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DNA Barcoding and Phylogenetic Relationships of Selected South Indian Freshwater Fishes Based on mtDNA COI Sequences

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Abstract

DNA barcoding is an effective tool for the identification of species representing diverse taxa especially through the sequence analysis of mitochondrial cytochrome c oxidase subunit I (COI) gene. In the present study, DNA barcodes were generated from 46 species of freshwater fishes covering the Orders *Cypriniformes*, *Siluriformes*, *Synbranchiformes* and *Perciformes* representing 30 genera under 9 families. All the samples were collected from diverse sites which also includes some endemic species. A total of 47 COI sequences were generated. After amplification and sequencing of 678 base pair fragment of COI, primers were trimmed which invariably generated a 635 base pair barcode sequence. The average Kimura two-parameter (K2P) distances within-species, genera, families, and orders were 0.32%, 8.40%, 14.50%, and 18.65%, respectively. DNA barcode discriminated congeneric species without any confusion. The present study strongly supports the efficiency of COI as an ideal marker for DNA barcoding of selected freshwater fishes.

Keywords: Barcoding; COI; Indian freshwater fishes; mtDNA; Phylogeny

Introduction

India is rich in fishery resources as it inhabits about 2508 fish species [1] of which the 856 are freshwater occupants [2,3]. The fishes are the most diverse vertebrate in world and about 40% of them live in freshwater. India is one of the mega biodiversity countries in the world and occupies the ninth position in terms of freshwater mega biodiversity and contributed 11.72% of the globe fish biodiversity [4]. However, the actual number of fish species found in India is still not accurately documented because of prevailing taxonomic confusion [5] due to inadequate exploration, indiscernibility among cryptic forms coupled with species ambiguity in the taxonomic keys [6]. As a result, many species have been considered as cryptic and some of which may also be dormant [6,7]. Therefore, for legible characterization of Indian freshwater fishes, there is a vital need of species scrutiny using advanced molecular methods. Hence, there is an urgent need for the assessment of Indian freshwater fish species through DNA barcoding.

DNA barcoding is a promising technique for species identification using a short mitochondrial DNA sequence of COI gene [8]. This technique involves the analysis of the sequence diversity of a 50 segment mitochondrial COI gene to identify species [9]. Of late, DNA barcoding method has been extensively followed for species identification as well as species discovery in various groups of organism [10,11]. Effectiveness of DNA barcoding has now been validated for many groups of animals [12] and among them fishes being one of the most extensively studied groups [13,14].

In recent years, several such molecular studies have been conducted on members of this group to better understand their relationships and to develop more accurate taxonomic classifications based on phylogeny [15-25]. Use of COI gene for barcode is considered to be suitable marker to discriminate the closely related species of fishes [26-29]. But the challenge in use of small DNA barcode (only 655bp) based phylogenetic study is selection of a nearly perfect nucleotide substitution model for the dataset, so that the weakest evolutionary signal can be correctly detected [30]. However, a comprehensive assessment of DNA barcodes of Indian freshwater fishes is limited, though a similar study has been done for the selected Indian freshwater fishes by Lakra [8]. Therefore, the present study reported additional DNA barcoding of 46 commercially important Indian freshwater fish species belonging to 30 genera under 9 families and 4 orders.

Materials and Methods

Sample collection and morphological identification

The tissue and voucher specimens of 46 species (9 families) were collected from different riverine systems of south India namely from Cauvery and Bhavani river systems. Approximately 100 mg of muscle tissue and fin clips from two to five individuals of each species were preserved in 95% ethanol until used. The species identification and confirmation were carried out using standard literature [31,32]. The valid nomenclatural names were adopted as per the Catalogue of Fishes of the California Academy of Sciences [1,33]. The live specimens were photographed with Canon 1100 Digital SLR Camera and later preserved in 7% formalin solution for future reference. Table 1 represents specimen details and GenBank accession numbers.

