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DNA BARCODING AND PHYLOGENETIC RELATIONSHIP OF RAY-FINNED FISH FAMILY *SERRANIDAE* (GENUS *EPINEPHELUS*) FROM THE COAST OF GWADAR BALUCHISTAN

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ABSTRACT

DNA barcoding is inevitable for identification and effectively replacing the traditional pattern of species taxonomy around the world. Species of the family ***S**erranidae* are commercially important and play a vital role in Pakistan economy. This family is in high demand in both domestic and international markets due to its white, tender, and flavorful meat, as well as its appealing texture, generating significant revenue. Many species exhibit close morphological characteristics, leading to confusion in accurate identification. The objectives of present study are to identify the grouper species based on morphological and molecular approaches. *Epinephelus* fish samples were collected with the assistance of local fisherman from the coast of Gawadar, Baluchistan. Besides the morphological identification approaches, the barcoding method became an integral tool for identification in by years and it replacing the traditional taxonomic approaches worldwide. In present study we became successful to identify the four species of *Epinephelus*, however the genetic distance revealed a diversity range of 0.00 to 0.19. Furthermore, the phylogenetic analysis detailed the close relationship between the species from China, Iraq, Iran, Malaysia, Bangladesh, India, Qatar, Saudi Arabia, Thailand, Canada and Tehran.

Key words: DNA barcoding, Phylogenetic relationship, *Epinephelus*, N.J. (neighbor joining), Kimura distance, Gwadar.

INTRODUCTION

The Family *Serranidae* (*genus Epinephelus*) represents the largest group of ray-finned fishes (*Actinopterygii)*. These fish possess rays and spines in their fins and exhibit remarkable diversity in shape, size and coloration. The family *Serranidae* has 234 species across 32 genera, with *Epinephelus* species commonly known as groupers. Groupers are among the most valuable fish found in tropical and sub-tropical waters across all oceans (Tupper andSheriff, 2008).

Groupers hold significant economic value due to their high market demand and excellent taste. They exhibit a fast growth rate, resilience to physicochemical environmental stress, and high food conservation efficiency (Craig and Hasting, 2007). Several *Epinephelus* species, are distributed throughout the Arabian Sea (Aamir *et al*., 2022), with key species in the Arabian Gulf including *Epinephelus Coioides*, *E. Polylepis*, *E. latifasiatus* and *E. dicanthus* However, there is a lack of genetic diversity studies on grouper species along the Arabian coast of Baluchistan.

Traditionally, fish taxonomy has been based on morphological characteristics such as body color, scale patterns, fin rays, fin spine, lateral lines, and body proportions (Strauss and Bond, 1990). However, this method requires taxonomic expertise and become challenging when morphological traits changes over a species life cycle. Fish are highly diverse aquatic organisms, and their morphological traits undergo onto genetics metamorphosis, further complicating species, classification (Zhang and Hanner, 2011).

Groupers are protogynous hermaphrodites, meaning they begin life as females and later transition into males. This natural sex transformation makes it difficult to catch mature males, thereby limiting artificial breeding and production (Oh *et al*., 2013). Moreover, morphological changes during this transformation can lead to misidentification. Such as, *Epinephelus* tauvina is often resembled with *E. coioides, E. malabaricus* (Rimmer & Glamuzina, 2019). Besides, it the relationships among this group of species are declining due to non-social behaviors, pollution, overfishing and habitat destruction (Martinez *et al*., 2018). It is long believed that cytochrome oxidase sub-unit I molecular marker brought the accuracy in identifications and it is proven to be a powerful approach for identification, diversity assessment and taxonomic classification (Hebert *et al*., 2003). Therefore, barcoding approach is considered as a highly reliable under various environmental conditions. (Meyer & Paulay 2005). Because of its rapid and robust approach of identification, the taxonomists now are widely using the method. The investigation is preliminary study of DNA barcoding and genetic relationship of *Epinephelus* fishes through COI gene to identify the groupers fishes (*Serranidae*, geneus *Epinephelus*) from coastal area of Gwadar and established their phylogenetic relationship, which will contribute the reference library and facilitate conservation and management of groupers fishes.

