DNA Barcoding of venomous stonefish (*Pseudosynanceia melanostigma*) from the Persian Gulf

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Abstract
Stonefish (*Pseudosynanceia melanostigma*), a species of the family Synanceiidae, which is considered the most venomous fish and a source of potential pharmaceuticals, is found in the muddy bottoms of the shallow waters of the Persian Gulf. This study presents the first barcode sequences of *P. melanostigma* in global gene databases from the Persian Gulf. The specimens were identified based on morphological characteristics as well as DNA barcoding based on the COI gene. The present record of this species as a reference in gene databases adds to our knowledge of the biodiversity of fish globally and could be used as an effective tool in the future for the accurate identification of fish at any stage of the life cycle or even for the identification of this species in other regions.

Keywords: *Pseudosynanceia melanostigma*, Morphology, COI marker, Mangrove forest.

INTRODUCTION

Stonefish are known as one of the most venomous fish in the world. They are typically small to medium-sized fish, moderately compressed to robust fish, usually basslike in appearance, mouth moderate to large, terminal and oblique, and equipped with a set of dorsal spines located on its back, and these spines are also directly linked to venom glands (Saggiomo et al. 2021). Stonefish are predominantly nocturnal and use their venomous spines to capture small fish, crustaceans, and other invertebrates. They are capable of camouflaging themselves among rocks and coral because of their varied coloration, from brown, gray, or yellow to olive or mottled shades (Osmany & Moazzam, 2018). Currently, this family comprises 141 species assigned to 52 genera (Fricke et al. 2023). Eagderi et al. (2019), based on prior studies in the Persian Gulf, reported six species of this family: *Choridactylus multibarbus*, *Minous dempsterae*, *Minous monodactylus*, *Synanceia nana*, *Synanceia verrucosa*, and *Pseudosynanceia melanostigma*.

Pseudosynanceia melanostigma Day, 1875, known as blackfin stonefish, sang-mahi-e-khal-syah, and Firyalah, is a benthic species found on muddy beds in marine shallow waters and estuarine environments. It is native to the western Indian Ocean and coastal regions of the Persian Gulf (Eagderi et al. 2019; Osmany & Moazzam 2018). It is a species of the family Synanceidae and is considered the most venomous fish. As its main defense weapon, the stonefish has dorsal spines that produce a very potent venom (Babaie et al. 2019). The venom glands in its fin spines represent a valuable source of pharmacological compounds that may be a potential candidate for cancer treatment and the development of anticoagulant drugs (Mirshamsi et al. 2017; Babaie et al. 2019). Most studies on *P. melanostigma* have focused primarily on the cardiovascular and neuromuscular pharmacological effects of this venom (Vazirizadeh et al. 2014; Ramezani et al. 2021).

Although this species has intrinsic value, no documented molecular studies have been conducted to confirm the morphological identity of this species and register its gene in the databases. One of the goals of gene registration in gene databases is to contribute to the discovery and monitoring of fish biodiversity and to the development of reference libraries to accurately identify species at any developmental stage (Endo et al. 2022).

