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Morphological and molecular analysis of *Otolithoides pama* (Hamilton, 1822) (Perciformes: Sciaenidae) from Hooghly-Matlah estuarine system of West Bengal, India

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Morphological characters of Pama croaker *Otolithoides pama* were established in the present study. The partial sequence of the fish was generated which is an important record of the species available in the Hooghly-Matlah estuarine system of West Bengal, for any further comparisons. The constructed Neighbor-Joining (NJ) tree based on Kimura's 2 parameter distance model indicated that *O. pama* showed an identical phylogenetic relationship with 28 sciaenid species. The tree showed high bootstrap support values with identical topology. The phylogenetic tree constructed for *O. pama* exhibited high similarity with *Panna microdon*, *Otolithoides biauritus* and *Pama pama*. Within the three closely related species, the overall mean distance between the individuals was found to be 10.30 %. The genetic distance value was highest between *Panna microdon* and *Pama pama* (15.60 %) and the lowest genetic distance was observed between *Otolithoides biauritus* and *Panna microdon* (0.30 %). The study revealed that for the identification of respective fish species based on nucleotide sequences, Cytochrome c oxidase subunit 1 (COI) genes can be used as barcodes.

[**Keywords**: COI gene, Hooghly-Matlah estuary, Morphological characters, *Otolithoides pama*]

Introduction

Sciaenids commonly known as croakers are small to moderately sized fishes mainly found in the muddy bottom of coastal waters and they belong to the family Sciaenidae and order Perciformes. Family Sciaenidae includes many commercially and recreationally important species occurring worldwide in temperate and subtropical marine, estuarine, and freshwaters[1]. Mohanraj *et al*.[2] described 300 species of sciaenids with 70 genera distributed globally including about 30 species from Indian waters. Sciaenid fishes are represented by 49 species, belonging to 22 genera, in the Indian Ocean of which 40 species belonging to 20 genera, inhabit the seas around India[3]. According to Joseph & Jayaprakash[4], 30 species of sciaenids belonging to 14 genera are distributed in Indian waters of which 20 are commercially important species. Most of the sciaenid species globally occur within marine and estuarine waters, while 28 species occur in freshwater[5]. Two large-sized species, namely *Macrospinosa cuja* (Hamilton-Buchanan) and *Daysciaena albida* (Cuvier) contribute significantly to the fisheries in Indian estuarine waters, while two fairly small species, *viz. Otolithoides pama* and *Johnius gangeticus* form a significant part of the sciaenid fishery in the Ganga river and its estuarine system[3]. The main Indian states contributing considerably to the sciaenid catch are Gujarat, Maharashtra, West Bengal, Tamil Nadu, Odisha, Andhra Pradesh, and Kerala.

In general, fishes of the family Sciaenidae are commonly identified through conventional techniques, mainly based on morphometric, meristic, and anatomical characters[3,6]. The existing ambiguities in sciaenid identification are due to morphological closeness among few species of sciaenids, especially of genus *Johnieops* and *Johnius* being similar as mentioned in the FishBase[7]. However, accurate identification of externally appearing similar species is essential to clinch the species identity, for successful population estimation and management of commercially important fish stocks[8,9]. Application of suitable molecular tools like DNA barcoding may provide need-based knowledge for species identification and validation of systematic positions[10].

The DNA barcoding techniques allow efficient and very fast means of recognition of fish and are also useful in the identification of larvae and even egg

