Journal of Materials and Environmental Science
ISSN : 2028-2508
e-ISSN : 2737-890X
CODEN : JMESCN

J. Mater. Environ. Sci., 2023, Volume 14, Issue 02, Page 210-223

http://www.jmaterenvironsci.com

Molecular characterization of demersal marine fish species *Pseudorhombus arsius* (Hamilton, 1822), *Psettods erumei,* (Bloch & Schneider, 1801) and *Cynoglossus cynoglossus* (Hamilton, 1822) from Sindh coasts, Pakistan through DNA barcodes

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Received *19 Jan 2023,*
Revised *16 Feb 2023,*
Accepted *18 Feb 2023*

Keywords:
- ✓ *DNA barcoding*
- ✓ *Demersal marine species*
- ✓ *Sindh coast, Pakistan*

Citation*: Khan P., Ali Q. M., Ahmed Q., Bat L. (2023) Molecular characterization of demersal marine fish species Pseudorhombus arsius, Psettods erumei, and Cynoglossus cynoglossus from Sindh coasts of Pakistan through DNA barcodes, J. Mater. Environ. Sci.,14(2), 210-223.*

Abstract: External morphological characteristics have traditionally been used to identify fish species. However, it can often be challenging to distinguish fishes by their morphological characteristics, particularly given their many developmental stages. Molecular-based identification creates a quick, precise, and affordable system for identifying species through DNA barcoding. To establish sequence profiles of known species versus sequences of unknowns that can be matched and subsequently identified, it uses short, conventional gene targets. *Pseudorhombus arsius*, *Psettods erumei* and *Cynoglossus cynoglossus* specimens were collected from two stations I: Kharochan, (67◦34'42' N, 24◦04'31E); II: Jhangisir (67◦37'34' N, 24◦11'18E); Sindh coast, Pakistan, during July 2020 to March 2021. The sequence of *P. arsius* DNA barcoding indicated (597 bases in 7258 scans); base spacing: 15.571008; (G:128 A:159 C:212 T:201); *C. cynoglossus* DNA barcoding indicated (681 bp in 7258 scans); Base spacing: 15.571008; (G:228 A:169 C:112 T:171); and *P. erumei* DNA barcoding indicated (680 bp in 7258 scans); Base spacing: 15.571008, (G:182 A:129 C:242 T:127). This is the first study that provides information on morphological and molecular-based identification from Pakistan's coasts of the Arabian Sea. DNA-based identification techniques provide a significant analytical complement or possibly a substitute for inventory for future biologists.

1. Introduction

Molecular characterization of demersal marine fish species is important for a variety of reasons. It can help with the accurate identification and classification of species, which is crucial for conservation and management efforts. It can also aid in understanding the genetic diversity and population structure of a species, which can inform breeding and stock management practices. Additionally, molecular characterization can be used to study the evolutionary relationships between different species and aid in understanding the origin and diversity of fish in marine ecosystems.

Fish species *Pseudorhombus arsius* (Family-Paralichthyidae), *Psettods erumei* (Family-Psettodidae), *Cynoglossus cynoglossus* (Family-Cynoglossidae) are marine brackish demersal fishes, distributed throughout the Indo-West Pacific region (Munroe, 2001; Froese, and Pauly, 2022). These

fish species are found in shallow waters, estuaries, and on muddy and sandy bottoms, and juveniles are commonly found in brackish water (Amaoka and Hensley, 2001). Identifications based on morphological characters are particularly difficult and time-consuming because fishes have enormously wide-ranging morphological characteristics as they transition through ontogenetic metamorphism, and thus, morphometric characteristics alteration during the process of ontogenetic development, thus convergent and divergent variations impose further task in the fish identification process (Ward *et al.,* 2009). In many fishes, high diversity in phenotypic plasticity and morphological features used to discern taxa can be so subtle for different developmental stages that it is very difficult to identify species by using morphological characteristics alone, even for trained experts (Victor *et al.,*2009; Zhang and Hanner, 2012).

The accurate identification of fish species is a pivotal component to protect the extant ichthyo-faunal biodiversity and to perform regular assessments of local fish faunas for conservation planning (Ahmed *et al.,* 2019). Correct and accurate identification of morphologically similar species is important for fisheries management and population studies. DNA-sequenced-based methods provide an opportunity to construct additional taxon diagnosis systems that employ DNA sequences as 'barcodes' to identify organisms (Xu *et al.,* 2019).

Traditional morphology-based identification systems rely mostly on expert experience and the integrity of samples (Li *et al.* 2017), Furthermore, some taxa show a variety of complex characteristics, such as sexual dimorphism or developmental variability of larvae (Webb *et al.,* 2006; Kenchington *et al.,* 2017; Batta-Lona*et al.,* 2019). Thus, the applications of molecular tools contributed significantly to intra-specific as well as inter-specific level identification. Mitochondrial genes have been extensively employed in phylogenetic studies due to their rapid evolution, lack of introns, and recombination. Partial sequences of mitochondrial genes such as 16SrDNA (Hillis and Dixon 1991), cytochrome b (Lydegard and Roe, 1997) and cytochrome oxidase subunit 1 (COI) (Hebert *et al.,* 2003a) have been successfully used to resolve species ambiguity and to estimate the chronology of different taxa (Hall 1999). Molecular approaches for the identification of fish species have been suggested to ease and the confines associated with morphological-based identification systems and the dearth of local fish identification expertise. More importantly, COI evolution is sufficiently rapid to allow the discrimination of very closely related species in most groups, as well as taxonomically significant intra-specific variation associated with a geographic structure (Bucklin *et al.,* 2011).