In recent years, the DNA barcoding molecular technique has become the golden standard for species identification and adding them to the inventory of
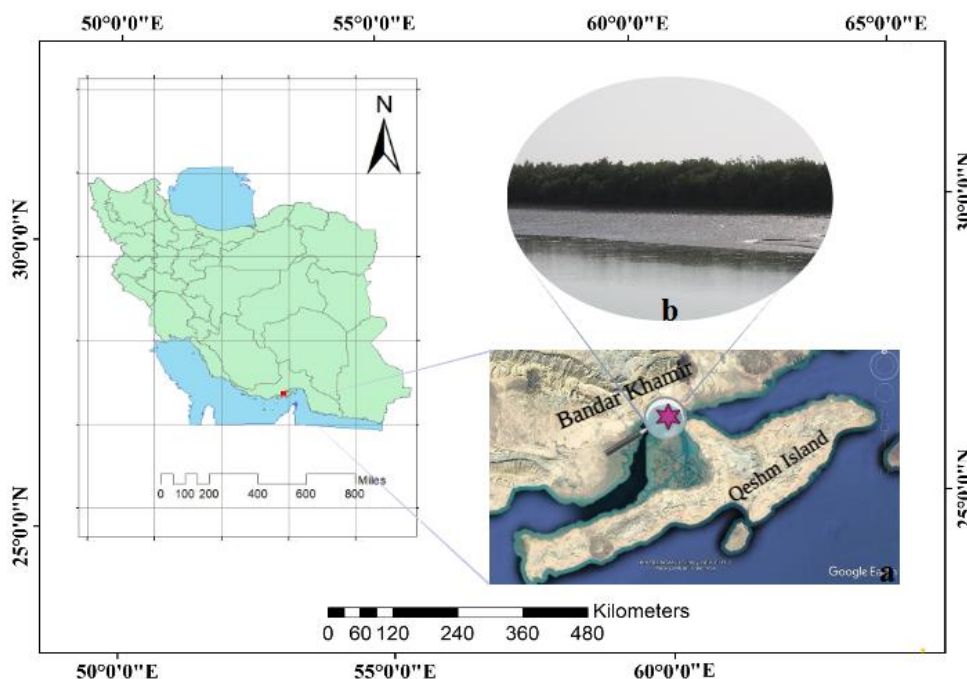
Fig.1. a) Sampling site of *P. melanostigma*, b) Natural habitat of this species: mangrove forest of Khuran Strait from Bandar Khamir.

global biodiversity in gene reference databases because of its rapid and accurate output based on the cytochrome c oxidase subunit I (COI) gene (Shirak et al. 2016; Fadli et al. 2020). Hence, the aim of this study was to provide morphological and molecular characteristics of *P. melanostigma* from the Persian Gulf and record the CO1 gene of this species in databases as a reference sequence.

MATERIALS AND METHODS

Study area and specimen collection: From the mangrove forest in the Khuran Strait near Bandar Khamir, one specimen was caught using the stake net method in the northern Persian Gulf (Fig. 1). Bandar Khamir was designated as an "International Lagoon" by the Ramsar Convention in 1974 (Harrington 1976). Once the live photographs of the specimen had been taken, it was then preserved in 96% ethanol for subsequent morphological and molecular analyses.

Morphological study: Morphological identification was conducted according to FAO species identification sheets for Western Indian Ocean (fishing area 51) and the valid references (Carpenter 1997; Fischer & Bianchi 1984).

DNA extraction, amplification of mitochondrial COI, and sequencing: DNA was extracted from the fin of the specimen preserved in ethanol using TOP General Genomic DNA Purification Kit (Cat. NO. TGK1003-T0PAZ Gene Research Company) following the manufacturer's protocol. Amplification of the barcode region of the mitochondrial cytochrome C oxidase subunit I (COI) gene sequence was achieved using the primer pair dgLCO1490 (5'GGTCAACAAATCATAAAGAYATYGG-3') and dgHCO2198 (5'TAAACTTCAGGGTGACCAAARAAYCA-3') (Meyer, 2003).

The T100 Thermal Cycler from BIO RAD was utilized for the amplification process. Polymerase chain reaction (PCR) was conducted in a 50 µL reaction mixture in 0.2-mL small reaction tubes comprised of 25 µL of Taq 2x Master Mix Red (Tris-HCl pH 8.5, $(NH_4)2SO_4$, 3 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM dNTPs, 0.2 units/µL Tag DNA polymerase [REDiant 2X PCR Master Mix, Denmark]), 20 µL of ultrapure water, 0.5µL of each forward and reverse primer, and 4µL of template DNA. The PCR condition profile consisted of a preheating at 94°C for 3 min in the five cycles and then followed by 35 cycles of denaturation at 94°C for 40s, annealing at 45°C for 1.5 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min

Table 1. Morphological and meristic characteristics of *P. melanostigma* collected from the mangrove forest, Khuran Strait, Bandar Khamir, Persian Gulf.

