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# DNA barcoding of some commonly exploited fishes from the northern Western Ghats, India

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ABSTRACT

The Western Ghats, being very rich in freshwater fish diversity, has recently been confirmed as a globally significant centre of diversity and endemism for freshwater species and comprise one of the 34 global biodiversity hotspots. Owing to its extreme ichthyofaunal diversity, the present study was designed to generate cytochrome oxidase I (COI) DNA barcodes for the identification of some commonly exploited fishes from the west-flowing rivers of northern Western Ghats. Twenty-three fish specimens representing 6 families and 10 species were barcoded from the major west-flowing rivers of the northern Western Ghats. The obtained barcodes discriminated all the species with sufficient barcode gap. The average Kimura two parameter (K2P) values for within species, the genus and family distances were 0.37, 17.74 and 18.51% respectively. The neighbour-joining tree revealed distinct clusters corresponding to the taxonomic status of the species. Generated barcodes are expected to provide the much-needed baseline reference for the ichthyofaunal biodiversity of the global biodiversity hotspot.

**Key words**: DNA barcoding, Freshwater fish, COI, Molecular phylogeny, Western Ghats

The Western Ghats, one of the 34 global biodiversity hotspots for conservation, is extraordinarily rich in biodiversity. It runs along the west coast of India extending from 08°19'08"–21°16'24" N to 72°56'24"–78°19'40" E covering a total area of 136,800 km$^2$ (CEPF 2007). The Western Ghats and the associated river drainages are rich in freshwater fish diversity (Kottelat and Whitten 1996). Originally designated for high diversity and endemism of plant species, the Western Ghats have recently been confirmed as a globally significant centre of diversity and endemism for freshwater species (Dahanukar *et al.* 2011). The northern region of the Western Ghats within the Konkan region of Maharashtra has a lower documented freshwater diversity than the southern region probably owing to inadequate surveys in the freshwater ecosystems of the west flowing rivers of the northern Western Ghats (Dahanukar *et al.* 2004).

Rivers of the Western Ghats can broadly be divided into the west-flowing rivers which are relatively small, originating in the Western Ghats and draining into the Arabian Sea and the east-flowing rivers which are relatively larger and which originate in the Western Ghats and finally flow into the Bay of Bengal. The Western Ghats region has not been investigated in its entirety in a standardized manner with respect to ichthyofaunal diversity. There are several areas, especially, in the northern Western Ghats region of Maharashtra, which are relatively under- and/or unexplored. This is particularly true for the rivers of the central and northern Western Ghats (Dahanukar *et al.* 2004) where the west-flowing rivers are poorly studied.

Accurate species identification has long been dependent on morphological analyses performed by taxonomists. It is, however, known that morphological approaches to species identification have limitations, mainly since morphological similarities between closely related organisms create challenges to discriminate them from each other. Morphological identification methods are also often dependent on gender and life stages of the species (Hebert *et al.* 2003a), which may lead to difficulties in recognition, for example, of juvenile specimens. DNA-based approaches for taxon diagnosis exploiting DNA sequence diversity among species can be used to identify fishes and resolve taxonomic ambiguity including the discovery of new and cryptic species (Hebert *et al.* 2003a). DNA barcoding is the derivation of short DNA sequence(s) that enables species identification, recognition, and discovery in a particular

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domain of life. The most frequently used gene for DNA barcoding is the mitochondrial Cytochrome c oxidase subunit I (COI) (Hebert *et al.* 2003a).This barcode region has been shown to exhibit a marked divergence in the genetic distance within species (typically <3%) versus that between species (typically 10–25%) (Hebert *et al.* 2003b).

A wide variety of protein- and DNA-based methods have been used for the genetic identification of fish species (Ward and Grewe 1994). In India, several studies have developed DNA barcodes for marine fishes (Lakra *et al.* 2010) and freshwater fishes (Chakraborty and Ghosh 2014, Lakra *et al.* 2015). Keeping in view the diverse ichthyofauna of ecologically rich and under-explored northern Western Ghats, the present study was undertaken with an aim to create a reference library of DNA barcodes for the highly valuable ichthyofauna of the region which is an important global biodiversity hotspot.

