Molecular Phylogeny of a Hillstream Fish Garra Gotyla Gotyla using mtDNA Cytochrome Oxidase I (COI) Gene

Poonam Tiwari[1], Arun Bhatt[2], and S N Bahuguna[3]

[1,3] *(Dept. of Zoology & Biotechnology, HNB Garhwal University, Srinagar Garhwal- 246174, Uttarakhand)*

[2] *(Dept. of Biotechnology, GBPE College, Ghurdauri, Pauri Garhwal- 246194, Uttarakhand)*

Abstract: *Garra gotyla gotyla is a small species of ray-finned fish inhabiting pool and run areas of streams. They are also known as sucker head. This genus consists of a group of species that are remarkably similar in general morphology. These species are often difficult to distinguish based on external morphological approach. Therefore, an attempt has been made to study the phylogenetic relationships of available G. gotyla gotyla using mtDNA COI gene sequence data, to resolve the existing uncertainty about the relationships and groups of these fishes. The sequences were submitted to NCBI GenBank to establish and validate the taxonomical identification of the samples.*

Keywords: *COI gene, Cyprinid, Garra gotyla gotyla, Molecular Phylogeny, mtDNA*

I. INTRODUCTION

Genetic relationship was established between members of different taxonomic categories by using Molecular techniques. Based on molecular data, Fitch and Margoliash (1967) made the first phylogenetic tree, which is so close to vertebrate phylogenetic trees. Taxonomists then categorized molecular techniques more significant over other traditional methods and molecular evidence gave final or confirmatory evidence.

COI is one of the most conserved protein-coding mitochondrial genes in animals (Mueller, 2006) and therefore shows a better phylogenetic signal (Hebert et al., 2003). COI has been used as a barcoding marker in many animal taxa with tremendous success (Hebert et al., 2003; Hogg and Hebert, 2004; Ward et al., 2005; Hajibabaei et al., 2006; Hubert et al., 2008). Barcoding varies from molecular phylogeny in that the fundamental goal isn't to decide relationship patterns however to distinguish an obscure example from a pre - existing classification (Kress et al., 2005).

Phylogenetic interrelations of cyprinid subfamilies have been investigated from a long past, from both morphological and molecular perspectives. Chen et.al (1984) first proposed a hypothesis based on morphological data of cyprinid interrelationships. The example fish under examination in the present study is *Garra gotyla gotyla* (Gray). These are slope stream fishes of Western Himalayas and are inspected from Garhwal region of Uttarakhand. As the Garhwal locale is uneven having steep and rough shores so these fishes are found in bounty in riverine frameworks of this region. To support in these humming natural surroundings, these fishes have experienced certain morphological alterations.

Hillstream fishes are often difficult to distinguish based on external morphological approach. A DNA barcoding approach may be useful for the identification of taxa. For these reasons, COI barcode sequence for the identification of these fishes were tested with a goal of whether DNA barcoding can achieve unambiguous species recognition in fishes.

Materials and Methods

Samples of *G. gotyla gotyla* were procured from fishermen near the Alaknanda river, a snowfed torrential stream at the Srinagar Garhwal (30.22°N 78.78°E), at an average elevation of 560m asl (1837 feet). The procured fishes were transported live to the laboratory, and were kept in a well-aerated hatchery at 20-24°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; Jingran 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, and were kept in cyroboxes at 4°C for further use.

Total genomic DNA was isolated from muscle tissues using standard phenol/chloroform procedures (Sambrook et al., 1989). Partial sequence of Cyt c oxidase I (COI) was amplified by PCR (Eppendorf, Master cycler gradient) using sets of primers:

FishF1:5'-TCAACCAACCACAAAGACATTGGCAC-3'; and

FishR1: 5'TAGACTTCTGGGTGGCCAAAGAATCA-3' (Ward et al., 2005).

Each PCR mixture (25 µL) included 2µl template DNA, 2.5 µL 10X Taq Assay Buffer, 2.5 µL dNTPs (2 mM each), 1.8-2 µL $MgCl_2$ (25 mM), 0.5 µL of each primer (10 µM), 0.15-0.2 µl (1 U) Taq DNA polymerase. The following cycling protocols were used to amplify the MCR gene: an initial denaturation at

94ºC for 4 min, 35 cycles of denaturation at 94ºC for 40 secs, annealing at 55ºC for 45 secs and extension at 72ºC for 45 secs, and a final extension at 72ºC for 20 min. Sequencing of amplified PCR products were done from outside agency: Xcelris Labs Limited, Ahmedabad.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA 7 (Kumar et al., 2016). The evolutionary history was inferred using the Neighbor-Joining method. In the chosen subgroups of fishes, bootstrapping was performed with 500 replications.