Morphometric characters
1. Body brownish; mottled with dark pigment on a lighter background
2. pectoral, pelvic, and caudal fins tipped with white
3. a paler area on the body, usually well marked, between the segmented part of the dorsal and anal fins
4. eyes that are just slightly elevated and separated apart
5. each segmented ray is often branched and coated in thick skin
6. its pectoral fins reach at most to the middle of the anal fin
7. the caudal fin is rounded
Meristic characters
1. dorsal fin with fifteen almost equal-length spines
2. pectoral fins with 18 rays

Fig.2. One specimen of *P. melanostigma*, collected from the mangrove forest in the Khuran

(Hebert et al. 2003). PCR samples with a single and clear visible band were chosen. Automated sequencing (ABI DNA analyzer) at Microsynth Inc. (Switzerland) was carried out following the Sanger standard method, utilizing the same PCR primers.
Data analysis: To read and edit the DNA chromatograms, the Seq Man program v7.2.1 (DNA Star software) was applied. The COI Sequences were checked using BLAST search engine supplied by The Barcode of Life Data Systems (BOLD) and National Center for Biotechnology Information (NCBI) as the main public repositories of DNA barcode sequences. Clustal W procedure in MEGA11 software (Tamura et al., 2021) was employed for sequence alignment.

The Kimura-2-parameter (K2P) model (Kimura 1980) was used to calculate the Pairwise distance of specimen collected from the Persian Gulf with other species recorded from stonefishes in NCBI. The phylogenetic tree was constructed using the neighbor-joining (NJ) methods and K2P evolution model. The reliability of the branching tree was tested by bootstrap analysis using 1000 replicates in MEGA11 software (Tamura et al. 2021).

RESULTS
Specimen examined: 78 mm SL, Iran: Persian Gulf, Khuran Strait, Bandar Khamir, mangrove forest (26°58'41.0"N 55°38'50.5"E).
Morphological characteristics of *P. melanostigma*: Morphological and meristic characteristics of *P. melanostigma* used for identification (based on identification keys) is described in Table 1. The specimen of *P. melanostigma* collected from the mangrove forest in the Khuran Strait in Bandar Khamir, Persian Gulf, Iran is presented in Figure 2.
BLAST analysis: After sequencing and removing the