S. no.	Order	Family	Species	Voucher No.	Accession no.
1	*Cypriniformes*	*Cyprinidae*	*Salmophasia bacaila* (Hamilton, 1822)	PUMNH 08/2013	KX266823
2			*Barilius canarensis* (Jerdon, 1849)	PUMNH 30/2013	KX230848
3			*Barilius gatensis* (Valenciennes, 1844)	PUMNH 31/2013	KX230845
4			*Barilius bendelisis* (Hamilton, 1807)	PUMNH32 /2013	KX230846
5			*Barilius bakeri* (Day, 1865)	PUMNH01 /2013	KX230847
6			*Danio rerio* (Hamilton, 1822)	PUMNH76 /2013	KX266821
7			*Danio rerio* (Hamilton, 1822)	PUMNH77 /2013	KX266822
8			*Devario aequipinnatus* (McClelland, 1839)	PUMNH 04/2013	KX289313
9			*Devario malabaricus* (Jerdon, 1849)	PUMNH02 /2013	KX529835
10			*Rasbora daniconius* (Hamilton, 1822)	PUMNH 34/2013	KX239494
11			*Esomus danricus* (Hamilton, 1822)	PUMNH06 /2014	KX266826
12			*Amblypharyngodon mola* (Hamilton, 1822)	PUMNH 28/2013	KX266827
13			*Tor khudree* (Sykes, 1839)	PUMNH 47/2013	KX550003
14			*Neolissocheilus hexagonolepis* (McClelland, 1839)	PUMNH 48/2013	KX266828
15			*Systomus sarana* (Hamilton, 1822)	PUMNH 49/2013	KX239499
16			*Dawkinsia filamentosa* (Valenciennes, 1844)	PUMNH 50/2013	KX230844
17			*Dawkinsia arulius* (Jerdon, 1849)	PUMNH 06/2013	KX239496
18			*Puntius amphibius* (Valenciennes, 1842)	PUMNH 07/2013	KX529836
19			*Puntius sophore* (Hamilton, 1822)	PUMNH03 /2014	KX289308
20			*Haludaria fasciata* (Jerdon, 1849)	PUMNH 51/2013	KX550002
21			*Pethia narayani* (Hora, 1937)	PUMNH 07/2014	KX289310
22			*Barbodes carnaticus* (Jerdon, 1849)	PUMNH 52/2013	KX239492
23			*Hypselobarbus dubius* (Day, 1867)	PUMNH 53/2013	KX266817
24			*Hypselobarbus curmuga* (Hamilton, 1807)	PUMNH 13/2014	KX266819
25			*Hypselobarbus kurali* (Menon & Rema Devi, 1995)	PUMNH 23/2014	KX266820
26			*Hypselobarbus kolus* (Sykes, 1839)	PUMNH 39/2014	KX266818
27			*Hypselobarbus micropogon* (Valenciennes, 1842)	PUMNH 72/2014	KX266813
28			*Hypselobarbus periyarensis* (Raj, 1941)	PUMNH 93/2014	KX266814
29			*Osteochilichthys nashii* (Day, 1868)	PUMNH 62/2013	KX239498
30			*Osteochilichthys thomassi* (Day, 1877)	PUMNH 19/2014	KX239497
31			*Osteobrama cotio* (Hamilton, 1822)	PUMNH 23/2014	KX550004