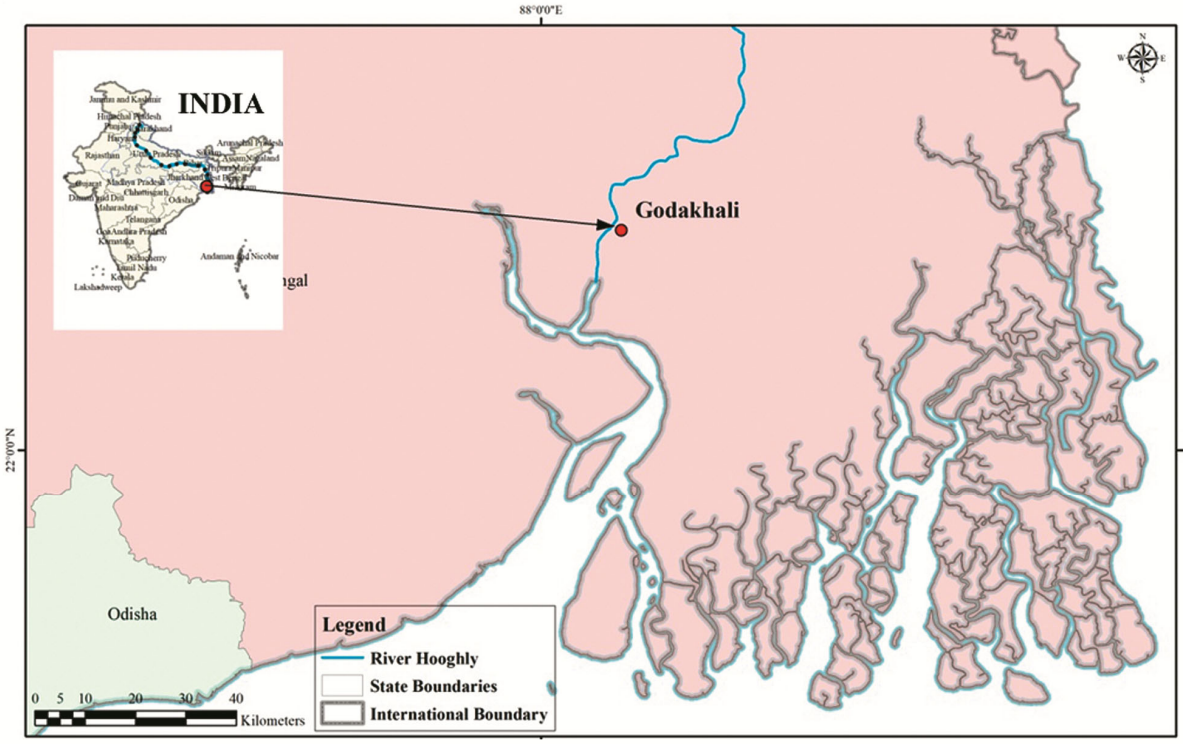

Fig. 1 — Study area under Hooghly-Matlah estuarine system

stages of fish species[11]. The mitochondrial genes were established to be promising areas for the identification of fish species compared to the nuclear genes[12] due to several special features like they are present in high copy numbers, and their mutation rate is greater than that of nuclear genes. For the majority of the animal kingdom COI mitochondrial protein-coding gene has been accepted widely as a species-level barcode[13]. In global biodiversity assessment and conservation programmes, DNA barcoding is found to facilitate rapid identification of potentially unidentified taxa[14]. Further, for discrimination between closely related species across diverse animal phyla, COI region is appropriate[15]. There are few reports available on molecular identification of Indian sciaenids based on 16S rRNA and COI mitochondrial genes[16-18]. Menezes *et al*.[19] studied the genetic characterization of four sciaenid species from the Arabian Sea around Goa. In the present study morphological analysis as well as mitochondrial DNA barcoding was done for *O. pama* occurring in the Hooghly-Matlah estuary from the Indian waters for the first time.

Materials and Methods

Samples collection

Otolithoides pama specimens were collected from Godakhali landing sites under the Hooghly-Matlah estuarine system of West Bengal, India (Fig. 1). The samples were identified following the synopsis of Indian sciaenid fishes[3,20,21]. For the morphological study, a total of 100 specimens were collected from January to July 2016. For DNA barcoding, approximately 100 mg of white muscle tissue along with fins from selected samples were collected. Fresh specimens were photographed before taking the tissue samples (Fig. 2). Tissue samples were kept in sterile Eppendorf tubes containing 95 % ethanol and after proper sealing with parafilm were kept at room temperature. Specimen details and GeneBank accession numbers are represented in Table 1.

