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RESEARCH PAPER

Deciphering Identification of Inland Fishes of Gujarat Using DNA Barcoding

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Abstract

The freshwater fishes of Gujarat have been largely untouched owing to the principal focus on the large marine sectors. In this paper, taxonomic identification has been done for few of the species and DNA barcoding has been attempted to strengthen the identification. This modern approach led us to the present study in which the classification of 52 species of the freshwater species found in selected six districts of Gujarat. 38 species of freshwater fishes, all belonging to the class Actinopterygii, were discriminated sequences (barcoded) for a 655bp region of the mitochondrial cytochrome oxidase subunit I gene (cox1). The samples were appropriately identified morphologically using standard available keys. Hence this will provide an insight of fish diversity and will take it to a further step, to carry out future molecular investigations which cannot be done if the sequences of the organisms are not known.

Keywords: Taxonomy, freshwater fishes, COI gene.

Introduction

India has a rich natural heritage and nurtures a unique bio-diversity, placing it among the 12 most bio diverse countries. Like other fauna, it is rich in fishery resources and comprises of around 2508 fish species (Eschmeyer & Fricke, 2012) of which 856 are freshwater inhabitants (Froese & Pauly, 2012; Menon, 1999). Indian freshwater fishes represent about 8.9% of the known fish species of the world and occupy the ninth position in terms of freshwater fish diversity (Levêque *et al.,* 2008). About 40% of the fresh water fishes of India are widely distributed in the North eastern part of the country (Ponniah & Sarkar, 2000). However, the actual number of fish species found in India is still not accurately known because of taxonomic impediments (Hoagland, 1996) arising due to lack of exploration, imperceptiblity among some alike species, and taxonomic ambiguity in the established keys. As a result, there is a large probability of cryptic species and many of which may also be undiscovered (Darshan *et al.*, 2010a; Pethiyagoda & Kottelat, 1994). Furthermore, due to lack of proper morphological description with respect to sexual dimorphism, geographically isolated populations, etc., the statuses of a few species have been disputable (Darshan *et al.,* 2010b; Kottelat & Lim, 1995; Vishwanath & Linthoingambi, 2007). Therefore, for proper identification of Indian freshwater fishes, there is an utmost need of inspection of fishes using advanced molecular methods.

Among the most widely used molecular methods used for species analysis, DNA barcoding has been extensively used for species identification as well as species discovery in various groups of organisms (Hajibabaei *et al.,* 2012). Efficacy of barcoding has now been approved for many groups of animals (Waugh, 2007), both invertebrates and vertebrates (Hajibabaei, *et al.,* 2006; Herbert, *et al.,* 2004; Hernández- Dávila, *et al.,* 2012), with fishes being one of the most extensively studied groups among them (Becker, *et al.,* 2011; Ward, 2012). Many successful nationwide studies on ichthyofaunal diversity have been undertaken using this method for both marine and freshwater fishes (Lakra, *et al.,* 2011; Bhattacharjee, *et al.,* 2012; Chakraborty and Ghosh, 2014). Furthermore, these studies have also generated a large scale of barcode data that are available in BOLD (Ratnasingham & Hebert, 2007a) and NCBI (http://www.ncbi.nlm.nih.gov). However, the reference library of barcodes is still incomplete as many geographical locations, particularly in Asia, are yet to be exhaustively covered. In India, in addition to the absence of an updated compiled checklist of freshwater fishes, the identification keys for many

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valid species have not been updated since, the study of Talwar and Jhingran (1991) and KC Jayaram (2006).

In this context, there is an urgent need for the assessment of freshwater fish species of Gujarat through morphological analysis and DNA barcoding. Few sequences of freshwater fishes from this part of the country have been submitted to the database and few studies have addressed problems specific to certain groups. Regarding the taxonomical evidences of the freshwater fishes found in Gujarat, an annual report by Zoological Survey of India, Devi and Indra (2012) reports about 120 freshwater fishes in Gujarat state. According to books by authors, Dholakia (2004), Patel and Chhaya (1990), a total of 96 freshwater fishes are present in the state of Gujarat. The other major literature resource available for freshwater fishes indicates work done by Goswami and Mankodi (2010) and Gohil and Mankodi (2013) on Nyari-II reservoir and Mahi River where they found fifteen and twenty-six species of fishes respectively. However, a comprehensive assessment of DNA barcodes of freshwater fishes of Narmada River, Gujarat has been done for freshwater fishes (Khedkar *et al.,* 2014). But still many species are left which is to be dealt with for molecular aspects.

