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## Article

# DNA Barcoding of Mulletts (Family Mugilidae) from Pakistan Reveals Surprisingly High Number of Unknown Candidate Species

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**Abstract:** The mulletts are a widespread group of ecologically and economically important fishes of disputed taxonomy due to their uniform external morphology. Barcoding and phylogenetic studies from various locations around the world largely highlighted the species diversity underestimation using morphological criteria used to establish the taxonomy of the family. Here, we investigated the mullet species diversity from Pakistan, a biogeographic area where nearly no mullet species were genetically characterized. Morphological examination of 40 mulletts reveals 6 known species (*Planiliza macrolepis*, *P. klunzingeri*, *P. subviridis*, *Crenimugil seheli*, *Ellochelon vaigiensis*, and *Mugil cephalus*). Using a references DNA barcode library, the DNA barcode-based species identification flagged eight molecular operational taxonomic units (MOTUs) belonging to five genera (*Crenimugil*, *Ellochelon*, *Mugil*, *Osteomugil*, and *Planiliza*). Among these MOTUs, only one was already present in Barcode of Life Data system, all other representing new Barcode Index Numbers (BIN). These results emphasize the importance of the recognition of cryptic species and the necessity to re-evaluate the overall diversity by the genetic characterization of different species of this family. DNA barcoding is an effective tool to reveal cryptic species that need to be considered in conservation and management measures of fisheries in Pakistan.

**Keywords:** Mugilidae; Cytochrome Oxidase I; Arabian Sea; cryptic species; sequence divergence



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## 1. Introduction

It is largely acknowledged that the current fish taxonomy based on the variation of morpho-anatomical characters greatly underestimated the species diversity [1,2]. Numerous phylogeographic, phylogenetic, and DNA barcoding studies flagged independent evolutionary lineages in widely geographically distributed species that are more and more recognized as cryptic species or at least as candidate species pending further taxonomic investigations [1,3–9]. The proper delineation of species is essential not only for better management and conservation of biodiversity [10,11] but also helps us to understand the causes of different evolutionary processes [12]. From a more pragmatic perspective, incorrect identification of commercially important species may lead to overexploitation and contribute to fish stock depletion [13].

In this context, the DNA barcoding method proved to be a useful and independent approach based on the variation of morphometric and meristic characters for species identification [14]. Instead of observable and definite morphological differences, mitochondrial cytochrome c oxidase subunit I (COI) gene has been used to resolve many taxonomic ambiguities [15,16].

The family Mugilidae currently consists of about 27 genera and 77 recognized species [17]. Mulletts are important in marine fisheries and aquaculture in many temperate and tropical

countries [18]. Owing to the significant morphological conservatism, the delimitation of mullet species is arduous, as a result, mullet species are often inadequately represented in field guides [19]. For this reason, several studies have been carried out to solve the phylogenetic relationship and identification of mullet species using different molecular methods [20–27]. Over the past decades, molecular phylogenetic studies have evidenced the presence of many species complexes within the Mugilidae family [2,19,28–32]. These species complex, often presented as complex of cryptic species due to the absence of evident diagnostic morphometric and meristic characters, are usually sibling species and thus with much more limited distribution range than described for the morpho-species [2]. Among those species *Mugil cephalus* Linnaeus, 1758, is a good example as the morpho species present in nearly all tropical, subtropical, and temperate waters of the world [33] consists of 14 molecular operational taxonomic units (MOTU's) with a distribution range generally limited to a biogeographic province [18]. In this context, it is important to better estimate the species diversity of the Mugilidae family and their distribution range to barcode most mullet species in the different biogeographic provinces. While some DNA barcoding or phylogenetic studies have been realized in various regions of the world such as Europe [21–23,34–42], Africa [43], South America [32,44], Asia [20,26,31], and India [45,46], no studies have been considered species diversity present in Pakistan or, more generally, in the Arabian sea.

Based on morphological variations, a variable number of mullet species have previously been reported from Pakistan: 6 species by Qureshi [47], 12 by Bianchi [48], 7 by Fahmida [49], 10 by Froese and Pauly [50], and 12 species by Psomadakis et al. [51]. Since DNA barcodes are not available for Pakistani mullet species, it is not possible to know if these morpho-species belong to already identified MOTU's (such as those listed in [29]) or represent cryptic diversity.

The present study was designed to evaluate the divergence threshold and barcoding gap for the accurate molecular delimitation of mullet species present in Pakistan and flag new MOTU's using sequences of the mitochondrial COI gene. The results will significantly contribute to BOLD systems and GenBank databases with new DNA barcodes and provide an overview of species diversity of mullets from Pakistan in comparison with species elsewhere.