## MATERIALS AND METHODS

*Sample collection:* Fish samples were collected from the west-flowing rivers and/or their tributaries of the northern Western Ghats. All samples were kept on ice during the entire time between collection of the specimen and tissue (muscle) sampling to avoid degradation of the DNA. While the left side of the fishes was photographed, the muscle tissues for DNA extraction were taken from the right side. Taxonomic identification of the collected fish specimens up to species level was done according to morphological and meristic characters (Jayaram 1981, Talwar and Jhingran 1991).

Tissue collection was done using sterilized surgical grade blades to prevent any contamination. The fresh blade was used for tissue collection from every individual. Approximately 100 mg of tissue sample per fish was collected from the dorsal muscle and were preserved in 95% ethanol (Omnis, Jebsen and Jessen GmbH and Co. Germany) in 2 ml Eppendorf tubes and held at – 40°C until used. Tissues were collected from a total of three to five individuals per species. Voucher specimens were prepared by preserving the sample in 8% formalin with proper labeling and deposited in the Fish Museum at the Department of Fisheries Biology, College of Fisheries, Ratnagiri (Maharashtra, India).

*DNA extraction:* DNA extraction from alcohol-preserved tissue was carried out as described by Bentzen *et al.* (1990) with minor modifications. The concentration of isolated DNA was estimated using a Nanodrop ND-1000 UV spectrophotometer (JH Bio Innovations Pvt. Ltd.). Extracted DNA was diluted to a final concentration of 100 ng/l.

*PCR and sequencing:* The primers (Ward *et al.* 2005) used for the amplification of the COI gene were FishF1 - 5'TCAACCAACCACAAAGACATTGGCAC3' and FishR1-5'TAGACTTCTGGGTGGCCAAAGAATCA3'. The COI gene was amplified in a 50 µl volume containing 5 µl 10× Taq polymerase buffer, 2 µl $MgCl_2$ (50 mM), 0.25 µl of each dNTP (0.05 mM), 0.5 µl each primer (0.01 mM), 0.6 U Taq polymerase and 5 µl genomic DNA. Amplifications were performed using a Thermal cycler 2720 (Applied Biosystems). The thermal regime consisted of an initial step of 5 min at 94°C followed by 35 cycles of 45 sec at 94°C, 45 sec at 54°C, and 1 min at 74°C with final extension of 15 min at 74°C followed by holding at 4°C. PCR products were visualized on 2% agarose gels and the most intense products were selected for sequencing. Products were labelled using the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) and sequenced bidirectionally using an ABI 3730 capillary sequencer following manufacturer's instructions.

*Sequence analysis:* Approximately 500–650 bp were amplified from the 5' region of the COI gene from mitochondrial DNA. Obtained sequences were assembled, trimmed and edited for quality using DNA Star software (DNASTAR, Inc.) as per manufacturer's instructions. The genetic distances within and between the species was determined by Kimura 2 parameter (K2P) method (Kimura 1980) by MEGA (v6.06) and BOLD (v4). Neighbour-joining (NJ) trees of K2P distances were created to provide a graphic representation of the patterning of divergence between species (Saitou and Nei 1987). The substitution patterns and rates were estimated under the Tamura-Nei model (Tamura *et al.* 2004). The phylogenetic tree was constructed based on NJ method using MEGA with 1000 pseudo-replications.