32			*Laboe bata* (Hamilton, 1822)	PUMNH 12/2013	KX289314
33			*Labeo rohita* (Hamilton, 1822)	PUMNH 14/2013	KX266835
34			*Garra mullya* (Sykes, 1839)	PUMNH 57/2013	KX239490
35			*Garra bicornuta* (Narayan Rao, 1920)	PUMNH 15/2013	KX289309
36			*Garra mcClellandi* (Jerdon, 1849)	PUMNH 56/2013	KX239495
37		*Cobitidae*	*Lepidocephalichthys thermalis* (Valenciennes, 1846)	PUMNH 54/2013	KX266825
38			*Lepidocephalichthys guntea* (Hamilton, 1822)	PUMNH 18/2014	KX266824
39			*Botia striata* Narayan Rao, 1920	PUMNH 13/2014	KX575850
40			*Bhavania australis* (Jerdon, 1849)	PUMNH 12/2014	KX289311
41		*Balitoridae*	*Nemacheilus guentheri* Day, 1867	PUMNH 15/2014	KX289312
42	*Siluriformes*	*Bagridae*	*Mystus bleekeri* (Day, 1877)	PUMNH 19/2013	KX266834
43		*Siluridae*	*Ompok bimaculatus* (Bloch, 1794)	PUMNH 22/2013	KX239493
44		*Sisoridae*	*Glyptothorax gracilis* (Gunther, 1864)	PUMNH 29/2014	KX289315
45	*Synbranchiformes*	*Mastacembelidae*	*Mastacembelus armatus* (Lacepede, 1800)	PUMNH 38/2013	KX575851
46	*Perciformes*	*Nandidae*	*Nandus nandus* (Hamilton, 1822)	PUMNH 70/2013	KX266833
47		*Gobiidae*	*Glossogobius giuris* (Hamilton, 1822)	PUMNH 43/2013	KX239491

Table 1: List of freshwater fish species barcoded along with GenBank accession numbers.

Amplification and sequencing

Genomic extractions were taken from fin clips, preserved in >95% ethanol using Invitrogen's "Pure Link Genomic DNA Mini Kit" following the manufactures instructions. COI amplification was carried out in 25-µL reaction mixtures containing 1 µl template DNA, 1X reaction buffer, 2.5 mM $MgCl_2$, 2.5 mM dNTPs, 0.5 µl of each primer, and 0.2 U TaqDNA polymerase in a PTC-200 (Bio-Rad, USA) PCR machine. The reaction mixtures were preheated at 94°C for 5 min, followed by 50 cycles of amplification (94°C for 45 sec, 48°C for 45 sec, and 72°C for 60 sec), and a final extension at 72°C for 6 min. The COI gene was amplified using the universal primer set: The primers used for the amplification of the COI gene were: Fish F1-5'-TCAACCAACCACAAAGACATTGGCAC-3' and Fish R1-5'-TAGACTTCTGGGTGGCCAA AGAATCA-3' [34]. Sequencing was performed using Big Dye Terminator on ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). The PCR products were visualized on 1.2% agarose gels and the most intense products were selected for sequencing. Products were labeled using the BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA) and sequenced bidirectionally using an ABI 3730 capillary sequencer following instructions of the manufacturer. One individual of each species was used for the nucleotide sequence analyses.

Sequence analysis

Sequences were aligned using Clustal W [35] and then submitted to GenBank. The extent of sequence difference between species was calculated by averaging pair-wise comparisons of sequence difference across all individuals. Pair-wise evolutionary distance among haplotypes was determined by the Kimura 2-Parameter method [36] using the software program MEGA 3 (Molecular Evolutionary Genetics Analysis, MEGA Inc., Englewood, NJ) [37]. The Neighbor-Joining (NJ) tree was constructed using MEGA 3 and to verify the robustness of the internal nodes of NJ tree, and bootstrap analysis was carried out using 1000 pseudo replications.

Results

Genetic divergence and phylogenetic analysis

A total of 47 sequences were generated from 46 freshwater fish species. Sequence alignment of COI gene after trimming of primers yielded 635 nucleotide base pairs per taxon. All the sequences showed simplicity and un-ambiguity, and no insertions, deletions, or stop codons were observed in any of the sequences. The sequence analysis revealed average nucleotide frequencies as A=26.00%, T=29.80%, G=26.4%. and C=17.90%. The average K2P distances in percentage among the different taxonomic levels were analyzed (Table 2). The average transitional pairs (si¼72) were more frequent than average transversional pairs (sv¼56) with an average ratio of 1.30. The average genetic distances within order, family, genus and species were 18.65%, 14.50%, 8.40%, and 0.32% respectively. The overall average genetic distance among all the species was 23.90%.