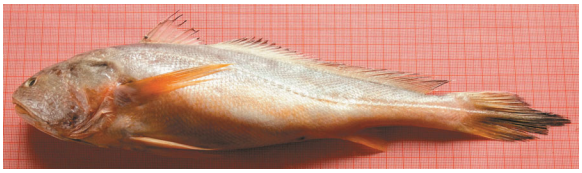

Fig. 2 — Specimen of *O. pama* collected from Hooghly-Matlah estuarine system of West Bengal

Genomic DNA extraction and quantification

Standard Proteinase-K/Phenol-Chloroform-ethanol method was used after few minor modifications for total genomic DNA extraction from the stored tissue samples. In 2 ml centrifuge tubes, 5 mm × 5 mm of tissue (stored in 95 % ethanol) from the specimen was

Table 1 — List of the sciaenids species being used for phylogenic analysis

Sl. No.	Name of the species	Common name	Gene Bank Accession No.	Location
1	*Argyrosomus hololepidotus*	Southern meagre	DQ107811	Australia
2	*Argyrosomus japonicus*	Japanese meagre	JF492880	South-Africa
3	*Atrobucca nibe*	Blackmouth croaker	JF492918	South-Africa
4	*Chrysochir aureus*	Reeve's croaker	KY118817	Bangladesh
5	*Daysciaena albida*	Bengal corvine	KP722719	Taiwan
6	*Dendrophysa russelii*	Goatee croaker	EU148580	India
7	*Johnius belangerii*	Belanger's croaker	FJ347917	India
8	*J. carouna*	Caroun croaker	KP722726	Taiwan
9	*J. carutta*	Karut croaker	FJ265843	India
10	*J. dussumieri*	Sin croaker	FJ347915	India
11	*J. elongatus*	Spindle croaker	EF534123	India
12	*J. macropterus*	Large-fined croaker	KP722727	Taiwan
13	*J. macrorhynus*	Big-snout croaker	KU893041	Taiwan
14	*Macrospinosa cuja*	Cuja bola	JX260908	India
15	*Nibea chui*	Chu's croaker	KP722744	Taiwan
16	*Nibea maculata*	Blotched croaker	EU014247	India
17	*Nibea soldado*	Soldier croaker	HQ219157	India
18	*Otolithes cuvieri*	Lesser tigertooth croaker	FJ347924	India
19	*O. ruber*	Tigertooth croaker	FJ237586	India
20	*Otolithoides biauritus*	Bronze croaker	EF534127	India
21	*Pama pama*	Pama croaker	MF611579	Bangladesh
22	*Panna microdon*	Panna croaker	JX983436	India
23	*Pennahia anea*	Bigeye croaker	KY024209	Bangladesh
24	*Pennahia macrocephalus*	Big-head pennah croaker	KU944112	Taiwan
25	*Pennahia ovate*	-	KX778086	Malaysia
26	*Protonibes diacanthus*	Blackspotted croaker	KX778092	India
27	*Pterotolithus maculatus*	Blotched tiger-toothed croaker	KP722772	Taiwan
28	*Umbrina canariensis*	Canary drum	JF494762	South Africa
29	*Otolithoides pama*	Pama croaker	MG787254	Hooghly-Matlah estuary

taken and ethanol was evaporated by keeping the tubes open. Incubation buffer (10 mM Tris, 1 mM EDTA, 0.4 M NaCl, 10 % SDS and Proteinase K) was added to ensure cell lysis and was incubated at 55 °C for two hours. After incubation, by successive extraction with phenol: chloroform: isoamyl alcohol (25: 24: 1) and chloroform: isoamyl alcohol (24: 1), the DNA was purified, and then centrifuged at 10,000 rpm for 15 minutes. The supernatant was transferred to a fresh tube and the DNA was precipitated by adding $1/10^{th}$ volume of 3 M sodium acetate and twice the volume of ice-cold absolute ethanol.

