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SHORT COMMUNICATION

Misidentification of three mullet species under family Mugilidae due to differential pigmentation pattern

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ABSTRACT

The mullet species under the family Mugilidae, considered to be a commercially important teleost, are mostly found in coastal waters throughout the world, distributed in tropical, subtropical and temperate areas. In terms of taxonomic study, biogeography and distribution pattern of mullets remain unclear due to difficulty in separating the species based on morphological characters. Thus, there is a need to study the taxonomy of Mugilidae. Further, phylogeny of family Mugilidae also exceptionally obscure at inter and intraspecific levels challenges exist in species under the family. The present study, has brought a new observation in form of temporary black dots (patrial pigmentation abnormality), especially in three species of Mugilidae were observed. Sometimes these pigmentation pattern can lead to misidentification or identification as different species. Further, DNA Barcoding (COI gene) and morpho-meristic analysis performed to resolve the ambiguity in the species identification, confirmed these species as *Mugil cephalus*, *Planiliza* sp., *Osteomugil perusii*. Present study will help to avoid the misidentification of species, which will assist biologists and managers for acquiring more information their distribution and life history pattern.

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Introduction

Mugilidae commonly known as mullets are most common teleost fishes in the coastal waters, especially in estuaries of the tropical, subtropical and temperate zones of the world (Thomson, 1996; Murgia et al., 2002; Rahman et al., 2013). Family Mugilidae, which was classified under order Perciformes (Menezes et al., 2015), is now placed under the order Mugiliformes (Froese & Pauly, 2022). Hence, this group needs thorough study and revision. The previous taxonomic revision has included varied number of valid genera and species between 14 and 20 genera and 62 valid species (Thomson, 1997); 72 species under 17 genera (Nelson, 2006); 81 species under 18 genera (Zubia et al., 2015; Hassanien et al., 2020); 78 species under 26 genera (Froese & Pauly, 2022). Family Mugilidae relies upon high proportion of monotypic genera which reflect upon general difficulty in classifying mullet species on the basis of few diagnostics or synapomorphic characters. Most of the species are reported under genera *Liza*, *Mugil* and *Valamugil*. Among them, *Liza* and *Mugil* are comprised of 40% of the species richness (Eschmeyer & Fricke, 2011). Morphological characters have been used by fishery biologists to measure discreteness and find out relationship among various taxonomic categories. Mullets are considered to be one of the most difficult taxonomic groups, hence there are conflicts regarding taxonomy of mullets at the generic as well as the species level (Krishnan, 2018). These groups are having highly conservative morphology and therefore, identifying them using only morphometric characters, has proven to be a tedious and complex task. First thorough taxonomic revision on Mugilidae (Schutz, 1946) was based on the mouth anatomy, especially lips and teeth, as supporting evidence. The taxonomy of adult and fry of mullets relies upon external morphology mainly (mouth, teeth, scale, lips, preorbital bones, jaws), meristic count and internal organs (intestine, stomach, pyloric caeca, pharyngo-branchial organs), pigmentation and melanophore pattern (Thompson, 1997; Krishnan, 2018).

DNA Barcoding, using COI gene, has been used for delineation as well as identification of species. In addition to species diagnosis using internal and external characters, in the present study, peculiarity of temporary pigmentation (ecotype) has been observed for three species (*Osteomugil perusii*, *Mugil cephalus* and *Planiliza* sp.). Though some literatures have cited about the pigmentation in fry stages (Minos et al., 2002) and body surface pigmentation (You-jun et al., 2014), there is no clear statement about the pigmentation pattern. Thus, the present study was designed not only to prevent the misidentification of species in the future research, particularly for this groups but also confirms different ecotypes rather than considering them as different species. The results of the present study will have significant contribution towards database with DNA Barcode and an awareness of existing ecotypes in natural environment rather avoid considering them as different species.

Material and Methods

Fishes under family Mugilidae were collected from coast of Eastern Arabian Sea Region (18°18.837 N, 73°45.750 E) (Figure 1), Maharashtra, during November 2021 to March 2022. A total 10 fishes were collected from local fishermen operating gillnet and dolnet, a type of Set bag net (traditional net) which is, 25 ft long and operated at a depth of 7-8 ft in Bhayander estuary. Specimens were brought to the laboratory under fresh condition and identified up to species level by using fish identification key (Luther, 1973; Rahman et al., 2013; Froese & Pauly, 2022; Bhatt & Mankodi, 2023). Tissue samples of the specimens were preserved in absolute alcohol, while whole fish specimens were preserved in formalin. The meristic (7) and morphometric (19) characters were measured with digital callipers to the nearest 0.1 mm precision and recorded in Microsoft® Excel 2019. The data was analysed using software PAST (version 4.30).