Many studies show the effectiveness of the COI gene for species identification in diverse animals (Hebert *et al.,*2003 a,b), including fish (Ward *et al.,* 2005; McCusker *et al.,* 2013). Hebert *et al.* (2003a and 2004) using a standard DNA sequence that is DNA barcoding to identify species and uncover biological diversity advocated using a standard DNA sequence that is DNA barcoding to identify species and uncover biological diversity. Therefore, the use of DNA barcodes as an accurate and effective method of species identification is currently favored by an increasing number of researchers. Correct identification of morphologically similar species is important for fisheries management and population studies. DNA-sequenced-based methods provide an opportunity to construct additional taxon diagnosis systems that employ DNA sequences as 'barcodes' to identify organisms (Xu et *al.,* 2019).

Molecular identification of *Pseudorhombus arsius* reported by Ahmed *et al.* (2021) from Bangladesh coastal waters, Cox's Bazar, and Patuakhali regions. Lü *et al.* (2021) provided the large-scale sequencing of flatfish including *Psettodes erumei* and other species *Trinectes maculates* (Bloch & Schneider, 1801), *Chascanopsetta lugubris* (Alcock, 1894), *Brachirus orientalis* (Bloch & Schneider, 1801), *Paraplagusia blochii* (Bleeker, 1851), *Colistium nudipinnis* (Waite, 1911),

Pseudorhombus dupliocellatus (Regan, 1905), *Platichthys stellatus* (Pallas, 1787). Safina (2016) analysed DNA barcoding in families (Family: Achiridae, Bothidae, Citharidae, Cynoglossidae, Paralichthodidae, Paralichthyidae, Pleuronectidae, Poecilopsettidae, Rhombosoleidae, Samaridae, Scophthalmidae, Soleidae and Psettodidae. Rahman *et al.,* (2019) molecular identification [cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial] of *Cynoglossus cynoglossus* from Bangladesh. Zhang *et al.,* (2010) reported the characterization of the mitochondrial control region sequence of *Psettodes erumei* and the phylogenetic analysis of Pleuronectiformes. Omir *et al.* (2020) also reported DNA barcoding in *Psettodes erumei* fish. From Pakistan (Ghouri*et al.,* 2020) reported the identification of fresh and marine water fish species including (*Scomberomorus commerson, Pampus argenteus, Scomberoides commersonianus, Carangoides malabaricus,* and *Tenualosa ilisha)* through DNA Barcoding. Hassan *et al,* (2021) reported DNA Barcoding in Mullets species, morphological examination of 40 mullets reveals 6 known species (*Planiliza macrolepis, P. klunzingeri, P. subviridis, Crenimugil seheli, Ellochelon vaigiensis,* and *Mugil cephalus*). No work has been done on DNA Barcoding in *Pseudorhombus arsius*, *Cynoglossus cynoglossus* and *Psettods erumei*from the Pakistan coasts of Arabian Sea. These species are economically important for the fishing industry, but it is not known about their population structure and genetic diversity on Pakistan's coasts. The main purpose of this study on the molecular characterization of *Pseudorhombus arsius, Psettodes erumei* and *Cynoglossus cynoglossus* through DNA barcoding (using COI) is to provide a more accurate and efficient way of identifying and classifying these species, understand their population structure, genetic diversity, evolutionary relationships, and to fill the knowledge gap on these species in the region.

2. Methodology

2.1. Study Area

The Sindh coastal region is in the South-Eastern part of the country between the Indian borders along Sir Creek on the east to Hub River along the Balochistan coast on the west (Fig. 1). The Sindh coast can further be subdivided into two parts, the Indus Delta Creek system, and the Karachi Coast. The Indus Delta (approx. 1000 sq. miles) is the most prominent ecological feature of the Sindh Coast (covers 85% of the coastal belt in Sindh) the coastal morphology of which is characterized by a network of tidal creeks and mudflats occupied by mangrove vegetation (MFF Pakistan, 2016). Fishing is one of the most vital activities along Sindh's coastline (WWF, 2005). This coastline is 352 Km and contains 71% of Pakistan's fisheries resources. The coastal waters are well-matched for fisheries production by using of accurate salinity and temperature characteristics. This coastline is an extremely productive area endowed with great biodiversity due to the combination of riverine flow into the Indus delta and the sub-tropical environment. Furthermore, recent assessments show that 70% of the total marine resource production for Pakistan came from this coastline (ADB, 2004, 2006). Sindh's coastline plays a significant role in the total marine catch of the country Pakistan is gifted with a wide variety of fisheries resources including 150 commercially important species (WWF, 2005; FAO, 2016).