MATERIALS AND METHODS

Specimen Collection: Samples were collected from Gawadar, Jiwani, Pasni, and Ormara (Fig 1). The individuals were photographed, and immediately transported to Center of Excellence in Marine Biology, University of Karachi. Morphological identifications were performed using several renowned identification keys like (Talwar & Kacker, 1984; Talwar & Jhingran, 1991).

DNA Extraction: Genomic DNA was extracted from 30 mg of fish muscle tissue using the standard Proteinase K-Phenol-Chloroform-Isoamyl Alcohol method (Sambrook & Maniatis, 1989). The COI gene was amplified using universal COI primers (Ward *et al*., 2005) as follows:

- FishF1: **5′-**TCAACCAACCACAAAGACATTGGCAC**-3′**
- FishR1: **5′-**TAGACTTCTGGGTGGCCAAAGAATCA**-3′**

The polymerase chain reaction (PCR) was performed in a 25 µL reaction mixture**,** which included: 1.25 µL of DNA template, 1.25 µL of each primer, 12.5 µL of Es Taq polymerase, 8.75 µL of double-distilled water.

PCR conditions were set as follows: Initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, final extension at 72°C for 7 minutes. Successful amplification was confirmed via 1.2% agarose gel electrophoresis stained with ethidium bromide.

Sequencing and Phylogenetic Tree Construction: BIOEDIT and MEGA 6 software (Tamura *et al*., 2013) were used to edit and align the obtained sequences. The sequences were analyzed for similarity using NCBI BlAST option for identification accuracy. The phylogenetic analysis were performed using MEGA-6 software with Kimura 2- parameter (K2P) ((Kimura *et al*., 1980), model and the neighbor joining tree was constructed.

RESULTS

DNA Barcoding and Identification of Grouper Species: This investigation based on molecular evidences re-confirmed four species of grouper in Pakistani waters. The photographic morphology of the samples is shown in (Fig. 2). The identified species are:

***Epinephelus latifasciatu*s** (Temminck & Schlegel, 1842): Characterized by prominent black spots and streaks on the dorsal and caudal fins.

Epinephelus polylepis (Randall & Heemstra, 1991): Displays a pale head with fins and body covered in small, closely spaced dark brown spots. The caudal fin features a white line and a row of blackish-brown spots.

Epinephelus dicanthus (Valenciennes, 1828): Exhibits a pale brownish-grey body with five distinct dark vertical bars, four of which extend to the dorsal sides, and the fifth located on the peduncle region.

***Epinephelus coioides* (Hamilton, 1822):** Has a tan dorsal side and a whitish ventral side with numerous orange to reddish-brown spots on the head. The body features five irregular brown oblique bars that bifurcate ventrally.

Molecular identification was conducted using fragments of the cytochrome oxidase subunit I (COI) gene. Amplification of the COI gene was performed through the Sanger sequencing method in China. The confirmed sequences were submitted to NCBI under Accession Numbers ON705637, ON706970, OQ423220, OQ423221, ON706969, and ON706971 (Table 1).

The nucleotide composition analysis revealed sequence lengths ranging from 633 to 655 base pairs. The AT content (52.23%) was found to be higher than the GC content (46.23%) among the four grouper species (Fig. 3). For species identification, the NCBI Nucleotide BLAST and BOLD v4 system were utilized, with the highest similarity percentage being selected (Fig. 4). The identified grouper species exhibited 97% to 100% similarity within the same species. The four *Epinephelus* species from the southwestern coast of Pakistan revealed genetic distances ranging from 0.00 to 0.19, with an average of 0.12 (Table 2). Notably, this study is the first molecular based report of three grouper species—*E. coioides*, *E. polylepis*, and *E. latifasciatus* in Pakistan.

Phylogenetic Analysis: A neighbor-joining phylogenetic tree was constructed using COI gene sequences, which clustered the identified species with reference sequences from various geographical regions. The phylogenetic relationships of *Epinephelus* species from Gwadar showed close genetic similarities with populations from Qatar (QA), Iran (IR), Iraq (IQ), Bangladesh (BD), China (CN), India (IN), Indonesia (ID), Malaysia (MY), Thailand (TH), Saudi Arabia (SA), and Canada (CA) (Fig. 5).