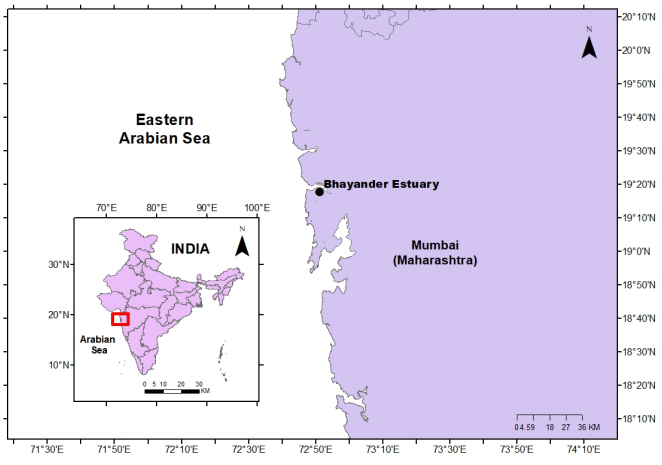

Figure 1. Black dot showing study area (Bhayander Estuary) Eastern Arabian Sea, Maharashtra, India

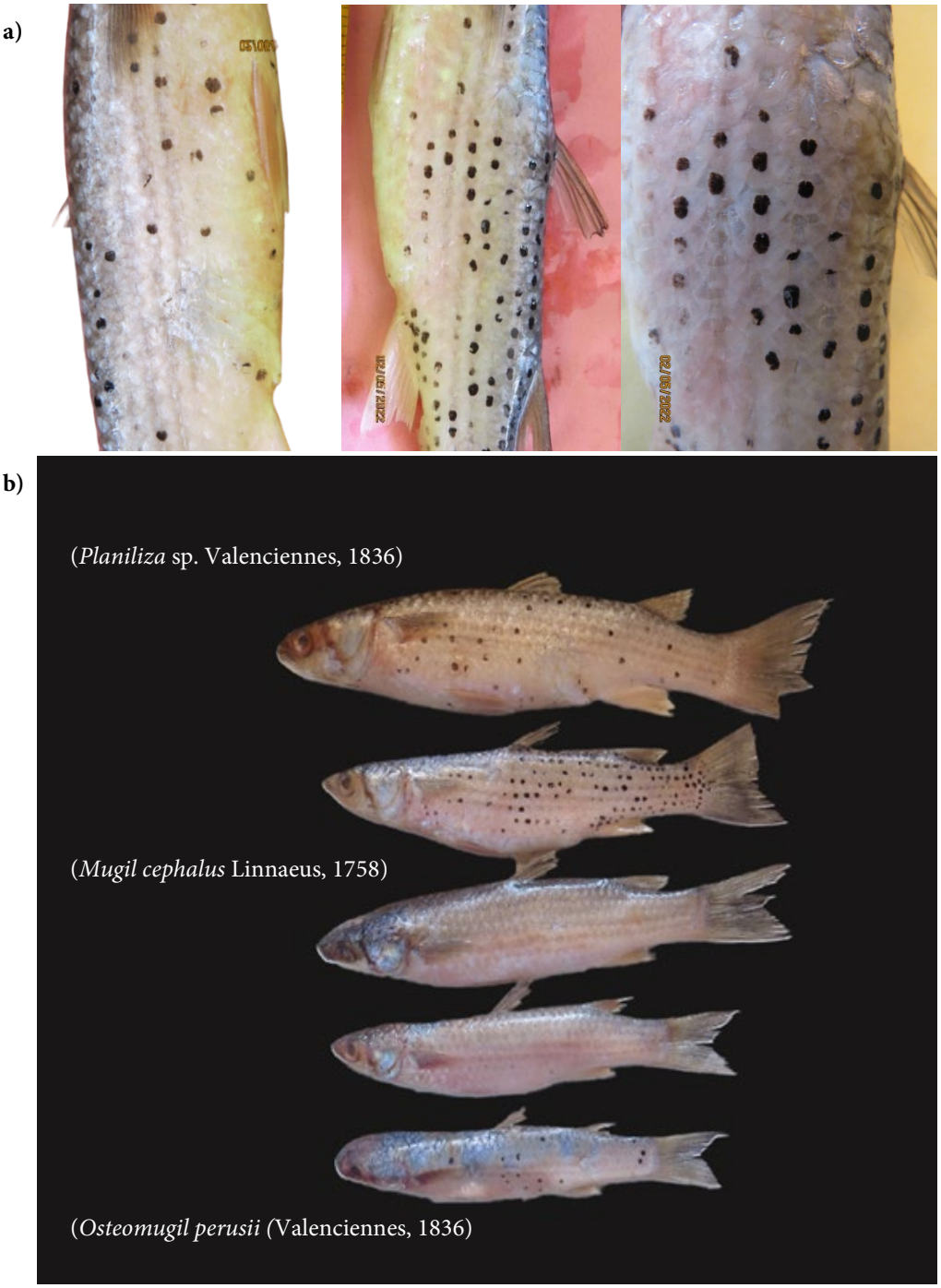

Figure 2. a) Spotted specimens with different pigmentation pattern. b) Observed specimens under 3 different species (Top to bottom- *Planiliza* sp. Valenciennes, 1836); (*Mugil cephalus* Linnaeus, 1758; (*Osteomugil perusii* (Valenciennes, 1836)

DNA Extraction, COI Gene Amplification and Sequencing

DNA was extracted by following Phenol chloroform method (Sambrook & Russell, 2005). Purity of concentration of DNA was checked by nano drop. Further, template DNA was been subjected to PCR amplification of specific region (600 bp) by using primers (F1R1; F2R2) (Ward et al., 2005). Thermal cycling was performed in BIORAD PTC-200, with the following conditions: 95°C for 300s, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 10 