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A phylogenetic study of a tropical sea urchin, *Echinometra* sp. EZ (Echinoidea: Camarodonta: Echinometridae) from the Persian Gulf

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Research Article

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Abstract

Sea urchins have important effects on marine ecosystems such as rocky shores and coral reefs across the world. However, species diversity and molecular phylogeny of most echinoid taxa are poorly known in Iran. In this study, the phylogenetic relationships of one of the most abundant species of the genus *Echinometra* in the Persian Gulf were examined. Echinoids were collected from the intertidal zone of Qeshm Island and Lengeh Port on March and December 2017. Morphological criteria based on valid identification keys combined with molecular analysis of a fragment of the mitochondrial cytochrome c oxidase subunit I (COI) protein-coding gene were used to delineate *Echinometra* species. Our analyses showed that all specimens (N = 15) belong to *Echinometra* sp. EZ. Tree topologies indicated that our individuals from two sampling sites formed a distinct monophyletic clade with *E.* sp. EZ, demonstrating high support values. This is the first phylogenetic analysis of *E.* sp. EZ from Iran.

Introduction

Sea urchins of the genus *Echinometra* have a pan-tropical distribution across the Pacific, Atlantic and Indian Oceans (Palumbi & Metz, 1991; Moulin *et al.*, 2015). The number of valid species in this genus has been debated in the scientific literature for over 180 years (Bronstein & Loya, 2013). Two species, *Echinometra mathaei* and *Echinometra oblonga*, were first described by Blainville (1825). Döderlein (1906) elevated *E. oblonga* to a separate genus, *Mortensia oblonga* based on the gonad spicule morphology, but Mortensen (1943) claimed this species is a morph of *E. mathaei* and named it *Echinometra mathaei oblonga*. Kelso (1970) did an extensive study on the ecological distribution and morphological characteristics of these two morphs; *E. mathaei* and *E. mathaei oblonga* in Hawaii. He strongly suggested that they were separate species, *E. mathaei* and *E. oblonga*.

Further morphological and molecular studies on *Echinometra* from Okinawa and the Indo West Pacific (Uehara *et al.*, 1986; Matsuoka & Hatanaka, 1991; Palumbi & Metz, 1991; Arakaki *et al.*, 1998; Landry *et al.*, 2003) revealed the presence of four *Echinometra* species in these areas. They were distinct, but very closely related species and originally referred to as *Echinometra* species A, B, C and D. Studies on both morphological characteristics and genetics of these species (Motokawa, 1991; Arakaki *et al.*, 1998; Landry *et al.*, 2003) asserted that *E.* sp. B and *E.* sp. D in Okinawa are indeed *E. mathaei* and *E. oblonga*, respectively. Palumbi (1996) showed that the genetic and morphological differences among these closely related tropical sea urchins were small, but their reproductive isolation was strong. As such, *Echinometra* makes a valuable group for studies of marine speciation. The genus *Echinometra* currently comprises nine species, three of them still undescribed (Bronstein & Loya, 2013). As species-level taxonomy of this genus is yet to be completed, more research is needed to clarify the obscure relationship between these species.

Echinometra mathaei is an important species of this genus that has been called the world's most abundant sea urchin (Palumbi & Metz, 1991) with a large geographic distribution from Hawaii and Tahiti throughout the Indo West Pacific (IWP), to the Western Indian Ocean (WIO), the Persian Gulf and the Red Sea (Clark & Rowe, 1971; Russo, 1977; Price, 1983; Lawrence, 1983; Palumbi & Metz, 1991; McClanahan & Muthiga, 2001). This species is ecologically important because it can control algal growth, and high densities of it can prevent recovery of fish and coral populations following a disturbance (McClanahan *et al.*, 1996). It has been mentioned as one of the most important bioeroder sea urchin species which can play a major role in bioerosion and herbivory on coral reefs and reduction of net accretion on these ecosystems (Downing & El-Zahr, 1987; Bak, 1990; McClanahan & Kurtis, 1991; Carreiro-Silva & McClanahan, 2001; Bronstein & Loya, 2014). New studies on the genus *Echinometra* in recent decades have revealed that some populations of *Echinometra* species had been previously mistaken for *E. mathaei*. One of these species is *Echinometra* sp. EZ which was identified in Zanzibar (WIO) and Eilat (Gulf of Aqaba/Eilat, northern Red Sea) by Bronstein & Loya (2013) for the first time.