By centrifugation at 10,000 rpm for 15 minutes, the precipitated DNA was pelleted. The DNA was air-dried and re-suspended in 20 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) after a wash with 70 % ethanol. Purity and concentration of extracted DNA was determined using UV Vis spectrophotometer (Aquarius, CECIL, CE7500) at 260 and 280 nm. The isolated genomic DNA quality was assessed electrophoretically, by running 2 µl of the DNA stock on 0.8 % agarose gel (Bio-Rad, USA) electrophoresis.

PCR amplification and sequencing

The mtDNA COI gene was amplified in a final concentration of 25 µl volume reactions containing 10× assay buffer (100 mMTris, 500 m MKCl, 0.1 % gelatin, pH 9.0) with 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 10 p moles/µL of primer mix, 10 m MdNTPs (Genei, Bangalore, India), 1.5 U *Taq*DNA polymerase and 20 ng of template DNA. Negative control was set up omitting template DNA from the reaction mixture to check DNA contamination. The reaction mixture was initially denatured at 95 ^{o}C for 5 minutes followed by a total of 30 cycles (94 ^{o}C for 45 seconds, 54 ^{o}C for 30 seconds and 72 ^{o}C for 45 seconds). The reaction was then subjected to a final extension at 72 ^{o}C for 5 minutes. By using different

combinations of two pairs of primers approximately 655 bp nucleotide was amplified from the 5' region of the COI gene from mtDNA.

Fish F1: 5'-TCAACCAACCA CAAAGACATT GGCAC-3' and

Fish R1: 5'-TAGACTTCTGG GTGGCCAAA GAATCA-3'[ref. 22].

3 µl PCR product along with the marker (100 bpDNA ladder, Genei, Bangalore, India) was run on 1.2 % agarose gel with 1× TAE buffer for 30 minutes and stained with ethidium bromide. The gel was visualized under UV trans-illuminator and documented using Image Master VDS (Biorad, USA).

Sequence analysis

Sequences were edited using DNA Bazer software and were NCBI BLAST analyzed. Finally, the sequences were submitted to NCBI (Table 1). By using MEGA 6.0 (Molecular Evolutionary Genetics Analysis) an NJ tree was constructed[23]. Bootstrap analysis was carried out using 1,000 pseudo-replicates, to verify the robustness of the internal nodes of the NJ tree[24]. For the construction of phylogenetic relationships, a total of 28 COI gene sequences of family Sciaenidae were retrieved from the National Center for Biotechnology Information (NCBI) and were included in the group (Table 1).

Nucleotide sequence analysis

By using Genomic Workbench 8.5.1 software, DNA sequence information of COI gene was translated to amino acid sequences and all codon positions were identified. Among the Indian sciaenids, *O. pama* cytochrome c partial sequence showed high similarity (100 %) with *Panna microdon* COI gene, partial cds (NCBI Acc. No. JX983436) and *Otolithoides biauritus* COI gene, partial cds (NCBI Acc. No. EF534127). *Otolithoides pama* COI gene, partial cds (NCBI Acc. No. MG787254) sequences show 97 % similarity with the same species *Pama pama* COI gene, partial cds (NCBI Acc. No. MF611579) collected from Bangladesh as well as 100 % similarities with the species *Panna microdon* and *Otolithoides biauritus*. Genomic Workbench 8.5.1 software was used for the comparisons of multiple sequences of *Otolithoides pama* cytochrome c partial sequence with other sciaenid species recorded from Indian water bodies and worldwide.

Phylogenetic tree analysis

For the molecular phylogenetic analysis, 28 numbers of nucleotide sequences from the family Sciaenidae (14 species from Indian water bodies, 14 from outside of India) were derived from NCBI Gene Bank for the present work (Figs. 3 and 4). The cladogram was drawn by tree construction method