DNA barcoding is a concept in which a short nucleotide sequence of mitochondrial genome will act as a DNA barcode for species identification of animals and it is proven to be a rapid and enhanced tool for precise identification of animal species. DNA barcoding works under the principle that inter-species variations are greater than the intraspecies variations, allowing one to distinguish the species using nucleotide sequences. Six-fifty nucleotide bases of 5′ cytochrome c oxidase subunit I gene (COI) have been accepted as a universal barcode to delineate animal life of this planet.

Indeed, the very characteristic that makes the COI gene a candidate for high-through put DNA barcoding highly constrained amino acid sequence and thus broad applicability of primers (Hebert *et al.,* 2003) also limits its information content at deeper phylogenetic levels (Russo, *et al.,* 1996; Zardoya & Meyer, 1996). Finally, while superficially appealing, the very term DNA barcoding is unfortunate, as it implies that each species has a fixed and invariant characteristic like a barcode on a supermarket product.

Currently, records are available for 10868 fishes belonging to the class Actinopterygii on the Barcode of Life Data Systems; BOLD (Ratnasingham & Hebert, 2007b). By utilizing the advance in analysis technology, barcoding is going to help investigators for quick and efficient recognition of known species and also to retrieve any particular information about them. This technique will also speed up the discovery of species yet to be named by helping in comparison with nearest found species. Thus, this technology will provide vital for appreciating and managing any species biodiversity in the earth.

The present study has been mainly focused on development of DNA barcode database along with proper morphological identification for some of the less available freshwater fishes of Gujarat, a rarely studied part of fauna in the state. This study was undertaken keeping in mind the increased conservation needs of the depleting fauna. It is to also to be known that only few fishes have been successfully barcoded in this attempt although more such work for the fresh water fishes is very much necessary.

Materials and Methods

Random sample collections of about 52 different species of fishes were collecetd from various perennial water sources, i.e., rivers and few ponds in the districts mainly Vadodara, Bharuch, Mehsana, Rajkot, Panchmahal and Banaskantha in the post monsoon months from August to December, 2014. The number of species collected and other details of the collection have been shown in map with appropriately tagged GPS (GARMIN OREGON 650) locations (Figure 1).

Digital photographs of all the fishes were taken immediately and the fish were stored and preserved at -20ºC. The composition of samples is multi species, signifying the characteristic feature of the freshwater fisheries. All the fishes were identified morphometrically, with the help of Day's volume I and II (1888), Talwar and Jhingran (1991), Freshwater Fishes of India (Daniels, 2002) and Leibniz Institute of Marine Sciences (IFM-GEOMAR) in Kiel, Germany managed website www.fishbase.org, a global species database for fishes.

DNA Isolation and Sequencing

Genomic DNA was extracted from the stored muscle and gill tissue samples by the standard available QIAGEN DNeasy Blood & Tissue Kit. The COI gene approximately 650 bp length located in the mitochondrial genome was amplified using three different sets of primers for different species in Thermal cycler (Table 1)

The processed sequencing plate was loaded on an automated 3500xL Genetic Analyzer using POP 7 for sequencing. The sequencing was done both in the forward and reverse directions.

Sequence Analysis

Sequence analysis was done using sequencing analysis software version 5.4 (Applied Biosystems) and BioEdit, biological sequence alignment editor (Ibis Biosciences). Consensus sequences generated after aligning gene sequences from forward and reverse primers. These sequences were subjected to

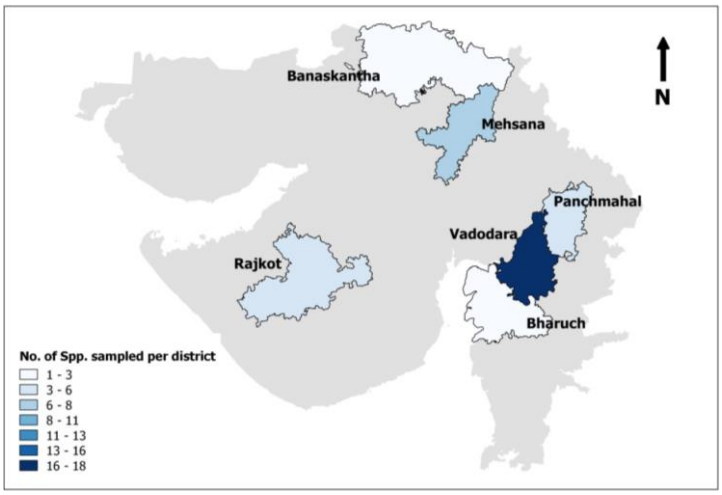

Figure 1. Map representation of the sampling sites with the number of samples.
*The map shows the various districts visited in the state of Gujarat for collection of samples and also the number of total samples collected per district.

