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Determination of DNA barcodes of six commercial fish species in Iran

Nahavandi R.[1*]; Tamadoni Jahromi S.[2]; Pourmozaffar S.[3]

Received: June 2023 Accepted: September 2023

Abstract

Understanding the genetic structure of economically significant species in the Persian Gulf is essential prior to implementing conservation and enhancement measures for their populations. This study involved the extraction of total DNA from at least five samples of the swimming fins of the species under investigation, following standard methodologies. Specifically, a segment of the mitochondrial DNA (mtDNA) known as cytochrome c oxidase subunit I (COI) was successfully amplified and sequenced for six species prevalent in the Persian Gulf, identified locally as Rashgo, Sobeity, Sangsar, Shoride, Mish, and Halvasefid, and scientifically as *Eleutheronema tetradactylum*, *Sparidentex hasta*, *Pomadasys kaakan*, *Otolithes ruber*, *Protonibea diacanthus*, and *Pampus argenteus*. The amplified sequences ranged from approximately 600 to 650 base pairs. Phylogenetic relationships among the studied species were analyzed using a Neighbor Joining tree based on cladistic and phenetic data, which facilitated the assessment of genetic relationships and distances among the species. The findings indicated that *Otolithes ruber* and *Protonibea diacanthus* clustered within the same clade as sister groups, exhibiting low genetic divergence compared to samples from Taiwan and Canada. Additionally, *Sparidentex hasta* and *Pampus argenteus* formed sister groups with *Protonibea diacanthus* in the first clade, drawing samples from China, America, Canada, and India. Notably, *Protonibea diacanthus* specimens from the western Persian Gulf displayed a 200% genetic divergence when compared to typical specimens from Hormozgan province, indicating distinct clades. Conversely, *Pomadasys kaakan* was situated in a separate branch with greater genetic divergence relative to the previously mentioned species and included samples from Saudi Arabia. Furthermore, *Eleutheronema tetradactylum* was identified as a distinct haplotype alongside samples from Malaysia, Indonesia, India, and Bangladesh within the first clade and exhibited sister group relationships with *Pomadasys kaakan*. A significant observation was the presence of *Protonibea diacanthus* Mangout in the Khuzestan region compared to common *Protonibea diacanthus* found in Hormozgan province. This suggests a potential dominance of the Mangout morphotype in the western Persian Gulf relative to its counterpart in the eastern region. Should this hypothesis be validated through further research, it may indicate that this morphotype represents a new endemic species or variant specific to the Persian Gulf region.

Keywords: Barcoding, *Pampus argenteus*, *Eleutheronema tetradactylum*, *Pomadasys Kaakan*, *Protonibea diacantus*, *Otolithes ruber* and *Sparidentex hasta*, Persian Gulf

1-Animal Science Research Institute of Iran (ASRI), Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
2-Persian Gulf and Oman Sea Ecology Research Center, Iranian Fisheries Sciences Research Institute, Agricultural Research Education and Extension Organization (AREEO), Bandar Abbas, Iran
3-Persian Gulf Mollusks Research Station, Persian Gulf and Oman Sea Ecology Research Center, Iranian Fisheries Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Bandar Lengeh, Iran
*Corresponding author' Email: Rezanahavandi91@gmail.com

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[DOI: 10.22034/INJBIR.1.2.47]

