

ORIGINAL ARTICLE

Morphological analysis and DNA barcoding confirm presence of a cryptic fish species, the blotched triplefin, *Enneapterygius ventermaculus* (Teleostei: Blenniiformes: Tripterygiidae), at Qeshm Island, Persian Gulf

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Abstract

The small, cryptic, blotched triplefin, *Enneapterygius ventermaculus* (Tripterygiidae), is reported based on 10 specimens collected at the Qeshm Island, Persian Gulf, Iran, in February 2021. The identification was confirmed using integrated morphological and molecular methods. *Enneapterygius ventermaculus* is characterized by its dorsal-fin rays III+XII-XIII+9-10; anal-fin rays I,17; pectoral-fins rays 14; lateral line with 13 pored and 22-26 notched scales, starting from the second scale row below the end of the pored series; total lateral scale rows 35-39; transverse scale rows 2+1+6; mandibular pores 3+1+3. *Enneapterygius ventermaculus* is distinguished from the similar species, *E. pusillus* by having a lower total number of anal-fin soft rays (17 vs. 20-21), a higher number of total lateral scale rows (35-39 vs. 29-30), a higher number of transverse scale rows (2/6 vs. 2/4), and a different number of mandibular pores (3+1+3 vs. 2+2+2). The morphological identification was confirmed by a molecular analysis based on mitochondrial COI barcode region sequences. *Enneapterygius ventermaculus* showed a sister-group relationship to *E. pusillus*. An average K2P genetic distance of 21.5% also supported the closer phylogenetic relationship of the two species.

Keywords: Western Indian Ocean, Triplefins, Mitochondrial COI sequence, Morphology-based identification.

INTRODUCTION

Tripterygiids (triplefins or threefin blennies) are a group of benthic-living blenniiform fishes (Nelson et al. 2016), inhabiting subtidal and intertidal rocky pools and coral reefs of cold, temperate, tropical, and subtropical seashores and offshore islands down to slopes below 500 metres depth (Fricke 1997, 2017). A total of 183 valid species belonging to 29 valid genera are known worldwide (Fricke et al. 2022b). All species are characteristically small and do not exceed 151 mm SL [this length is reached by the New Zealand Giant Triplefin *Blennodon dorsale* (Clarke, 1879)], but most of them are less than 30 mm SL (Fricke 1997). Tripterygiids are characterised by having three dorsal fins and the loss of the posteriormost spine of the second dorsal fin (Fricke 1994a, 1997, 2017).

Studies on the southern coasts of the Persian Gulf and the Gulf of Oman have confirmed the presence of several species of the family, including three species of *Enneapterygius*, two species of *Helcogramma*, and

a single species of *Norfolkia* (Randall 1995; Carpenter et al. 1997; Bishop 2003; Eagderi et al. 2019). So far, only the species *Helcogramma ellioti* (Herre, 1944) has been reported from the Iranian waters based on the collected specimens (Sharafiniya et al. 2021). Fishes of the tripterygiid genus *Enneapterygius* Rüppell, 1835 are characteristically small, most species being less than 25 mm long. They are generally coral or rocky reef inhabitants, their colouration and small size rendering them cryptic. There are currently 63 valid species recognised in the genus (Fricke et al. 2022a). *Enneapterygius ventermaculus* Holleman, 1982 was originally described from a rock pool about 10 km south of the Sodwana Bay, KwaZulu-Natal, South Africa, southwestern Indian Ocean. It is known to occur in the southern Red Sea and the western Indian Ocean from East Africa, Transkei (South Africa) and the southern Persian Gulf to the Sea of Oman and Pakistan (Randall 1995; Holleman 2005; Holleman & Bogorodsky 2012; Fricke et al. 2022a). Randall

