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A Molecular Phylogenetic Analysis of the Red Sea Goatfish *Parupeneus forsskali*: An Overlooked Confirmed Invader along the Libyan Coast (Southern Mediterranean Sea)

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ABSTRACT

Here we confirm the occurrence of population of the Red Sea goatfish, Parupeneus forsskali (Fourmanoir & Guézé, 1976), in the Libyan coastal waters of Benghazi. A brief description of the collected individuals is presented, and the species' geographical expansion and phylogenetic structure along the southern Mediterranean coasts and the description of the phylogenetic structure in order to explain the route of its arrival to the southern Mediterranean is discussed.

تحليل تطور السلالات الجزيئي لسمكة عنز البحر *Parupeneus forsskali* في البحر الأحمر: نوع غازٍ مؤكَّد ومغفَل على طول الساحل الليبي (جنوب البحر الأبيض المتوسط)

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نؤكد هنا وجود سمكة الماعز البحر الأحمر Parupeneus forsskali (Fourmanoir & Guézé, 1976)، في المياه الساحلية الليبية لبنغازي. يُقدَّم وصف موجز للأفراد التي جُمعت، ويناقش التوسع الجغرافي لهذا النوع وبنيته التطورية على طول سواحل جنوب البحر الأبيض المتوسط، بالإضافة إلى وصف بنيته التطورية لتفسير مسار وصوله إلى جنوب البحر الأبيض المتوسط.

Introduction

Goatfish is the common name used to indicate fishes of the family Mullidae, a group of carnivorous marine fish distributed in the Atlantic, Indian, and Pacific oceans. The family comprises 6genera and about 85 species (Uiblein and McGrouther, 2012; Yamashita *et al*., 2011; Nelson *et al*., 2016). They are recognized as some of the most significant commercial fish in the world (Whitehead *et al.* 1986; Evagelopoulos *et al.* 2020). Today, a total of 6goatfish species are reported in the Mediterranean Sea. The surmullet, Mullus surmuletus (Linnaeus, 1758), and the red mullet, Mullus barbatus (Linnaeus, 1758) (Whitehead *et al*., 1986), are native species widespread across the basin and are historically targeted by demersal fishery(Capapé *et al*., 2018). The West African goatfish, Pseudupeneusprayensis (Cuvier, 1829), is an Atlantic species that entered naturally from Gibraltar and is occasionally recorded in the western and eastern Mediterraneans (Azzouz *et al*., 2011; Evagelopoulos *et al*., 2020). Two Upeneus species have been introduced via the Suez Canal (Lessepsian migrants), namely: goldband goatfish Upeneus moluccensis (Bleeker, 1855) and Por's goatfish Upeneus pori (Ben-Tuvia&Golani, 1989). They are currently common in the Eastern Mediterranean and represent an important fishery resource (EastMed 2010). Finally, the Red Sea goatfish, Parupeneusforsskali (Fourmanoir&Guézé, 1976) (Bariche *et al*., 2015), was first recorded in the early 2000s along the Levantine coasts of Turkey (Çinar *et al*., 2006) and was originally regarded to be a rare species with no commercial value (Bariche *et al*., 2013; Avşar *et al.* 2016). Parupeneusforsskali can be found all over the Indo-Pacific Ocean (Nelson *et al.* 2016; Golani *et al*., 2021). Furthermore, Bogorodsky and Randall (2019) placed the species among the Red Sea endemic fishes, which are also found in the Gulf of Aden, where it is particularly numerous at depths of 30 m (Ben-Tuvia and Kiss, 1988). It is one of the most commonly fished goatfish in the Red Sea, particularly with gill and trammel nets (Farrag *et al*., 2018). Parupeneusforsskali can reach a maximum total length of 28 cm in its native region (Sabrah, 2015), a maximum lifetime of 5 years (Mehanna *et al*., 2018), and a maturity length of 16 cm (Farrag *et al*., 2018). It dwells on sandy bottoms and coral reefs, and like other goatfish species, it hunts for prey by probing the sand with its sensory organs-carrying hyoid barbels. Benthic invertebrates are the major prey of Parupeneus species in their natural regions (Hobson 1974; Wahbeh and Ajiad 1985). The purpose of this paper is to document and confirm the occurrence of population of theP. forsskali along the Libyan coasts as well as the description of the phylogenetic structure in order to explain the route of its arrival to the southern Mediterranean