Introduction

Fish represent the largest clade of vertebrates, exhibiting a remarkable diversity in their characteristics and biological adaptations. Distinctive morphological traits serve as primary criteria for differentiating fish species from other vertebrate groups. However, the presence of numerous intra-species variations and inter-species morphological overlaps poses significant challenges for taxonomists striving for accurate identification. This complexity underscores the broader phenomenon of phenotypic diversity among vertebrates, which manifests at various developmental stages. Consequently, relying solely on morphological traits is insufficient for the comprehensive identification of all fish species. Numerous fish species, including North American ornamental fish, Canadian freshwater fish, and Australian marine fish, can be identified using the mitochondrial DNA gene of cytochrome oxidase subunit one as a barcode (Sureandiran *et al*., 2023). Hebert and associates presented the application of this gene as a diagnostic tool for animals (Mahboobeh *et al*., 2023). Identifying fish and their products—from eggs to mature fish—accurately and unambiguously will enhance ecosystem protection and support sustainable fishery stock management. Consequently, fish species can be successfully identified using the DNA barcoding technique. A standardized reference database of all fish sequences has been produced by the International Fish Barcoding Campaign for Species Identification (IBOL), a project of the International DNA Barcoding Consortium. Barcoding is a quick and accurate way to identify and classify taxa at the species level. It has offered a fresh viewpoint on the taxonomy, ecology, and biodiversity of fishes across different regions. The ability to accurately identify a variety of fish species, including whole fish, pelagic and benthic fish (Habib *et al*., 2023), filefish (Haung *et al*., 2023), fins and larvae (Ram *et al*., 2020), eggs, and even cooked fish (Kang *et al*., 2023), as well as any tissue from which DNA can be extracted, is one of barcoding's most significant benefits. Therefore, it appears necessary to establish a genetic reference library using DNA coding or genetic identification techniques for aquatic animals (Habib *et al*., 2022) in order to identify and list the nation's valuable and endangered aquatic species (Fadli *et al*., 2023). Therefore, scientific and precise methods have not been conducted for the country's valuable and ornamental fish because there is insufficient genetic information about them. In order to study six species, found in the Persian Gulf, this project successfully designed and implemented the use of a particular region of the mtDNA genome known as COI. Locally, these species are referred to as silver pomfret, Tigertooth croaker, Blackspotted croaker, Javeling runter, Sobaity seabream, and fourfinger

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threadfin. Their scientific names are *E. hasta, P. tetradactylum, S. Kaakan, O. Ruber, P. Diacantus, P. Argenteus*.

Materials and methods

The primary and most important unique habitats of the six species examined in this project in Iran are the target and target areas, where the sampling was carried out. In order to perform molecular work on the common gene of cytochrome oxidase subunit one, at least five pieces of the swimming fin of the species under study were extracted from each sample using the standard procedure (Kang *et al*., 2023). This was carried out in order to precisely identify the species and give each one a unique genetic ID. They were then taken to the Persian Gulf and Oman Sea Ecology Research Institute for molecular testing after being submerged in pure ethyl alcohol. In accordance with the manufacturer's instructions, DNA was extracted using a DNA extraction kit. Using a 1 percent agarose gel and horizontal electrophoresis, the extracted DNA's quality was assessed.

Additionally, a biophotometer (Eppendorf, Germany) was used to measure the samples' light absorbance at wavelengths of 260 and 280 nm in order to calculate the amount of DNA present (Panprommin *et al*., 2023). DNA samples were stored until the polymerase chain reaction at -20°C.

A 25 microliter reaction containing roughly 100 nanograms of extracted DNA was used for the polymerase chain reaction (PCR). According to Ward et al., there were 0.6 microliters of forward and reverse primers in the reaction (5'-TCAACCAACCACAAAGACATTGG CAC-3' and 5'-TAGACTTCTGGGTGGCCAAACAA TCA-3', respectively, five units of Tag DNA polymerase (mixed in Amplicon master mix made in South Korea), 10 mM dNTP, 25 mM MgCl2, and 5X PCR buffer. The first step involved providing the thermal range in order to determine the optimal annealing temperature for each primer to the template strand in order to maximize the PCR operation. The PCR product was then optimized by varying the concentrations of MgCl2, genomic DNA, and dNTP in order to produce the best and most distinct bands while removing undesired bands. The thermal cycle consists of 40 cycles after an initial step at 94 degrees for 4 minutes and 30 seconds. Each cycle includes 30 seconds of denaturation at 94 degrees, 45 seconds of annealing at 54 degrees, and 1 minute of extension at 72 degrees. Lastly, the last elongation step was carried out for five minutes at 72 degrees. A 100 bp molecular marker (Fermentas GmbH, Germany) and five microliters of the PCR product from each sample were loaded onto a 2 percent agarose gel for electrophoresis. 50 microliters of the primer (concentration: 10 pmol) and the remaining PCR product were sent to Amitis Gene Company's molecular division for purification and use as

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sequencing template DNA. The BigDye kit (BigDye kit v3.1, Applied Biosystems, Foster City, CA, USA) and the DNA analyzer model XL3730 (Applied Biosystems, USA) were used to carry out DNA sequencing reactions with the forward primer. The degree of homology was determined after the obtained sequences were sequenced using the NCBI database's Blastern procedure, BioEdit software, and the potent Blast tool. Following receipt of the sequences, we used Chromas 2.23 software to review related sequences. Clustal W software was used to align the sequence samples in order to determine the differences between the sequences (Haung *et al*., 2023).The Neighbor-Joining approach, which was based on the Kimura 2-parameter model, was used to build the evolutionary tree. MEGA 4 software was used for this (Chen *et al*., 2022). A median-joining haplotype network and DnaSp software were used to calculate haplotype diversity (Kang *et al*., 2023).

