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DNA Barcoding of a Bottom Feeder Hill Stream Sisorid Fish *Glyptothorax pectinopterus* Using mt DNA Cytochrome Oxidase I (COI) Gene

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Abstract *Glyptothorax pectinopterus* is a small, benthic Hill Stream fish inhabiting pool and run areas of streams. 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. DNA barcoding differs from molecular phylogeny in that the main goal is not to determine patterns of relationship but to identify an unknown sample in terms of a preexisting classification. DNA barcoding can serve a dual purpose as a new tool in the taxonomists toolbox supplementing their knowledge as well as being an innovative device for non -experts who need to make a quick identification. Therefore, an attempt has been made to study the Phylogenetic relationships of available *Glyptothorax* spp., using mtDNA sequence data, to resolve the existing uncertainty about the relationships and groups of these frogs. COI mtDNA gene was used for barcoding of the samples. The sequences were submitted to NCBI GenBank to establish and validate the taxonomical identification of the samples.

Keywords : Barcoding, COI gene, *Glyptothorax pectinopterus*, mtDNA, Sisorid.

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Introduction

Phylogenetic and molecular systematic study, based on DNA, exploits diversity among sequences of DNA, and can also be used for species identification and delineation. Mitochondrial DNA (mtDNA) is a small, double-stranded, circular molecule consisting of approximately 37 genes coding 22 tRNAs, two rRNAs and 13 mRNAs (Brown et al. 1979, Cantatore and Saccone 1987). DNA barcoding has been established as a mature biodiversity science field that fills the gap between traditional taxonomy and various fields of molecular systematics.

Tautz et al. (2003) contended for a taxonomic system that relies on DNA. It has been used for identification of species for over three decades. Mitochondrial DNA gene cytochrome oxidase subunit I (COI)

has been well accepted as a global bioidentification system for barcoding of animals. This gene region is a 650bp fragment of mtDNA and is sufficient to differentiate all or atleast a majority of animal species (Herbert 2003).

Glyptothorax pectinopterus is a small, benthic hill stream fish inhabiting pool and run areas of streams. Species of this genus belongs to family Sisoridae, and order Siluriformes (fishbase 2019). 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. 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. pectinopterus* were procured from fishermen near the Alaknanda river, a snowfed torrential stream at the Srinagar Garhwal (30.22°N, 78.78°E) (Fig. 1), 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, 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°C for further use.

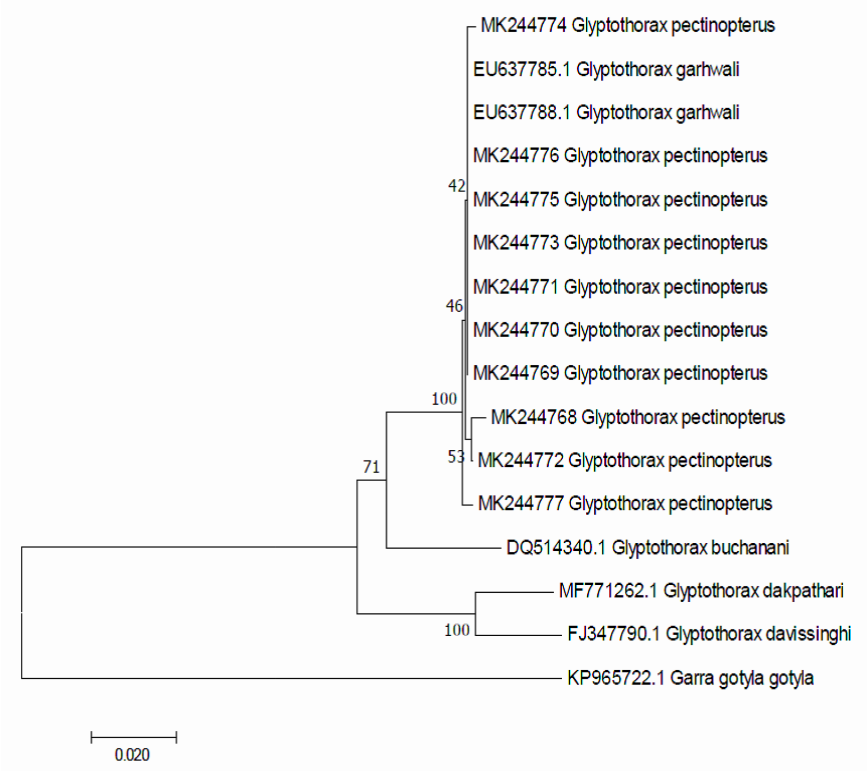

Fig. 1. Molecular phylogenetic analysis of *Glyptothorax pectinopterus* by Neighbor-Joining method.