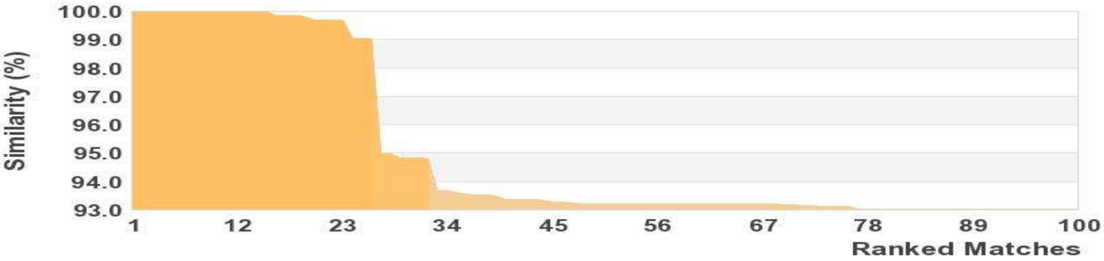
Supplementary Material

Figure 1. Map showing the major fishing station of Sample from Gwadar, Pasni, Ormara and Jiwani, Balochistan, Pakistan.

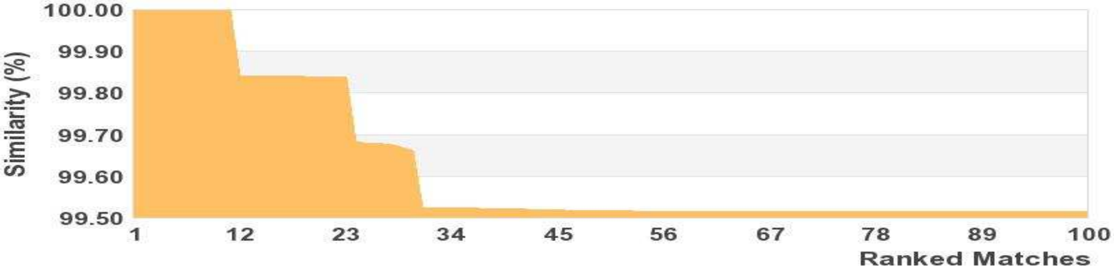
Fig.2. The Morphological characters of four Grouper species collected from Coast of Gwadar, Balochistan, Pakistan. (A). *Epinephelus polylepis.* (B). *E. coioides.* (C). *E. latifasciatus.* (D). *E. dicanthus.*

Fig.3. GC% and AT% content of Epinephelus marine fishes from the coast of Gwadar.

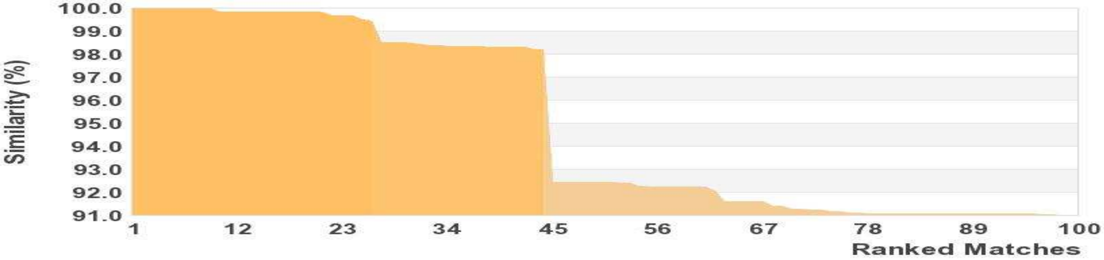
A. ***Epinephelus polylepis*** **ON706969**



B. ***Epinephelus coioides*** **ON706971**



C. ***Epinephelus latifasciatus*** **ON706970**



D. ***Epinephelus dicanthus*** **OQ423220**

Figure 4. Higher similarity score of top 100 matches gained from BLAST and BOLDv4 system of A. *Epinepheleus polylepis* B. *E. coioide*s, C. *E. latifasciatus, D. E.dicanthus.*