(1995) reviewed the tripterygiids of the Gulf of Oman, describing two additional species of *Enneapterygius*, *E. hollemani* and *E. melanospilus*; he also extended the range of *E. ventermaculus* to the Gulf of Oman and the northwest coast of India. Holleman (2005) and Jawad et al. (2015) reported *E. pusillus* Rüppell, 1835 from Saudi Arabia and Kuwait in the Persian Gulf, respectively. The genus *Enneapterygius* is represented by five species in the southern Persian Gulf and southern Sea of Oman: *E. abeli* (Klausewitz, 1960), *E. hollemani*, *E. melanospilus*, *E. pusillus* and *E. ventermaculus*, of which *E. abeli*, *E. pusillus* and *E. ventermaculus* also occur the Red Sea (see Holleman & Bogorodsky 2012). Although several triplefin species are widespread in the Indo-Pacific, there is just a single record of the family from the northern Persian Gulf, i.e., *E. pusillus* (see above). Records of two triplefin species by Eagderi et al. (2019) is based on the previous records of these species from the southern Persian Gulf and have not been confirmed based on collected specimens. This study aims to (i) report and provide morphological characteristics of the collected specimens of *E. ventermaculus* from the northern Persian Gulf, (ii) generate and analyses the first mitochondrial COI sequences (DNA barcoding) of this species, and (iii) present a key to the known species of genus *Enneapterygius* in the region.

MATERIAL AND METHODS

Study area and specimen collection: Ten specimens were collected from the coastal waters of the Qeshm Island, Persian Gulf (26.927 N, 56.255 E) at low tide using a hand net (Fig. 1). After preparing live photographs (Fig. 2) of each specimen anesthetized using a 1% clove oil solution, eight individuals were fixed in 5% formaldehyde and were later stored in 70% ethanol, while two specimens were fixed in 96% ethanol for molecular analysis. All individuals were deposited in the Zoological Museum, Collection of Biology Department, Shiraz University (ZM-CBSU). **Morphological analysis:** The specimens were identified based on their morphological characters, following Holleman (2005) and Holleman &

Bogorodsky (2012). The genus and species classification follows Fricke et al. (2022a); the family classification follows Laan et al. (2014). Measurements follow Fricke (1997). The head and standard lengths are abbreviated as HL and SL, respectively. Due to the small size of the specimens, the measurements were made using ImageJ 1.52a (Schneider et al. 2012). Counts follow the methods presented by Fricke (1983) using a ZEISS stereomicroscope. Body scales are deciduous and often lost, especially in small specimens; lateral line (LL) scales were counted on the left side where scales were present, and scale pockets were included where these could be distinguished. The mandibular pore pattern is given as the number of pores on the left side + the number at the symphysis + the number on the right dentary side (see Williams & McCormick 1990; Fricke 1994b; Holleman 2005). The colour pattern was described from live and preserved specimens. Maximum, minimum, mean and standard deviation of morphometric characters were calculated using IBM SPSS 25.

DNA extraction, PCR amplification and sequencing: Total genomic DNA was extracted from the fin/tissue samples using a salt method protocol described in Bruford et al. (1992). The mitochondrial cytochrome C oxidase subunit I (COI) gene sequence (the barcode region) was amplified using a primer pair, FishF1 (5'-TCAACCAACCACAAAGACATTGGCAC-3') and FishR1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') (Ward et al. 2005). The amplification was performed using a Bioer XP Thermal Cycler (Bioer Technology Co. Ltd., Hangzhou, China) with a thermal profile for amplification of COI consisting of an initial step of denaturation at 95°C for 5 min., followed by 35 cycles of denaturation (1 min. at 94°C), annealing (45 secs. at 58.4°C), and extension (1 min. at 72°C), followed by a final extension step at 72°C for 7 min. The quality of the PCR products was assayed using a 1% agarose gel was stained with a green fluorescent dye (DNA Green Viewer™). After purification with the ExoSAP-IT® (usb) kit, the PCR products were Sanger