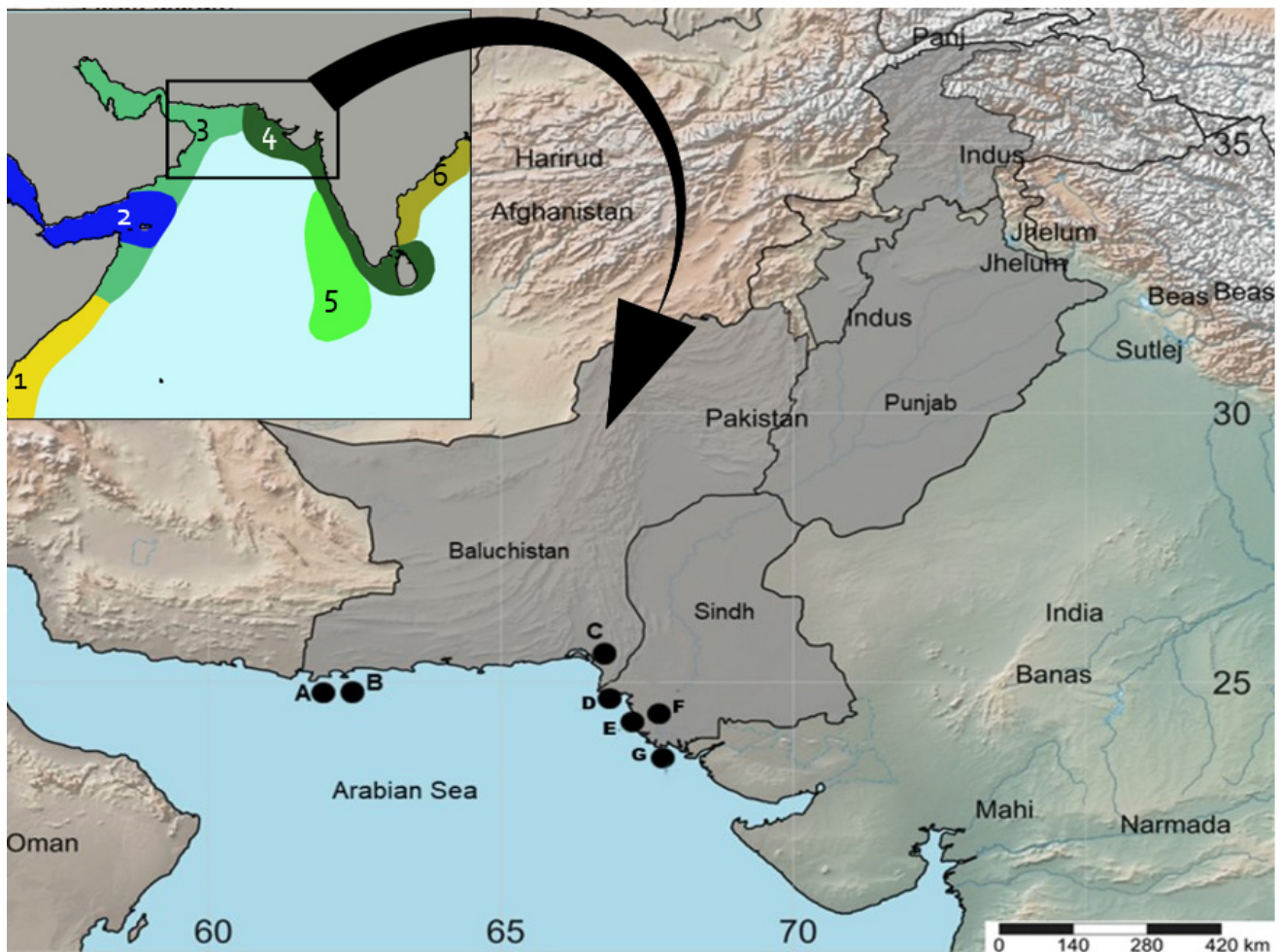
## 2. Materials and Methods

### 2.1. DNA Barcode Reference Library and Taxonomical Nomenclature

The reference library used in this study originates from Durand et al. [28], Shen and Durand [29], and Delrieu-Trottin et al. [31]. This library consists of 76 DNA barcode records trimmed to 556 base pairs representing all the species and BIN diversity of genera *Planiliza*, *Ellochelon*, *Crenimugil*, *Osteomugil*, and *Mugil*. These DNA barcodes have been selected as reference for the DNA barcode-based species identification since most of specimens barcoded are stored in museum and have been identified by taxonomic experts of the Mugilidae family [2,19,28] (Table S1). They have been used in a number of phylogenetic studies dealing with the taxonomy of the Mugilidae family by several authors [2,19,29–32]. The nomenclature proposed by Durand et al. [52] and Xia et al. [30] was used for genera while cryptic or unidentified species followed the interim taxonomical nomenclatures established by Durand and Borsa [2]. However, in a state of clarity and traceability, we also mentioned the Barcode Index Numbers (BINs) that can also represent an interim taxonomical nomenclature when no clear species name can be assigned to a barcode. The two interim taxonomical nomenclatures are largely redundant since Durand et al. [19] demonstrated a large overlap of barcode gaps recovered with COI marker (used to establish the BIN by the BOLD system) or a longer marker composed of COI, 16S, and cytochrome b fragment (such as in Durand et al. [28] used by Durand et al. [2] for their interim taxonomical nomenclature). However, the advantage of BIN is to have using the BOLD system with a direct and dynamic vision of the distribution range of the putative species.

## 2.2. Sample Collection and Identification

Mullet fish samples were collected from the landing sites and fish markets of Pakistan located along with Sindh (Karachi Fish Harbor, Kakapir, and Keti Bunder) and Baluchistan coasts (Somniani, Pasni, Gwadar, and Jiwani) (Figure 1). All samples were morphologically identified using different identification keys [48,51] and other available literature [53,54]. Each specimen was photographed and fin clipped; then, all the samples were stored individually in an Eppendorf vial with 70% ethanol, and later, they were stored at  $-20\text{ }^{\circ}\text{C}$ .



**Figure 1.** Biogeographic provinces present and surrounding the Pakistan marine region [55]: 1 = Western Indian Ocean, 2 = Red Sea and Gulf of Aden, 3 = Somali/Arabian, 4 = West and South Indian Shelf, 5 = Central Indian Ocean Islands, and 6 = Bay of Bengal. (B) Map showing the sampling locations of mullets analyzed in this study. A = Jiwani; B = Gwadar, C = Somniani, D = French Beach, E = Kakapir, F = Karachi Fish Harbor, G = Ibrahim Haideri and H = Keti Bunder.