II. RESULTS AND DISCUSSION

Thirty morpho-meristic characters were analysed (Table 1) for correct identification and taking morpho-metric data at species level based on Tilak and Hussain (1977) and Jingran (1975). Altogether 10 samples of *G. gotyla gotyla* were used and sequenced for mitochondrial DNA partial sequence analysis. Sequences were submitted to NCBI GenBank (Accession numbers: MK450491- MK450500). In total 18 sequences were analysed for preparing phylogenetic tree.

Maximum composite likelihood estimate of the pattern of nucleotide substitution was estimated using MEGA 7 (Table 2). Rates of different transitional substitutions was shown in bold and those of transversional substitutions are shown in italics. The nucleotide frequencies are 27.68% (A), 28.93% (T/U), 25.85% (C), and 17.54% (G). The transition/transversion rate ratios are $k1$ = 14.926 (purines) and $k2$ = 13.938 (pyrimidines). The overall transition/transversion bias is R = 7.133, where R = [A*G*$k1$ + T*C*$k2$]/[(A+G)*(T+C)].

The Ti/Tv ratio observed was 7.133. The results in the present study showed conformity with previous studies in other fishes (Lakra et al., 2009, Lasker et al., 2018). The transitional bias suggests that this is a recently evolved group or slowly evolving genes. A transition bias in these genes means that there are few multiple substitutions and that the data therefore have phylogenetic signal (Simon et al., 1994). Overall the lower rate of transversions should lead to better resolution of deep divergence events because of low saturation effects. The study suggested that the transition was higher than transversion, which is consistent with conclusions of other authors (Liu HY et al., 2004; Yang et al., 2008; Peng et al., 2010; Li et al., 2012).

Pairwise distance of 18 sequences viz., 10 from present study and 8 retrieved from GenBank showed the overall average distance was 0.159 (Table 3). The tree confirms the species as *Garra gotyla gotyla*. Least intera-genus genetic distance of the present study samples was found with *G. litanensis* (0.068), followed by *G. lamta* (0.090). Average evolutionary divergence showed that none of the species had enough divergence to be divided into two species as well all sample species had enough similarity to be united with other species. Average evolutionary divergence within and between species and pairwise K3P distances have shown to be the best parameter for phylogenetic analyses, clearly putting boundaries between the species. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.35350756 is shown (Fig. 1).

A C-terminal fragment of the mitochondrial gene for cytochrome oxidase subunit I (COI) has been proposed as universal marker for this purpose among animals. The use of short DNA sequences for the standardized identification of organisms has recently gained attention under the terms DNA barcoding or DNA/Molecular taxonomy (Floyd et al., 2002; Hebert et al., 2003; Tautz et al., 2003). Although, it is not a fundamentally new technique (Moritz and Cicero, 2004), DNA barcoding is promising because technical progress has made its large-scale, automated application feasible (Blaxter, 2004; Tautz et al., 2003), which may accelerate taxonomic progress (Wilson, 2004). Finally, it is concluded that partial sequence information of the mitochondrial gene COI can be used as a diagnostic molecular marker in identification and resolution of taxonomic ambiguities as well as establishing molecular Phylogenetic relationships.

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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Table 1 Measurements (in cm) and counts of *Garra gotyla gotyla* (Gray).