Sea urchins have a significant effect on coral reefs and the intertidal zone in marine ecosystems of Iran, but the taxonomy and phylogeny of most taxa are poorly known. Therefore, the phylogenetic relationships of one of the most abundant species of *Echinometra* in the area were studied here to illuminate more details about it. Because of the variation in morphological criteria of *Echinometra* species, different molecular studies have been performed (Palumbi & Metz, 1991; Palumbi, 1996, 1997; Landry *et al.*, 2003; Bronstein & Loya, 2013; Nakano *et al.*, 2019). Consequently, we studied the phylogeny of this echinoid, combining molecular analysis and morphology.

Materials and methods

Sample collection and morphological measurements

Echinoids were collected from the intertidal zone of Qeshm Island (26°87′N 56°15′E) and Lengeh Port (26°18′N 54°30′E), both within the Hormozgan Province in the Northern Persian Gulf of Iran (Figure 1). Sampling was carried out in March and December 2017. A total of 15 individuals were sampled and preserved in 96% ethanol in the laboratory. The specimens were first morphologically identified according to Mortensen's criteria (1943), Clark & Rowe (1971), Arakaki *et al.* (1998) and Bronstein & Loya (2013). The morphological characters used to identify echinoids in this study were colour of spines, colour of milled rings and skin around the peristome, shape of spicules in the gonads and the number of pore-pairs per ambulacral plate. The test size was measured by Vernier callipers after removing the spines. Measurements were performed to the nearest 0.5 mm. Spicules of the gonad were photographed under a light microscope and analysed using the software ImageJ (Abràmoff *et al.*, 2004). The number of pore-pairs on ambulacral plates (10 columns per individual) was counted from the apical system to the oral plates under a dissecting microscope.

DNA extraction, amplification and sequencing

DNA was extracted from the gonads based on the CTAB protocol following Baker (1999). DNA concentrations were then assessed using a spectrophotometer UV/VIS (biophotometer, RS-232C). A fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was amplified using the primers COI-F (5′-GGTCA CCCAGAAGTGTACAT-3′) and COI-R (5′-AGTATAAGCGT CTGGGTAGTC-3′) as suggested by Lessios *et al.* (2012). These primers can amplify up to 670 nucleotides of the COI region. A polymerase chain reaction (PCR) was performed in 25 µl total volume (9.5 µl ddH_2O, 12.5 µl Mastermix (Taq DNA Polymerase Master Mix Red – 1.5 mM MgCl2, Ampliqon), 1 µl of each primer (10 pmol) and 1 µl of DNA template (~7 ng μl^{-1})). Amplifications were conducted with the following temperature profile: an initial denaturation step at 94 °C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 45 s and finished with final elongation of 10 min at 72°C. PCR products were purified using a Thermo Scientific Genomic DNA purification kit and sequenced using the forward PCR primer on a genetic analyser 3130xl sequencer using sequencing analysis software v5.2 (Applied Biosystems).

Data analysis

For analysing the sequence data, chromatograms were checked and edited manually using ChromasPro v2.6.4 (Technelysium Pty Ltd). New COI sequences were deposited in GenBank. Accession numbers of the new sequences are shown in Table 1. For comparison with the other known *Echinometra* species, additional sequences were obtained from GenBank (Table 2). Sequences were aligned using ClustalW and genetic distances within and among taxa were calculated using the Tamura 3-parameter model (Tamura, 1992) in Mega X v10.0.5 (Kumar *et al.*, 2018). The phylogenetic tree reconstruction was drawn using both Maximum likelihood (ML) and Bayesian inference (BI) analyses. ML analysis was conducted using Mega X applying 1000 bootstrap replications. The best-fit evolutionary model identified for the ML tree was T92 + G + I, which was selected based on the results from Mega X. Bayesian analysis was performed using MrBayes v3.2.7 (Ronquist *et al.*, 2012) with the generalized time-reversible model GTR + I, which was identified using the Akaike Information Criterion (AIC) in MrModeltest v2 (Nylander, 2004). The BI analysis was conducted with two runs and four chains and sampling every 100 generations. The sampling continued until 5,000,000 generations. The first 25% of the total number of generations was discarded as burn-in and a 50% majority rule consensus tree was calculated from the remaining trees.