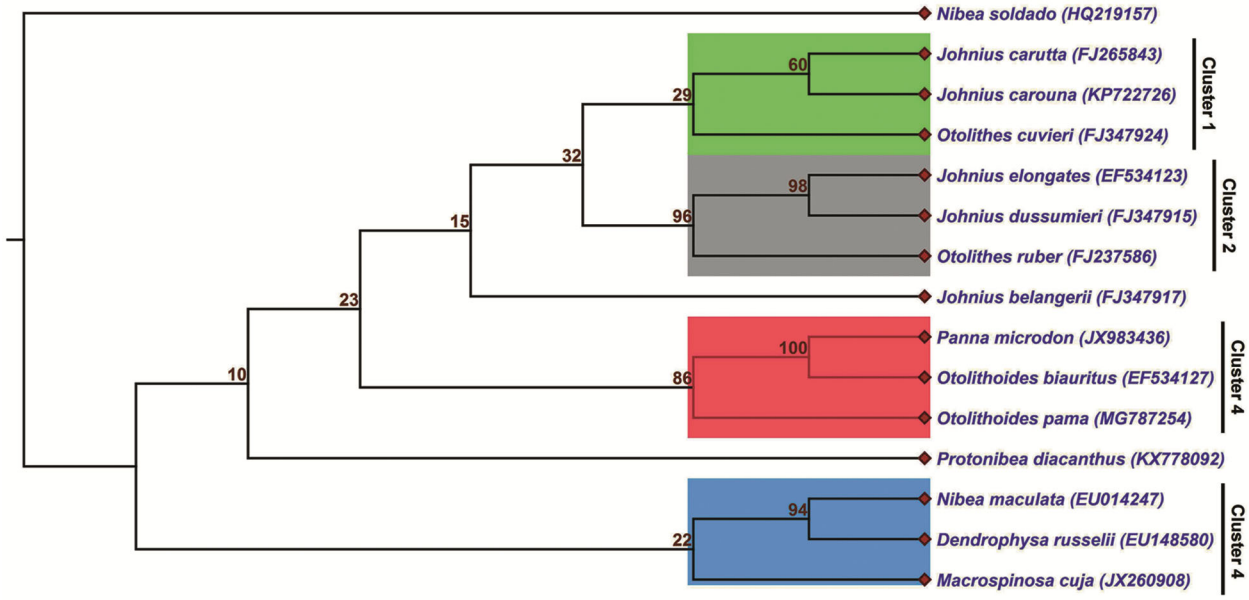

Fig. 3 — Molecular phylogenetic analysis of 15 sciaenids species (Indian region) by NJ tree of c oxidase I gene sequences derived from the family Sciaenidae by using K2P (Kimura 2-parameter) distance