Table 2. K$_2$P pairwise genetic distance matrix of Synanceidae

|	Scientific name	1	2	3	4	5	6	7	8	9	10
1	*Pseudosynanceia melanostigma*										
2	*Trachicephalus uranoscopus*	0.230									
3	*Synanceia horrida*	0.166	0.203								
4	*Choridactylus multibarbus*	0.189	0.217	0.171							
5	*Minous monodactylus*	0.232	0.220	0.199	0.220						
6	*Synanceia verrucosa*	0.227	0.229	0.213	0.242	0.239					
7	*Inimicus didactylus*	0.163	0.192	0.154	0.163	0.195	0.217				
8	*Inimicus caledonicus*	0.168	0.187	0.163	0.162	0.189	0.213	0.035			
9	*Inimicus japonicus*	0.169	0.207	0.165	0.159	0.227	0.219	0.103	0.104		
10	*Inimicus filamentosus*	0.187	0.192	0.177	0.188	0.212	0.199	0.121	0.115	0.125	
11	*Inimicus sinensis*	0.168	0.187	0.155	0.162	0.194	0.213	0.041	0.019	0.100	0.127
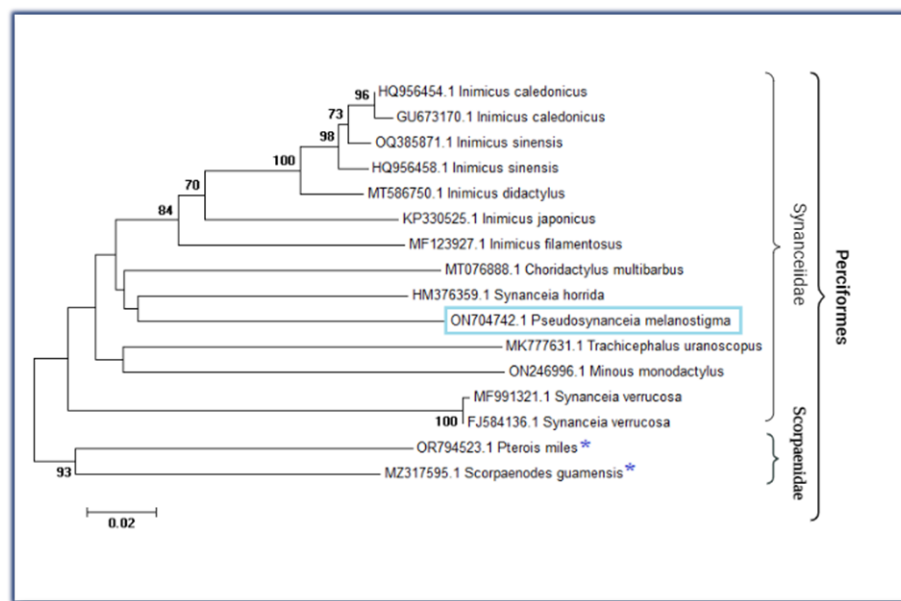
Fig.3. Stonefish Neighbor-joining (NJ) phylogeny tree based on COI gene sequences generated in MEGA 11. The labels at the branch tips refer to the species name and accession number. The numbers on the branches refer to bootstrap values for each node. The *Scorpaenodes guamensis* and *Pterois miles* were selected as out groups.

ambiguous nucleotides near primer ends, we obtained a sequence of 685 bp, which was blasted against the GenBank and BOLD databases. Our query sequence showed a high identity (84.99%) with *Inimicus sinensis* (accession numbers: MT585142.1) deposited in the GenBank database. In addition, the same high sequence identity was obtained with the *Inimicus didactylus* sequence deposited in the BOLD database (id: AEG0968) of the same family. Therefore, *P.melanostigma* registered for the first time in gene databases (NCBI: ON704742.1 and BOLD: AFH3872).

Genetic distance analysis: We compared the COI sequences of *P. melanostigma* from the Persian Gulf with 10 other sequences (Belonging to 10 species) of the family Synanceiidae retrieved from GenBank. The pairwise distance of the COI sequence revealed intraspecific variation ranging from 0.163-0.232 between the *P. melanostigma* specimen and the other species retrieved from GenBank. Table 2 shows the K2P pairwise genetic distance matrix of Synanceidae. Column 1 of this table shows the genetic diversity between *P. melanostigma* and the other species of Synanceidae retrieved from GenBank.

Phylogenetic analysis: The NJ tree (Fig. 3) based on 685 COI sequences revealed that the 13 species were clearly divided into two main clades: the clade of Synanceiidae and the clade of Scorpaenidae as

outgroups (*Scorpaenodes guamensis*, *Pterois miles*). All species formed distinct clusters in the trees, and there was no overlap between species.

DISCUSION

The morphological characteristics of *P. melanostigma* were in accordance with FAO species identification sheets by Fischer & Bianchi (1984), showing almost conservative morphology. The molecular results and sequences BLAST in the BOLD showed 84.99% identity with *I. didactylus* and 85% identity with *I. sinensis* when blasted in NCBI. DNA barcoding studies indicate that genetic distances greater than 3% are not related to intra-species or inter-population diversity. Therefore, the genetic distance of more than 3% between two sequences indicates that the two species are separate (Hebert et al. 2004; Steinke et al. 2009).

