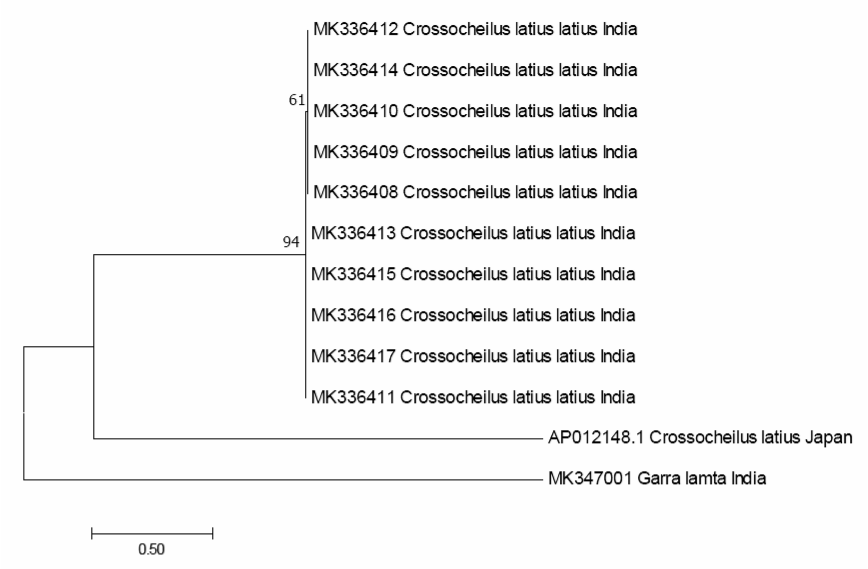

Fig. 1. Molecular phylogenetic analysis of *Crossocheilus latius latius* by Maximum Likelihood method.

The control region is noted for its non-protein coding and a faster rate of evolution, as it is a unique and highly variable area in the mitochondrial DNA. However, there has been no reported study about the complete or partial sequence of the mitochondrial control region of the *Crossocheilus latius latius*. For these reasons, the objective of this research was to establish the molecular characterization of this species based on control region.

Materials and Methods

Samples of *C. latius latius* were procured from fishermen near the Alaknanda river, a snowfed torrential stream at the Srinagar Garhwal (30.22^{0}N 78.78^{0}E) (Fig.1), at an average elevation of 560meter above sea level (1837 feet). The procured fishes were transported live to the laboratory, and were kept in a well-aerated hatchery at 20-24^{0}C before analysis to get acclimatized to the existing conditions. After correct identification and taking morpho-metric data at species level (Tilak and Hussain 1977, Jhingran 1975), the specimens were properly cleaned and different tissues were taken out by sacrificing the fish. Tissue (Muscle) samples were collected and preserved in 95% v/v ethanol in 2ml cryo-preservatory vials. These vials were kept in cyroboxes. The cyroboxes containing tissue samples were then kept at 4^{0}C for further use.

The standard phenol/chloroform procedures (Sambrook et al. 1989) were used to isolate the total genomic DNA. A partial sequence of the MCR was amplified by PCR (Eppendorf, Master cycler gradient) using sets of primers DLL: 5'-CCACTAGCTCCCAAAGCTA-3' and DLH: 5'- ACTTTCTAGGGTCCATC-3' (Bernatchez et al. 1992). Each PCR mixture (25µL) included 2µl template DNA, 2.5µL 10 X Taq Assay Buffer, 2.5 µLdNTPs (2mM each), 1.8-2 µL $MgCl_2$ (25 mM), 0.5µL of each primer (10 µM), 0.15-0.2µl (1 U) Taq DNA polymerase. The following cycling protocols were used to amplify the MCR gene : An initial denaturation at 94^{0}C for 3 min, 35 cycles of denaturation at 94^{0}C for 30 secs, annealing at 49^{0}C for 45 secs and extension at 72^{0}C for 1 min and a final extension at 72^{0}C for 10 min. Sequencing of amplified PCR products were done from outside agency: Xcelris Labs Limited, Ahmedabad.

Phytogenetic and molecular evolutionary analyses were conducted using MEGA 7 (Kumar et al. 2016). The Maximum Likelihood method tree was

Table 1. Measurements (in cm) and counts of *Crossocheilus latius latius* (Hamilton-Buchanan).