min. PCR products were purified with Qiagen Gel Extraction kit and Promega PCR purification kit following manufacturer's guidelines and sequenced in both directions using a Genetic Analyzer (Massey University Genome Sequencing Service). The purified samples were sent for sequencing to Eurofins Genomics. The Forward and backward sequences were analysed by using MEGA-X software.

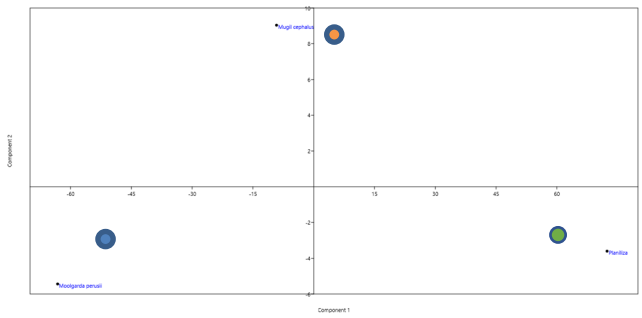

Figure 3. Principal component analysis for *Mugil cephalus* , *Osteomugil* (*Moolgarda) perusii* , *Planiliza* sp.)

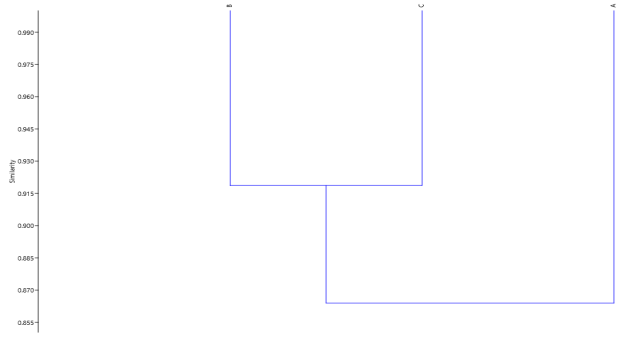

Figure 4. Cluster diagram showing Bray-Curtis similarity Index; (B- *Mugil cephalus*, A-*Planiliza* sp. and C- *Osteomugil (Moolgarda) perusii*

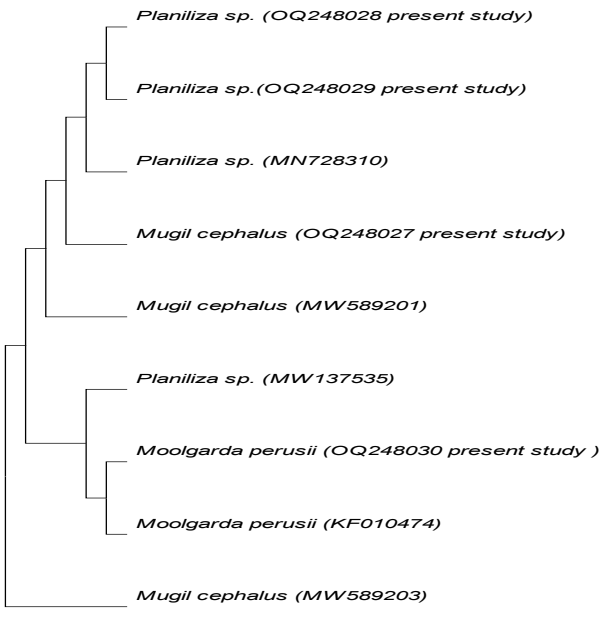

Figure 5. Maximum Likelihood phylogenetic tree for the generated sequence for *Mugil cephalus*, *Planiliza* sp. and *Osteomugil* (*Moolgarda*) *perusii*