Results

Morphological observations

The morphological characters of specimens studied in this research included test height, test length, spine length, colour of

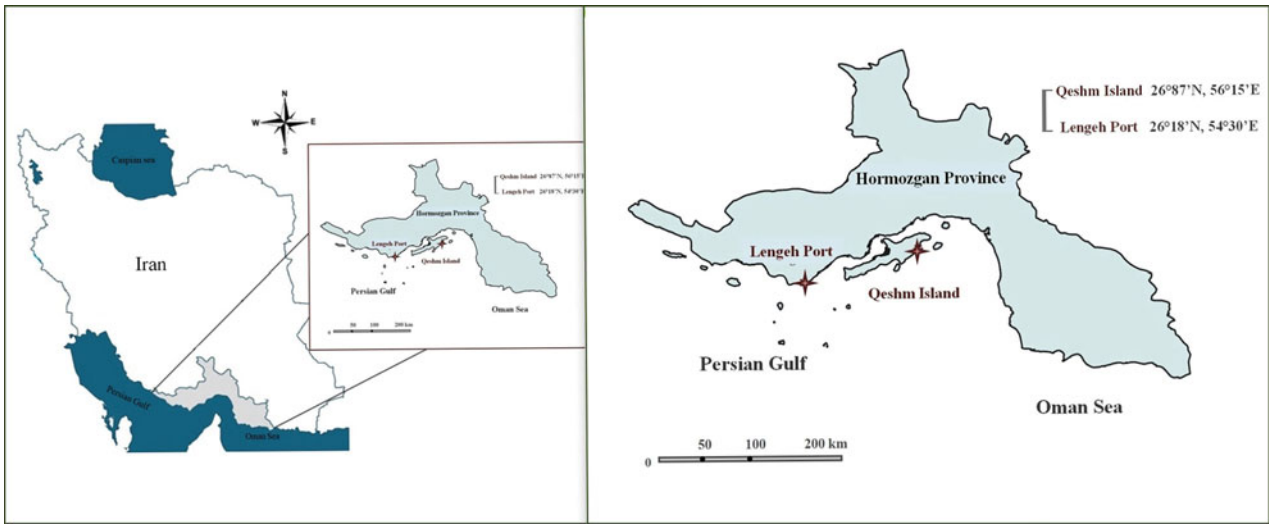

Fig. 1. Sampling sites of *Echinometra* specimens in the Persian Gulf: Qeshm Island and Lengeh Port.

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Table 1. GenBank accession numbers of COI sequences of *Echinometra* sp. EZ examined in this study

Species	Locality	Voucher	Accession no.
Echinometra sp. EZ	Lengeh Port	*E.* sp. EZ Lengeh 1	MT976151
	Lengeh Port	*E.* sp. EZ Lengeh 2	MT815722
	Lengeh Port	*E.* sp. EZ Lengeh 4	MT821457
	Lengeh Port	*E.* sp. EZ Lengeh 5	MT826942
	Lengeh Port	*E.* sp. EZ Lengeh 6	MT828885
	Lengeh Port	*E.* sp. EZ Lengeh 7	MT991673
Echinometra sp. EZ	Qeshm Island	*E.* sp. EZ Qeshm 1	MT826946
	Qeshm Island	*E.* sp. EZ Qeshm 2	MT826925
	Qeshm Island	*E.* sp. EZ Qeshm 3	MT826947
	Qeshm Island	*E.* sp. EZ Qeshm 4	MT826943
	Qeshm Island	*E.* sp. EZ Qeshm 5	MT826944
	Qeshm Island	*E.* sp. EZ Qeshm 6	MT826945
	Qeshm Island	*E.* sp. EZ Qeshm 7	MT994482
	Qeshm Island	*E.* sp. EZ Qeshm 8	MT826941