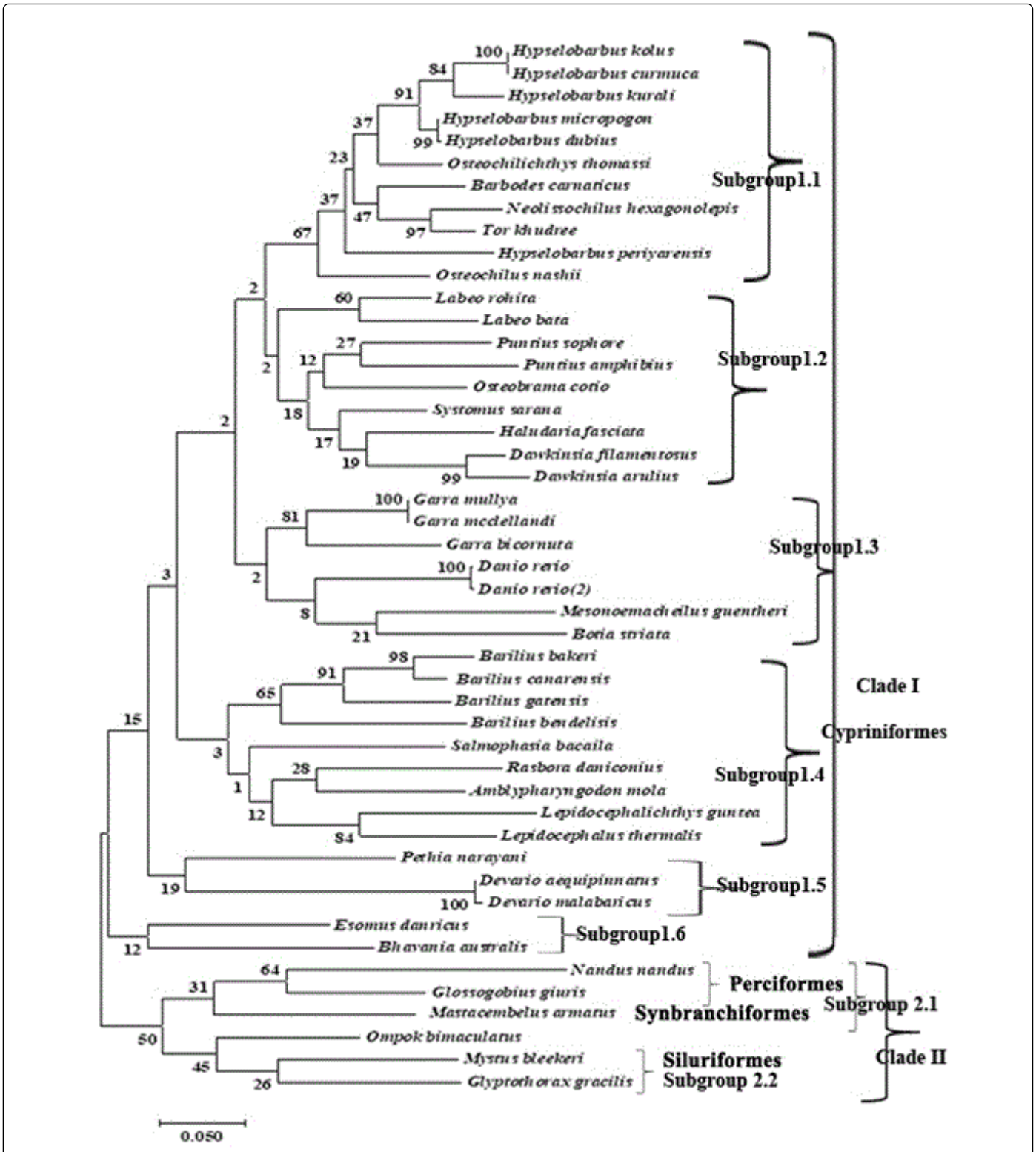

Figure 1: The Neighbor-joining tree for 47 species of freshwater fishes based on COI sequences. Note: Scale bar indicates the genetic distance.