Table 1. Measurements (in cm) and counts of *Glyptothorax pectinopterus* (McClelland).

Sl. No.	Morphometric and meristic characters	Mean	Range
1.	Total length	11.44	9.5-13.3
2.	Standard length	9.42	7.8-11.0
3.	Head length	2.46	2.0-2.8
4.	Snout length	1.26	1.1-1.4
5.	Eye diameter	0.30	0.2-0.35
6.	Length of caudal peduncle	1.74	1.5-2.1
7.	Height of caudal peduncle	1.12	1.0-1.2
8.	Maximum body depth	1.88	1.6-2.2
9.	Intra orbital length	1.44	1.2-1.7
10.	Fork length	10.12	8.3-11.8
11.	Head depth	1.82	1.5-2.1
12.	Pre pectoral length	2.62	2.2-3.0
13.	Pre dorsal length	3.28	2.7-3.8
14.	Pre ventral length	4.72	3.8-5.5
15.	Pre anal length	6.74	5.6-7.9
16.	Height of dorsal fin	1.76	1.5-2.0
17.	Height of anal fin	1.54	1.3-1.718.
18.	Height of caudal fin	1.4	1.2-1.6
19.	Length of dorsal fin	1.48	1.2-1.6
20.	Length of anal fin	1.38	1.1-1.5
21.	Length of caudal fin	2.02	1.7-2.3
22.	Barbells number	4 pairs	4 pairs
23.	Caudal fin	Deeply forked	Deeply forked
24.	No. of lateral line scales	-	-
25.	No. of L.tr.scale	-	-
26.	Dorsal fin ray	7 (1/6)	7 (1/6)
27.	Pelvic fin ray	9.4 (1/8.4)	9-10 (1/8-9)
28.	Ventral fin ray	6	6
29.	Anal fin ray	9 (2/7)	9 (2/7)
30.	Caudal fin ray	17	17

Total genomic DNA was isolated from muscle tissues using standard phenol/chloroform procedures (Sambrook et al. 1989). Partial sequence of cytochrome oxidase I (COI) was amplified by PCR (Eppendorf, Master cycler gradient) using sets of primers Fish F1 : 5'-TCAACCAACCACAAAGACATTGGCAC-3' and Fish R1 : 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3' (Ward et al. 2005). Each PCR mixture (25 µL) included 2µl template DNA, 2.5µL10XTaq Assay Buffer, 2.5µL dNTPs (2mM each), 1.8-2 µL $MgCl_2$ (25mM), 0.5 µL of each primer (10µM), 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 94°C for 4 min, 35 cycles of denaturation at 94°C for 40 secs, annealing at 55°C for 45 secs and extension at 72°C for 45 secs and a final extension at 72°C 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.

Results and Discussion

Thirty morpho-meristic characters were analyzed (Table 1) for correct identification and taking morpho-metric data at species level based on (Tilak and Hussain 1977, Jhingran 1975). Altogeher of *G. pectinopterus* were used and sequenced for mitochondrial DNA partial sequence analysis. Sequences were submitted to NCBI GenBank (Accession numbers : MK244768-MK244777). In total 16 sequences were analyzed 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 was shown in bold and those of transversional substitutions are shown in italics. The nucleotide frequencies were 26.13% (A), 28.65% (T/U), 28.38% (C) and 16.84% (G). The transition/transversion rate ratios was kl=5.677 (purines) and $k2$ = 1.062 (pyrimidines). The overall transition/transversion bias was R=1.372, where R=[A*C*kl +T*C*$k2$] / [(A+G)*(T+C)].

The Ti/Tv ratio observed was 1.372. 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

Table 2. Maximum composite likelihood estimate of the pattern of nucleotide substitution for *Glyptothorax pectinopterus.*

	A	T	C	G
A	-	5.68	5.62	**18.95**
T	5.18	-	**5.98**	3.34
C	5.18	**6.03**	-	3.34
G	**29.4**	5.68	5.62	-

Table 3. Estimate of evolutionary divergence (K3P) in sequence pairs between species in COI gene sequences. The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal.