2.2. Sample collection and Morphological identification of Fish

Pseudorhombus arsius (7 specimens), *Psettods erumei* (13 specimens) and *Cynoglossus cynoglossus* (11 specimens) were collected from two stations; I: Kharochan, (67◦34'42' N, 24◦04'31E) and II: Jhangisir (67◦37'34' N, 24◦11'18E) during July 2020 to March 2021. Obtained fish samples were washed carefully with distilled water and immediately transferred to the laboratory in polythene

bags and labeled. Samples were stored at –20°C until the DNA extraction process. The length and weight of *P. arsius* (30.4-32.2cm; 290-360gm), *P. erumei* (23.5-25.4cm; 218-266 gm), *C. cynoglossus* (28.5-30.5 cm; 276-372 gm) were measured. For morphological identification, morphometric and meristic characters were measured. Field identification Guide to the Living Marine Resources of Pakistan (FAO, 2016) was used for the identification of specimens at the species level.

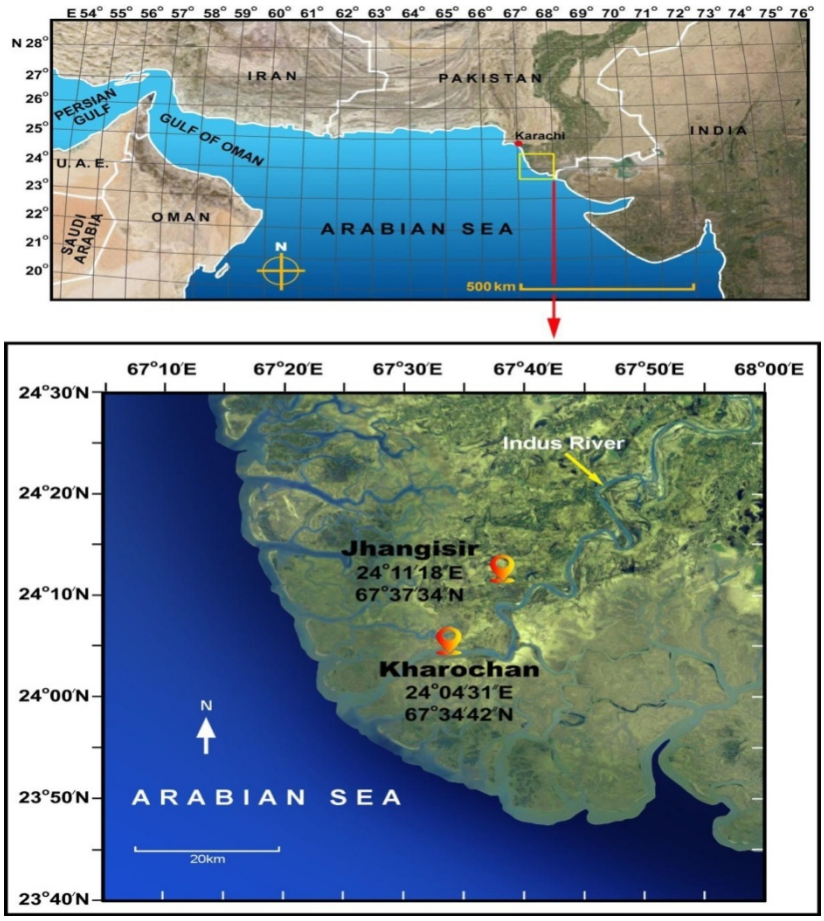


Figure 1. Map of the sampling area.

2.3. DNA extraction, amplification, and sequencing

Genomic DNA was extracted from dorsal tissue samples dissected from the dorsal muscle, and genomic DNA was extracted according to the standard Barcode of Life (Ivanova et *al.,* 2006). Frozen fresh tissues were crushed to a fine powder using a mortar and pestle and then suspended in 1 2 ml/100 mg tissue of digestion in a buffer (100 mM NaCl, 10 mM TRIS-Cl, 25 mM EDTA, SDS, pH8-4), containing 01 mg/ml proteinase K (Sigma). The sections were then scraped into microfuge tubes, suspended in digestion buffer (about200pl/10 sections), and incubated at 37°C for a variable period. Nucleic acid was purified by an organic extraction step. An equal volume of phenol: chloroform isoamyl alcohol (25:24:1) was added to the proteinase K digests, mixed thoroughly, and centrifuged to separate the aqueous and organic layers. The upper aqueous layer containing the nucleic acid was collected and the extraction was repeated. A final extraction was carried out using an equal volume of chloroform: iso-amyl alcohol (24:1). The nucleic acid was then precipitated with two volumes of cold ethanol (- 20°C) and one-tenth volume of 3M sodium acetate (pH 5 2) at - 20°C for one hour, spun

down (13000 rpm, for 10 minutes, dried, and re dissolved in TRIS-EDTA buffer. Two sets of primers fish primers were used Fish.