## 2.3. DNA Amplification and Sequencing

Genomic DNA was isolated from fins using the G-Spin Total DNA extraction mini kit (iNtRON Biotechnology, Jungwon-gu, Gyeonggi, Korea) following the manufacturer's recommendations. Approximately, 652 base pairs (bp) of the cytochrome oxidase subunit I (COI) were amplified using primers FishF1+ FishF2/FishR1 [56]. Polymerase Chain Reaction (PCR) was conducted in the total volume of 40  $\mu\text{L}$  containing 20  $\mu\text{L}$  of MyTaq PCR Mastermix (Bioline, London, UK), 16  $\mu\text{L}$  of ultrapure water, 0.8  $\mu\text{L}$  of BSA (Euromedex, Souffelweyersheim, France), 0.6  $\mu\text{L}$  of each primer (3  $\mu\text{M}$ ), and 2  $\mu\text{L}$  of DNA template. The conditions used during PCR reaction were as follows: initial denaturation temperature at  $92\text{ }^{\circ}\text{C}$  for 5 min followed by 35 cycles of strand denaturation at  $92\text{ }^{\circ}\text{C}$  for 1 min, primer annealing at  $52\text{ }^{\circ}\text{C}$  for 45 s, primer extension at  $72\text{ }^{\circ}\text{C}$  for 1.5 min, and a final extension at  $72\text{ }^{\circ}\text{C}$ .

°C for 5 min. Sequencing was performed by Macrogen Inc. (Seoul, Korea). All nucleotide sequences were deposited in GenBank. The accession numbers are given in Table 1.

**Table 1.** List of mullet species (names inferred from morpho-anatomical keys), code numbers, locality information and GenBank's accession numbers.

Morpho Species	Code No.	Location	Co-Ordinates	Accession No.
<i>Mugil cephalus</i>	792	Kakapir, Karachi	24°50'42" N 66°54'01" E	MT943713
	794	Kakapir, Karachi	2450'42" N 66°54'01" E	MT943714
	PMNH-55212	Jiwani, Baluchistan	25°10'59" N 61°46'24" E	MN511974
	PMNH-55368	Gwadar, Baluchistan	24°06'53" N 62°19'41" E	MN511975
<i>Planiliza macrolepis</i>	PMNH-54728	Ibrahim Haideri, Karachi	24°47'39" N 67°08'31" E	MN512028
<i>Planiliza subviridis</i>	PAK Mu 851	Somniani, Baluchistan	25°09'25" N 66°43'25" E	MT943724
	806	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943723
	840	Keti Bunder, Sindh	24°07'49" N 67°27'10" E	MT943722
	PMNH-55121	Ibrahim Haideri, Karachi	24°47'39" N 67°08'31" E	MN511966
<i>Planiliza klunzingeri</i>	PAK Mu 884	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943743
	PAK Mu 804	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943734
	824	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943727
	816	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943737
	830	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943735
	828	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943736
	PaK Mu 3	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943740
	PAK Mu 872	Keti Bunder, Sindh	24°07'49" N 67°27'10" E	MT943730
	PAK Mu 870	Keti Bunder, Sindh	24°07'49" N 67°27'10" E	MT943731
	PAK Mu 881	Keti Bunder, Sindh	24°07'49" N 67°27'10" E	MT943729
PAK Mu 882	Keti Bunder, Sindh	24°07'49" N 67°27'10" E	MT943728	

Table 1. Cont.

Morpho Species	Code No.	Location	Co-Ordinates	Accession No.
	PAK Mu 883	Keti Bunder, Sindh	24°07'49" N 67°27'10" E	MT943744
	802	Keti Bunder, Sindh	24°07'49" N 67°27'10" E	MT943742
	827	Keti Bunder, Sindh	24°07'49" N 67°27'10" E	MT943726
	811	Keti Bunder, Sindh	24°07'49" N 67°27'10" E	MT943739
	812	Keti Bunder, Sindh	24°07'49" N 67°27'10" E	MT943738
	832	Gwadar, Baluchistan	25°06'53" N 62°19'41" E	MT943725
	PAK Mu 821	Gwadar, Baluchistan	25°06'53" N 62°19'41" E	MT943733
	823	Gwadar, Baluchistan	25°06'53" N 62°19'41" E	MT943732
	PMNH 55125	Gwadar, Baluchistan	25°06'53" N 62°19'41" E	MN12027
<i>Crenimugil seheli</i>	819	Gwadar, Baluchistan	25°06'53" N 62°19'41" E	MT943705
	PMNH-55231	Gwadar, Baluchistan	25°06'53" N 62°19'41" E	MN512029
<i>Osteomugil sp.</i>	829	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943716
	PAK Mu 866	Somniani, Baluchistan	25°09'25" N 66°43'25" E	MT943715
	PMNH-55122	Gwadar, Baluchistan	25°06'53" N 62°19'41" E	MN512031
	810	Karachi Fish harbor	24°50'57" N 67°27'10" E	MT943717
	PAK Mu 864	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943718
	PAK Mu 862	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943719
	PAK Mu 2	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943721
	PAK MU 885	Karachi Fish harbor	24°50'57" N 66°58'35" E	MT943720
<i>Ellochelon vaigiensis</i>	805	Keti Bunder, Sindh	24°07'49" N 67°27'10" E	MT943712
	PMNH-55070	French beach, Karachi	24°50'32" N 66°48'53" E	MN511887