Therefore, in the present study, we reported the DNA barcode (CO1 gene sequence) of *P. melanostigma* species for the first time in the world. Many similar studies have highlighted the importance of completing biodiversity inventories in gene databases by integrating morphology and DNA barcoding as increasingly prominent techniques in species identification and discovery (Batta-Lona et al. 2019; Afrand et al. 2020; Ude et al. 2020; Esmaeili et al. 2022; Afrand & Sourinejad 2023; Kainama et al. 2023; Zhang et al. 2023).

Recording the CO1 gene sequence of this species in the NCBI and BOLD databases can be useful as a reference sequence both to identify it in all life stages, including eggs, larvae, and juveniles, which is often difficult to do using traditional methods, and to identify this species in different regions of the world. In phylogenetic analysis, the phylogenetic tree of the stonefish species found in the Persian Gulf and different geographic regions derived from NCBI based on the COI gene showed that all species formed distinct clusters in the trees, which are divided into two main clades. The first main clade consists of genera: Choridactylus, Inimicus, Minous, Synanceia, Trachicephalus, and Pseudosynanceia genera of the Synanceidae family, whereas the second clade consists of genera of the Scorpaenidae family: Scorpaenodes, Pterois (as an outgroup). All species formed distinct clusters in the trees, and *P. melanostigma* as expected, was related to the species of the family Synanceiidae. The exact classification of the species was checked according to the World Register of Marine Species database, WoRMS (Froese & Pauly, 2024).

According to our analysis, the genetic distance between our specimen and the Synanceiidae species downloaded from NCBI ranged from 0.163 to 0.232. Our specimens had the lowest genetic distance with *Inimicus didactylus* (0.163), *Synanceia horrida* (0.166), and other species of Inimicus (0.168-0.187) and Choridactylus (0.189), respectively. This issue is clearly evident in the phylogeny tree classification. In the phylogeny tree, *P. melanostigma* forms a joint subclade with these genera. No phylogenetic studies have been conducted on *P. melanostigma* so far. Over the last years, there have been multiple attempts to resolve the phylogenetic relationships between families in suborder the scorpaenoids (MYu et al. 2022; Smith et al. 2018; Imamura 2004). It seems that the limited studies have focused on the phylogenetic relationships among species in the family Synanceiidae of this suborder (Kim et al. 2020; Kim et al. 2019). Herein, we reported the first molecular phylogeny of this species and other species of the same family (Synanceiidae) in the gene bank.

In conclusion, this study was the first to register the COI sequence of the Persian Gulf venomous stonefish *P. melanostigma* in BOLD and GenBank databases. In fact, morphological and DNA-barcoding approaches were combined to exactly identify the species. In the phylogeny tree, *P. melanostigma* formed a subclade with the genera of Synanceiidae. The identified sequence of this species would be of great value for future studies on phylogeny, management and conservation of this valuable species.

FUNDING

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مقاله کامل

DNA بارکدینگ سنگ ماهی سمی (*Pseudosynanceia melanostigma*) خلیج فارس

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چکیده: سنگ ماهی سمی (*Pseudosynanceia melanostigma*)، گونهای از خانواده Synanceiidae که سمیترین ماهی و منبع دارویی بالقوه محسوب میشود، در اعماق گلی آبهای کم عمق خلیج فارس یافت میشود. این مطالعه ثبت اولین توالی های بارکد *P. melanostigma* از خلیج فارس را در پایگاههای ژن جهانی ارائه میکند. نمونهها براساس ویژگیهای ریختشناسی و همچنین بارکد DNA براساس ژن COI شناسایی شدند. ثبت این گونه بهعنوان مرجع در پایگاههای ژنی به دانش ما از تنوع زیستی ماهی در سطح جهانی میافزاید و میتواند بهعنوان ابزاری مؤثر در آینده برای شناسایی دقیق ماهیها در هر مرحله از چرخه زندگی یا حتی برای شناسایی این گونه در مناطق دیگر استفاده شود.

کلمات کلیدی: *Pseudosynanceia melanostigma*، ریختشناسی، نشانگر COI، جنگل مانگرو.