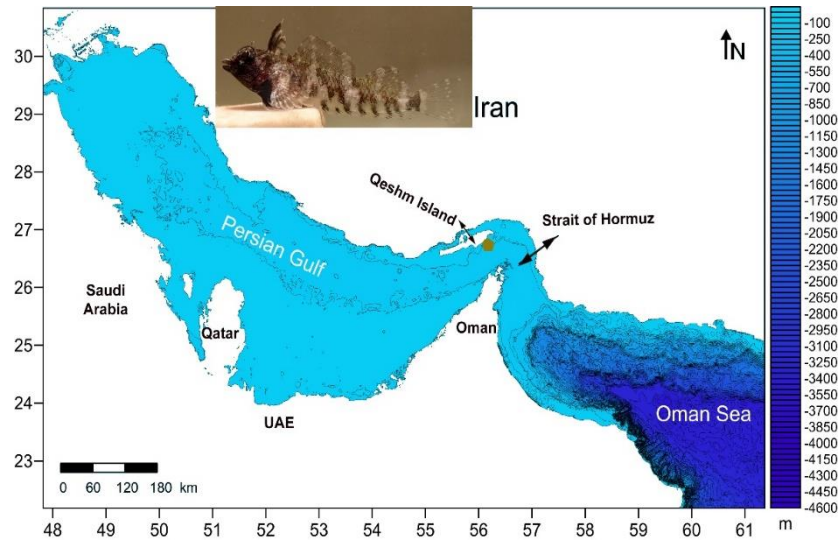


Fig.1. Collection site of *Enneapterygius ventermaculus* in the Persian Gulf.

sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) by the Faghihi Laboratory, Shiraz, Iran.

Phylogenetic analysis: BioEdit 7.1 (Hall 1999) was used to read and edit the DNA chromatograms, while ClustalW procedure in Mega 7.0 (Kumar et al. 2016) was used for sequence alignment. The new COI sequences are deposited in GenBank (Genbank Acc. No.: OM792190 and OM792191). A total of 66 COI sequences representing different tripterygiid species were obtained from GenBank. After alignment with the new sequences as well as a gobiesocid species sequence as outgroup (*Gobiesox juradoensis*; GenBank Acc. No. Y656423), GTR+I+G was selected as the best nucleotide substitution model based on the BIC model selection criterion in jModelTest 2.1.10 (Darriba et al. 2012). A Bayesian phylogeny was reconstructed using the Bayesian inference (BI) method in MrBayes 3.2 (Ronquist et al. 2012) with default priors and MCMC proposal distributions, run for 40,000,000 generations and sampled every 1000 generations. As burn-in, the first 20% of the generations were discarded.

Results

Key to the Persian Gulf and Oman Sea Species of *Enneapterygius*:

1a. Caudal peduncle with a distinct, broad dark bar with a white band at base of caudal fin; body with 6 distinct vertical dark bars.

.....
E. melanospilos

1b. Caudal peduncle without distinctive bars or markings; body without well-defined bars..... 2

2a. Supratemporal sensory canal C-shaped, running in a curve in front of the first dorsal-fin spine..... *E. abeli*

2b. Supratemporal sensory canal U-shaped, curving around the first 1-2 dorsal-fin spines..... 3

3a. Anal fin with basal spots absent; black preanal spot present..... *E. pusillus*

3b. Anal fin with basal spots present; black preanal spot present or absent..... 4

4a. Pectoral-fin rays 15; second dorsal-fin spines 13; first dorsal fin not higher than second in males, white in life; caudal peduncle with a quadrangular dusky mark. *E. hollemani*

4b. Pectoral-fin rays 14; second dorsal-fin spines usually 12; first dorsal fin higher than second in males, cream-yellow in life; caudal peduncle with a triangular dusky mark.....

E. ventermaculus

Taxonomy

Family: Tripterygiidae Whitley, 1931

Enneapterygius ventermaculus Holleman, 1982

Enneapterygius ventermaculus Holleman, 1982: 123, fig. 7 (northern KwaZulu-Natal, South Africa); Randall 1995: 32, fig. 5 (Gulf of Oman).

Enneapterygius nasimae: Hoda, 1983: 116, figs. 1-3 (Karachi coast, Pakistan).