#### 2.4. DNA Barcode-Based Species Identification

Species identification based on specimen morphology was confronted to an independent species identification using DNA barcodes. All DNA barcodes generated in this study were uploaded on the BOLD system that assigned these barcodes to molecular

operational taxonomic units, (MOTUs) called Barcode Index Numbers (BINs) using the RESL algorithm. [57]

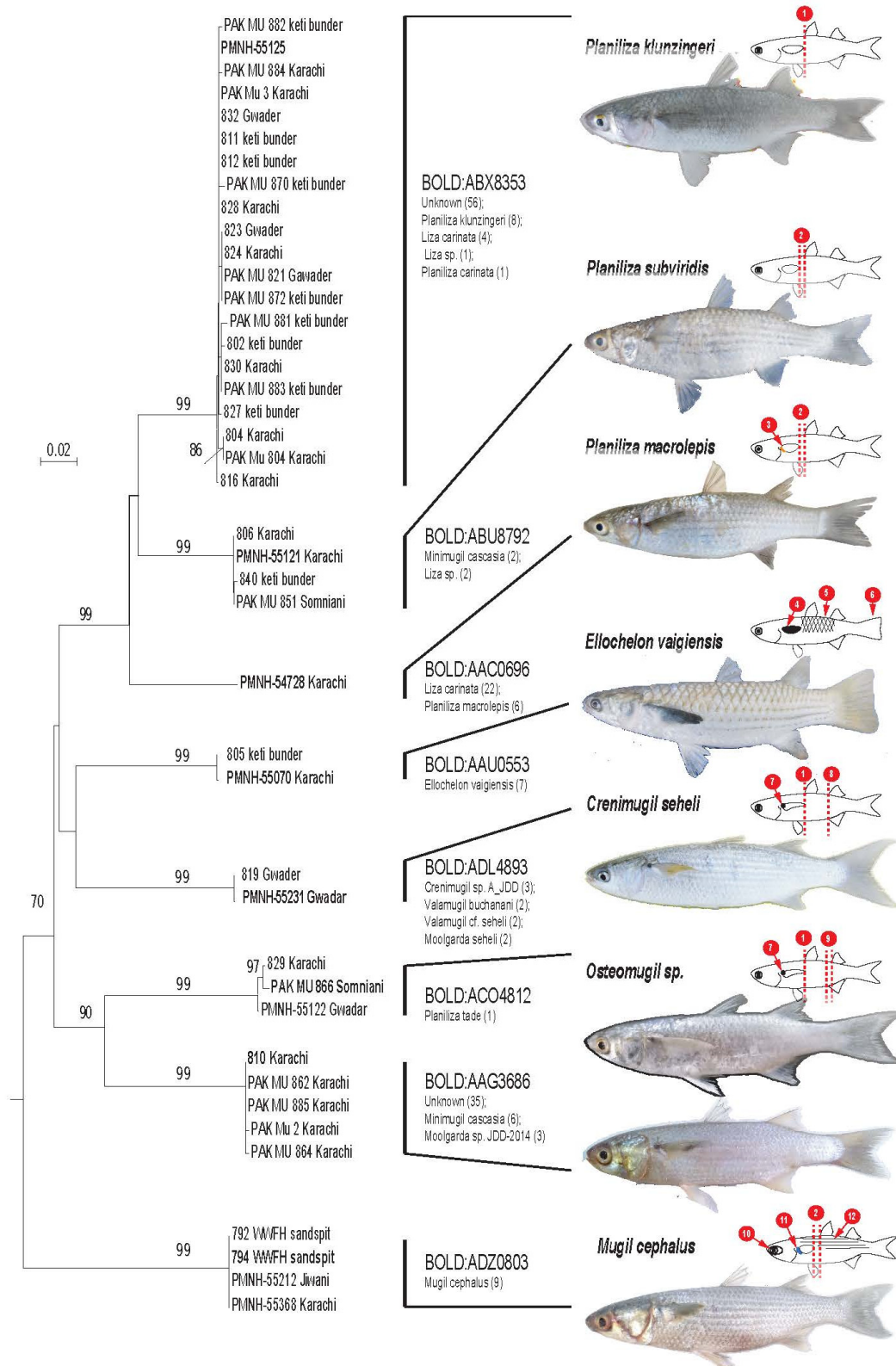
This algorithm flag MOTU's boundaries by clustering DNA barcodes with high sequence similarity and connectivity using all DNA barcodes of the BOLD's library. BINs are used to confirm the concordance between species designations and barcode sequence clusters [57].

The composition and variations of nucleotides were analyzed by Mega V. 7.0 [58]. For the calculation of genetic distances between and within the species of mullets, Kimura-2-parameter (K2P) model was used [59]. A Neighbor-Joining tree was constructed with bootstrap analysis (500 replicates) to evaluate the reciprocal monophyly of species. To reveal and discriminate various species present in our sampling, we constructed phylogenetic tree using all COI barcodes generated in this study and secondarily with mullet reference barcodes. All trees were rooted using as outgroup a sequence of *Abudefduf vaigiensis* (Perciformes: Pomacentridae).

### 3. Results

A total of 41 specimens were successfully barcoded. All data relative to these specimens as well as their DNA barcodes were uploaded in BOLD's project PAKF. Among these specimens, 33 were morphologically identified at the species level. (Figure 2). These species are *Planiliza macrolepis*, *P. klunzingeri*, *P. subviridis*, *Crenimugil seheli*, *Ellochelon vaigiensis*, and *Mugil cephalus* (Table 1). The remaining eight specimens were identified at the genus level only: the *Osteomugil* genus. At exception of *Osteomugil* species, they were easily distinguishable using following criteria: length of the pectoral fin in regard to the birth of the first dorsal fin, presence of dot or blotch at the birth of the pectoral fin, presence and importance of adipose eyelid, color of the pectoral fin, position of the second dorsal in regard to the anal fin, form of the caudal fin, and scale margin (Figure 2).