Results

The 10 pigmented specimens were collected and identified under 3 different species (Figure 2) under 3 different genera as *Mugil cephalus, Osteomugil perusii* and *Planiliza* sp. based on morpho-meristic traits (Table 1) and molecular barcoding. The highest proportions among these morphometric variables were obtained (Table 1) for eye diameter and pre-orbital length in % HL and inter dorsal space, pre-anal length, and body depth in % SL in case of *Mugil cephalus*. While in *Planiliza* sp., the proportion of caudal peduncle depth in SL was also high. The three species have been clearly differentiated based on morphometric variables through principal component analysis (Figure 3). Further, to understand the Bray-Curtis similarity index based on the morphometric measurements cluster analysis has been performed (UPGMA Method) (Figure 4a, Figure 4b). The least value of similarity index was observed between *Planiliza* sp. and *Osteomugil perusii* (0.82) in comparison with other values among other species (Table 2). Initially, the black dots covering whole fish body, appeared to be natural body colour. But after a long storage in freezer the dots were found to be diminishing. Further, the three identified species were confirmed through molecular barcoding. The maximum likelihood tree has been constructed (Figure 5) by aligning sequences for *Mugil cephalus, Osteomugil* (*Moolgarda*) *perusii* and *Planiliza* sp., by using generated sequences in this present study and downloaded from NCBI.

Discussion

In this present study, characteristics of Mugil *cephalus*, *Osteomugil perusii* (*Moolgarda perusii*) (Rahman et al., 2013) and *Planiliza* sp. were examined and compared with the published records (Table 3). There were insignificant variations in meristic characters in between the specimens (pelvic fin-1 spine, 5 ray, 1st dorsal fin-4 spine, 2nd dorsal fin-8 ray, anal fin spine-2, anal fin ray-9, pre-dorsal scale-18), pectoral fin ray-15 except for *Moolgarda perusii* (pectoral fin ray-14). In addition to this, ambiguity in identification of this group of species has found due to overlapping of characters and pigmentation over the body surface. Melanophore pigmentation was also reported from head of different stages of the fry of 5 grey mullet species (Teleostei: Mugilidae) *Mugil cephalus*, *Liza aurata*, *Liza ramada*, *Chelon labrosus*, *Liza saliens* (Minos et al., 2002) without any taxonomic concern.

Table 1. Morphometric and meristic parameters of species of *Mugil cephalus*, *Osteomugil perusii and Planiliza* sp. expressed as a percentage of standard length and head length

Parameters	***Mugil* Species**		
	Mugil cephalus **SL – 22.1 cm**	***Osteomugil perusii*** **SL – 18.9 cm**	***Planiliza* sp.** **SL – 27.2 cm**
Characters (% of HL)			
Pr-OL	19.22%	20.86%	18.61%
ED	25.51%	23.48%	21.25%
Po-OL	58.27%	55.24%	56.79%
Characters (% of SL)			
HL	24.04%	23.22%	24.03%
PPtL	24.91%	25.35%	25.01%
PPvL	37.17%	36.51%	37.54%
PVL	13.34%	14.55%	16.05%
PAL	71.0%	68.76%	69.63%
AL	15.44%	14.54%	15.91%
PtL	17.32%	16.86%	16.04%
PDL1	48.69%	50.36%	50.05%
PDL2	73.94%	75.33%	76.38%
DFL1	15.49%	16.22%	14.26%
DFL2	15.57%	16.14%	15.92%
DFB1	6.19%	8.43%	10.02%
DFB2	8.62%	8.63%	9.44%
BD	26.53%	24.40%	22.96%
CPD	11.32%	12.45%	16.83%
IDS	22.04%	19.34%	17.69%

Table 2. Values of similarity in between *Planiliza* sp., *Mugil cephalus*, *Osteomugil perusii*

Species	***Planiliza* sp.**	***Mugil cephalus***	***Osteomugil perusii***
Planiliza sp.			
Mugil cephalus	0.9026		
Osteomugil perusii	0.8253	0.9187	