For testing phylogenetic similarities and validations of obtained COI sequences, the homologous sequences of nine species from the southern Western Ghats and other parts of India were included (mined from GenBank database). These species were *Puntius sophore* (Accession numbers: KJ936845, JX983465.1), *Etroplus suratensis* (Accession numbers: KP939359, KF442194.1), *Mastacembelus armatus* (Accession numbers: JX983364.1, JX260909.1), *Glossogobius giuris* (Accession numbers: JX983309.1, JX260877.1), *Garra mullya* (Accession numbers: JX983296.1, JX293005.1), *Puntius chelynoides* (Accession number: JN965207.1), *Puntius sarana* (Accession numbers: JX181867.1, JX260951.1), *Mystus malabaricus* (Accession number: HQ219113.1) and *Mystus oculatus* (Accession number: HQ009493.1).

## RESULTS AND DISCUSSION

Twenty-three COI consensus sequences were obtained for ten species (Table 1). The sequences obtained in the present study were compared with the sequences reported in public databases (e.g., GenBank and BOLD) using BLAST to confirm the species identity. Simplicity and unambiguity was observed among all obtained sequences. The COI sequences were checked for indels and stop codons to verify their functionality. No NUMTs (transfers of mtDNA cox1 gene sequences into the nuclear genome) were observed. On the contrary, some of the invertebrates have been reported to contain NUMTs (Williams *et al.* 2001) whereas most of the Actinopterygii did not show any NUMTs (Bensasson *et al.* 2001, Ward *et al.* 2005, Lakra *et al.* 2010). All the COI sequences were submitted

to NCBI GenBank database. The GenBank Accession numbers for the COI sequences of the investigated species are given in Table 1. Sequences and trace files are also available at College of Fisheries, Ratnagiri upon request to the authors.

*COI gene nucleotide frequency:* The COI sequence composition was estimated across all collected specimens. Sequence analysis revealed average nucleotide frequencies of 25.52% (A), 29.48% (T), 27.48% (C) and 17.52% (G). Mitochondrial genomes show wide variation in their GC content. A strong correlation has been reported between mitochondrial genome-wide shifts and COI gene (Clare *et al.* 2008). In the present study, the GC content of partial COI gene was on average 45.00%. Nearly similar GC content in fishes has been reported earlier based on complete mtDNA genome ranging from 38.4–43.2% and 42.2–47.1% with COI alone (Ward *et al.* 2005), which was mostly attributable to 3rd base variation. Substantially, more nucleotide changes at the 3rd codon position followed by 1st codon position were observed. This reflects the fact that most synonymous mutations occur at the 3rd position, with a few at the 1st position and none at the 2nd.

In a significant number of species, it has been reported that the transition frequencies are more than the transversion frequencies (Brown *et al.* 1982, Curtis and Clegg 1984). In the present study, the average number of transitions for COI gene was more than the mean number of transversions. The transition/transversion rate ratios were $k_1$ = 2.15 (purines) and $k_2$ = 2.644 (pyrimidines). The overall transition/transversion bias was $R$ = 1.264.

Table 1. GenBank Accession numbers of COI sequences of species studied

| Species | River | GenBank Accession Number |
|---|---|---|
| *Garra mullya* | Amba | KX228709 |
| *Garra mullya* | Bhogwati | KX352734 |
| *Garra mullya* | Vashisthi | KX228707 |
| *Garra mullya* | Oros | KX352735 |
| *Garra mullya* | Muchkundi | KX352740 |
| *Puntius chelynoides* | Savitri | KX228702 |
| *Puntius chelynoides* | Gad | KX228701 |
| *Puntius sophore* | Amba | KX228708 |
| *Puntius sophore* | Vashisthi | KX228706 |
| *Mystus malabaricus* | Bhogwati | KX228710 |
| *Mystus malabaricus* | Vashisthi | KX352739 |
| *Mystus malabaricus* | Gad | KX352736 |
| *Mystus oculatus* | Amba | KX228700 |
| *Mestacembelus armatus* | Vashisthi | KX228705 |
| *Mestacembelus armatus* | Gad | KX352737 |
| *Mestacembelus armatus* | Kundalika | KX371833 |
| *Glossogobius giurus* | Muchkundi | KX228704 |
| *Glossogobius aureus* | Amba | KX352738 |
| *Glossogobius aureus* | Savitri | KX228703 |
| *Etroplus suratensis* | Savitri | KX371828 |
| *Etroplus suratensis* | Vashisthi | KX371830 |
| *Puntius sarana* | Bhogwati | KX371825 |
| *Puntius sarana* | Muchkundi | KX371832 |