Table 1. List of universal primers used for sequencing

Sr No.	Primers Used	Sequence	Reference
1.	FishF2_t1 (Forward)	TGTAAAACGACGGCCAGTCGACTAATCAT AAAGATATCGGCAC	(Ivanova *et al*.,2007)
	FishR2_t1 (Reverse)	CAGGAAACAGCTATGACACTTCAGGGTGA CCGAAGAATCAGAA	(Ivanova *et al*.,2007)
2.	LCO1490 (Forward)	GGTCAACAAATCATAAAGATATTGG	(Folmer *et al.*,1994)
	HCO2198 (Reverse)	TAAACTTCAGGGTGACCAAAAAATCA	(Folmer *et al.*,1994)
3.	FISH-BCL (Forward)	TCAACYAATCAYAAAGATATYGGCAC	(Baldwin *et al.*, 2009)
	FISH-BCH (Reverse)	ACTTCYGGGTGRCCRAARAATCA	(Baldwin *et al.*,2009)

*The above table shows the list of universally available and standard primers for fish DNA barcoding and sequencing.

Sequence match analysis using Basic Local Alignment Search Tool (BLAST) on NCBI. Consensus sequences which showed significant match with the earlier identified data on NCBI were submitted to BOLDSYSTEMS according to the guidelines provided onto BOLD website (http://www.boldsystems.org). For few species where NCBI data was not available, they were subjected to detailed and thorough morphological analysis and have been submitted to BOLD.

Results and Discussion

This study of identification of freshwater fishes from Gujarat was based on the morphological investigation followed by DNA barcoding approach revealed 38 different species of freshwater fishes. In a few cases, morphological species keys were difficult to discern. The DNA barcoding approach resolved some identification issues and explained the actual species composition in the region (Table 2).

A broad species identification of the studied freshwater fishes was developed based on BOLD and NCBI databases. Most of the identified fishes could be verified from the present database. The rest of the unavailable species have been grouped as per their barcoding availability from Gujarat, India and world. Species (like *Wallago attu, Glossogobius giuris, Labeo gonius, Labeo calbasu, Mystus cavasius, Colisa fasciata, Sperata seenghala, Nandus nandus, Mastacembelus armatus, Puntius sophore, Catla catla, Labeo bata, Heteropneustes fossilis, Channa punctata* and *Clarias gariepinus)* have been barcoded for the first time from Gujarat. Species like *Sicamugil cascasia* and *Cyprinus carpio* are primarily available in BOLD from India with this study only. And most importantly species like *Labeo ariza, Puntius burmanicus, Gobius personatus* and *Cirrhina cirosus* had no match with any of species listed the database. They have been enlisted with proper accession numbers in BOLD.

The summarised form of the Neighbour joining tree of cytochrome oxidase I gene sequences of the 38 different freshwater fishes is shown (Figure. 2).

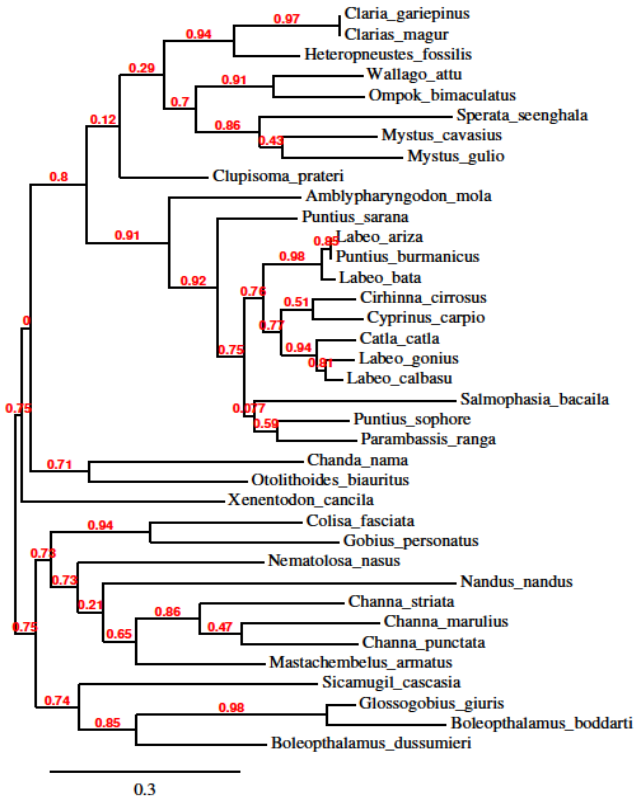

Figure. 2. Summary of Neighbour joining tree of cytochrome oxidase I gene sequences derived from 38 fish species.
*The neighbour joining tree comprising of the cytochrome oxidase I gene sequences derived from 38 barcoded fish species has been constructed using available standard online software tools.