All barcodes obtained in this study have been assigned in BOLD to 8 BINs (Figure 2). For specimens identified at the species taxonomic level, only one BIN has been recovered. The generated COI sequences were compared with the available COI sequences [2,19] and BOLD system revealed the presence of at least seven unknown candidate species.



**Figure 2.** Phylogenetic relationships among Mugilidae specimens collected along Pakistan shores recovered using 622 bp of the COI and the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [3] and are in the units of the number of base substitutions per site. Each



leaf of the tree corresponds to an individual sampled in Pakistani water, and leaves in bold correspond to the specimen's picture on the right. BINs provided from BOLD is mentioned for each clade as well as species name of specimen belonging to the BIN (in parentheses number of specimen). Species identified using morpho-anatomical criteria are indicated on the right of the figure. Fish draws highlight main morphometrical criteria that discriminate species collected in Pakistan. 1. Tip the pectoral fin at vertical of the birth of the first dorsal fin, 2. Tip the pectoral fin not reaching vertical of the birth of the first dorsal fin, 3. Presence of a golden blotch at the birth of the pectoral fin, 4. Pectoral fin black, 5. Scales with black margin, 6. Fin tail truncated, 7. Presence of a black dot at the birth of the pectoral fin, 8. Birth of the second dorsal fin at vertical of the birth of the birth of the anal fin, 9. Birth of the second dorsal fin not at vertical of the birth of the anal fin, 10. Large adipose eyelid, 11. Presence of a blue blotch at the birth of the pectoral fin, and 12. Black stripes on flank. Pictures provided by Ariba Hasan & Shabir Ali Amir (copyright).

#### 4. Discussion

##### BOLD:AAC0696/*Planiliza macrolepis* (morphology)

Specimen from Pakistan identified morphologically as *Planiliza macrolepis* belongs to this BIN as well as Durand and Borsa's [2] reference sequences of *Planiliza macrolepis* (Figure 3A). Specimens identified morphologically as *P. macrolepis* belongs to two lineages with parapatric geographic distribution: one located in the East Indian Ocean (South Africa, Seychelles, and Oman) and one from the Central Indian Ocean to the Pacific Ocean (Maldives, Sri Lanka, Taiwan, Japan, New Caledonia, and Fiji) [28]. However, because the type locality of *P. macrolepis* Smith 1948 is in South Africa, Durand and Borsa [2] proposed to keep this name only for the NW Indian lineage, the second one being provisionally designated as *Planiliza* sp. H. Present data precise *P. macrolepis* (BOLD:AAC0696) distribution range eastward and, more importantly, its geographic limit with its sibling species *Planiliza* sp. H, to date, situated in India [29].

##### BOLD:ABX8353/*Planiliza klunzingeri* (morphology)

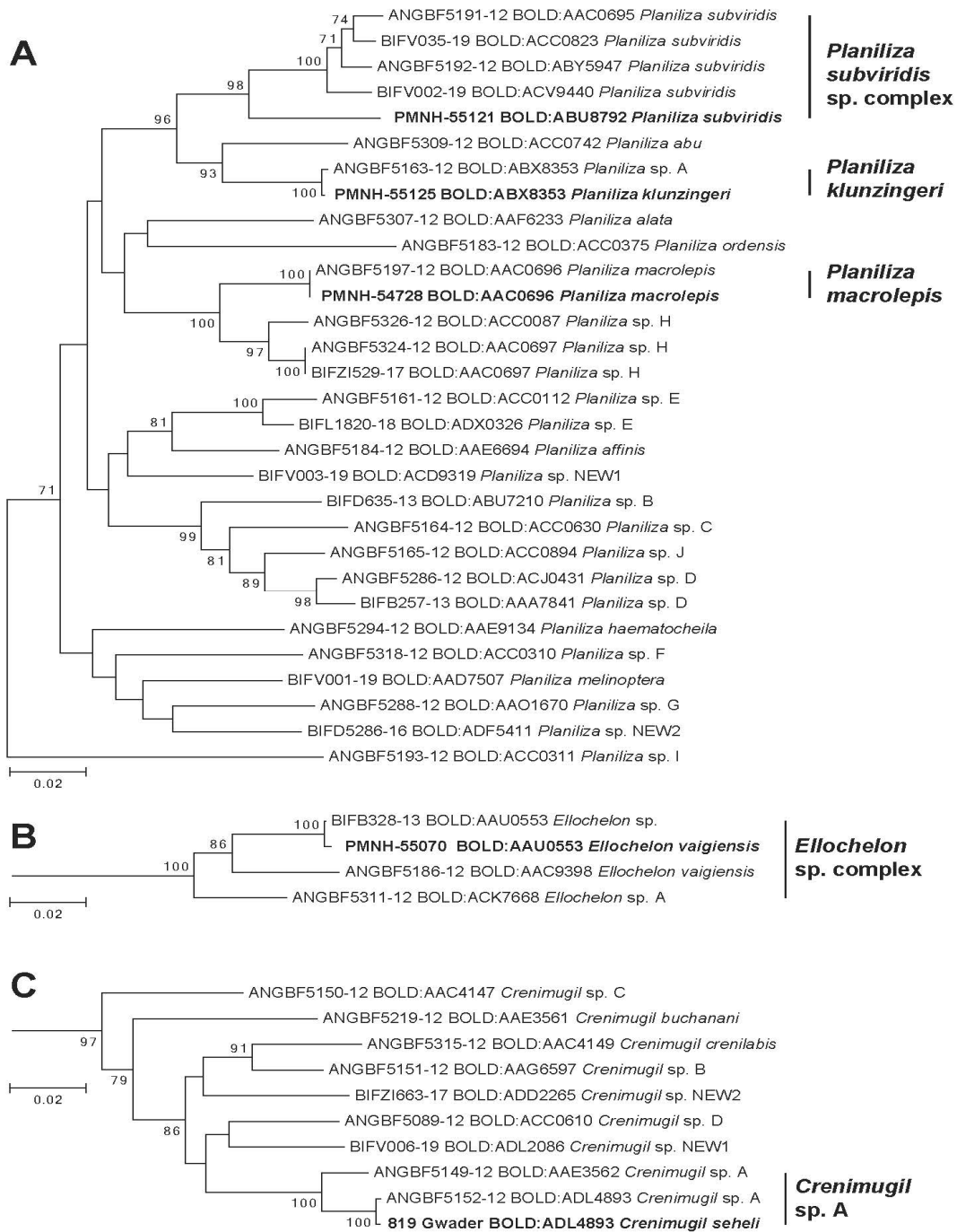
Specimens from Pakistan identified morphologically as *Planiliza klunzingeri* assigned to the BIN BOLD:ABX8353 that also included reference sequences of *Planiliza* sp. A of Durand and Borsa [2] (Figure 3A). There is no doubt that mullet specimens collected in the Persian Gulf and named "*Planiliza* sp. A" by Durand and Borsa [2] is actually *Planiliza klunzingeri* considering morphological characters records on our specimen as well as previous barcoding studies [29,60] The distribution range of *Planiliza klunzingeri* encompasses the Persian Gulf eastward to the coast of Karachi and Bombay [61]. Eastward distribution limits have been confirmed by [62], which provided DNA barcodes of *P. klunzingeri* (BOLD:ABX8353) collected in the Narmada River (NW India). Interestingly, *P. klunzingeri* (BOLD:ABX8353) and *P. macrolepis* (BOLD:AAC0696) probably shared the same eastward distribution limit, which suggests the presence of a biogeographic barrier in NW India and not along Pakistani shores as suggested by Spalding et al. [55] (Figure 1).