Results

The ideal temperature for primer annealing is 48 degrees Celsius, according to the results of optimizing the PCR reaction for amplifying the COI gene using a temperature gradient of 48–60 degrees Celsius. The COI gene segment, which is roughly 645 bp long, was amplified thanks to the primers. The figure below displays the PCR product's banding pattern on a 2 percent agarose gel (Figs. 1 and 2).

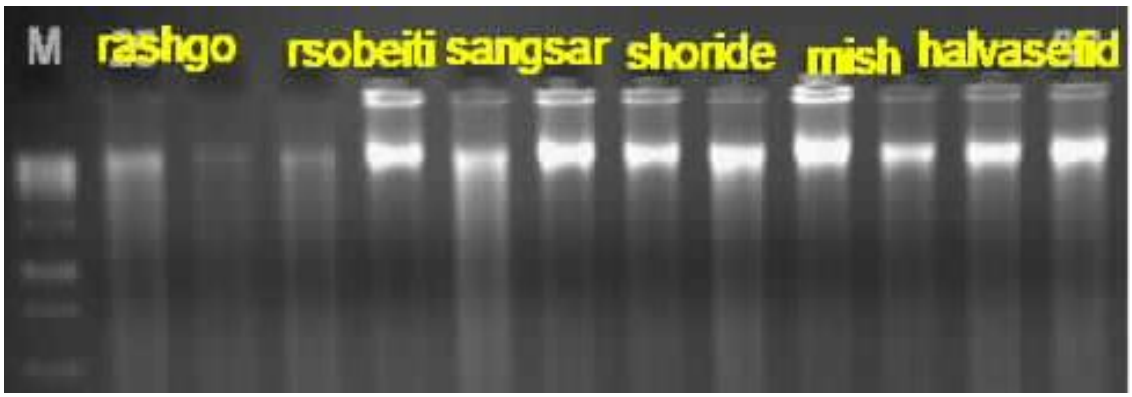

Figure 1: Six target species' pectoral fins were used for DNA electrophoresis using the phenol-chloroform method.

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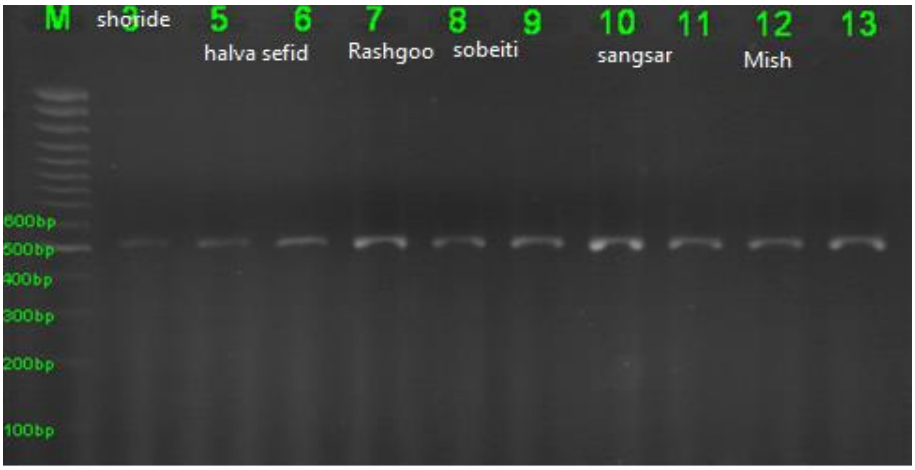

Figure 2: PCR product of six species.

A 2 percent agarose gel, ethidium bromide staining, and UV exposure were used to assess the PCR product. The PCR machine's thermal cycle consists of 40 cycles after an initial step at 94 degrees for 4–30 minutes. Each cycle includes 30 seconds of 94-degree denaturation, 45 seconds of annealing at a particular primer temperature (56 degrees for most species), 1 minute of extension at 72 degrees, and 5 minutes of final annealing at 72 degrees (Table 1).

Table 1: PCR program.