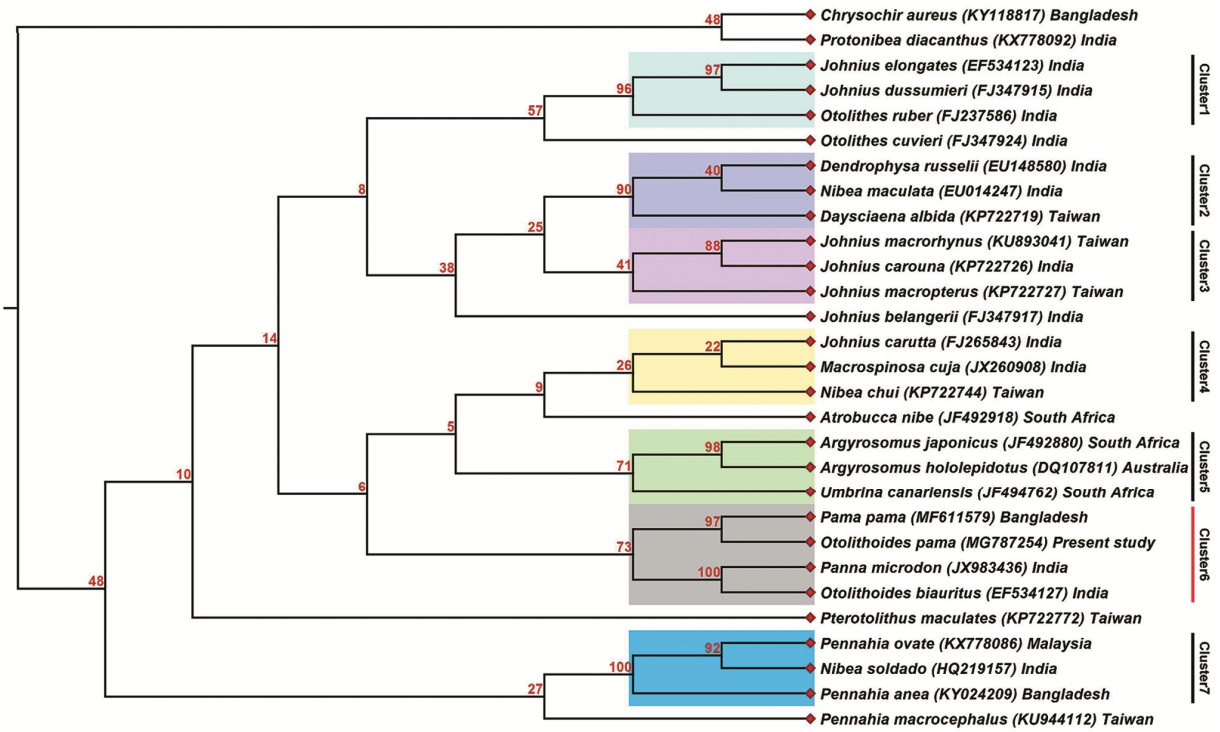

Fig. 4 — Molecular phylogenetic analysis of 29 sciaenids species (world) by NJ tree of COI gene sequences derived from the family Sciaenidae by using K2P (Kimura 2-parameter) distance

(NJ) by keeping boot-step value 1,000 through Genomic Workbench 8.5.1 software. From the NJ tree, it was found that all sciaenids species exhibited an identical phylogenetic relationship. The tree showed high bootstrap support values with identical topology. Alignment of multi-nucleotide sequences in COI gene, of 29 sciaenids collected worldwide and with the present sequence is presented in Figure S1.

Results

Adults of *O. pama* are light brownish in colour at the backside and silvery-white or white on belly portion; head usually short in size compared to the body length and golden in colour. Fins are slightly yellowish and the upper half portion of the dorsal fin is grey. Scales usually cycloid on the head portion and on most of the body parts are ctenoid. Scales in the lateral line were found to be more than 45 in numbers. Scales above the anterior part of the lateral line were much smaller, 10 to 11 between the origin of the dorsal fin and lateral line. Dorsal fin weakly notched; dorsal spines are weak, 3rd and 4th are the longest ones. The second anal spine is short and weak. Pectoral fins are pointed, and length extended up to head. Caudal fins are very long and rhomboid in shape. Gas bladder is carrot-shaped; a pair of tubules originates near its posterior end and extends forward into the head forming several branches. Teeth are well-differentiated in both jaws, with 1 or 2 pairs of canniform teeth in the upper jaw and sometimes a pair of strong teeth in the lower jaw.

Table 2 — Morphometric parameters of *O. pama* collected from the Hooghly-Matlah estuary system of West Bengal

Morphometric parameters	Values in mm		
	Min	Max	Average (± SD)
Total length	167	287	212.76 ± 19.68
Standard length	138	232	165.86 ± 15.58
Body weight	39.2	195.4	71.81 ± 21.15
Pre-anal length	94	167	119.66 ± 12.01
Pre-dorsal length	35	59	43.26 ± 3.89
Pre-pectotal length	35	54	42.58 ± 3.79
Pre-pelvic length	37	65	46.57 ± 4.31
Head length	37	59	45.06 ± 3.93
Maximum body depth	35	44	41.97 ± 4.06
Caudal depth	10	18.50	12.59 ± 1.43
Caudal length	34	61	47.76 ± 4.38
Snout length	9	19	11.30 ± 1.24
Post-orbital length	25	49	32.96 ± 3.51
Intra-orbital length	12	19	14.72 ± 1.19
Eye diameter	5	9	6.89 ± 0.59