Specimens examined (morphology): ZM-CBSU VY101-108, 8, 15.0-18.9 mm SL, Iran: Qeshm Island,



Fig.2. Live specimens of *Enneapterygius ventermaculus*, collected from the Qeshm Island. Top, male; bottom, female.

Persian Gulf (26.927 N, 56.255 E).

Specimens examined for DNA barcoding: ZM-CBSU C3273-1, 16 mm SL, Iran: Qeshm Island, Persian Gulf (26.927 N, 56.255 E); ZM-CBSU H2136BC-1, 15 mm SL, Iran: Qeshm Island, Persian Gulf (26.927 N, 56.255 E).

Diagnosis: Third dorsal fin-rays 9-10; 6-8 conspicuous black spot at the base of anal fin, and one anterior to anus.

Description: Dorsal-fin rays III+XII-XIII+viii,1-ix,1 (usually III+XII+viii,1); anal-fin rays I,xvi,1; pectoral-fins rays i,4,ix (total rays 14). Lateral line with 13 pored and 22-26 notched scales [22 (2 specimens), 24 (1), 25 (3) and 26 (2)]; notched scale series starting at second scale row below end of pored series (Fig. 2); total lateral scale rows 35-39 (usually 38-39, but 35-36 for Oman specimens, 31-34 for the western Indian Ocean); transverse scale rows 2+1+6. Mandibular pores 3+1+3). Head length 24.9-29.9% SL, head depth at opercle 20.7-23.6% of SL; eye

diameter 29.8-31.9% of HL, snout 22.6-23.7% of HL, and interorbital distance 7.3-8.2% of HL.

Body moderately robust. Mouth small and nearly horizontal, reaching vertical through anterior margin of orbit. Orbital cirrus moderate and lobate. Supratemporal sensory canal U-shaped, bending around first dorsal-fin spine. First dorsal fin slightly higher than second in males, slightly lower in females. Pelvic-fin rays united by membrane for about half length of shorter ray, longer ray reaching to pre-anal mark when laid back. No scales on nape, base of first dorsal fin and belly.

Colouration in life: (Fig. 2) (see Randall 1995; Holleman 2005; Holleman & Bogorodsky 2012):

Male: Body greenish, scale margins with brown stipples and orange edge, except ventrally; black pre-anal mark present, ranging from small spot to boomerang shape half around vent; three white blotches along midside, first under end of pectoral fin, second below junction between second and third

Table 1. Morphometric characteristics of eight collected specimens of *Enneapterygius ventermaculus* from the Qeshm Island, Persian Gulf.

Character	Min-Max	Mean \pm SD
Total length (mm)	18.1-22.9	19.26 \pm 1.6
Standard length (mm)	14.8-18.9	15.90 \pm 1.3
In % standard length		
Head length	24.9-29.8	27.54 \pm 1.76
Head depth at opercle	20.6-23.5	22.54 \pm 1.0
Head depth at nape	18.1-19.6	19.07 \pm 0.57
Caudal-peduncle depth	8.2-8.8	8.5 \pm 0.3
Prepelvic-fin length	19.7-21.6	20.6 \pm 0.8
Prepectoral-fin length	27.6-31.7	30.1 \pm 1.6
Preanal-fin length	44.1-52.4	48.1 \pm 2.9
Predorsal(1)-fin length	19.7-22.1	20.9 \pm 0.9
Predorsal(2)-fin length	32.6-35.1	33.6 \pm 1.0
Predorsal(3)-fin length	64.5-70.9	67.7 \pm 2.2
Snout length	5.7-6.9	6.43 \pm 0.5
Postorbital distance	8.7-10.7	9.75 \pm 0.68
Interorbital distance	2.0-2.2	2.1 \pm 0.1
Eye diameter	7.4-9.5	8.5 \pm 0.7
Upper-jaw length	6.7-8.5	7.8 \pm 0.5
In % head length		
Head depth at opercle	78.4-84.5	81.9 \pm 2.0
Head depth at nape	63.7-72.8	69.0 \pm 3.2
Snout length	22.6-23.7	23.3 \pm 0.4
Postorbital distance	34.5-36.1	35.4 \pm 0.7
Interorbital distance	7.3-8.2	7.8 \pm 0.3
Eye diameter	29.8-31.9	30.8 \pm 0.8
Upper-jaw length	26.9-29.3	28.3 \pm 0.8