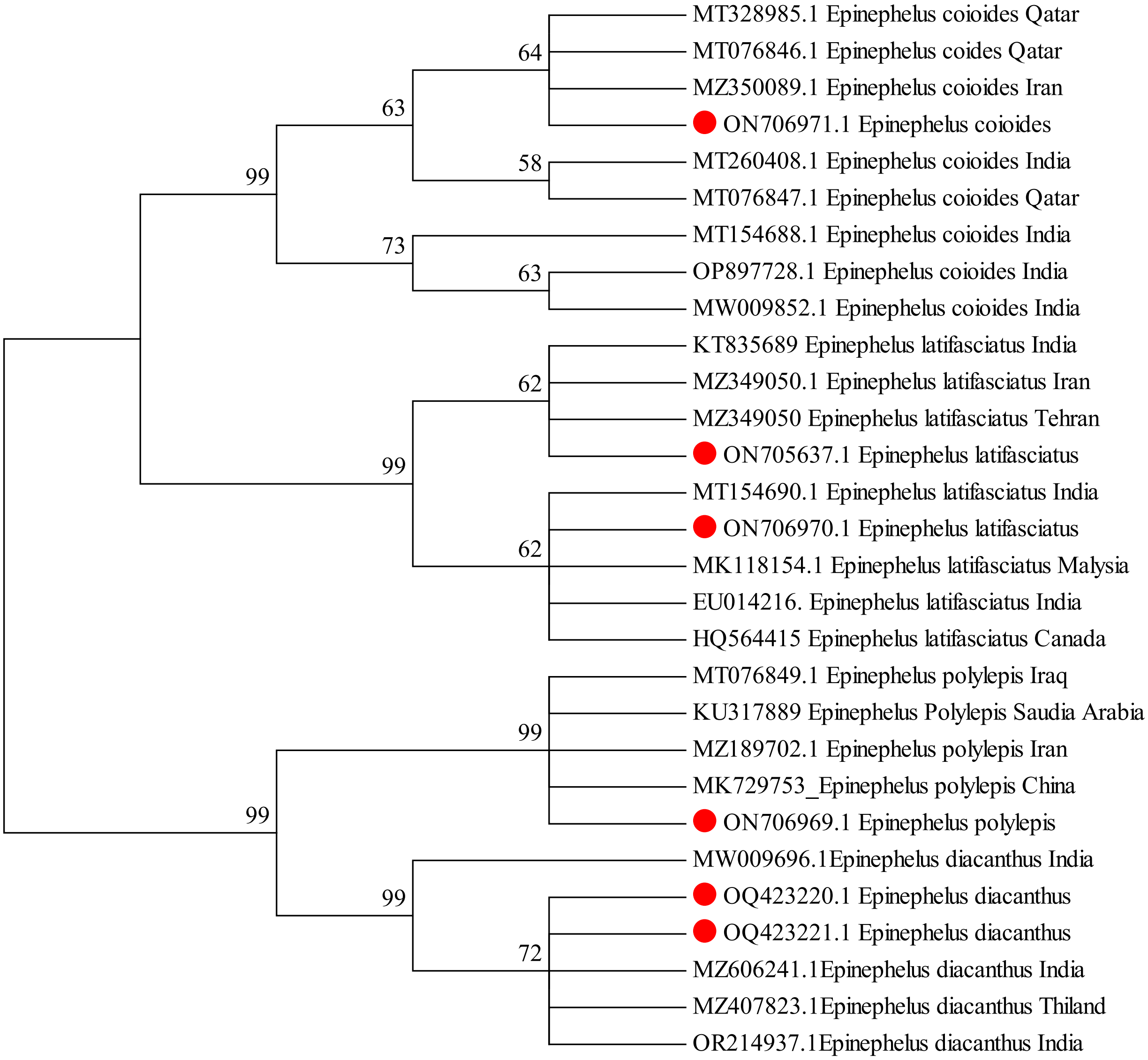


Figure 5. Neighbor Joining tree of CO1 barcode for Epinephelus species only bootstrap value (1000) replicates greater than 70 % are shown with samples collected from the coast of Gwadar, Balochistan.

Tab.1. Fish species identified with their families and Gene Bank acquired sequences.