Table 3. Comparison of morph-meristic characters of *Mugil cephalus*, *Osteomugil perusii and Planiliza* sp. reported by different authors

Parameters	**Species**					
	Mugil cephalus		***Osteomugil perusii***			***Planiliza* sp.**
Author	*Present Study (n=2)*	*Zubia et al. (2015)*	*Present Study (n=1)*	*Rahman et al. (2013)*	*Aaron et al. (2018)*	*Present Study (n=1)*
PrOL	8.32	-	7.46	-	-	9.66
ED	11.04	12.5±0.24	8.4	14.8±0.25	5.71±2.6	11.03
PoOL	25.22	-	19.76	12.4±0.28	21.18±4.1	29.48
HL	43.28	51.44±1.36	35.77	33.8±0.19	34.46±4.7	51.91
PPtL	44.84	-	39.04	-	38.64±4.6	54.03
IDS	39.68	-	29.78	-	-	38.22
PVL	24.02	-	22.41	-	16.00±1.1	34.66
PAL	128.34	-	105.89	-	103.48±10.5	150.4
AL	27.8	35.5±0.25	22.39	-	-	34.37
PtL	31.18	-	25.97	-	33.03±2.4	34.64
BD	47.75	-	37.58	-	37.48±3.0	49.6
CPD	20.38	25.4±0.59	19.17	-	-	36.35

Note: **PrOL** = Pre-Orbital Length, **ED**= Eye Diameter, **PoOL**=Post-Orbital Length, **HL**= Head Length, **PPtL**= Pre-Pectoral Length, **IDS**= Inter Dorsal Space, **PVL**=Pelvic fin Length, **PAL**=Pre-Anal Length, **AL**=Anal Fin Length, **PtL**= Pectoral fin Length, BD=Body Depth, **CPD**=Caudal Peduncle Dept

The genetic variation or ecological factors or interactions between them may also be responsible for morphological differentiation (Krishnan, 2018) and sometimes variation in pigmentation pattern (Jawad et al., 2022). Genetic variations and reproductive isolation of species may result in local adaptation. That is expressed in the morphology, physiology, behaviour and other traits of life (Pakkasmaa & Piironen, 2001; Santos et al., 2016; Hassanien et al., 2020). Pigmentation pattern in animals is noted as a highly variable phenotypes in both inter and intra-specific level, thus suggest to study both genetics of species diversification and adaptation (Mills & Patterson, 2009; Wittkopp & Beldade, 2009; Hubbard et al., 2010). Earlier studies have revealed several reasons for pigmentation such as protective measure from UV ray (Kumar et al., 2023) and water pollution (Bolker & Hill, 2000; Carnikián et al., 2006; Bukola et al., 2015; Jawad et al., 2022). Literatures have also cited pigmentation pattern as a kind of abnormalities categorized under either patrial or hyperpigmentation (Jawad et al., 2022), The pigmentation observed in the present study can be named as under patrial category of pigmentation. Even though morphological traits are the primary requirement for species identification, the molecular traits are confirmatory results. Morphological characteristics, classical taxonomy has made significant contributions in species classification, however, due to morphological plasticity, cryptic species and traditional taxonomy cannot accurately distinguish all species, particularly for similar and closely related species (Pigliucci, 2005; Wang et al., 2020). Therefore, new methods of supporting species identification with classical taxonomy methods are needed. Tautz et al. (2002) first suggested DNA sequencing, namely, DNA taxonomy as the main platform for biological classification. The generated sequences using COI gene has been submitted in NCBI-Gene bank repository (*Mugil cephalus*: OQ248027; *Planiliza* sp.: OQ248028, OQ248029; *Osteomugil* (*Moolgarda*) *perusii*: OQ24803). The information on the differentiation of taxonomic units based on molecular evidence authenticating its phylogenetic status was confirmed by constructing maximum likelihood tree (Figure 5). Further, the sequences deposited in NCBI without confirming morphometry, allows taxonomical complications which need to be verified (Silpa et al., 2021; Behera et al., 2022). This part of information is crucial in establishing a genetic database which will assist in the conservation and efficient management towards fisheries resources in Indian waters.

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Compliance With Ethical Standards

Authors' Contributions

AB, BB & AKJ: Manuscript design
AB: Data collection
AB, SB: Drafting, Writing
AB, APK & AKJ: Data analysis and management
All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Data Availability Statements

The specimens were stored in the museum repository in ICAR-CIFE, Mumbai.

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