dorsal fins, third below end of third dorsal fin; caudal fin base with triangular dark area, apex anterior, base darkest, followed by narrow white band at base of caudal fin; head bottle-green above with orange-brown markings, darkest on sides below eyes, on thorax and to pelvic-fin bases and on pectoral-fin bases. First dorsal fin finely speckled with brown and orange, but without pigment on membrane between third spine and dorsum; second dorsal spines orange with alternating areas of orange and dense, dark brown stippling along fin margin and band of stipples along base; third dorsal fin banded with dark brown on the rays and orange stipples on membranes anteriorly; anal fin with 6-8 black broken bars with white and transparent areas in between, and 6-8 black basal spots which continue subcutaneously; caudal fin rays

greenish, upper with some brownish semi-bands, lowermost with black marks; pelvic fins yellow; pectoral fins with white and dark brown, lower rays with yellow, and two white spots between base of pectoral fin and base of pelvic fin, which may be largely obscured in darkly marked specimens.

Female: Dorsally translucent pale green with orange margins to scales; no black or brown on ventral part of head and thorax, but small clusters of melanophores below midline more prominent than in males; a dark streak in front of each pelvic fin, another on side of thorax and a third from lower pectoral-fin base, latter two continuing a little below branchiostegal membrane (Fig. 2).

Sexual dimorphism: Males with blackish head, first dorsal fin slightly higher than second. Females without black or brown on ventral part of head and thorax, first dorsal fin slightly lower than second (Fig. 2).

Comparisons: *Enneapterygius ventermaculus* is distinguished from the similar species, *E. pusillus* by having a lower number of anal-fin soft rays (17 vs. 20-21), a higher number of total lateral scale rows (35-39 vs. 29-30), a higher number of transverse scale rows (2+1+6 vs. 2+1+4), and by the number of mandibular pores (3+1+3 vs. 2+2+2).

Phylogenetic placement: The morphological identification was confirmed by a molecular analysis carried out using the COI barcode region sequences. We compared the new COI sequences of *E. ventermaculus* from the Persian Gulf, with several other triplefin fish sequences retrieved from GenBank (Fig. 4). Bayesian inference of the COI dataset placed *E. ventermaculus* as a monophyletic sister clade to the morphologically similar species, *E. pusillus*, with an average K2P genetic distance of 21.5%, confirming a closer phylogenetic relationship with this species. The maximum average K2P distance was 25.7% for *E. elegans*, another triplefin species distributed in the Indo-West Pacific Ocean with 6-9+1+6-9 mandibular pores, 30-31 total lateral scales; 3+1+5 transverse scales, a crescent-shaped supratemporal sensory canal, and a scaled nape (see Holleman 2005).