Family	Species Name	Character	Accession No.
Serranidae	*Epinephelus latifasciatus*	They are grey shading to ventrally white, two black edged white longitudinal bands.	ON705637 ON706970
	Epinephelus dicanthus	The body is pale greyish brown, usually having five dark vertical bars. Fins dusky grey, anal fin with three spines.	OQ423320 OQ423321
	Epinephelus coioides	Prominent numerous small brownish orange spots, dark bars which bifurcates ventrally H shaped. Eleven dorsal spines and three anal spines.	ON706971
	Epinephelus polylepis	The body is pale with a small, closed set of dark brown spots. Spots are closer on dorsal parts of head and body as compared to the ventral side of the body. Having three anal spines.	ON706969

Table 2. Genetic distance of Epinephelus nucleotide sequence of CO1 gene with other related sequences retrieved from NCBI.

OQ423220																												
OQ423221	0.00																											
MZ60624	0.00	0.00																										
MZ407823	0.00	0.00	0.00																									
MW009696	0.00	0.00	0.00	0.00																								
OR214937	0.00	0.00	0.00	0.00	0.00																							
ON706971	0.16	0.16	0.16	0.16	0.16	0.16																						
OQ376707	0.16	0.16	0.16	0.16	0.16	0.16	0.01																					
OP897728	0.16	0.16	0.16	0.16	0.16	0.16	0.01	0.00																				
MT260408	0.16	0.16	0.16	0.16	0.16	0.16	0.00	0.00	0.00																			
MW009852	0.17	0.17	0.17	0.17	0.17	0.17	0.01	0.00	0.00	0.01																		
MZ350089	0.16	0.16	0.16	0.16	0.16	0.16	0.00	0.01	0.01	0.00	0.01																	
MT076847	0.16	0.16	0.16	0.16	0.16	0.16	0.00	0.00	0.00	0.00	0.01	0.00																
MT328985	0.16	0.16	0.16	0.16	0.16	0.16	0.00	0.01	0.01	0.00	0.01	0.00	0.00															
MT076846	0.15	0.15	0.15	0.15	0.15	0.15	0.00	0.01	0.01	0.00	0.01	0.00	0.00	0.00														
MT154688	0.16	0.16	0.16	0.16	0.16	0.16	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01													
ON705637	0.18	0.18	0.18	0.18	0.18	0.18	0.11	0.11	0.11	0.11	0.12	0.11	0.11	0.11	0.11	0.11												
ON706970	0.18	0.18	0.18	0.18	0.18	0.18	0.11	0.12	0.12	0.11	0.12	0.11	0.11	0.11	0.11	0.11	0.00											
MK118154	0.18	0.18	0.18	0.18	0.18	0.18	0.11	0.12	0.12	0.11	0.12	0.11	0.11	0.11	0.11	0.11	0.00	0.00										
EU014216	0.18	0.18	0.18	0.18	0.18	0.18	0.11	0.12	0.12	0.11	0.12	0.11	0.11	0.11	0.11	0.11	0.00	0.00	0.00									
HQ564415	0.18	0.18	0.18	0.18	0.18	0.18	0.11	0.12	0.12	0.11	0.12	0.11	0.11	0.11	0.11	0.11	0.00	0.00	0.00	0.00								
MZ349050	0.18	0.18	0.18	0.18	0.18	0.18	0.11	0.11	0.11	0.11	0.12	0.11	0.11	0.11	0.11	0.11	0.00	0.00	0.00	0.00	0.00							
KT835689	0.18	0.18	0.18	0.18	0.18	0.18	0.11	0.11	0.11	0.11	0.12	0.11	0.11	0.11	0.11	0.11	0.00	0.00	0.00	0.00	0.00	0.00						
ON706969	0.15	0.15	0.15	0.15	0.15	0.15	0.17	0.18	0.18	0.18	0.19	0.17	0.18	0.17	0.17	0.18	0.19	0.19	0.19	0.19	0.19	0.19	0.19					
MT154690	0.18	0.18	0.18	0.18	0.18	0.18	0.11	0.12	0.12	0.11	0.12	0.11	0.11	0.11	0.11	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19				
MT076849	0.15	0.15	0.15	0.15	0.15	0.15	0.17	0.18	0.18	0.18	0.19	0.17	0.18	0.17	0.17	0.18	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.00	0.19			
MZ189702	0.16	0.16	0.16	0.16	0.16	0.16	0.18	0.18	0.18	0.18	0.19	0.18	0.18	0.17	0.18	0.18	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.00	0.00	0.19		
KU317889	0.15	0.15	0.15	0.15	0.15	0.15	0.17	0.18	0.18	0.18	0.19	0.17	0.18	0.17	0.17	0.18	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.00	0.00	0.00	0.19	
MK729753	0.15	0.15	0.15	0.15	0.15	0.15	0.17	0.18	0.18	0.18	0.19	0.17	0.18	0.17	0.17	0.18	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.00	0.19	0.00	0.00	0.00