##### BOLD:ABU8792/*Planiliza subviridis* (morphology)

Pakistani specimens assigned to this BIN have been morphologically identified as *Planiliza subviridis* but does not correspond to any reference sequences nor *Planiliza subviridis* sensu Durand and Borsa [2] that are assigned in BOLD to 4 different BINs (Figure 3A). If specimens from Pakistan share a common ancestor with *P. subviridis* sensu Durand and Borsa [2], the divergence of Pakistani specimen with other *P. subviridis* specimen (6.9% K2P) largely exceeds divergence observed among *P. subviridis* sensu Durand and Borsa [2] (1.8% K2P).

In BOLD, the BIN BOLD: ABU8792 consists of only 4 specimens (ANGEN 113-115, DBFN284-12, DBFN295-12, and GBMINI126937-17) collected at two localities: Gujarat, India, and the Persian Gulf, Iran. These barcodes are labeled as *Liza* sp. or *Minimugil cascasia*, which indicate nomenclature mistake, i.e., *Liza* is no longer considered as valid [52], or misidentification, *Minimugil cascasia* is endemic to rivers of northern Bengal and present a very different phylogenetic position [63]. However, the barcode's geographic distribution origins describe a geographic distribution for this MOTU (BOLD: ABU8792) similar to *P. klunzingeri*, from the Persian Gulf to NW India. This distribution is fully parapatric to the distribution of *P. subviridis* sensu Durand and Borsa [2] as BIN assigned to this last

species consists of specimen sampled from West to East in India (Maharashtra and Kochi to Puducherry, (BOLD: AAC0695); Indonesia and Malaysia (BOLD: ACC0823); Indonesia and Papua New Guinea (BOLD: ACV9440); Philippines, Taiwan, and China (BOLD: ABY5947). Considering the type locality of *P. subviridis*, Valenciennes 1836 Ganges River, Malabar, India, the name *subviridis* should be maintained only for the BIN BOLD: AAC0695, the other close candidate species being named “*Planiliza cf. subviridis*”. In the case of the MOTU present in Pakistan, we assigned provisional species name *Planiliza cf. subviridis* (BOLD: ABU8792) pending further morpho-anatomical investigation to determine potential diagnostic feature.



**Figure 3.** Phylogenetic tree of species in Genera *Planiliza* (A), *Ellochelon* (B), and *Crenimugil* (C). Leaves in bold represent a representative MOTU/BIN identified in Pakistan waters.

BOLD:AAU0553/*Ellochelon vaigiensis* (morphology)

Pakistani specimens assigned to the BIN BOLD:AAU0553 have been identified morphologically as *Ellochelon vaigiensis*. This BIN also includes the reference sequence from Indonesia labeled as *Ellochelon* sp. by Delrieu-Trottin et al. [31] but none of those depicted in Durand et al. [28] that identified two MOTUs in *Ellochelon vaigiensis* morpho species (Figure 3B). Later, based on the level of divergence that largely exceeds interspecific diversity, Durand et al. [2] proposed to provisionally name these MOTUs as *Ellochelon* sp. A for the lineage (BOLD:ACK7668) observed only in Australian specimens and maintained the name for the lineage (BOLD:AAC9398) present from Indonesia to French Polynesia. Following this logic, this third lineage corresponding to the BIN BOLD:AAU0553 with the divergence of 6.2% and 5.8% with *Ellochelon* sp. A and by *Ellochelon vaigiensis*, respectively, and is temporarily designated as *Ellochelon* cf. *vaigiensis* (BOLD:AAU0553). This MOTU is observed in specimens collected in Pakistan, as well as Iran, Malaysia, and Indonesia (BOLD, consultation 01/20/21). No significant phylogenetic relationship has been recovered in the COI phylogenetic tree; all MOTUs corresponding to *Ellochelon vaigiensis* sensu [2] descended from the same common ancestor (Figure 3B). A larger sampling scheme in the Indo-Pacific targeting *Ellochelon* spp. is necessary to better delineate the geographic structure of this species complex as well as its evolutionary history.