Cycle	Time (min or sec)	Temperature (^{0}C)	PCR cycle
1	3 min	95	Initial Denaturation
30	30 sec 45 sec 60 sec	95 56 72	Denaturation Annealing Extension
1	5 min	72	Final Extension

The findings of the COI genome-based genetic analysis of the fish under study revealed that *O. ruber and A. hololepidotus* (Azahar *et al*., 2022). Two sister groups were formed when *A. hololepidotus* were grouped together in a clade. Comparing them to samples from Taiwan and Canada revealed few genetic differences, suggesting that the migration of this species from higher latitudes had little effect on the noticeable genetic variations. Species that are less evolved than other species in the area were grouped together in one branch for this study. The species *S. hasta* and *P. argenteus*, a sister group of *A. hololepidotus* which are found in places like China, America, Canada, and India. In the first Clyde, *A. hololepidotus* were seen in two different groups. This result was fairly close to what was anticipated. Even though *P. diacanthus*, which grows in the Persian Gulf's western regions, and an A sample of *A. hololepidotus* from

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Hormozgan province, which are both members of the same clade and differ by 0.02% (Figs. 3 and 4; Table 2). However, the common stonefish with a greater difference from the other four species mentioned was placed together with a sample from Saudi Arabia. In addition, the species *E. tetradactylum* is presented in the first group as a separate haplotype along with samples from Malaysia, Indonesia, India and Bangladesh. In addition, *P. kaakan* samples are found in two sister groups. A notable point in this context is the identification of a new haplotype with genetic differences from other registered samples identified in the World Gene Bank. Meanwhile, the notable observation was the discovery of *P. diacanthus* in the Khuzestan region during sampling, in contrast to a sample of *A. hololepidotus* in Hormozgan Province. This finding suggests that *P. diacanthus* is more common in the western part of the Persian Gulf, while *A. hololepidotus* is dominant in the eastern part of the Persian Gulf.