The tree created using appropriate software tools and the DNA sequence obtained from the species suggests the genetic differences between the species. The base 0.3 suggests the nucleotides per sites in the alignment. The neighbour joining tree constructed with the obtained nucleotides of the specimens shows the inter relationship between them. It is clearly seen that separate clade has been formed for different families. The first major clade shows the family Siluridae with their respective distances between the species of the Cyprinidae family. The families of Siluridae and Cyprinidae are distanly related by 8% which shows their relative closeness in characters. Further, species of families like Channidae and Gobiidae are forming a clade at the end of the tree which shows their maximum distances from the others. The unique characteristics of the family Channidae, fishes also known as the 'Snakehead fishes' owing to their structure of the head resembling to that of the snake and also the body structure is very much unique for the freshwater fishes. Similarly, the characteristics of the family Gobiidae differentiates itself from the rest of the species by forming a separate clade though it was observed that *Sicamugil cascasia*, a species of mullet belonging to the eustarine region was 74% related to Gobiids. The similarity of characters presently surviving in the same ecological niche can be connected with the closeness of both the species of both the groups.

Conclusion

Thus, it can be concluded that 38 freshwater fish species after being primarily identified through morphological identification with various keys have been confirmed and validated using DNA barcoding. Occurrence of the four newly barcoded species was evident in the study but still discussions on their doubtful status can be done though taxonomical classification of the same has been very well taken care of. However, the remaining of the studied species representing 13 families can be seen convincing and requires no further assessment. The universal fish primers were used for all the 38 species. The barcode sequences were clearly able to differentiate between the different species. Though if the present database is augmented with multiple sequences for a target species of the same range of distribution, the species taxonomy would be further strengthened and assessment of biodiversity would be correct and much easier in future.

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Table 2. List of the 38 species barcoded along with BOLD accession numbers

Sr. No.	Order	Family	Species Name	Bold Accesson Numbers
1		Cyprinidae	*Labeo gonius*	ANGEN110-15
2		Cyprinidae	*Labeo calbasu*	ANGEN111-15
3		Cyprinidae	*Labeo ariza*	ANGEN118-15
4		Cyprinidae	*Labeo bata*	ANGEN264-15
5		Cyprinidae	*Cyprinus carpio*	ANGEN270-15
6		Cyprinidae	*Catla catla*	ANGEN262-15
7	Cypriniformes	Cyprinidae	*Puntius sophore*	ANGEN260-15
8		Cyprinidae	*Osteobrama cotio*	ANGEN261-15
9		Cyprinidae	*Puntius burmanicus*	ANGEN119-15
10		Cyprinidae	*Puntius sarana*	ANGEN263-15
11		Cyprinidae	*Cirrhina cirosus*	ANGEN273-15
12		Cyprinidae	*Salmophasia bacaila*	ANGEN270-15
13		Cyprinidae	*Amblypharyngodon mola*	ANGEN302-15
14		Siluridae	*Wallago attu*	ANGEN106-15
15		Siluridae	*Mystus gulio*	ANGEN116-15
16		Siluridae	*Mystus cavasius*	ANGEN112-15
17		Siluridae	*Sperata seenghala*	ANGEN115-15
18	Siluriformes	Siluridae	*Heteropneustes fossilis*	ANGEN265-15
19		Siluridae	*Ompok bimaculatus*	ANGEN266-15
20		Siluridae	*Claria gariepinus*	ANGEN272-15
21		Siluridae	*Clarias magur*	ANGEN300-15
22		Schilbeidae	*Clupisoma prateri*	ANGEN120-15
23		Gobiidae	*Glossogobius giuris*	ANGEN108-15
24		Gobiidae	*Gobius personatus*	ANGEN267-15
25		Gobiidae	*Boleopthalamus dussumieri*	ANGEN270-15
26		Gobiidae	*Boleopthalamus boddarti*	ANGEN299-15
27	Perciformes	Ambassidae	*Parambassis ranga*	ANGEN298-15
28		Ambassidae	*Chanda nama*	ANGEN304-15
29		Nandidae	*Nandus nandus*	ANGEN117-15
30		Sciaenidae	*Otolithoides biauritus*	ANGEN303-15
31		Osphronemidae	*Colisa fasciata*	ANGEN114-15
32		Ophiocephalidae	*Channa striata*	ANGEN301-15
33	Symbranchiformes	Ophiocephalidae	*Channa marulius*	ANGEN107-15
34		Ophiocephalidae	*Channa punctata*	ANGEN268-15
35	Beloniformes	Belonidae	*Xenentodon cancila*	ANGEN109-15
36	Synbranchiformes	Mastachembelidae	*Mastachembelus armatus*	ANGEN259-15
37	Mugiliformes	Mugilidae	*Sicamugil cascasia*	ANGEN113-15
38	Clupeiformes	Clupeidae	*Nematolosa nasus*	ANGEN269-15

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