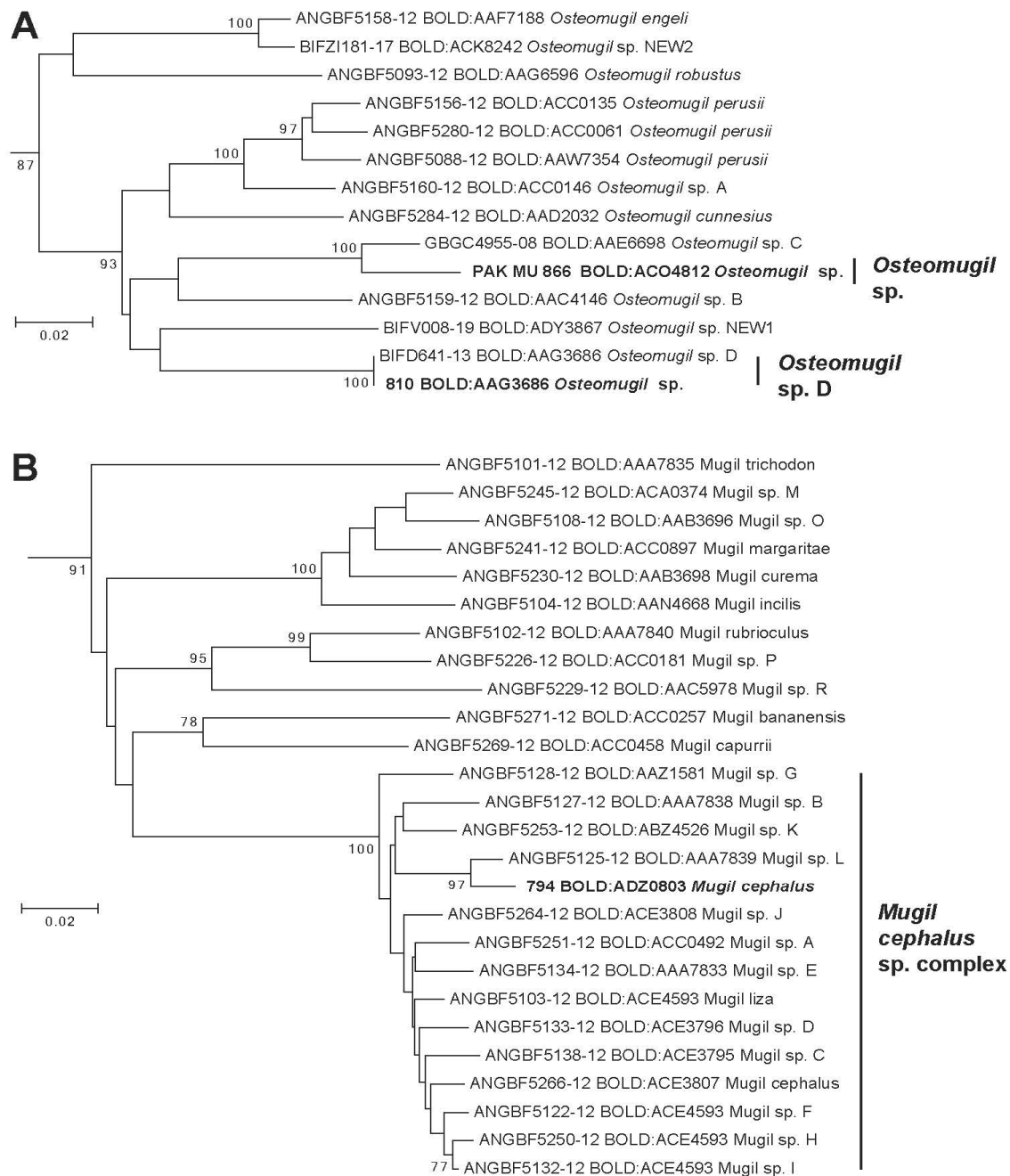
BOLD:ADL4893/*Crenimugil seheli* (morphology)

Specimen from Pakistan identified morphologically as *Crenimugil seheli* is assigned to the BIN BOLD:ADL4893 also included reference sequences of *Crenimugil* sp. A of Durand and Borsa [2] (Figure 3C). [28] Durand et al. [28] identified in *Crenimugil seheli* three lineages that occur sympatrically in the Indo-West Pacific; *Crenimugil* sp. A sensu Durand and Borsa [2] is one of this lineages. In BOLD, barcodes identified as *Crenimugil* sp. A by Durand and Borsa [2] are assigned to two BINs; BOLD:ADL4893 and BOLD:AAE3562 (Figure 3C). In BOLD, the BIN BOLD:ADL4893 is composed of barcodes observed in 9 specimens collected in the NW Indian Ocean (Iran, Saudi Arabia, Oman, and Seychelles), while BOLD:AAE3562 is composed of 50 specimens collected in the Indo-Pacific region (Reunion Island, Maldives, West Papua, China, Taiwan, Saipan, Australia, New Caledonia, and Fiji). Geographic distribution of these two MOTUs appears parapatric suggesting that these three are sibling species; the species present in Pakistan being assigned to provisional species name *Crenimugil* cf. *seheli* (BOLD:ADL4893).