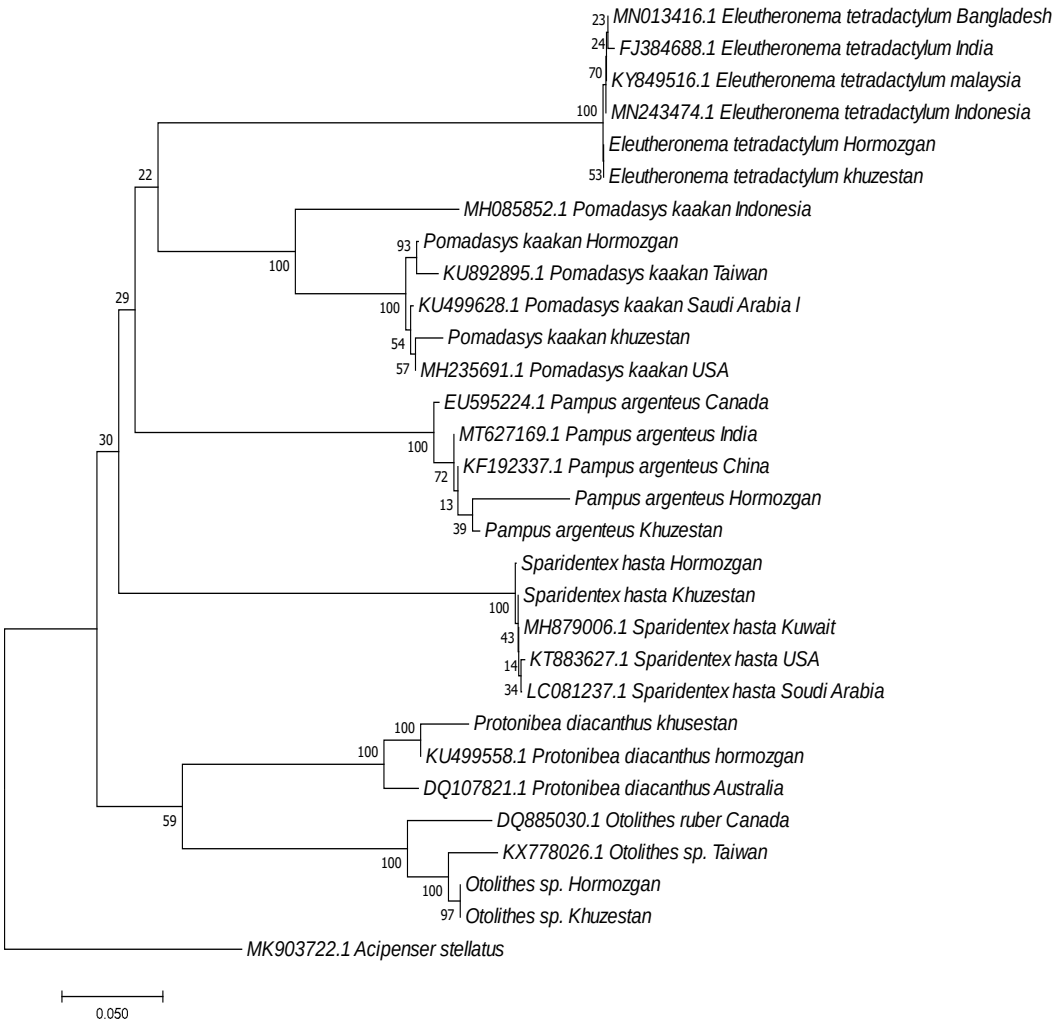

Figure 3: Using the neighbor-joining method, a phylogenetic tree of the COI gene from six species was created.

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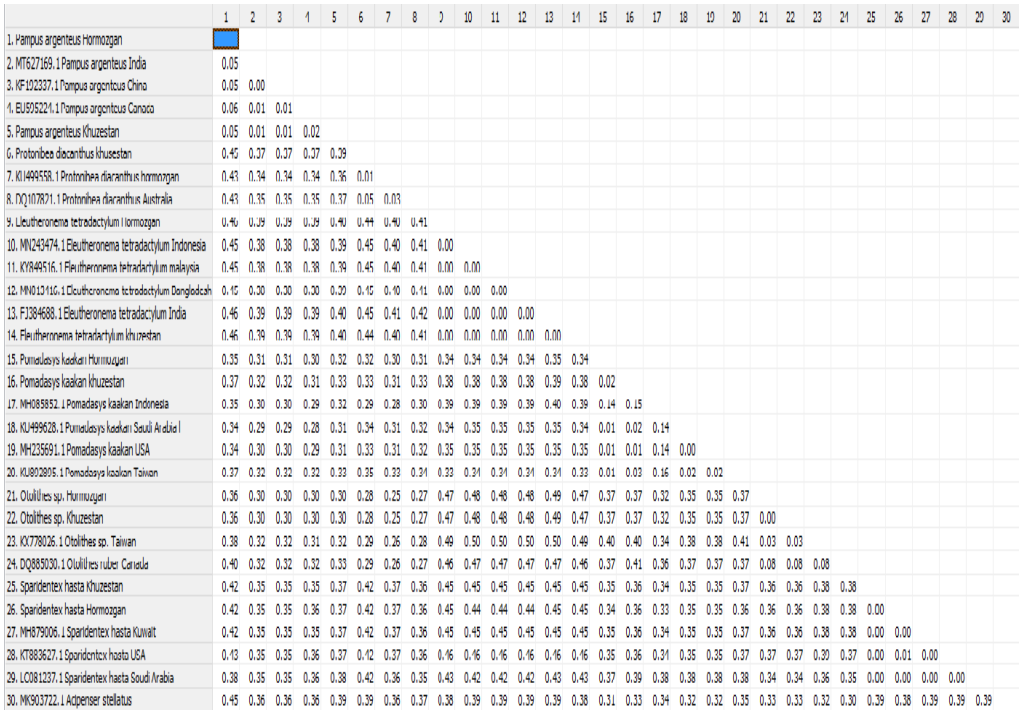