Sl. No.	Morpho-Meristic characters	Mean	Range
1.	Total length	21.42	15.1-25.6
2.	Standard length	17.04	11.2-20.4
3.	Head length	3.38	2.9-3.8
4.	Snout length	1.42	1.1-1.7
5.	Eye diameter	0.68	0.55-0.8
6.	Length of caudal peduncle	3.68	3.3-4.
7.	Height of caudal peduncle	1.86	1.7-2.0
8.	Maximum body depth	3.52	2.9-4.4
9.	Intra orbital length	2.14	1.9-2.4
10.	Fork length	18.56	12.3-21.5
11.	Head depth	2.36	2.0-2.6
12.	Pre pectoral length	3.32	3.0-3.7
13.	Pre dorsal length	7.36	6.3-8.0
14.	Pre ventral length	8.4	7.2-9.2
15.	Pre anal length	12.56	9.1-14.7
16.	Height of dorsal fin	4.68	3.8-5.7
17.	Height of anal fin	3.16	3.0-3.4
18.	Height of caudal fin	3.4	2.5-5.0
19.	Length of dorsal fin	2.62	2.1-3.4
20.	Length of anal fin	1.3	1.0-3.4
21.	Length of caudal fin	4.38	3.9-5.2
22.	Barbells number	2 pairs	2 pairs
23	Caudal fin	Deeply forked	Deeply forked
24.	No. of lateral line scales	38.4	38-40
25.	No. of L. tr. scales	6/6	6/6
26.	Dorsal fin ray	10.4(3/7.4)	10-11/(3/7-8)
27.	Pelvic fin ray	15	15
28.	Ventral fin ray	09	09
29.	Anal fin ray	7 (2 /5)	7 (2/5)
30.	Caudal fin ray	19	19

created to provide a graphic representation of the patterning of divergence between species.

Results and Discussion

Thirty morpho-meristic characters were analyzed (Table 1) for correct identification and taking morpho-metric data at species level based onTilak and Hussain (1977), Jhingran (1975), Altogether 10 samples of *C. latius latius* were used and sequenced for mitochondrial DNA partial sequence analysis. Sequences were submitted to NCBI GenBank (Accession numbers : MK336408-MK336417). In total 12 sequences were analyzed for preparing phylogenetic tree. The sample details used for analysis along with their GenBank accession numbers are mentioned in Table 2.

Table 2. Species name and GenBank accession numbers of species included in this study (*present study sequences and sequences retrieved from GenBank #).

Sl. No.	Species	Accession number	Reference
1-10	*Crossocheilus latius latius,* India (10)*	MK336408-MK336417	Present study
11	*Crossocheilus latius,*Japan #	AP012148.1	Yang L.et al. (2013)
12.	*Garralamta,* India (Outgroup) #	MK437001	Tiwari et al. (2019)

Maximum composite likelihood estimate of the pattern of nucleotide substitution was estimated using MEGA 7 (Table 3). Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. The nucleotide frequencies were 33.20% (A), 44.00% (T/U), 8.60% (C), and 14.20% (G). The transition/transversion rate ratios werk $k1$ = 3.656 (purines) and $k2$ = 0.051 (pyrimidines). The overall transition/transversion bias was R = 0.699, where R = [A*G*k1+T*C*k2]/[(A+G)*(T+C)].

The nucleotide composition of the mtDNA control region of the *C. latius latius* was also calculated, in which the average A, T, C, and G contents were 37.2, 47.6, 3.9, and 11.30%, respectively, and the base distribution shower no apparent difference among populations. The results showed that the G and C bases were relatively low. The A + T content was higher than the G + C content among the sequences examined, which was consistent with previous findings that the control region is an A+ T-rich region of the mitochondrial genome (Brown et al. 1986, Saccone et al. 1987, Cheng et al. 2010, Li et al. 2012). For the populations, the average transition/transversion ratio was 0.699, which suggest that the transition was higher than transversion, which was consistent with

Table 3. Maximum composite likelihood estimate of the pattern of nucleotide substitution.

	A	T	C	G
A	-	11.7	2.29	**13.81**
T	8.83	-	**0.12**	3.78
C	8.83	**0.6**	-	3.78
G	**32.28**	11.7	2.29	-

conclusions of other authors (Liu et al 2004, Yang et al. 2008, Peng et al. 2010, Li et al. 2012).

The evolutionary history was inferred by using the Maximum likelihood method based on the Tamura 3-parameter model. The tree confirms that the studied samples are of genus *Crossocheilus*. The sequences show the close proximity with the sequences from Japan of genus *Crossocheilus.* The mtDNA control region or displacement-loop (D-loop) region, is located between tRNAPro and tRNAPhein mtDNA. This region being a non-protein coding region ideal marker for phylogenetic studies. The present study was a preliminary step toward assessing the molecular characterization of *C. latius latius* using control region from India. However, to better resolve phylogenetic relationships of this species with other members of cyprinids based on control region, more taxon sampling is needed for future studies (Tamura 1992, Tamura and Kumar 2002).

Acknowledgement

The authors extend their deepest thanks to Bhim Dutt Joshi and Ashish Kr. Chowdhary for technical assistance in accomplishing this research work. The authors sincerely thanks to Xcelris Labs Limited, Ahmedabad for sequencing purposes.

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