In the present study the number of spines and branched dorsal-fin rays varied from IX-X+I and 44 to 45, respectively (Table 2). The number of pectoral-fin rays was I 16. A total of five pelvic fin

Table 3 — Pair-wise genetic distance matrix of *O. Pama*

Species	1	2	3	4
MG787254 *Otolithoides pama* (present collection)	-	0.017	0.017	0.003
Ef534127 *Otolithoides biauritus*	0.149	-	0.002	0.018
JX983436 *Panna microdon*	0.149	0.003	-	0.018
MF611579 *Pama pama*	0.005	0.155	0.156	-

rays and seven anal-fin rays were recorded. The caudal fin rays were recorded from 18 to 20. The number of gill rakers on the lower limb of the first-gill arch was found from 19 to 21. The total number of lateral line scales was observed between 48 and 52.

For the molecular phylogenetic analysis, 15 numbers of nucleotides sequences (including the present sequence) from the family Sciaenidae collected from NCBI Gene Bank were used (Fig. 2). From the NJ tree, it was observed that there was an identical phylogenetic relationship existed among all sciaenids species. A total of four major clusters were derived from the tree with the first cluster from three species of two genera, namely *Johnius* (*J. carutta* and *J. carouna*) and *Otolithoides* (*O. cuvieri*). The second cluster was formed by the two congeneric genera, *i.e. Johnius* (*J. elongatus* and *J. dussumieri*) and *Otolithes* (*O. ruber*). The third cluster was formed by three species namely *Panna microdon* and *Otolithoides* (*O. biauritus* and *O. pama*) whereas, the fourth cluster was formed by *Nibea maculates*, *Dendrophysa russulii*, and *Macrospinosa cuja*. In the NJ tree, all of the four clusters were supported by high bootstrap values (60 to 100 %). The species like *Nibea soldado*, *Johnius belangeri*, and *Protonibea diacanthus* did not make any clusters in primary as well as in the secondary nodes. The tree generated using NJ depicted that species *O. pama* showed high similarity with *Panna microdon*, *Otolithoides biauritus* and *Pama pama*.

The pair-wise genetic divergence values (K2P method) based on COI sequences were calculated using MEGA 6.0 software and given in Table 3. The overall mean distance between individuals was found to be 10.30 %. Genetic distance was observed highest between *Panna microdon* and *Pama pama* (15.60 %) and that of lowest between *Otolithoides biauritus* and *Panna microdon* (0.30 %).

Discussion

The number of fish species reported in the world is about 34,725 and out of them, 49 were from sciaenids[25]. A total of 2523 species are reported in Indian waters which includes 958 species of freshwater and 1623 species of saltwater habitats[26]. Out of 257 commercially important fish species available from Indian waters, nine belong to sciaenids such as *Daysciaena albida*, *Dendrophysa russelii*, *Johnius coitor*, *Johnius gangeticus*, *Johnius glaucus*, *Kathala axillaris*, *Otolithes cuvieri*, *Otolithoides pama* and *Pennahia anea*. Order Perciformes contributes a lion's share of all fish orders and constitutes around 160 families which comprise very similar but poorly separated families[27-29].

In the present study, maximum number of meristic characters were found similar to the earlier studies with little differences in the number of gill rakers and the total number of lateral line scales[30,31]. Such variations might be due to environmental factors like temperature, salinity, oxygen, pH, food availability, and growth rate, which were noticed in many species[32,33]. For the sustainable utilization of biodiversity and IPR protection in the country, a species-level characterization is must. Moreover, only a small fraction *i.e.*, 1.5-1.8 million species have been formally identified and documented[34] out of an estimated 10 million species across the world.

As mitochondrial 16S rRNA gene and the protein-coding COI gene are highly conserved, the mtDNA has been extensively studied in fish phylogenetics[35]. The mitochondrial DNA partial cds gene enables researchers to discriminate animal life. In DNA barcoding several approaches such as distance, character, and phylogeny have been proposed and used to discriminate species[36]. Such mitochondrial genes have been sequenced in other fishes and reported[18]. For species-level identifications, DNA barcoding aims to provide an efficient method using an array of species-specific molecular tags derived from COI gene[37,38].