The phylogram was divided into two main clades with high bootstrap support (>50%) (Figure 1). The clade I was subdivided into six separated subgroups: subgroup 1.1 includes 11 species belong to 6 genus (*Hypselobarbus*, *Osteochilichthys*, *Barbodes*, *Neolissochilus*, *Tor* and *Osteochilus*) of the family *Cyprinidae*.

Taxa	Min dist (%)	Mean dist (%)	Max dist (%)	Standard error (SE)
Within species	0	0.32	0.77	0.003
Within genus	0.12	8.4	14.32	0.007
Within family	0.73	14.5	25.6	0.01
Within order	5.2	18.65	29.5	0.016

Table 2: Summary of genetic divergences of different taxonomic levels (based on the K2P distance model).

Subgroup 1.2 includes 9 species belonging to 6 genera (*Labeo*, *Puntius*, *Osteobramma*, *Systomus*, *Haludaria*, *Dawkinsia*) of the family *Cyprinidae*. Subgroup 1.3 includes 6 species belonging to 4 genera (*Garra*, *Danio*, *Mesonemacheilus*, *Botia*) of the family *Cyprinidae*. Subgroup 1.4 includes 9 species belonging to 5 genera (*Barilius*, *Salmophasia*, *Rasbora*, *Amblypharyngodon*, *Lepidocephalichthys*) of the family *Cyprinidae*. Subgroup 1.5 includes 3 species belonging to 2 genera (*Pethia*, *Devario*) of the family *Cyprinidae*. Subgroup 1.6 consists of 2 species belonging to 2 genera (*Esomus*, *Bhavania*). At the genus level all the species showed monophyly with high BT support. The clade II was divided into two subgroups. The Subgroup 2.1 consists of 3 species from two orders, *Nandus nandus* and *Glossogobius giuris* belonging to the order *Perciformes* and *Mastacembelus aramatus* belonging to the order *Synbranchiformes*. Subgroup 2.2 consists of 3 species of the genera; *Ompak*, *Mystus*, *Glyptothorax* belonging to the order *Siluriformes*.

Discussion

During DNA barcoding by Hebert et al. [38], the sequencing of a ~650bp region of the mitochondrial cytochrome oxidase I gene (COI), has been proven to be extremely an effective method for discriminating fish species [28,34,39]. Interestingly, recent research has illustrated some straightforward benefits from the use of standardized species-specific molecular tags derived from COI gene for species-level identifications [40]. DNA barcoding analysis has clearly discriminated freshwater fish species from India [8] Canada [28] and Mexico and Guatemala [39]. Presently, we have effectively used partial COI genes as DNA barcode in 46 freshwater fish species from south Indian waters representing 4 orders (*Cypriniformes*, *Siluriformes*, *Perciformes* and *Synbranchiformes*) representing 9 families and 30 genera. The universal primers amplified the target region in all 46 species, thus generating 47 COI barcodes of 635 bp and no insertions, deletions, stop codons or NUMTs were observed in any sequence, which support the hypothesis that all the amplified sequences derive from a functional mitochondrial COI sequences. And the present findings are in line with the previous reports [34]. Although the primary objective of DNA barcoding is to identify species, phylogeographic structure among COI sequences within species became evident. DNA barcoding pursues to provide a convenient, accurate and valid tool for species identification, and any candidate gene must suit this qualification. Use of a single, universal gene has many advantages, especially as barcoding applications expand to ecological questions and in the identification of illegally imported parts of organisms [41]. The study indicates that the standard barcoding marker, COI, can identify fish species [42,43,44]. The barcode sequences clearly discriminated all the studied freshwater fish species along with the apparent phylogenetic resolution. Although intra- and inter-specific genetic divergences overlap, tree-based methods can distinguish species in unidentified samples. For the ecologist and taxonomist alike, DNA barcoding would provide a powerful tool for the correct species identification, biodiversity assessments and locating the occurrence of cryptic species [45,46].

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