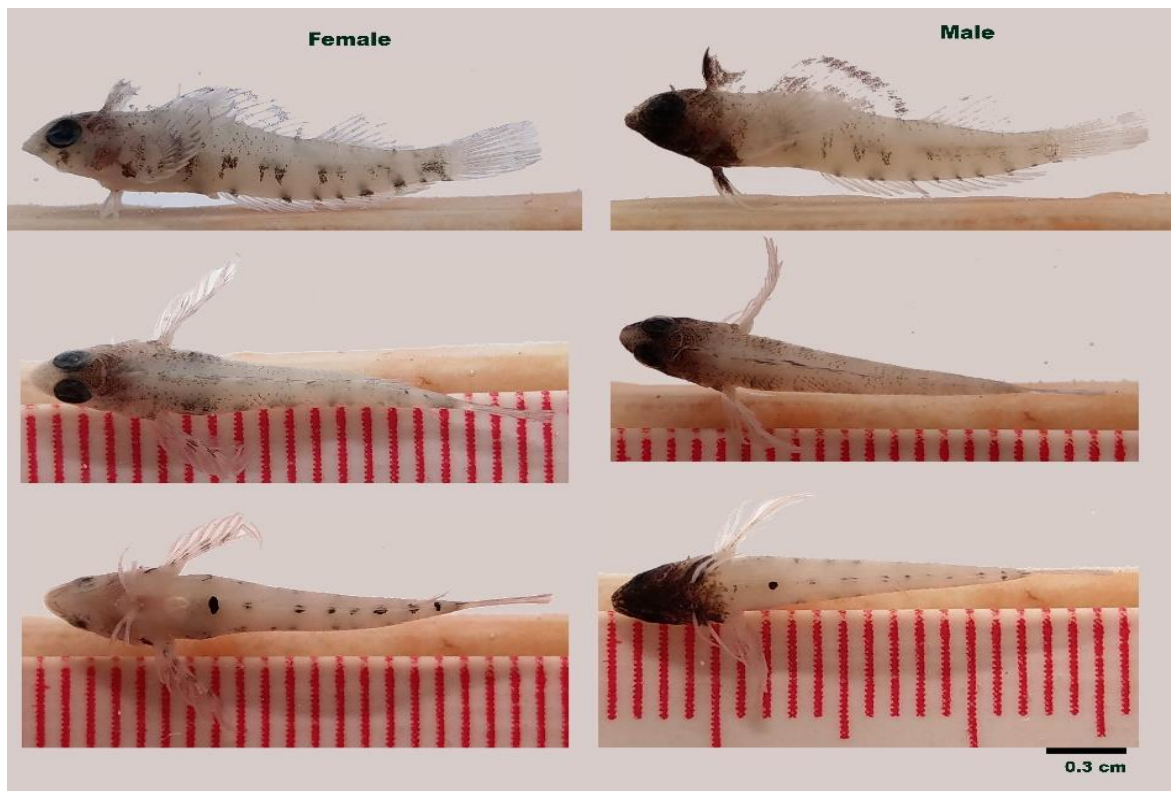


Fig.3. Lateral, dorsal and ventral views of female and male *Enneapterygius ventermaculus*, collected from Qeshm Island.

Table 2. Comparison of meristic characteristics of different populations of *Enneapterygius ventermaculus*.

Character	Persian Gulf	South Africa	Oman	Western Indian Ocean	Red Sea
Total dorsal-fin rays (D1+D2+D3)	III+XII-XIII+9-10 (usually III+XII+9)	III+XI-XIII+9-10 (usually III+XII+10)	III+XI-XIII+9-11 (usually 10)	III+XI-XIII+8-10 (usually III+XII+10)	III+XI-XIII+8-10
Total anal-fin soft rays	17	17-20 (usually 19)	17-20 (usually 19)	17-20 (usually 19)	17-21 (usually 19)
Total pectoral-fins rays	14	14	13-14 (usually 14)	13-14 (usually 14)	14
Lateral-line scales (pored)	13	13-16 (usually 15)	13-15 (usually 14)	13-16 (usually 15)	13-16 (usually 15)
Lateral-line scales (notched)	22-26 (usually 25)	21-25 (usually 23)	21-22	21-25 (usually 23)	21-25 (usually 23)
Total lateral-line scales	35-39 (usually 38-39)	32-34 (usually 33)	35-36	31-34	Usually 38
Mandibular pore	3+1+3	-	3+1+3	3+1+3	3+1+3
D1: D2 height (Male)	D1>D2	D1>D2	D1=D2	D1>D2	D1>D2
D1: D2 height (Female)	D1<D2	D1=D2	D1=D2	D1<D2	D1<D2
Black spot at base of anal fin	6-8	5-6	5-6	5-7	6-8
Reference	Present study	Holleman (1982)	Randall (1995)	Holleman (2005)	Holleman & Bogorodsky (2012)

DISCUSSION

Enneapterygius ventermaculus is distributed in the southern Red Sea and the western Indian Ocean (Randall 1995; Holleman 2005; Holleman and

Bogorodsky 2012; Fricke et al. 2021a, b), including the Persian Gulf. Table 2 summarizes the basic meristic and other characteristics for different populations of *E. ventermaculus* from the western Indian Ocean and adjacent water bodies.