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1. Pampus argenteus Hormozgan																														
2. MT627169.1 Pampus argenteus India	0.05																													
3. KF102337.1 Pampus argenteus China	0.05	0.00																												
4. EU595224.1 Pampus argenteus Canada	0.06	0.01	0.01																											
5. Pampus argenteus Khuzestan	0.05	0.01	0.01	0.02																										
6. Protonibea diacanthus khuestan	0.45	0.37	0.37	0.37	0.39																									
7. KU499558.1 Protonibea diacanthus hormozgan	0.43	0.34	0.34	0.34	0.36	0.01																								
8. DQ107821.1 Protonibea diacanthus Australia	0.43	0.35	0.35	0.35	0.37	0.05	0.03																							
9. Eleutheronema tetradactylum Hormozgan	0.40	0.39	0.39	0.39	0.40	0.44	0.40	0.41																						
10. MN243474.1 Eleutheronema tetradactylum Indonesia	0.45	0.38	0.38	0.38	0.39	0.45	0.40	0.41	0.00																					
11. KY849516.1 Eleutheronema tetradactylum malaysia	0.45	0.38	0.38	0.38	0.39	0.45	0.40	0.41	0.00	0.00																				
12. MN013116.1 Eleutheronema tetradactylum Bangladesh	0.45	0.38	0.38	0.38	0.39	0.45	0.40	0.41	0.00	0.00	0.00																			
13. FJ384688.1 Eleutheronema tetradactylum India	0.46	0.39	0.39	0.39	0.40	0.45	0.41	0.42	0.00	0.00	0.00	0.00																		
14. Eleutheronema tetradactylum khuzestan	0.46	0.39	0.39	0.39	0.40	0.44	0.40	0.41	0.00	0.00	0.00	0.00	0.00																	
15. Pomadasys kaakan Hormozgan	0.35	0.31	0.31	0.30	0.32	0.32	0.30	0.31	0.34	0.34	0.34	0.34	0.35	0.34																
16. Pomadasys kaakan khuzestan	0.37	0.32	0.32	0.31	0.33	0.33	0.31	0.33	0.38	0.38	0.38	0.38	0.39	0.38	0.02															
17. MH085852.1 Pomadasys kaakan Indonesia	0.35	0.30	0.30	0.29	0.32	0.29	0.28	0.30	0.39	0.39	0.39	0.39	0.40	0.39	0.14	0.15														
18. KU499628.1 Pomadasys kaakan Saudi Arabia I	0.34	0.29	0.29	0.28	0.31	0.34	0.31	0.32	0.34	0.35	0.35	0.35	0.35	0.34	0.01	0.02	0.14													
19. MH235691.1 Pomadasys kaakan USA	0.34	0.30	0.30	0.29	0.31	0.33	0.31	0.32	0.35	0.35	0.35	0.35	0.35	0.35	0.01	0.01	0.14	0.00												
20. KU902805.1 Pomadasys kaakan Taiwan	0.37	0.32	0.32	0.32	0.33	0.35	0.33	0.34	0.33	0.34	0.34	0.34	0.34	0.33	0.01	0.03	0.16	0.02	0.02											
21. Otolithes sp. Hormozgan	0.36	0.30	0.30	0.30	0.30	0.28	0.25	0.27	0.47	0.48	0.48	0.48	0.49	0.47	0.37	0.37	0.32	0.35	0.35	0.37										
22. Otolithes sp. Khuzestan	0.36	0.30	0.30	0.30	0.30	0.28	0.25	0.27	0.47	0.48	0.48	0.48	0.49	0.47	0.37	0.37	0.32	0.35	0.35	0.37	0.00									
23. KX778026.1 Otolithes sp. Taiwan	0.38	0.32	0.32	0.31	0.32	0.29	0.26	0.28	0.49	0.50	0.50	0.50	0.50	0.49	0.40	0.40	0.34	0.38	0.38	0.41	0.03	0.03								
24. DQ885030.1 Otolithes ruber Canada	0.40	0.32	0.32	0.32	0.33	0.29	0.26	0.27	0.46	0.47	0.47	0.47	0.47	0.46	0.37	0.41	0.36	0.37	0.37	0.37	0.08	0.08	0.08							
25. Sparidentex hasta Khuzestan	0.42	0.35	0.35	0.35	0.37	0.42	0.37	0.36	0.45	0.45	0.45	0.45	0.45	0.45	0.35	0.36	0.34	0.35	0.35	0.37	0.36	0.36	0.38	0.38						
26. Sparidentex hasta Hormozgan	0.42	0.35	0.35	0.36	0.37	0.42	0.37	0.36	0.45	0.44	0.44	0.44	0.45	0.45	0.34	0.36	0.33	0.35	0.35	0.36	0.36	0.36	0.38	0.38	0.00					
27. MH879006.1 Sparidentex hasta Kuwait	0.42	0.35	0.35	0.35	0.37	0.42	0.37	0.36	0.45	0.45	0.45	0.45	0.45	0.45	0.35	0.36	0.34	0.35	0.35	0.37	0.36	0.36	0.38	0.38	0.00	0.00				
28. KT883627.1 Sparidentex hasta USA	0.43	0.35	0.35	0.36	0.37	0.42	0.37	0.36	0.46	0.46	0.46	0.46	0.46	0.46	0.35	0.36	0.34	0.35	0.35	0.37	0.37	0.37	0.39	0.37	0.00	0.01	0.00			
29. LC081237.1 Sparidentex hasta Saudi Arabia	0.38	0.35	0.35	0.36	0.38	0.42	0.36	0.35	0.43	0.42	0.42	0.42	0.43	0.43	0.37	0.39	0.38	0.38	0.38	0.38	0.34	0.34	0.36	0.35	0.00	0.00	0.00	0.00		
30. MK903722.1 Acpenser stellatus	0.45	0.36	0.36	0.36	0.39	0.39	0.36	0.37	0.38	0.39	0.39	0.39	0.39	0.38	0.31	0.33	0.34	0.32	0.32	0.35	0.33	0.33	0.32	0.30	0.39	0.38	0.39	0.39	0.39	