Table 2. Distance (K2P) values (%) within various taxonomic levels

| Comparison | Taxa | Comparisons | Min. | Mean | Max. | S.E. |
|---|---|---|---|---|---|---|
| Within species | 21 | 21 | 0.00 | 0.73 | 4.63 | 0.07 |
| Within genus | 13 | 17 | 15.23 | 17.74 | 20.07 | 0.08 |
| Within family | 11 | 30 | 15.44 | 18.51 | 21.43 | 0.07 |

*Distance summary*: DNA barcodes can discriminate species based on the magnitude of difference between intraspecific and interspecific genetic distance value. The mean nucleotide diversity (Pi) among all the species was estimated to be 0.226. The average K2P distance values for COI gene increased from lower to higher taxa levels, first within species and genus and then the family. The intraspecific genetic distance varied from 0.00 to 4.63% whereas the interspecific distance ranged from 15.23 to 20.07%. The average genetic distance based on K2P within species, genus and family was 0.73%, 17.74% and 18.51% respectively (Table 2). In general, the average conspecific, congeneric and confamilial K2P distances were within the range observed for other Indian freshwater fish (1.6%, 7.16%, 16.66%, respectively) (Chakraborty and Ghosh 2014); for Carangids from the Kakinada coast (0.78%, 17.2%, 24.18%, respectively) (Persis *et al.* 2009); for Canadian fishes (0.27%, 8.37%, and 15.38%, respectively) (Hubert *et al.* 2008) and for Australian marine fishes (0.39%, 9.93%, and 15.46%, respectively) (Ward *et al.* 2005). The average interspecific distance value was ~25 times higher than average intraspecific distance, which clearly indicates the presence of DNA barcode gap among the sequenced species.These findings support previous observations. The DNA barcode gap of ~25 fold was higher than the 18 fold increase observed for other Indian freshwater fish (Chakraborty and Ghosh 2014). The rate of increase in the genetic distance declined in the higher taxonomic groups due to substitution saturation. Overlapping of conspecific and congeneric levels of divergence was not observed.

The distribution of K2P distance values showed <2% divergence for intraspecific comparisons. However, for *Mastacembelus armatus* (From River Kundalika and Vashisthi), the intra specific value was 4.6%±0.01 (Supplementary data available from the authors upon request).

In addition, DNA barcode gap was estimated as a test of there liability of barcodes for species discrimination, which enables the assignment of unidentified individuals to their species with a negligible error rate. Barcode gap analysis and nearest neighbour distance analysis showed absence of overlap between intraspecific and interspecific distance values (Table 3).

An optimum phylogenetic signal has been reported in COI sequence data in addition to the use in the delineation

of species boundaries. The NJ tree constructed based on COI genes revealed distinct phylogenetic relationship among the species. Distinct clusters were shared by congeneric species and the species belonging to the same family grouped together. All major nodes were supported by high bootstrap value (Fig. 1).

*COI gene geographic distance correlation*: The intraspecific variation increased after including conspecific sequences from other geographical locations. Mantel test showed a positive correlation between genetic distance and geographic distance (Table 4). Generally, species tend to accumulate mutations if the gene flow between populations is hampered for several generations and thus, may show allopatric divergence. In some species, the Mantel test *P*-values were higher which can be attributed to the non-availability of conspecific sequences in the BOLD database based on geographic locations. In the present study, the intraspecific variation increased several folds after including conspecific sequences from other geographical locations. Previously, few studies have examined the levels of COI divergences across broad geographic regions in a large number of taxa (Hebert *et al.* 2004, Bergsten *et al.* 2012) and showed that DNA barcodes could differentiate even as geographic coverage expanded.