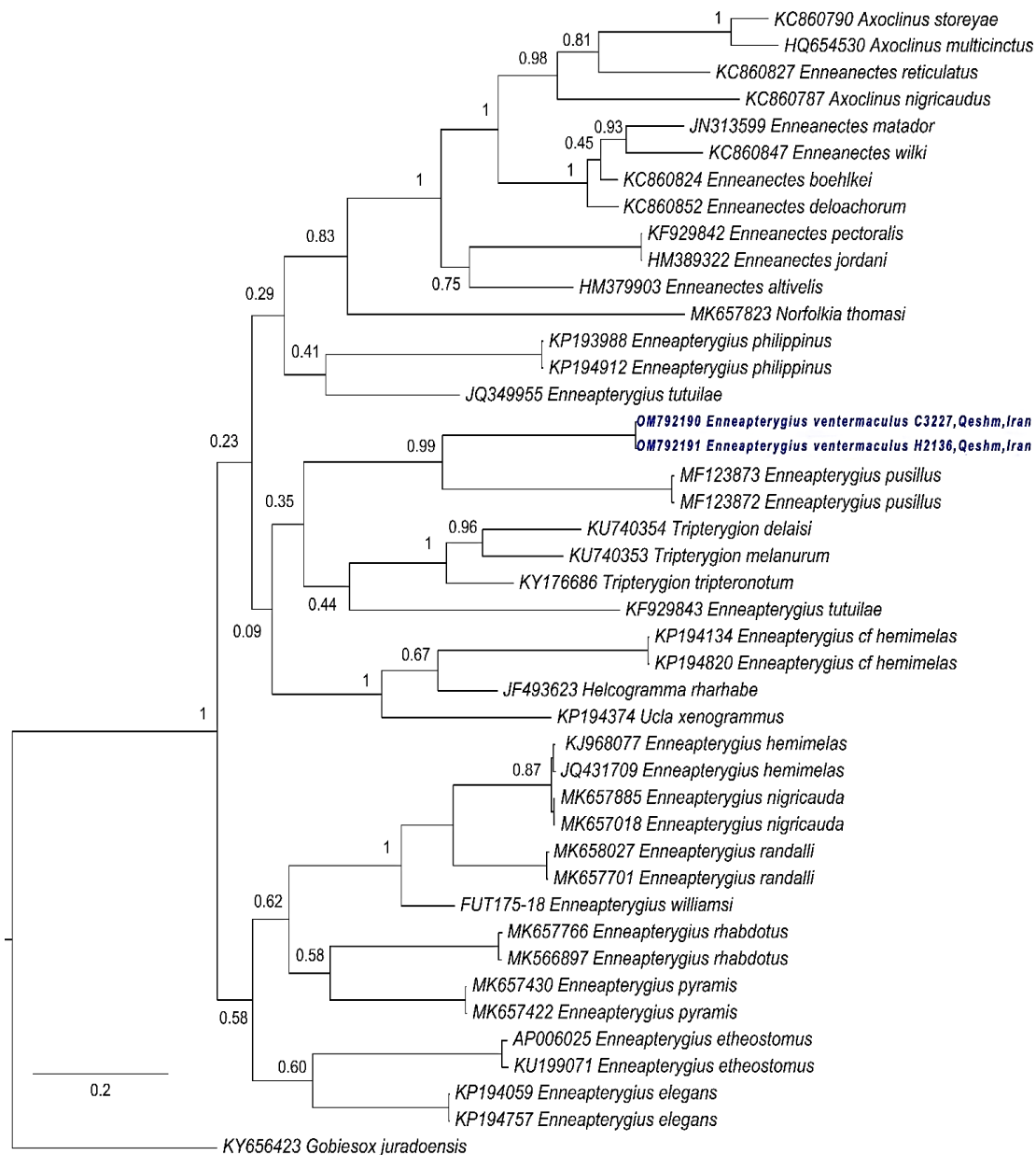


Fig.4. Bayesian phylogeny of triplefin species reconstructed based on COI gene sequences. The values beside the branches are BI posterior probability values. Accession number of each sequence is given before the fish's scientific name.