spines, colour of milled rings and skin of peristome, and gonad spicule types (summarized in Table 3). The milled rings and the skin colour around the peristome were dark in all of the individuals from the two sampling sites. Observation of the colour of spines showed variation from dark olive (green) to black (Figure 2). The spicules found in the gonads were comprised of four spicule types categorized into needle, triradiate, bihamate (C-shaped) and figure-eight shaped (Figure 3). The gonads presented various combinations of needle spicules with the other spicule types. The percentages of pore-pairs on ambulacral plates of individuals from each sampling site and total specimens are shown in Figure 4. The five-pore-pair percentage (40–75%) was the highest, while the four-pore-pair percentage (10–50%) was the second highest in the individuals of both studied areas. Furthermore, the percentage of five-pore-pairs in the Qeshm individuals was higher than in the Lengeh ones (Figure 4).

Phylogenetic relationship

After mitochondrial DNA sequencing of our specimens and sequence alignment, a portion of the COI gene corresponding to the interval between positions 5913–6456 of *Strongylocentrotus purpuratus* mitochondrial genome was obtained for each individual. Additional sequences of the other *Echinometra* species were obtained from GenBank. However, some COI sequences of this genus which correspond to positions 6400–7100 in the *Strongylocentrotus purpuratus* mitochondrial genome, including the sequences of *E.* sp. EZ of the Bronstein & Loya (2013) study, could not be used for our analyses. We could use complete mitogenome sequences or COI sequences which contained the first fragment or two overlapping fragments of the CO1 gene. Phylogenetic reconstruction of *Echinometra* specimens from the two sampling sites of this study indicated that the specimens belong to *Echinometra* sp. EZ. As both Maximum likelihood and Bayesian inference analyses produced the same tree topologies, the phylogenetic trees were depicted in Figure 5. The results of the phylogenetic tree drawn by Bayesian inference and Maximum likelihood methods showed that individuals from the two novel sampling sites were clustered into one of the main clades of *Echinometra* (Figure 5), corresponding to one of the nine known species in the genus. Our sequences formed a distinct monophyletic clade with *E.* sp. EZ. These sequences were separated from the other species of *Echinometra* by high support values (BSP = 99; PP = 1) (Figure 5). The results of genetic pairwise distances of the sequences indicated that intraspecific divergence of *E.* sp. EZ in the current study and that from GenBank was 0.41% and intraspecific divergence of our sequences was 0.39%. Interspecific divergence values between *E.* sp. EZ and the other *Echinometra* species showed that the genetic distance between *E.* sp. EZ and *E.* sp. A (2.20%) was smaller than to the others.

Table 2. Accession numbers of *Echinometra* species obtained from GenBank

Species	Locality	Accession no.
Echinometra mathaei	IWP	AY262861
	IWP	AY262912
	IWP	AY262914
	IWP	AY262924
	Japan	LC406271
	Japan	LC406272
	Japan	LC406276
	Korea	JQ742945
Echinometra sp. A	Japan	LC406260
	Japan	LC406262
	Japan	LC406264
	Japan	LC406259
	IWP	AY262884
	IWP	AY262886
Echinometra oblonga	IWP	AY262867
	IWP	AY262937
	IWP	AY262872
	Japan	LC406277
	Japan	LC406281
	Japan	LC406282
Echinometra sp. C	Japan	LC406284
	Japan	LC406286
	Japan	LC406290
	IWP	AY262875
	Japan	LC406283
	IWP	AY262940
Echinometra insularis	Easter Island	AY262902
	Easter Island	AY262904
	Easter Island	AY262905
	Easter Island	AY262908
	Easter Island	AY262911
Echinometra sp. EZ	Persian Gulf, UAE	MH685644
Echinometra vanbrunti	Eastern Pacific	AY262883
Heliocidaris crassispina	Korea	JN716400

IWP, Indo-West Pacific.