DNA barcoding has proved to be an efficient tool for documenting the fish diversity of northern Western Ghats.

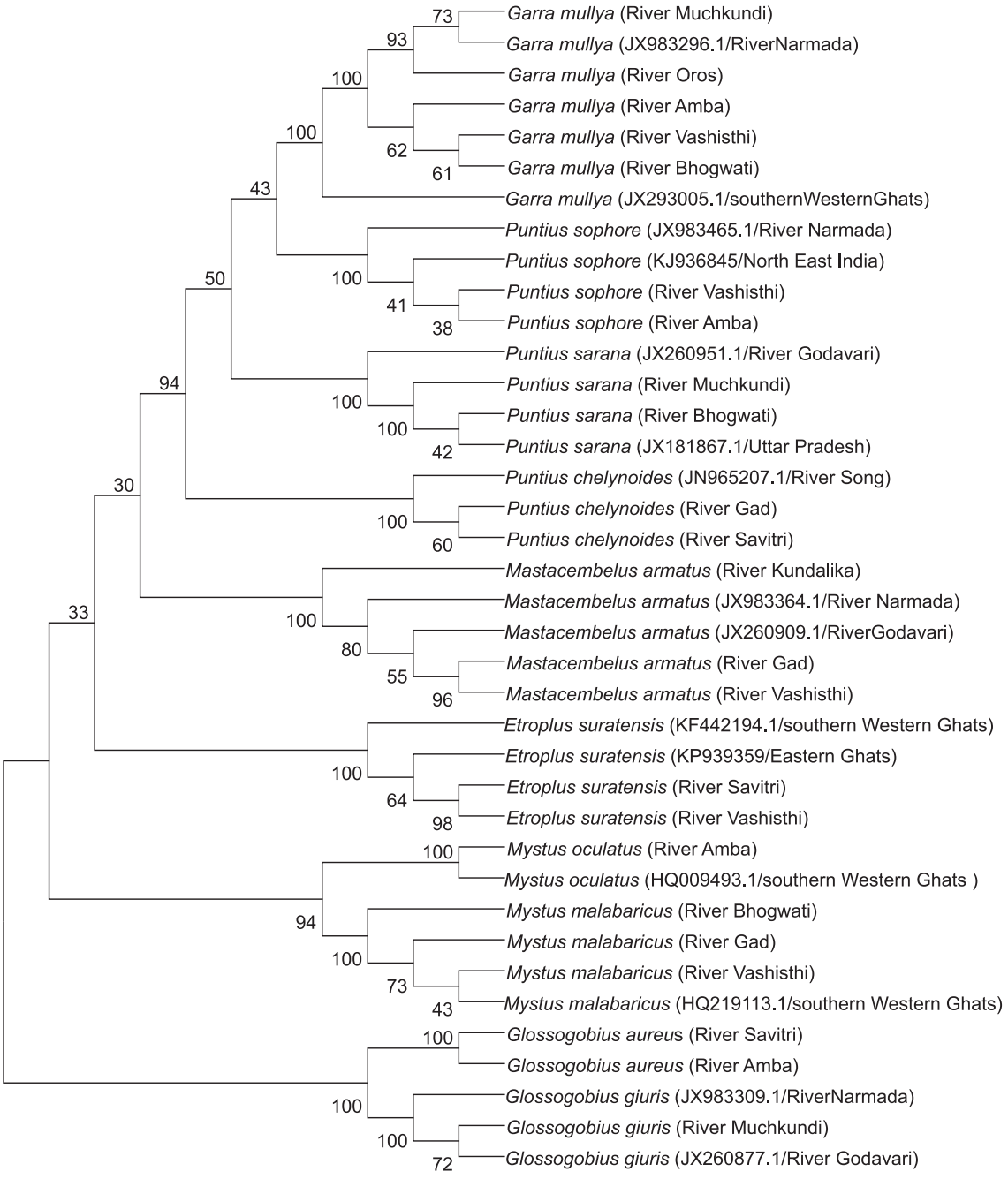


Fig. 1. K2P divergence based Neighbour-joining tree.