Figure 4: The six species and other species were compared in terms of the COI gene's genetic distance percentage.

Table 2: Six sequences from various species were registered with the NCBI.

Date of Registration	Verification of gene	Type of gene	Raw
2021	NCBI	*Eleutheronema tetradactylum* cytochrome oxidase subunit 1 (COX1) gene, partial cds; mitochondrial - MZ196221.1	1
2021	NCBI	*Otolithes ruber* cytochrome oxidase subunit 1 (COX1) gene, partial cds; mitochondrial - MZ191371.1	2
2021	NCBI	*Pampus argenteus* cytochrome oxidase subunit 1 (COX1) gene, partial cds; mitochondrial - MZ203316.1	3
2021	NCBI	*Pomadasys kaakan* cytochrome oxidase subunit 1 (COX1) gene, partial cds; mitochondrial - MZ191167.1	4
2021	NCBI	*Protonibea diacanthus* cytochrome oxidase subunit 1 (COX1) gene, partial cds; mitochondrial - MZ196508.1	5
2021	NCBI	*Sparidentex hasta* cytochrome oxidase subunit 1 (COX1) gene, partial cds; mitochondrial - MZ191899.1	

The isolation of haplotypes from the samples of six fish species in the region is an intriguing finding compared to the recorded samples. Mitochondrial DNA analysis showed high resolution of genetic divergence between species, allowing identification of species and determination of genetic affinity in the Persian Gulf region (Fig. 5).

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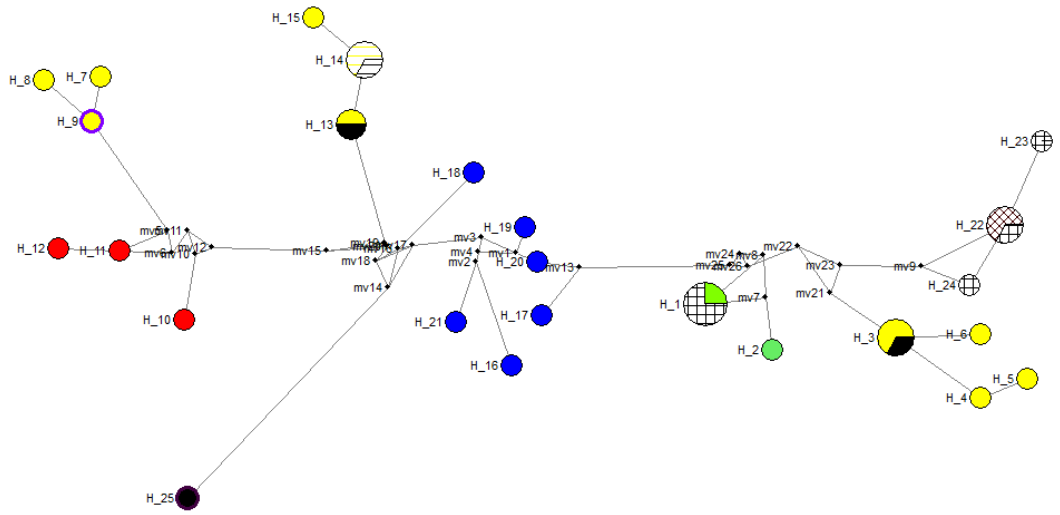

Figure 5: Haplotype diversity network using the COI gene of the six studied species.