DISCUSSION

The coast of Gwadar is a biodiversity hotspot, with Astola Island and mangrove ecosystems providing essential habitats for various marine species. DNA barcoding is a highly reliable molecular tool for accurate taxonomic identification, with the COI mitochondrial gene being widely used due to its high authenticity. This study confirmed the identification of four species from the family Serranidae (genus *Epinephelus*): *Epinephelus polylepis* (Small-scaled grouper), *Epinephelus latifasciatus* (Striped grouper), *Epinephelus dicanthus* (Spiny cheek grouper), *Epinephelus coioides* (Orange-spotted grouper). These species are economically important due to their high commercial value and demand in local and international markets, contributing significantly to Pakistan's fisheries industry. The species composition varied across different regions of Gwadar, with local fishermen unable to distinguish among different Serranidae species based solely on morphology. Prior to this study, only *E. dicanthus* was reported from Makran Coast (Aamir *et al*., 2022). The genetic distance of groupers from Gwadar (average 0.12) was comparable to global reports: Madura Island, Indonesia: *Epinephelus fasciatus* (0.15), *E. areolatus* (0.17), *E. coioides* (0.15) (Basith *et al*., 2021). Jayapura and Papua, Indonesia: *E. epistictus* (0.12), *E. merra* (0.16) (Fadli *et al*., 2021). Indian Waters: *E. epistictus*, *E. latifasciatus*, and *E. radiatus* (0.08–0.16) (Sachithanandam *et al*., 2022). Nigeria: *Caranx fischeri*, *Pseudotolithus senegallus*, *Sphyraena ensis* (0.17–0.19).

Phylogenetic analysis confirmed close genetic similarities between Gwadar groupers and populations from Iran (MZ189702), Qatar (MT329085), and India (OQ376707). The results validate COI as a universal marker for species identification.

Nucleotide Composition: The successfully amplified sequences ranged from 633 to 655 bp, with an average GC content of 46.23% and AT content of 52.23%. These values are comparable to: Pakistan: *GC 46.26%*, *AT 53.73%* (Ghouri et al., 2020). Indonesia (Aceh Waters): *GC 46.23%* (Fadli *et al*., 2021). Madura Island (Indonesia): *GC 47.06%* (Basith *et al*., 2021).

Global Average: *GC 47.1%* across 143 Osteichthyes species (Ward *et al*., 2005). Contrasting results were observed by Saccone *et al.*. (1999), who reported a GC content of 43.2% in bony fishes.

Conclusion: The coastal ichthyofauna of Gwadar exhibits a unique diversity of grouper species. Morphological identification alone is often inconclusive; therefore, DNA barcoding using the COI gene provides an effective molecular tool to differentiate similar species and resolve taxonomic ambiguities.

This study successfully sequenced the mtCOI gene from four *Epinephelus* species, marking the first submission of these sequences from Gwadar to NCBI, thus significantly contributing to the global fish barcode database.

The findings highlight the importance of DNA barcoding for accurate species identification, marine biodiversity conservation, fisheries management and sustainability and Economic benefits through improved fisheries monitoring

The COI gene proves to be a highly reliable and universal standard for identifying *Epinephelus* species worldwide.

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Ethical statement: Permission to undertake surveys in Gwadar district was granted by the Director General Fisheries Baluchistan, Quetta (BFD/DG/Misc/2021-22/613). In the present study we have strictly avoided sampling of endangered species.

Author's Contribution: **RK:** Sample collection, experimental analysis, writing original draft

investigation, and visualization.

AK, FM, MS: Supervision and reviewing.

FM, RK, NT: Experimental analysis.

AS : Identification of fishes and editing.

AAY: Helped in sample collection.

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