FishF1: 5′-TCAACCAACCACAAAGACATTGGCAC-3′

FishR1: 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′

Pro889U20: 5′-CCWCTAACTCCCAAAGCTAG-3′

TDKD1291L21:5′-CCTGAAATAGGAACCAAATGC-3′

Fifty picomoles of each were incubated with 1 pl of an extracted DNA sample in 25 ml of 1 x Polymerase Chain Reaction ((PCR) buffer (50 mMKCl, 10 mM TRIS-Cl, 1 5 mM MgCl2, 0.010o gelatine, pH8 3) for five minutes at 94°C in the Thermal cycler to denature fully the template DNA and to inactive any proteases contaminating the DNA before Taq polymerase was added. To this was added 25 pl of 1 x PCR buffer, containing 2 5 U of "AmpliTaq" (Cetus) and 0 4 mM each of dATP, dCTP, dGTP and dTTP. The reaction mixture was then incubated for 40 cycles of 48 seconds at 94°C, 48 seconds at 50°C, and one minute at 72'C, followed by 10 minutes at 72°C to ensure that the entire product was fully extended. Each PCR experiment included a negative control of distilled H_20 in place of DNA. The PCR products were analysed by running on a 2% agarose electrophoresis gel, stained with ethidium bromide in a DNA sub-cell (BioRad) at 150 V for one and a half hours.

2.4. Data Analysis

The original data obtained by sequencing were analysed by The Basic Local Alignment Search Tool (NCBI-BLAST). All high-quality sequences were compared with the NCBI, BLAST program to determine the species identification. The Basic Local Alignment Search Tool (BLAST) database is a highly efficient tool for determining sequence similarities with reference sequences from GenBank. The input sequences were compared with the maximum similarity data sets of fish species based on the lowest significant *E*-values for the pair wise generated alignment (Ghouri *et al.,* 2020). According to Wong and Hanner (2008) if sequence similarity greater than 98% was the criterion for identification at the species level, and a similarity lower than 98% was used for identification at the genus level. The accessioned sequences with the highest maximum identity to the amplicon sequence were downloaded from the GenBank sequence database.

3. Results and Discussion

3.1. Morphological and DNA identification of fish

Fish samples (a total of 31 specimens) were collected from our two selected sites, for morphological and DNA identification of fish. These specimens were identified as *Pseudorhombus arsius*, *Psettods erumei* and *Cynoglossus cynoglossus* through the identification guidebook (FAO, 2016), and showed in Table 1. The sequence of *P. arsius* DNA barcoding indicated (597 bases in 7258 scans); base spacing*: 15*.571008; (G:128 A:159 C:212 T:201); *C. cynoglossus* DNA barcoding indicated (681 bp in 7258 scans); Base spacing: 15.571008; (G:228 A:169 C:112 T:171); and *P. erumei* DNA barcoding indicated (680 bp in 7258 scans); Base spacing: 15.571008, (G:182 A:129 C:242 T:127) sequences were amplified Tables 2-4. 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. Molecular characterization of marine and coastal fishes of Bangladesh through DNA barcodes of *P. arsius,* (Voucher no. MH311283 MH230944 MH429297) was presented by (Ahmed *et al.,* 2021). This study describes the molecular characterization of marine and coastal fishes of Bangladesh based on the mitochondrial cytochrome c oxidase subunit I (COI) gene as a marker. Fig. 2 shows the phylogentic distance tree in between species. The accuracy of species identification through DNA barcoding mostly depends on both inter-specific and intra-specific divergence. In recent years, some progress has been made regarding the evolutionary origin of flatfishes and their morphological adaptations. According to current theories, flatfishes evolved from basally divergent percoids (Campbell *et al.,* 2013). The early exploration of the genetic origin of the specialized morphology of flatfishes was started by Inui and Miwa (1985) and continued by Hashimoto *et al*., (2002) and Dornbos *et al.,* (2005).

Lü *et al.,* (2021) observed that in both gene trees and species trees, *Psettodes erumei* is clustered with non-flatfish Perciformes rather than with Pleuronectoidei species provides strong support for the independent origins of Pleuronectoidei and Psettodoidei. Furthermore, reconstruction of ancestral chromosomes for the Pleuronectoidei and Psettodoideiline ages also shows that *Psettodes erumei*s hares specific chromosome rearrangements with *Toxoteschat areus* and *Polydactylussext arius*, rather than with Pleuronectoidei species, further supporting a polyphyletic origin for these two lineages. Also, the authors conclude that these phenotypical observations, combined with our results, provide strong support for a polyphyletic origin of flatfishes, with Psettodoidei and Pleuronectoidei, respectively, arising from two independent evolutionary events. To capture real evolutionary signals, we, therefore, split the previously known Pleuronectiformes into 'real flatfish Pleuronectoidei' (RFP) and 'flatfish-like Psettodoidei' (FLP) lineages in the following analysed.