S. No.	Morpho- Meristic Characters	Mean	Range
	Total length	17.18	10.2-20.1
	Standard length	14.04	8.1-16.0
	Head length	3.64	2.1-4.2
	Snout Length	1.74	1.1-2.2
	Eye diameter	0.64	0.4-0.7
	Length of caudal peduncle	2.34	1.3-2.5
	Height of caudal peduncle	2.02	1.2-2.4
	Maximum Body depth	3.32	2.0-3.9
	Intra Orbital Length	2.06	1.2-2.5
	Fork Length	16	9.1-18.4
	Head Depth	3.72	2.3-4.3
	Pre Pectoral length	3.76	2.2-4.3
	Pre Dorsal Length	6.32	3.7-7.5
	Pre Ventral Length	7.54	4.4-8.9
	Pre Anal Length	10.68	6.2-12.6
	Height of dorsal fin	2.72	1.7-3.8
	Height of anal fin	2.12	1.1-3.1
	Height of caudal fin	3.38	2.3-4.2
	Length of dorsal fin	2.1	1.3-2.5
	Length of anal fin	1.46	1.1-1.7
	Length of caudal fin	3.04	1.7-4.1
	Barbells Number	2 Pairs	2 Pairs
	Caudal fin	Slightly Emarginated	Slightly Emarginated
	No. of lateral line scales	33.4	33-34
	No. of L. tr. scales	4 ½/5	4 ½/5
	Dorsal fin ray	10.6	10-11 (2/8-9)
	Pelvic fin ray	10.8	15-16
	Ventral fin ray	9	9
	Anal fin ray	8 (2/6)	8 (2/6)
	Caudal fin ray	17	17

Table 2 Maximum Composite Likelihood estimate of the Pattern of Nucleotide Substitution for *Garra gotyla gotyla*

	A	T	C	G
A	-	*1.77*	*1.58*	**15.98**
T	*1.69*	-	**21.99**	*1.07*
C	*1.69*	**24.61**	-	*1.07*
G	**25.22**	*1.77*	*1.58*	-

Table 3 Estimate of evolutionary divergence (K3P) in sequence pairs between species in COI gene sequences.

*The overall average Distance is 0. 159

MK450491_Garra_gotyla_gotyla		0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.002	0.002	0.002	0.002	0.002	0.004	0.012	0.012	0.013	0.134
MK450492_Garra_gotyla_gotyla	0.002		0.002	0.002	0.000	0.000	0.003	0.003	0.000	0.000	0.000	0.002	0.002	0.004	0.011	0.011	0.013	0.137
MK450493_Garra_gotyla_gotyla	0.003	0.002		0.000	0.002	0.002	0.003	0.003	0.002	0.002	0.002	0.002	0.002	0.004	0.011	0.011	0.013	0.141
MK450494_Garra_gotyla_gotyla	0.003	0.002	0.000		0.002	0.002	0.003	0.003	0.002	0.002	0.002	0.002	0.002	0.004	0.011	0.011	0.013	0.141
MK450495_Garra_gotyla_gotyla	0.002	0.000	0.002	0.002		0.000	0.003	0.003	0.000	0.000	0.000	0.002	0.002	0.004	0.011	0.011	0.013	0.137
MK450496_Garra_gotyla_gotyla	0.002	0.000	0.002	0.002	0.000		0.003	0.003	0.000	0.000	0.000	0.002	0.002	0.004	0.011	0.011	0.013	0.137
MK450497_Garra_gotyla_gotyla	0.009	0.007	0.005	0.005	0.007	0.007		0.000	0.003	0.003	0.003	0.004	0.004	0.005	0.011	0.011	0.013	0.147
MK450498_Garra_gotyla_gotyla	0.009	0.007	0.005	0.005	0.007	0.007	0.000		0.003	0.003	0.003	0.004	0.004	0.005	0.011	0.011	0.013	0.147
MK450499_Garra_gotyla_gotyla	0.002	0.000	0.002	0.002	0.000	0.000	0.007	0.007		0.000	0.000	0.002	0.002	0.004	0.011	0.011	0.013	0.137
MK450500_Garra_gotyla_gotyla	0.002	0.000	0.002	0.002	0.000	0.000	0.007	0.007	0.000		0.000	0.002	0.002	0.004	0.011	0.011	0.013	0.137
KJ476785.1_Garra_sp.	0.002	0.000	0.002	0.002	0.000	0.000	0.007	0.007	0.000	0.000		0.002	0.002	0.004	0.011	0.011	0.013	0.137
KF550093.1_Garra_gotyla	0.003	0.002	0.003	0.003	0.002	0.002	0.009	0.009	0.002	0.002	0.002		0.002	0.004	0.012	0.012	0.013	0.137
KF550092.1_Garra_gotyla	0.003	0.002	0.003	0.003	0.002	0.002	0.009	0.009	0.002	0.002	0.002	0.003		0.004	0.012	0.012	0.014	0.137
KP965722.1_Garra_gotyla_gotyla	0.012	0.010	0.012	0.012	0.010	0.010	0.017	0.017	0.010	0.010	0.010	0.012	0.012		0.012	0.012	0.014	0.133
KT896679.1_Garra_litanensis	0.068	0.066	0.064	0.064	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.068	0.068	0.077		0.000	0.012	0.162
KT896678.1_Garra_litanensis	0.068	0.066	0.064	0.064	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.068	0.068	0.077	0.000		0.012	0.162
JX074157.1_Garra_lamta	0.090	0.088	0.086	0.086	0.088	0.088	0.084	0.084	0.088	0.088	0.088	0.090	0.090	0.100	0.075	0.075		0.166
MK244768_Glyptothorax_pectinopterus	1.183	1.195	1.207	1.207	1.195	1.195	1.232	1.232	1.195	1.195	1.195	1.196	1.195	1.182	1.261	1.261	1.286	