Discussion

Based on the results of this study, our specimens were identified as *Echinometra* sp. EZ, which has been previously described by

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Table 3. Morphological characteristics of *Echinometra* sp. EZ from two sampling areas in the Persian Gulf

Sampling Area	GenBank Voucher	Colour of spines	Spine length (mm)	Test length (mm)	Test height (mm)	Milled rings	Skin of peristome	Spicules in gonads
Persian Gulf	Qeshm 1	Black	21	32	18	Dark	Dark	Needle + 8 shaped
Qeshm Island	Qeshm 2	Dark olive	22	33	20	Dark	Dark	Needle
(N = 8)	Qeshm 3	Black	20	35	20	Dark	Dark	Needle + Bihamate
	Qeshm 4	Dark olive	21	33	20	Dark	Dark	Needle + Triradiate
	Qeshm 5	Black	29	48	29	Dark	Dark	Multiple
	Qeshm 6	Dark olive	20	36	20	Dark	Dark	Multiple
	Qeshm 7	Dark olive	23	50	27	Dark	Dark	Needle + Bihamate
	Qeshm 8	Black	25	40	25	Dark	Dark	Needle + Triradiate
Persian Gulf	Lengeh 1	Dark olive	24	45	24	Dark	Dark	Needle
Lengeh Port	Lengeh 2	Black	22	43	24	Dark	Dark	Multiple
(N = 7)	Lengeh 3	Black	28	42	25	Dark	Dark	Needle + Triradiate
	Lengeh 4	Dark olive	24	40	20	Dark	Dark	Needle + 8 shaped
	Lengeh 5	Dark olive	21	38	23	Dark	Dark	Multiple
	Lengeh 6	Dark olive	24	48	26	Dark	Dark	Multiple
	Lengeh 7	Black	22	45	25	Dark	Dark	Needle
Mean ± SD			23 ± 2.6	40.5 ± 5.7	23 ± 3.1			

Multiple: combination of three or more types of spicules; 8 shaped: figure-eight shaped; N: the number of individuals; SD, standard deviation.