DNA barcoding studies on bottom fish have come to the fore in recent years because they offer a powerful tool for species identification and classification. Traditional methods, such as morphological analysis, can be challenging for bottom fish due to their high degree of variability and similarity within and between species. DNA barcoding allows for the use of a standardized genetic marker to differentiate between species, which can be a quick and accurate method for identifying fish species (Abdalwahhab *et al.,* 2020a, b; Keskin and Atar, 2013). The present study is focused on DNA barcoding study targeting three commercially important demersal fish species of Sindh coasts of the Arabian Sea in Pakistan. This is done for the first time with this study due to limited research, identification challenges, advancements in technology, conservation concerns and the desire for a better understanding of these species and their populations in the region.

Sequence of *Pseudorhombus arsius* DNA barcoding (indicated 597 bp sequences were amplified).

AAAGATATCGGCACCCTATATTTAGTATTCGGTGCCTGAGCTGGAATGGTCGGCACAGCCCTTAGCCTGCTCATTCGAGCCGA
GCTCAGTCAACCTGGTGCCCTCCTTGGAGACGATCAGATTTATAATGTAATCGTCACCGCACACGCTTTTGTAATAATCTTCTTT
ATAGTTATACCAATTATGATTGGAGGCTTCGGAAACTGACTCATTCCGCTTATAGTAGGGGCCCCCGATATGGCCTTCCCACGA
ATAAATAACATGAGCTTCTGACTTCTCCCCCCTTCCTTCCTCCTACTTTTAGCATCTTCTGGTGTTGAGGCGGGGGCAGGAACA
GGGTGAACTGTTTATCCTCCACTAGCTGGCAATCTTGCCCACGCTGGAGCATCCGTTGACCTCACTATTTTCTCCCTACACCTT
GCTGGGATTTCTTCCATTCTTGGGGCAATTAACTTTATTACAACTGTCATCAATATGAAACCCCCTTCTGTTACCATATACCACAT
CCCCCTCTTTGTCTGAGCCGTCCTAATTACAGCCGTACTACTCCTACTTTCTCTACCAGTTCTGGCTGCAGGGATCACAATACTA
CTTACGGACCGTAACCTGAACACCACTTTCTTTGACCCTGCAGGAGGCGGAGACCCCATCTTATACCAGCACCTCTTCT
GATTCTTC

Table 1. Morphometric and meristic characteristics of Fish species

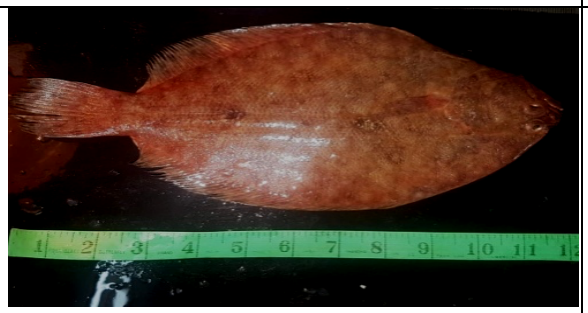
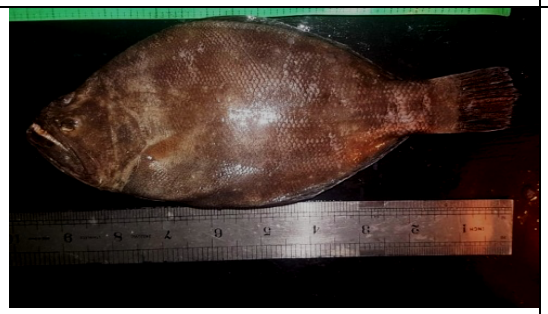
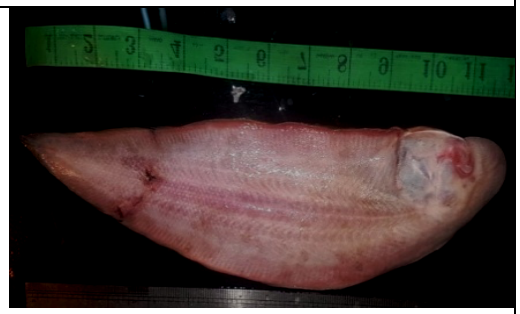
	Pseudorhombus arsius	***Psettods erumei***	***Cynoglossus cynoglossus***
Picture			
Common name	Large tooth flounder	Indian halibut	Tongue sole
Size	33.2 cm	25.4 cm	30.5 cm
Coloration	• Background colour of eyed side brownish with varying patterns of dark spots but always a large black blotch and 1 or 2 smaller dark spots along lateral line.	• Brownish grey, with 4 broad dark crossbars. • Dorsal, anal and caudal fin tips black; eyed side occasionally only partially coloured	• Eyed side uniformly brown-grey, with vague dark marbling.
Distinctive characteristics	• Body ovoid, • upper jaw teeth small, closely spaced posteriorly, becoming widely spaced and enlarged anteriorly; lower jaw teeth large, widely spaced, 8–18 on blind side lower jaw; • Gill rakers longer than broad, 9–13 on the lower limb of 1st gill arch; scales on eyed side ctenoid, those on blind side cycloid. • Dorsal soft rays (total): 71-84; Anal spines: 0; Anal soft rays: 53 – 62.	• Body oval and flat, but thicker than that in most other flat fishes; mouth large with strong canine teeth; pelvic fins with 1 spine and 5 soft rays; both eyes on left or right side; • Anterior rays of dorsal and anal fins spinous. • Dorsal soft rays (total): 38-45; Anal spines: 1; Anal soft rays: 33 – 43.	• Snout rounded; corner of mouth not reaching posteriorly beyond vertical through posterior. • No dark blotches or irregular cross bands on body. Margin of lower eye • Scales on both sides of body ctenoid; usually 10 caudal fin rays. • Dorsal soft rays (total): 95 102; Anal spines: 0; Anal soft rays: 72 - 78. The dorsol ateral line usually undulating.