Although the 138 presently known species of Tripterygiidae are distributed worldwide, but no representative of this family had to date been reported based on specimen collected from the Iranian part of the Persian Gulf, likely a result of no comprehensive studies in the area, the small size of the species and cryptic color patterns of members of this family. The present record of *Enneapterygius ventermaculus* adds to our knowledge of species biodiversity of Tripterygiidae in the Persian Gulf, and it is reasonable to assume that this water body contains several other

species found in the western Indian Ocean. During recent years, several new fish species have been described or recorded from the Persian Gulf and the Sea of Oman (e.g., Uiblein and Heemstra 2011; Yasemi & Nazari Bejgan 2014; Sadeghi et al. 2017, 2019a, 2019b; Mehraban & Esmaeili 2018; Sadeghi & Esmaeili 2019a, 2019b; Kovačić et al. 2020), indicating the Gulf's potential for harboring a high ichthyodiversity.

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ETHICAL APPROVAL

The research work was approved by the Ethics Committee of Biology Department (SU-9831511).

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مقاله پژوهشی

تجزیه و تحلیل ریخت‌شناختی و بارکدینگ DNA، تایید کننده حضور ماهی سه باله لکه‌دار *Enneapterygius ventermaculus* (Teleostei: Blenniiformes: Tripterygiidae) در جزیره

قشم، خلیج فارس

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چکیده: در این مطالعه حضور گونه کوچک و مرموز سه باله لکه‌دار، *Enneapterygius ventermaculus* (Tripterygiidae)، بر اساس ۱۰ نمونه جمع‌آوری شده از جزیره قشم، خلیج فارس، ایران، گزارش شده است. شناسایی با استفاده از روش‌های ریخت‌شناختی و مولکولی تایید شد. *Enneapterygius ventermaculus* با تعداد شعاع‌های باله ای پشتی 10-9+XII-XIII+III، تعداد شعاع‌های باله مخرجی I و ۱۷؛ تعداد شعاع‌های باله سینه‌ای ۱۴؛ خط جانبی با ۱۳ فلس شکاف‌دار و ۲۲-۲۶ فلس منفذدار که از ردیف دوم فلسی در زیر انتهای سری‌های منفذدار شروع می‌شود، مجموع ردیف‌های فلس جانبی ۳۹-۳۵، ردیف‌های فلس عرضی ۶+۱+۲، و تعداد منافذ فک پایین ۳+۱+۳ مشخص می‌شود. *Enneapterygius ventermaculus* از گونه مشابه، *E. pusillus* براساس تعداد کمتر کل شعاع‌های نرم باله مخرجی (۱۷ در مقابل ۲۱-۲۰)، تعداد بیشتر ردیف‌های فلسی جانبی کل (۳۹-۳۵ در مقابل ۳۰-۲۹)، تعداد ردیف‌های فلسی عرضی (۶/۲ در مقابل ۴/۲)، و تعداد متفاوت منافذ فک پایین (۳+۱+۳ در مقابل ۲+۲+۲) متمایز می‌شود. شناسایی ریختی، توسط آنالیز مولکولی براساس توالی ژن سیتوکروم C اکسیداز، زیر واحد I (COI) تایید شد. *Enneapterygius ventermaculus* گروه خواهری *E. pusillus* بوده، و میانگین فاصله ژنتیکی ۵/۲۱ درصد بر اساس توالی ژن COI از این خویشاوندی حمایت می‌کند.

کلمات کلیدی: اقیانوس هند غربی، سه باله لکه‌داران، توالی COI میتوکندری، شناسایی ریختی.