MK244768_Glyptothorax_pectinopterus		0.003	0.003	0.003	0.002	0.003	0.003	0.003
MK244769_Glyptothorax_pectinopterus	0.005		0.000	0.000	0.002	0.000	0.002	0.000
MK244770_Glyptothorax_pectinopterus	0.005	0.000		0.000	0.002	0.000	0.002	0.000
MK244771_Glyptothorax_pectinopterus	0.005	0.000	0.000		0.002	0.000	0.002	0.000
MK244772_Glyptothorax_pectinopterus	0.004	0.002	0.002	0.002		0.002	0.003	0.002
MK244773_Glyptothorax_pectinopterus	0.005	0.000	0.000	0.000	0.002		0.002	0.000
MK244774_Glyptothorax_pectinopterus	0.007	0.002	0.002	0.002	0.004	0.002		0.002
MK244775_Glyptothorax_pectinopterus	0.005	0.000	0.000	0.000	0.002	0.000	0.002	
MK244776_Glyptothorax_pectinopterus	0.005	0.000	0.000	0.000	0.002	0.000	0.002	0.000
MK244777_Glyptothorax_pectinopterus	0.005	0.004	0.004	0.004	0.005	0.004	0.005	0.004
EU637788.1_Glyptothorax_garhwali	0.005	0.000	0.000	0.000	0.002	0.000	0.002	0.000
EU637785.1_Glyptothorax_garhwali	0.005	0.000	0.000	0.000	0.002	0.000	0.002	0.000
DQ514340.1_Glyptothorax_buchanani	0.050	0.046	0.046	0.046	0.048	0.046	0.408	0.046
MF771262.1_Glyptothorax_dakpathari	0.078	0.072	0.072	0.072	0.074	0.072	0.074	0.072
FJ347790.1_Glyptothorax_davissinghi	0.080	0.074	0.074	0.074	0.076	0.074	0.076	0.074
KP965722.1_Garra_gotyla_gotyla	0.238	0.233	0.233	0.233	0.235	0.233	0.235	0.233

Table 3. Continued.

MK244768_Glyptothorax_pectinopterus	0.003	0.003	0.003	0.003	0.010	0.012	0.013	0.022
MK244769_Glyptothorax_pectinopterus	0.000	0.002	0.000	0.000	0.009	0.012	0.012	0.021
MK244770_Glyptothorax_pectinopterus	0.000	0.002	0.000	0.000	0.009	0.012	0.012	0.021
MK244771_Glyptothorax_pectinopterus	0.000	0.002	0.000	0.000	0.009	0.012	0.012	0.021
MK244772_Glyptothorax_pectinopterus	0.002	0.003	0.002	0.002	0.010	0.012	0.013	0.022
MK244773_Glyptothorax_pectinopterus	0.000	0.002	0.000	0.000	0.009	0.012	0.012	0.021
MK244774_Glyptothorax_pectinopterus	0.002	0.003	0.002	0.002	0.010	0.012	0.012	0.021
MK244775_Glyptothorax_pectinopterus	0.000	0.002	0.000	0.000	0.009	0.012	0.012	0.021
MK244776_Glyptothorax_pectinopterus		0.002	0.000	0.000	0.009	0.012	0.012	0.021
MK244777_Glyptothorax_pectinopterus	0.004		0.002	0.002	0.009	0.012	0.012	0.021
EU637788.1_Glyptothorax_garhwali	0.000	0.004		0.000	0.009	0.012	0.012	0.021
EU637785.1_Glyptothorax_garhwali	0.000	0.004	0.000		0.009	0.012	0.012	0.021
DQ514340.1_Glyptothorax_buchanani	0.046	0.048	0.046	0.046		0.013	0.013	0.022
MF771262.1_Glyptothorax_dakpathari	0.072	0.073	0.072	0.072	0.086		0.008	0.022
FJ347790.1_Glyptothorax_davissinghi	0.074	0.076	0.074	0.074	0.082	0.039		0.023
KP965722.1_Garra_gotyla_gotyla	0.233	0.232	0.233	0.233	0.237	0.251	0.256	

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 et al. 2004, Yang et al. 2008, Peng et al. 2010, Li et al. 2012).