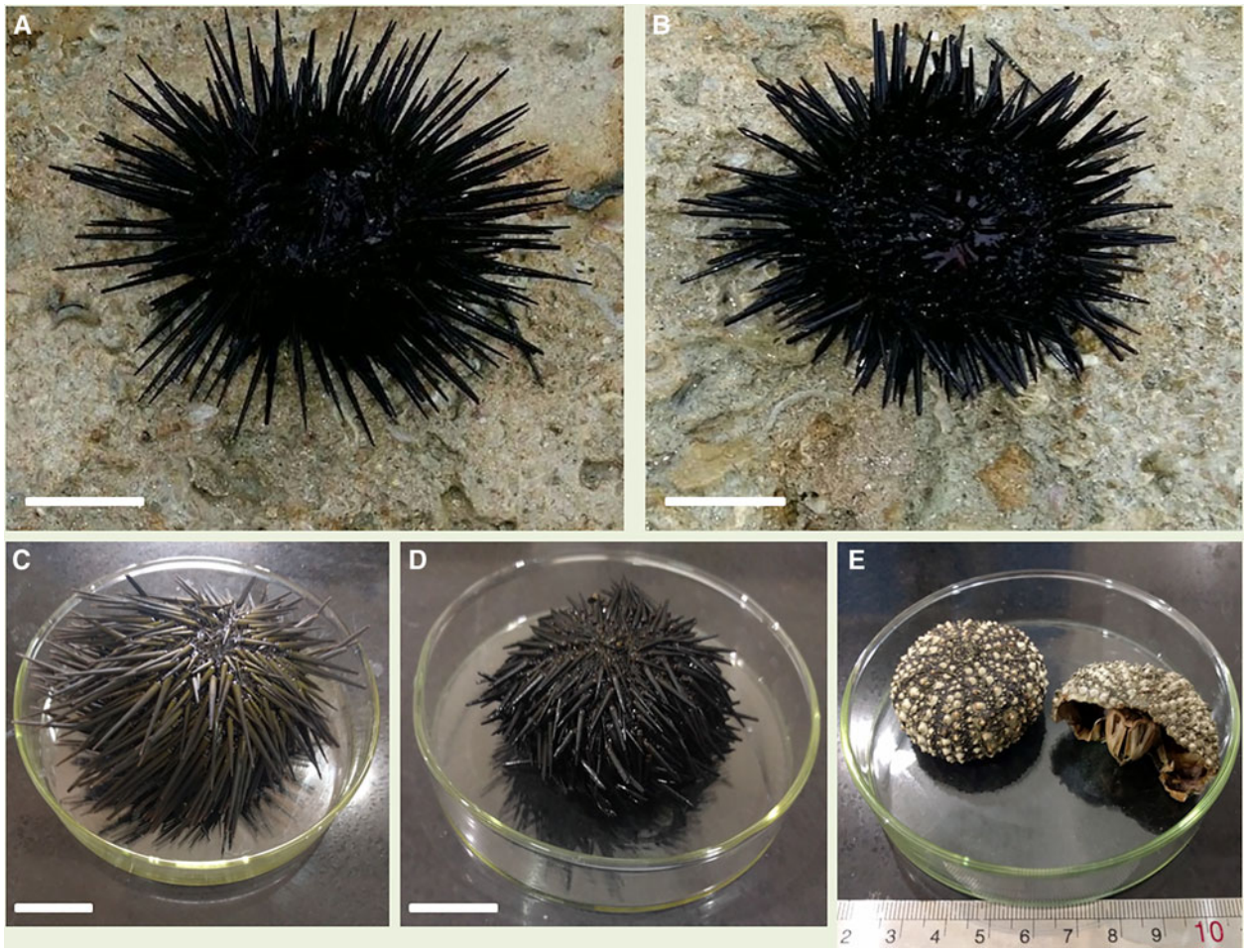

Fig. 2. Photographs of *Echinometra* specimens from the Persian Gulf. A and B represent aboral and oral sides of an individual in the sampling site, respectively; C and D: specimens with olive spine colour and black spine colour before fixation, respectively; E: test and Aristotle's lantern of an individual in the current study. Scale bars indicate 2 cm.

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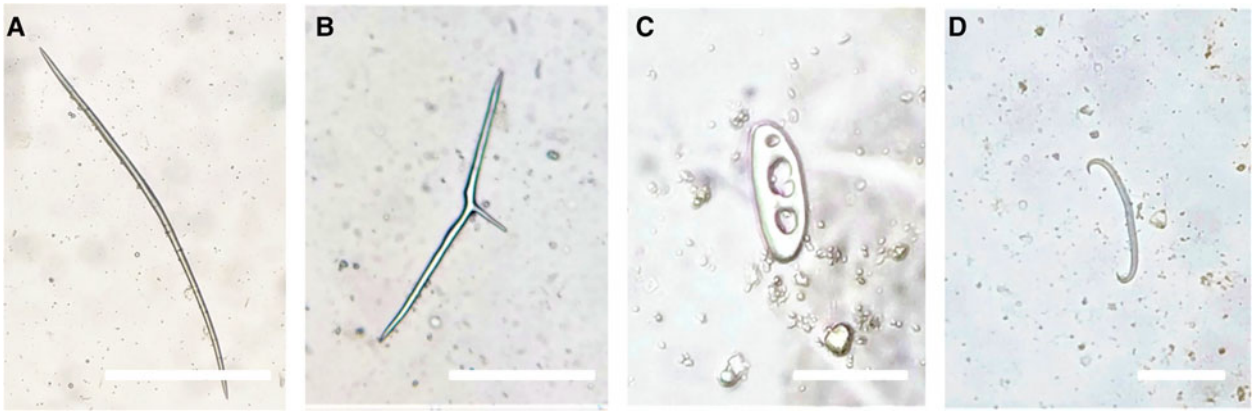

Fig. 3. Spicule types in the gonads of *Echinometra* individuals from the Persian Gulf. A: needle spicule, B: triradiate spicule type, C: 'figure-eight' shaped spicule and D: bihamate spicule. The longer (A, B) and shorter scale bars (C, D) indicate 50 and 20 μm, respectively.