Table 3. COI barcode gap between species

| Order | Family | Species | Average intra-sp | Max. intra-sp | Nearest neighbour (NN) species | Distance to NN |
|---|---|---|---|---|---|---|
| Cypriniformes | Cyprinidae | *Garra mullya* | 0.61 | 2.56 | *Puntius sophore* | 15.44 |
| Cypriniformes | Cyprinidae | *Puntius chelynoides* | 0 | 0 | *Puntius sarana* | 18.08 |
| Cypriniformes | Cyprinidae | *Puntius sarana* | 0 | 0 | *Puntius sophore* | 15.23 |
| Cypriniformes | Cyprinidae | *Puntius sophore* | 0 | 0 | *Puntius sarana* | 15.23 |
| Perciformes | Cichlidae | *Etroplus suratensis* | 0 | 0 | *Puntius sophore* | 20.43 |
| Perciformes | Gobiidae | *Glossogobius aureus* | 0 | | *Glossogobius giuris* | 17.2 |
| Perciformes | Gobiidae | *Glossogobius giuris* | 0 | 0 | *Glossogobius aureus* | 17.2 |
| Siluriformes | Bagridae | *Mystus malabaricus* | 0 | 0 | *Mystus oculatus* | 17.07 |
| Siluriformes | Bagridae | *Mystus oculatus* | 0 | 0 | *Mystus malabaricus* | 17.07 |
| Synbranchiformes | Mastacem-belidae | *Mastacembelus armatus* | 3.07 | 4.63 | *Puntius sarana* | 21.55 |

Table 4. Geo-distance correlation among fishes across different populations

| Species | Count | Linear regression $R^2$ | Linear regression slope | Gen Dist Max | Geo Dist Max (Km) | Mantel $R^2$ | Mantel *P*-value |
|---|---|---|---|---|---|---|---|
| *Glossogobius giuris* | 3 | 1 | 0.0004 | 0.491 | 1290.94 | 1 | 0.31 |
| *Puntius sarana* | 3 | 1 | 0.0004 | 0.491 | 1290.94 | 1 | 0.3 |
| *Puntius chelynoides* | 4 | 0.76 | 0.0003 | 0.491 | 1290.94 | 0.76 | 0.28 |
| *Garra mullya* | 6 | 0.47 | 0.0003 | 0.818 | 1290.94 | 0.47 | 0.18 |
| *Mastacembelus armatus* | 6 | 0.47 | 0.0003 | 0.818 | 1290.94 | 0.47 | 0.13 |
| *Mystus malabaricus* | 9 | 0.28 | 0.0002 | 0.818 | 1385.82 | 0.28 | 0.11 |
| *Puntius sophore* | 8 | 0.32 | 0.0002 | 0.818 | 1385.82 | 0.32 | 0.09 |
| *Glossogobius aureus* | 12 | 0.95 | 0.0048 | 21.967 | 4745.87 | 0.95 | 0.01 |

Increasing use of DNA barcoding can overcome the limitations of morphology-based identifications and help identify previously unidentified species by documenting the diversity of COI sequences within currently recognized species. These barcodes were the first barcode reference from the relatively poor studied west-flowing rivers of the northern Western Ghats. Given the vulnerability of Western Ghats to anthropogenic influences, the present information is expected to serve as a baseline for further studies. This is especially true in a sense that the present study had focused only on some common exploited species from the various west-flowing rivers of northern Western Ghats. A greater diversity is bound to be exhibited if one attempts to address the non-exploited species too.

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