Table 2. Significant alignments four (04) search results based on using the BLAST algorithm on the *Pseudorhombus arsius* GenBank database (NCBI) Sequences producing significant alignments.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudorhombus arsius voucher CEW0079 cytochrome c oxidase subunit 1 (COI) gene, partial cds; mitochondrial	*Pseudorhombus arsius*	1253	1253	100%	0.0	100.00%	678	KU236030.1
Pseudorhombus arsius voucher CEW0149 cytochrome c oxidase subunit 1 (COI) gene, partial cds; mitochondrial	*Pseudorhombus arsius*	1234	1234	99%	0.0	99.56 %	686	KU317884.1
Pseudorhombus arsius voucher EADF_267 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Pseudorhombus arsius*	1214	1214	97%	0.0	99.70 %	663	MT076773.1
P*seudorhombus arsius* voucher ADC 259.17-2 cytochromeoxidase subunit 1 (COI) gene, partial cds; mitochondrial	*Pseudorhombus arsius*	1155	1155	96%	0.0	98.62 %	652	JF494301.1

Sequence of *Psettods erumei* DNA barcoding (indicated 680bp sequences were amplified).

CTTTATCTAGTATTTGGTGCTTGAGCCGGCATAGTAGGCACAGCCCTGAGCCTGCTAATTCGGGCAGAACTTAGCAGCCCGGAA
CCCTCCTGGGAGATGACCAAATCTACAATGTCATCGTAACAGCACACGCCTTCGTGATAATTTTCGTTATAGTAATGCCTATCATG
ATCGGAGGCTTCGGAAACTGACTTATCCCCTAATAATCGGCGCCCCAGACATAGCATTCCCCCGTATGAATAACATGAGTTTCTG
ACTCCTTCCCCCTTCTTTCTTACTGCTACTTGCCTCTTCAGGAGTTGAGGCTGGTGCGGGTACTGGATGAACCGTTTATCCGCCT
CTGGCCGGCAACCTAGCCCACGCAGGAGCATCCGTTGACCTGGCTATCTTTTCCCTTCACCTGGCCGGAATCTCCTCAATTCTA
GGGGCCATTAATTTGATCACTACGATCATCAACATAAAACCCCCGACCGTCTCTATGTACCAAATCCCCCTCTTTGTGTGAGCCG
TGCTCATTACAGCTGTCCTGCTTCTCCTCTCTCTACCCGTCCTAGCTGCTGGCATGACAATGCTCCTAACAGATCGCAACCTTAA
C

Table 3. Significant alignments five (05) search results based on using the BLAST algorithm on the *Psettods erumei* GenBank database (NCBI) Sequences producing significant alignments.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Psettodes erumei haplotype 3 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Psettodes erumei*	1096	1096	100%	0.0	99.83%	597	MT905057.1
Psettodes erumei voucher EADF_556 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Psettodes erumei*	1057	1057	100%	0.0	98.66%	657	MT076807.1
Psettodes erumei voucher EADF_383 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Psettodes erumei*	1057	1057	100%	0.0	98.66%	663	MT076806.1
Psettodes erumei voucher EADF_382 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Psettodes erumei*	1057	1057	100%	0.0	98.66%	663	MT076805.1
Psettodes erumei voucher EADF_381 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Psettodes erumei*	1057	1057	100%	0.0	98.66%	663	MT076804.1