Bronstein & Loya (2013). The study of morphological characters indicated that most features of our specimens are consistent with previous descriptions in the only morphological study of this species (Bronstein & Loya, 2013). The colour of spines of our individuals was dark olive (green) or black. Bronstein & Loya (2013) also showed that *E.* sp. EZ specimens exhibited various colours, including black, light or dark brown, light or dark brown-green and violet. The skin colour around the peristome of our individuals was dark which was consistent with the results of Bronstein & Loya (2013) that indicated the presence of predominant dark-skinned specimens with only a few bright-skinned ones. In addition, the milled rings of our samples were all dark and in Bronstein and Loya's (2013) study, the milled rings of *E.* sp. EZ individuals were determined as bright, faded or dark. The spicules in the gonads were either of the needle type or various combinations of the needle type with three other spicule types. The observations of Bronstein & Loya (2013) also revealed that in the gonads of this species, needle type spicules were always present either solely or in combinations with other spicule types. Moreover, the figure-eight shaped spicules were presented in the gonads of our specimens as observed in *E.* sp. EZ specimens in Bronstein & Loya's (2013) study. The results of Bronstein &

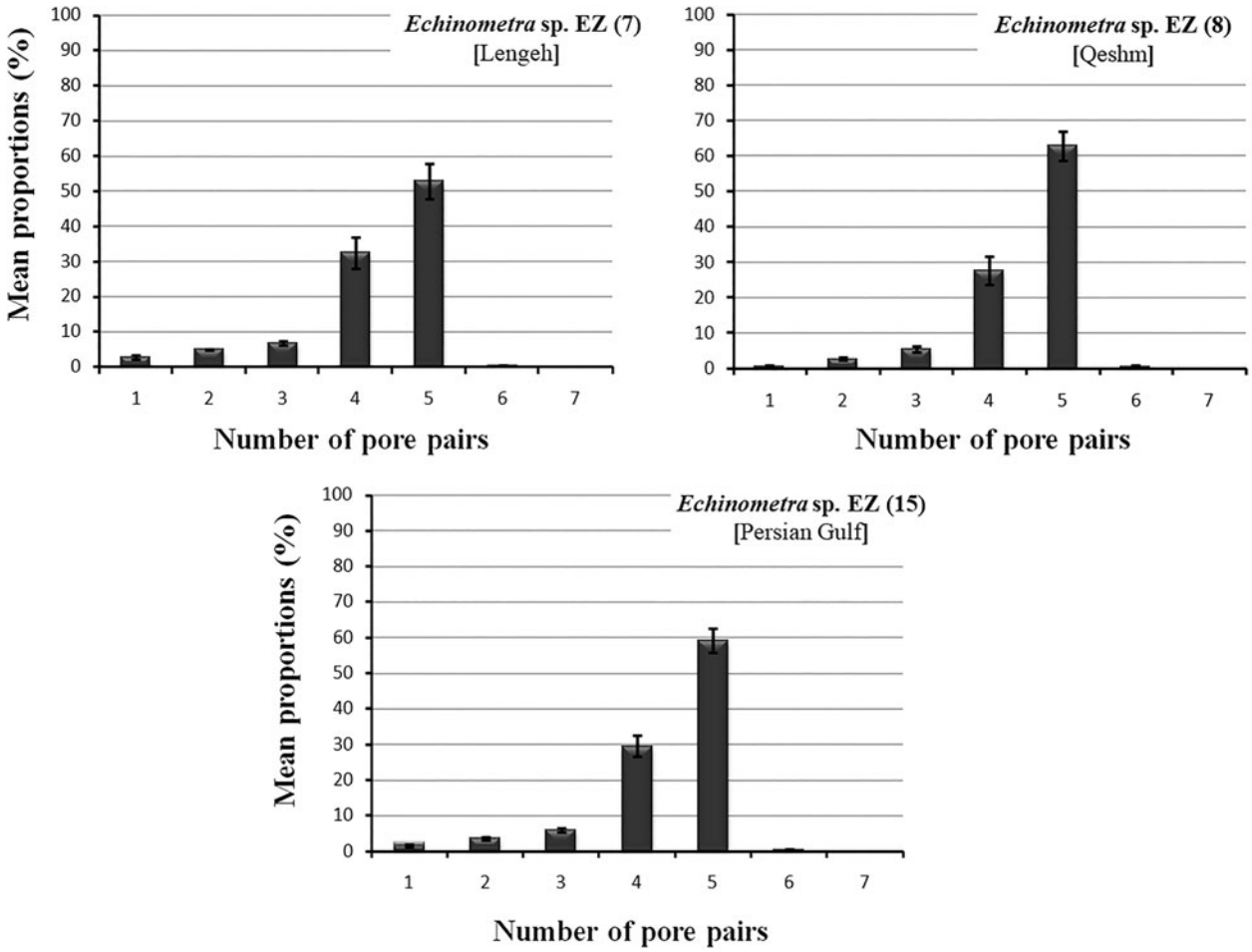

Fig. 4. Pore-pairs ratios of *Echinometra* sp. EZ from Qeshm Island and Lengeh Port in the Persian Gulf from all individuals analysed in this study (N = 15). The values represent mean proportions ± SE (%). The figures in parentheses indicate sample sizes.

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Fig. 5. Phylogenetic tree of COI sequences of *Echinometra* specimens analysed in the current study and sequences of *Echinometra* species obtained from GenBank. The phylogenetic trees were reconstructed by both Maximum-likelihood (ML) and Bayesian analysis. *Heliocidaris crassispina* (Echinodermata, Echinoidea) (GenBank accession number: JN716400) was used as outgroup. Support values (>50%) of 1000 bootstrap replications of the ML analysis and the posterior probabilities of the BI analysis are shown for each node, respectively.

Loya (2013) also indicated that in the other *Echinometra* species, these spicules were nearly absent. The results of the number of pore-pairs of the individuals we examined indicated the five-pore-pairs ratio was the highest in contrast to the results of Bronstein & Loya (2013) in which a four-pore-pair ratio was the highest in this species.

The small morphological differences may be due to regional differences in *E.* sp. EZ populations. Another reason for these