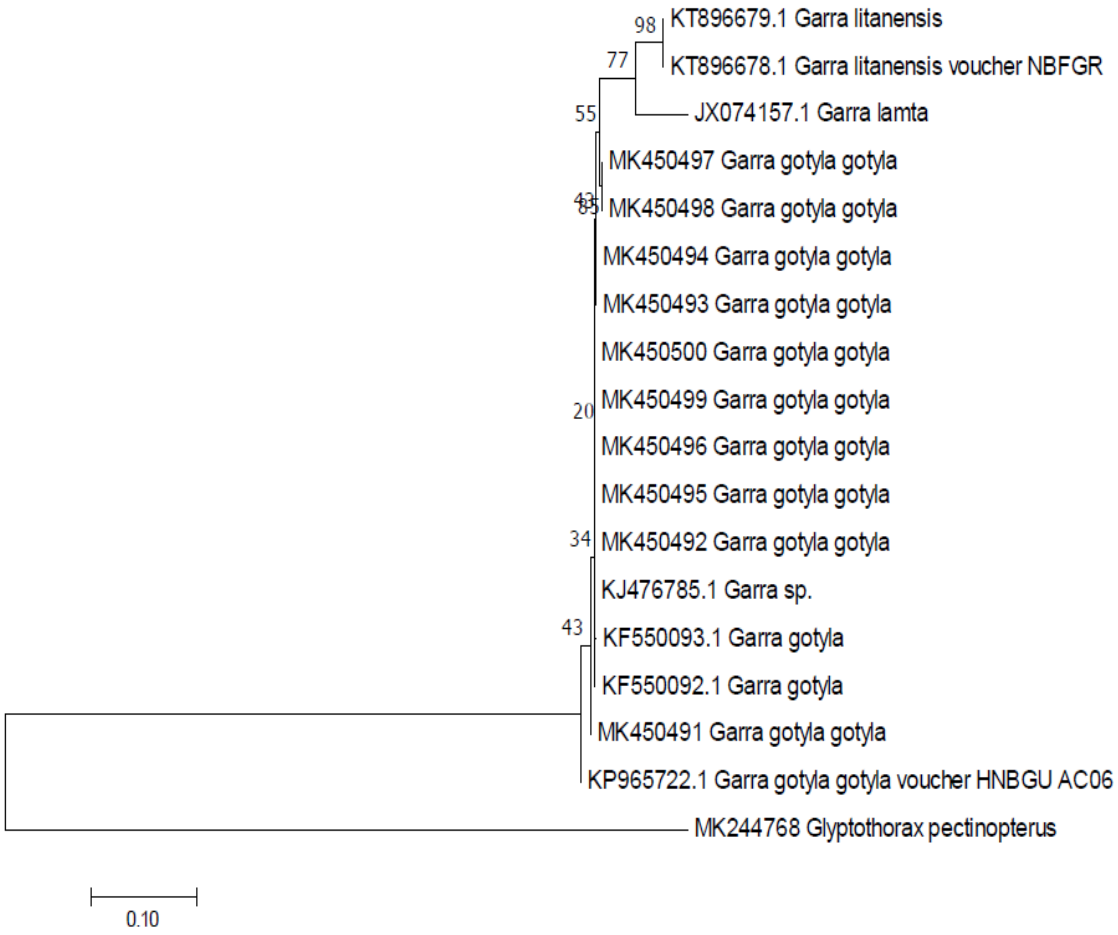

Figure 1 Molecular Phylogenetic analysis of Glyptothorax pectinopterus by Neighbor- Joining method.