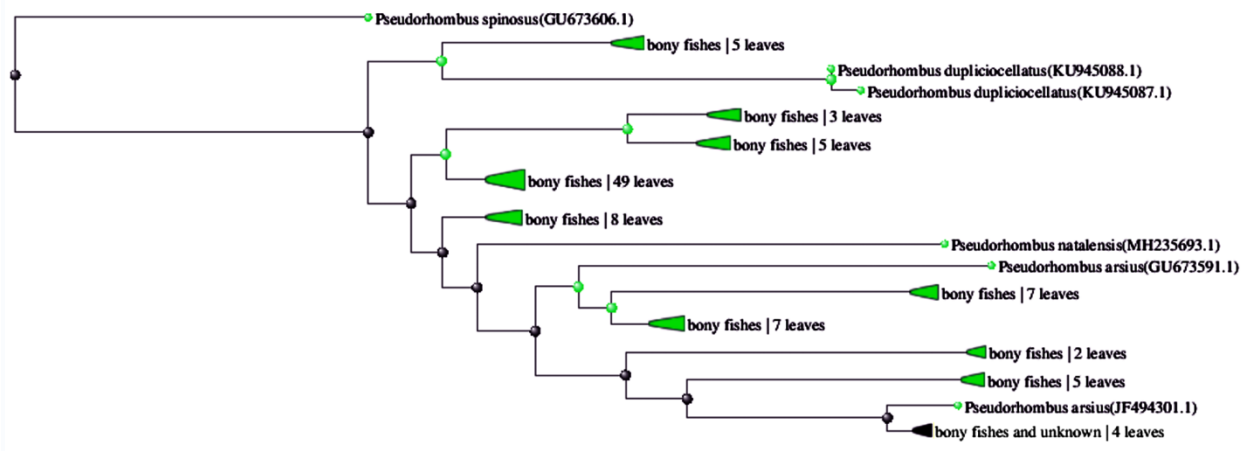
Sequence of *Cynoglossus cynoglossus* DNA barcoding (indicated 681bp sequences were amplified).

AAAGACATTGGCACCCTATACATAGTATTTGGTGCCTGGGCTGGAATAGTAGGAACTGCCCTCAGCCTACTTATTCGAGCAGAG
CTAAGCCAACCAGGAAGTCTGCTTGGTGATGACCAGATCCAATGTTATTGTGACCGCCCATGCATTTGTAATAATTTTCTTCATA
GTAATACCAATTATAATTGGAGGATTTGGAAATTGACTTATCCCCCTAATAATTGGAGCCCCTGACATAGCATTCCCACGAATAAA
TAATATAAGTTTCTGACTGCTCCCTCCTTCTTTTCTCCTTCTATTAGCTTCCTCCGCTGTAGAGGCAGGAGCTGGTACAGGCTGA
ACTGTTTACCCCCCTTTAGCAGGAAATCTAGCCCATGCAGGAGCATCTGTTGATCTAACAATTTTCTCACTCCACTTAGCAGGTG
TTTCGTCCATCCTAGGAGCTATTAACTTTATTACAACAGTTCTTAACATAAAACCTGAAGGGATAACAATATATCAAGTACCTCTAT
TTGTCTGAGCTGTATTTATTACAGCCATCCTCCTTCTCCTTTCACTTCCTGTTCTAGCCGCAGGGATTACTATACTACTGACAGAC
CGAAATTTAAACACCACATTCTTTGATCCTGCAGGAGGAGGGGACCCTATTCTCTACCAACACTTATTCTGATTCTTTGGC

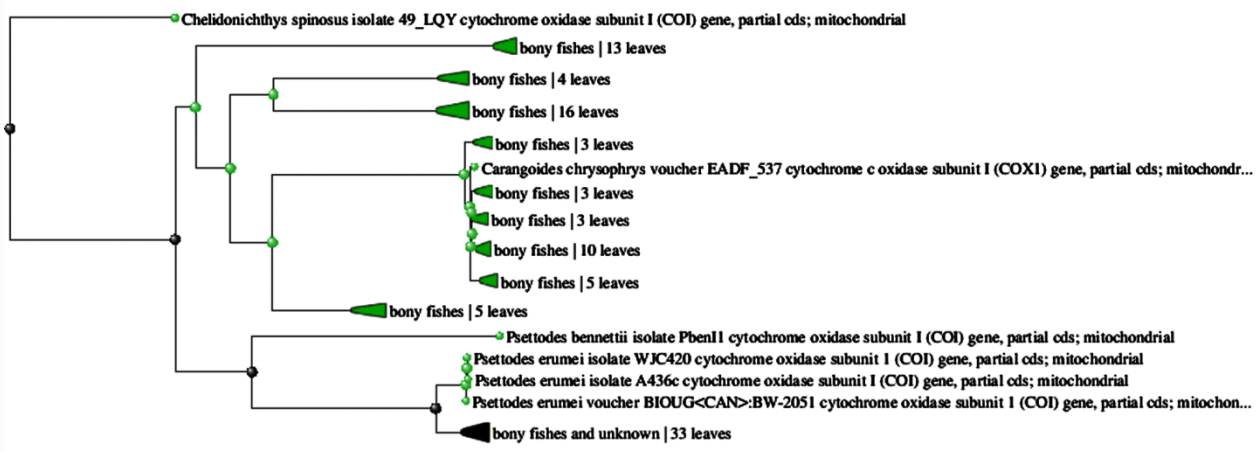
Table 4. Significant alignments five (05) search results based on using the BLAST algorithm on the *Cynoglossus cynoglossus* GenBank database (NCBI)Sequences producing significant alignments

Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cynoglossus cynoglossus from Bangladesh cytochromeoxidase subunit 1 (COI) gene, partial cds; mitochondrial	*Cynoglossus cynoglossus*	1245	1245	100%	0.0	99.71%	MK572144.1
Cynoglossus cynoglossus from Bangladesh cytochromeoxidase subunit 1 (COI) gene, partial cds; mitochondrial	*Cynoglossus cynoglossus*	1216	1216	97%	0.0	99.70%	MK572143.1
Cynoglossus sp. KAH-2017 voucher FBGN-SAU-Dhaka F1611Sb-123 cytochromeoxidase subunit I (COI) gene, partial cds; mitochondrial	*Cynoglossus sp. KAH-2017*	1133	1133	93%	0.0	98.90%	MF594608.1
Cynoglossus cynoglossus isolate F1812ME-50 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	*Cynoglossus cynoglossus*	1079	1079	88%	0.0	99.00%	MN703121.1

A. *Pseudorhombus arsius*



B. *Psettodes erumei*



C. *Cynoglossus cynoglossus*

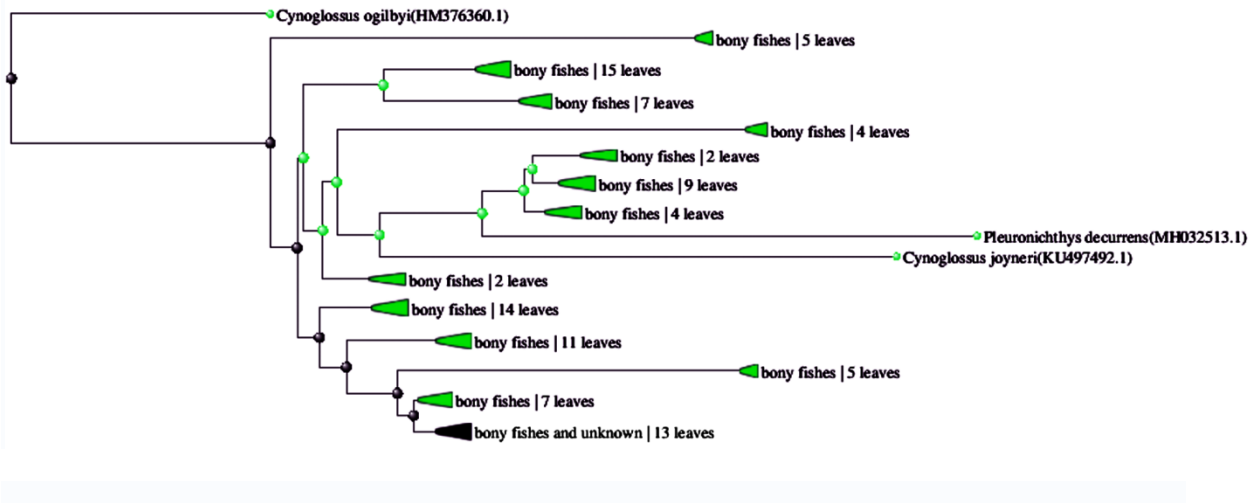


Figure 2. Distance tree in between species.

Conclusion

The present study being conducted for the first time is an important step in understanding the population structure and genetic diversity of *P. arsius, P. erumei* and *C. cynoglossus* from the Sindh coasts of Pakistan. This can provide important information for the conservation and management of these species. Additionally, the study can aid in understanding the evolutionary relationships between these species and other related species and can contribute to understanding the origin and diversity of fish in the marine ecosystems of Pakistan's coasts in the Arabian Sea as well as have practical implications for the fish industry in Pakistan.We believe that this study can contribute to future studies in several ways:

Baseline data on the population structure and genetic diversity of *P. arsius, P. erumei* and *C. cynoglossus* from the Sindh coasts of Pakistan, which can be used as a reference for future studies to track changes in the population over time.

It may identify genetic variation within and between populations of these species, which can inform future research on the adaptation and survival of these species in different coasts in Arabian Sea.

It may inform conservation and management strategies for these species, such as determining appropriate fishing quotas and identifying areas that may require protection in Pakistan coasts.

It may provide information on the evolutionary relationships between these species and other related species in the region, which can be used in comparative studies to understand the origin and diversity of fish in the marine ecosystems of the Arabian Sea. It may also give information on the population structure and genetic diversity of these species.

It may fill the knowledge gap on the population structure and genetic diversity of these species in the region, providing important information for future studies on the ecology, conservation, and management of fish populations in the Arabian Sea.

Suggestions

Larger sample sizes will support a more robust understanding of the population structure and genetic diversity of the species. This could involve sampling from different locations, seasons or different depths.

Integrating DNA barcoding data with other studies, such as population genetics and ecological data, can provide a more complete picture and comprehensive understanding of the population structure and conservation needs of the species on a global scale.

Monitoring the population over time to track changes in population structure and genetic diversity.

Collaboration with other researchers, institutions, and agencies can help to increase the sample size, improve data quality and increase the scope of the study.

Comparing the data obtained from the DNA barcoding study with the morphological identification and traditional methods and evaluating their accuracy, it is possible to identify potential conservation units that should be protected.

Acknowledgment: The authors want to acknowledge Higher Education Commission of Pakistan, (Grant No. IPFP/SRGP/HEC/2565) and MRC&RC for lab facilities, sample collection, sample preparation, and data analysis.

Disclosure statement: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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