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differences may be related to the number of samples. In Bronstein & Loya's (2013) study, a larger number of individuals were examined in comparison to the current study, potentially capturing more intraspecific variation. Furthermore, *Echinometra* may exhibit high morphological plasticity and the currently available morphological keys may be limited in their ability to delineate all species within this genus (Bronstein & Loya, 2013). Mortensen (1943) mentioned that *E. mathaei* represents extensive morphological variations in test shape and spine colour. Other studies also showed that *Echinometra* species exhibited various spine colours (Arakaki *et al.*, 1998; Bronstein & Loya, 2013).

Results of the phylogenetic tree supported by both ML and BI analysis indicated that the sequences of our specimens formed a clearly distinct monophyletic clade with the GenBank sequence of *E.* sp. EZ. Based on the results of the current study, degrees of divergence of the clade containing *E.* sp. EZ from the other *Echinometra* (2.2–5.5%) are well within the interspecific range for this genus. These interspecific relationships are consistent with previous molecular studies on *Echinometra* species (Landry *et al.*, 2003; Palumbi & Lessios, 2005; Bronstein & Loya, 2013; Nakano *et al.*, 2019). There are some differences in *Echinometra* tree topology between our analysis and previous phylogenetic studies of this genus. It can be due to a single and relatively short aim of the current study which was to define the phylogenetic relationship of only one species of *Echinometra* from the Persian Gulf. Both molecular data and morphological features suggest that the current *Echinometra* specimens from the northern Persian Gulf are *E.* sp. EZ. Currently, this species was only reported from Zanzibar (WIO) and Eilat in the northern Red Sea (Bronstein & Loya, 2013) and from the southern Persian Gulf (Ketchum *et al.*, 2018). It seems that these areas share similar echinoids which are distinct from the rest of the Indo-Pacific and Atlantic sea urchins. However, further phylogenetic studies on this species and the greater *Echinometra* species complex, across various regions and with additional loci (including nuclear genes) are needed to illuminate the obscure relationship between species of this genus. It will be valuable for increasing our knowledge about distribution, marine speciation and species diversity of *Echinometra*.

Author contributions. Seyedeh Mojgan Kalantarian: Her role in this study was to carry out the research, analyse the data, interpret the findings and write the article. Bita Archangi: Her role in this study was to formulate the research questions, design the study and support as supervisor 1. Tooraj Valinassab: His role in this study was to collect the specimens from the sampling sites. Hassan Rajabi-Maham: His role in this study was to analyse the data and interpret the findings. Rahim Abdi: His role in this study was to help supervisor 1 as supervisor 2.

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Conflict of interest. The authors declare none.

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