The constructed NJ tree with the other 14 sciaenid species (occurring in Indian waters) indicated a homogenous phylogenetic relationship among the species. The phylogenetic relationship established through 16S rRNA and COI gene sequences within the species of the same family found that similar species were clustered under the same nodes, while dissimilar species were clustered under separate nodes. It was found that the congeneric species were clustered together. A similar kind of congeneric species relationship was observed in four fishes namely, *Sillago sihama*, *Mene maculata*, *Seriolina*

nigrofasciata and *Minous dempsterae* collected from southwest coastal waters of India[39].

Talwar[3] has found 40 species of sciaenids in India, and as per the FishBase database, there are 39 species under the family Sciaenidae in Indian waters[26]. Out of 40 species, a total of 14 species were barcoded from Indian waters[16]. Lakra *et al*.[17] have described bar-coding of 115 marine finfishes species, belonging to 79 genera and 37 families inhabiting the Indian waters. Lakra *et al*.[8] observed three genetically distinct groups of six species of Indian sciaenids namely *Otolithes cuvieri*, *O. ruber*, *Johnius borneensis*, *J. dussumieri*, *Dendrophysa russelii* and *Nibea maculata* from the phylogenetic relationships based on 16S rRNA and COI.

Khedkar *et al*.[40] identified 85 fish species through DNA barcoding employing COI gene sequences out of 90 species reported from river Narmada. High-resolution clusters were generated in NJ trees which supports groupings of respective species according to their genera and families. By DNA barcoding techniques 10 selected estuarine species have been identified from the river Krishna region of Andhra Pradesh, India by Krishna *et al*.[41]. They did not find many variations while comparing the sequences of their findings with earlier barcoded species. Through the construction of a phylogram, they stated that CO1 gene sequences can perform as universal DNA markers for the recognition of fish species. Recently Velu & Uthandakalaipandian[42] identified an endangered fish *Dawkinsia tambraparaniei* collected from Tamiraparani river in Tamil Nadu with COI genes which were previously misidentified as *D. arulius*. The main purpose of DNA barcoding is to create reference DNA-barcode libraries for conceding species to be used as DNA-identifiers[42].

The NJ tree by Kimura 2-parameter (K2P) provides a graphic representation of divergence using Genomic Workbench 8.5.1 software. In the established phylogenic relationship among the species, similar species were clustered under the same nodes while dissimilar species were clustered under separate nodes and all the nodes were supported by high bootstrap values (90-100 %)[43]. In the present study, transitions outnumbered transversions compared to the previous reports on fish mtDNA analyzed by Santos *et al*.[44] and Vinson *et al*.[45]. The COI sequence generated in the present study is an important record of *O. pama* available from the Hooghly-Matlah estuarine system of West Bengal, for any further comparisons.

Conclusion

In recent years, DNA barcoding techniques have been found as a vital instrument for fisheries managers and certain regulatory agencies for species identification, conservation and resource management as well as for consumer health. To resolve enigmatic species complexes, both the genetic and DNA-based tools are making it possible to obtain more detailed and accurate assessments of biodiversity levels both within and between species. To catalogue biodiversity using molecular approaches, the DNA barcoding method now represents the largest effort. DNA barcoding based on COI gene sequence has been widely used for species identification of fishes. It also provides a basic framework for PCR-based assays which can differentiate various fish species. The present findings on morphological analysis as well as mitochondrial DNA barcoding were done for *O. pama* for the first time from the Indian waters in the Hooghly-Matlah estuary. It can be quite useful to uphold sustainable fisheries of this fish in the locality and thus promote welfare of fishers.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at http://nopr.niscair.res.in/jinfo/ijms/IJMS_50(03)219-227_SupplData.pdf

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Disclosure statement

The authors declare that there is no conflict of interest.

Author Contributions

The first author (DB) was involved in fish samples collection, morphological and molecular analysis, and manuscript writing, and the rest of the authors helped in manuscript reviewing and editing.

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