Pairwise distance of 16 sequences viz., 10 from present study and 6 retrieved from GenBank showed the overall average distance was 0.052 (Table 3). Least intera-genus genetic distance of the present study samples was found with *G.garhwali* (0.005), followed by *G. buchanani* (0.050), *G.dakpathari* (0.078) and *G.davissinghi* (0.080). 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=0.33491416 is shown (Fig .1). The present study validates the study of previous workers like (Singh et al. 2011) and confirms that *G.pectinopterus* is closely related to *G. garhwali* and

G. buchanani.

AC-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, Herbert 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.

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References

Blaxter ML (2004) The promise of DNA taxonomy. Philosoph Transac of Royal Soc of London B. Biol Sci 359 : 669−679.

Brown WM, George M, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Nat Acad of Sci 76 (4) : 1967−1971.

Cantatore P, Saccone C (1987) Organization, structure and evolution of mammalian mitochondrial genes. Int Rev Cyto 108 : 149−208.

Floyd R, Abebe E, Papert A, Blaxter M (2002) Molecular barcodes for soil nematode identification. Mol Ecol 11 : 839−850.

Herbert PDN, Cywinska A, Ball SL, Waard De JR (2003) Biological identifications through DNA barcodes. Proc R Soc B 270 : 313−322.

Jhingran VG (1975) Fish and fisheries of India. Hindustan Publishing Corporation (India), Delhi, pp 1−954.

Kumar S, Stecher G, Tamura K (2016) MEGA7 : Molecular evolutionary genetics analysis version 7.0 for bigger data sets. Mol Biol and Evol 33 : 1870-−1874.

Lakra WS, Verma MS, Goswami M, Lal KK, Mohindra V, Punia P, Gopalkrishnan A, Singh KV, Ward RD, Hebert P (2009) DNA barcoding Indian marine fishes. Mol Ecol Resour 11 : 60−71.

Laskar BA, Kumar V, Kundu S, Darshan A, Tyagi K, Chandra K (2018) DNA barcoding of fishes from River Diphlu within Kaziranga National Park in Northerast India. Mitochondrial DNA Part A : 1-9. (doi : 10.1080/24701394.2018.1463373).

Li HY, Xu TJ, Cheng YZ, Sun DQ, Wang RX (2012) Genetic diversity of *Setipinnataty* (Engraulidae) populations from the China Sea based on mitochondrial DNA control region sequences. Genet and Mol Res 11 (2) : 1230−1237.

Liu HY, Jing SG, Su TF, Gong SY (2004) Polymorphism study of the mitochondrial DNA D-loop gene sequences from *Sparus latus*. J Fish China 28 : 371−374.

Moritz C, Cicero (2004) DNA barcoding. Promise and pitfalls. PLoSBiol 2;e354.

Peng Zuogang, Wang J, He Shunping (2010) The complete mitochondrial genome of helment catfish *Cranoglanis bouderius* (Siluriformes : Cranoglanididae) and the phy zlogeny of otophysan fishes. Gene 376 (2) : 290−297.

Saitou N, Nei M (1987) The neighbor-joining method : A new method for reconstructing phylogenetic trees. Mol Biol and Evol 4 : 406−425.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning.* Cold spring harbor laboratory press, New York.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting , and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann Entomol Soc Am 87: 651−701.

Singh M, Lakra WS, Bahuguna SN, Kartavtsev YP (2011) Cytochrome b gene sequence divergence of seven sisorid species of catfish genus *Glyptothorax* (Siluriformes, sisoridae) from India. Mol Biol Rep DOI 10.1007/s11033-011-12143.

Tautz D, Arctander P, Minelli A, Thomas RH, Vogler AP (2003) A plea for DNA taxonomy. Trends Ecol Evol 18 : 70−74.

Tilak R, Hussain A (1977) A check list of the fishes of Himachal Pradesh. Zool J Syst Bot 104 : 265−301.

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert Paul DN (2005) DNA barcoding Australia's fish species. Philosoph Transac of the Royal Soc of London B : Biol Sci 360 (1462) : 1847−1857.

Wilson EO (2004) Taxonomy as a fundamental discipline. Philosoph Transac of the Royal Soc of London B : Biol Sci 359 : 739−749.

www.fishbase.se/summary/24870. Accessed on 29th March, 2019.

Yang B, Chen XY, Yang JX (2008) Structure of the mitochondrial DNA control region and population genetic diversity analysis of *Anabarilius grahami* (Regan). Zool Res 29 : 379−385.