Discussion

A portion of the mtDNA genome known as COI was successfully amplified and sequenced in this study, yielding an amplification of roughly 650 base pairs. 560 high-quality base pairs were chosen for phylogenetic analysis after the bases were filtered out. The relationships between the members of the studied species are ascertained using two distinct phylogenetic relationship analysis techniques: Neighbor-Joining and Maximum Parsimony trees. Cladistic and phenetic data serve as the foundation for these techniques. It was employed for genetic research and to calculate the genetic separation between the six species that were being studied. DNA barcoding is a molecular technique that uses short, standardized gene sequences to identify eukaryotes at the species level (Haung *et al*., 2023). Meanwhile, the results of genetic analysis using the COI genome showed that the fish species *O. ruber* and *P. diacantus* were clustered in the same group and formed two sister groups. Compared to samples from Taiwan and Canada, they showed minimal genetic differences. Therefore, it is evident that migration of this species from higher latitudes did not contribute significantly to the observed genetic differences. Although this species is critically endangered in the Persian Gulf, it is crucial to take the necessary measures to prevent the extinction of this species in the region. One of the important goals of genetic barcoding in the Persian Gulf region is to protect native and endemic species (Zhang *et al*., 2023). In this study, species that are evolutionarily lower than other species in the region were placed into a branch. Surprisingly, the species *S. hasta* and *P. argenteus* from the regions of China, America and Canada, together with a sister group of *P. diacantus* in the first group, appeared in two sister groups, which was unexpected. In addition, the spotted *P. diacantus* from the western parts of the Persian Gulf and an example of the common *P. diacantus* from Hormozgan

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Province, although they have a difference of 200%, are both placed in the next group. It is worth noting that moderate haplotype diversity was observed between the studied species. There are two possible haplotype diversity levels: zero, in which every member of the population has the same haplotype, and one, in which every member of the population has a different haplotype. Marine species typically exhibit moderate to high haplotype diversity (Habib *et al*., 2022; Habib *et al*., 2023). The large population size and widespread dispersal of marine species over great distances are frequently cited as reasons for this high diversity. Numerous distinct haplotypes are maintained throughout population growth and distribution as a result of these factors (Ram *et al*., 2020). Of course, a variety of factors can cause the population size in various geographic areas to fluctuate on a regular basis. These include a decline in population as a result of mortality and a rise in population as a result of individual growth and the arrival of juveniles. Consequently, genetic diversity levels also shift (Panprommin *et al*., 2023). Different patterns of genetic diversity may also arise from evolutionary dynamics (natural selection, genetic drift, and mutation) that impact the organism at different levels (Fadli *et al*., 2023). On the other hand, the common *P. kaakan* along with a sample from Saudi Arabia was placed in the next branch with a greater difference from the other four species mentioned. Of course, it should be noted in this context that, despite its numerous advantages, the DNA barcode also has some limitations. For example, this method requires a reliable DNA library in order to be able to compare newly obtained sequences with existing ones (Chen *et al*., 2022). Different levels of genetic diversity of a given species in different geographical areas can be attributed to the intensity of natural selection and the destruction and subsequent resurgence of the population as a result of environmental changes caused by both natural and human factors (Haung *et al*., 2023). Furthermore, *E. tetradactylum* is a distinct haplotype, along with samples from Malaysia, Indonesia, India and Bangladesh in the first group (Xiao *et al*., 2022). In addition, *P. kaakan* fish samples are found in two sister groups. A notable point in this context is the identification of a new haplotype with genetic differences from other registered samples identified in the World Gene Bank (Chen *et al*., 2022). At the same time, it is worth noting that there was an occurrence of spotted *P. diacantus* in the Khuzestan region, as opposed to a sample of *P. diacantus* found in Hormozgan Province (Mahboobeh *et al*., 2023). It demonstrates the dominance of stippling species in the western Persian Gulf compared to the common *P. diacantus* species in the eastern Persian Gulf. This species may be regarded as a novel and endemic species or morphotype of the Persian Gulf region if

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this hypothesis is validated. As a result, creating a gene bank of this kind is unavoidable. Assume, nevertheless, that the acquired data
accurately depicts the species' genetic c
omposition. In that instance, it can be said that the phylogenetic relationship and genetic composition of the species under investigation were not very different from what was anticipated.

Comparing the isolated haplotypes from the samples of six fish species in the area to the recorded samples, however, is highly intriguing. In the Persian Gulf region, species identification and genetic affinity determination were made possible by mitochondrial DNA analysis, which demonstrated a high resolution of genetic divergence between species.

Acknowledgment

The Iran National Science Foundation (INSF) provided financial support for the successful completion of this project, number 95849755.

Conflict of Interest

To the best of our knowledge, the named authors have no conflict of interest, financial or otherwise.

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