BOLD: ACO4812/*Osteomugil* sp. (morphology)

Pakistani specimens assigned to this BIN have been morphologically identified as an *Osteomugil* species but their barcode does not correspond to any reference sequences. BIN BOLD:ACO4812 is associated with only one public specimen (LQDWL-TIS-31-12-2013-011) collected in India, Gujarat, close to Pakistan's border.

This specimen LGEN074-14 has been identified as *Planiliza tade* but the picture of specimen available in BOLD System (consultation 05/23/2021) indicates that it is an *Osteomugil* species: presence of a black dot at the birth of the pectoral fin, pectoral fin long reaching to the first dorsal fin vertically, and birth of second dorsal fin not to the birth of anal fin vertically. The phylogenetic position in the tree of this BIN also confirms that it is an *Osteomugil* species with a sister relationship with *Osteomugil* sp. C also collected in India. (Figure 4A). This MOTU is assigned to a provisional species name *Osteomugil* sp. (BOLD:ACO4812).



**Figure 4.** Phylogenetic tree of species in Genera *Osteomugil* (A) and *Mugil* (B). Leaves in bold represent a representative MOTU/BIN identified in Pakistan waters.

Pakistani specimen assigned to the BIN BOLD:AAG3686 present morphological characters of an *Osteomugil* species but the exact species was not identified. This BIN includes a reference sequence labeled as *Osteomugil* sp. D by Shen et al. [29] and Delrieu-Trottin et al. [31] but none depicted in Durand and Borsa [2] (Figure 4A). These reference sequences have been observed in a specimen collected in India and Indonesia. In BOLD, this BIN is associated with some additional barcodes obtained from India, Bangladesh, and Malaysia. This species provisionally named "*Osteomugil* sp. D" (BOLD: AAG3686) appear to be endemic to the Indian Ocean largely distributed from Pakistan to Indonesia. More taxonomical investigations are necessary to identify this species among all the *Osteomugil* species diversity described in the past.

BOLD: ADZ0803/*Mugil cephalus* (morphology)

Pakistani specimens assigned to this BIN BOLD: ADZ0803 have been identified morphologically as *Mugil cephalus*. No reference sequences have been observed in this BIN, while Durand et al. [28] and later Durand et al. [52] depicted in this morpho-species up to 13 and 14 MOTUs, respectively. In BOLD, this BIN is composed of 3 public specimens, collected in Bangladesh (GBMNB8388-20), India (GBGC9983-09), and one from unknown origin but the sequence produced from a laboratory in India (ANGBF54236-19). Within the *Mugil cephalus* species complex, these new MOTUs presents significant phylogenetic relationships with *Mugil* sp. L (BOLD:AAA7893) (Figure 4B) observed in the Pacific Ocean [2,18,64]. *Mugil cephalus* is considered as a species complex consisting of 15 candidate species including *Mugil liza* in the West Atlantic [2,18]. In some parts of the world, most of these species present parapatric distribution ranges such as the present new species provisionally named "*Mugil* cf. *cephalus*" (BOLD:ADZ0803) which is, to date, limited to the North Indian Ocean where no other *M. cephalus* MOTUs have been identified. When sympatry occurred, such as in the NW Pacific where three MOTU are present, reproductive isolation has been demonstrated, which confirms the validity of their species status [27,65]. No clear phylogenetic structure has been observed in the phylogenetic tree (Figure 4B) as well as a previous phylogenetic tree that included more molecular markers [28]. The diversification of *M. cephalus sensu lato* occurred during 5 million years (MYA) [32]. The divergence between the Indian *Mugil* cf. *cephalus* (BOLD:ADZ0803) and Pacific *Mugil* sp. L probably has rating of less than 2 MYA, considering its low level of divergence (1.7% K2P) by comparing to other lineages (mean distance 2.9% K2P).

More taxonomic and phylogenetic investigations are necessary to highlight the evolutionary history of this species complex present on a worldwide scale.

## 5. Conclusions

DNA barcoding appears to be the most efficient method for species identification and its advantage in the detection of cryptic species, an appealing application for many taxonomists [66]. The increasing number of new species detected through DNA barcoding suggests that the biodiversity level is greatly underestimated using solely the classical system of morphology-based identification.

In the present study, the COI gene was successfully used for species identification. Delimitation of MOTUs within the members of family Mugilidae found along the Pakistani waters was determined for the first time. Here, we morphologically characterized six species, although our specimens correspond genetically to eight MOTUs. The comparison of COI sequences generated in this study with the sequences available from different geographical regions [2,19] and BOLD system uncovered the existence of at least seven unknown candidate species from as much as a species complex. Analysis of the geographic distribution of *Planiliza* species present in Pakistan in light of the genetic diversity stressed the importance of Pakistan as a biogeographic border or transition between the NW Indian fauna and the rest of the Indo-Pacific region. This study calls for more taxonomic and phylogenetic investigations to describe Pakistani species and highlight the biogeographic component of Pakistan ichthyofauna in the Indo-Pacific area. This study will help in the development of DNA barcode reference data for the mullets of Pakistan which in turn would help in the management and conservation of fisheries. Furthermore, the novel sequences generated in this study and deposited in BOLD/GenBank will be available for future reference and research.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/d13060232/s1>: Table S1: Mullet specimens list used as species reference from genera *Planiliza*, *Ellochelon*, *Crenimugil*, *Osteomugil* and *Mugil*.

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