Materials and Methods

Five specimens of P. forsskali were caught with trammel net by artisanal fishermen off the coast of Benghazi, Libya (32.06°N, 20.03°E, Figure 1) on July 15th, 2021, and three more individuals were caught on July 17th, 2021, with the same gear at a depth of 20 meters, over a sandy bottom. On November 28th, 2022 a total of about 31 specimens were caught with trawler off the coast of Alzwaitina (120 km west of Benghazi). The surmullets (M. surmuletus) accounted for the vast majority of the entire catches. The specimens were maintained on ice and transported together with the remainder of the haul to a local fish market. Specimens of P. forsskali were detected and brought by the first author to the Aquaculture and Fish Biology Lab to be photographed (Figure 2) and analyzed for morphometric-meristic characters. Finally, the specimens were preserved in formalin and deposited at the Museum Collection of the Department of Zoology, Faculty of Science, University of Benghazi under the accession code Z9365. Interviewed fishermen declared that individuals of P. forsskali species have been captured by them on a regular basis since April 2021, at almost the same depth and fishing area using different gears.

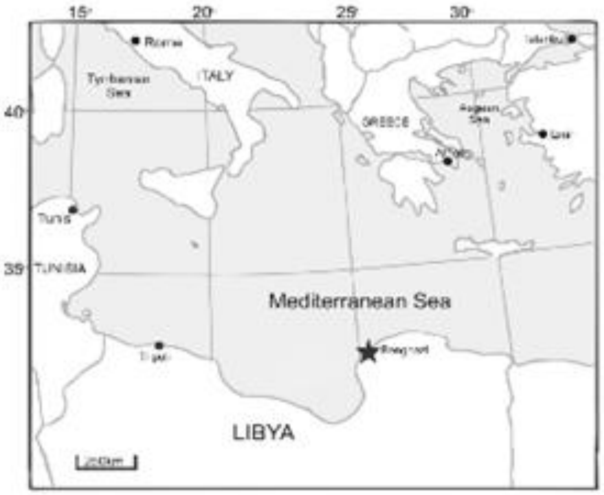

Figure 1. Map of the coast of Benghazi, Libya (southern Mediterranean) indicating the location that specimens of P. forsskali were caught (black star).

Figure 2.Specimen of Parupeneusforsskalifrom Benghazi, Libya (southern Mediterranean). Photo by: H. Elbaraasi.

Genomic DNA isolation

Total genomic DNA was extracted from a portion of the specimen's lateral muscle tissue using the Qiagen DNeasy Blood and Tissue Kit, as directed by the manufacturer (Qiagen, UK).. Thermo Fisher Scientific's NanoDrop TM 1000 Spectrophotometer was used for genomic DNA quantification and quality control.

Mitochondrial gene selection and PCR

The phylogenetic relationship among individuals was performed using the mitochondrial marker: 655 bp portion of cytochrome oxidase I (COI) (Ward *et al.* 2005). COI is the most widely accepted marker in the Barcode of Life Data System (BOLD). COI can exhibit a degree of genetic distance within species. Amplifications of mitochondrial COI genes were performed using the set of primers L1085 (5'-TAAACCAGGATTAGATACCC-3') and L1478 (5'-GAGAGTGACGGGCGATGTGT-3'; 375 bp; Miya and Nishida 2000), as well as FishF1 (5'-TCAACCAACCACAAAGACATTGGCAC-3') and FishR1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'; 655 bp; Ward *et al.* 2005), respectively. The reaction mixtures consisted of 5 µl of 5x MyTaq™ Reaction Buffer (Bioline), 0.5 µl of each primer (20 µM), 0.5 µl of MyTaq™ HS DNA Polymerase (5 U/µl concentration, Bioline), 1 µl of DNA template, and autoclaved Milli-Q water to bring the final volume up to 25 µl. The thermal cycling profiles consisted of an initial denaturation for 3 min at 95 °C, followed by 35 cycles for 30 s at 94 °C , 30 s 48 °C , 45 s at 72 °C, with a final extension of 10 min at 72 °C. The DNA amplification products were checked with 1% agarose gel electrophoresis using 10.000x GelRed® Nucleic Acid Gel Stain (Biotium), 0.4 g of agarose, 40 ml of TBE Buffer for the gel preparation, as well as 2 µl of 5x GelPilot® DNA Loading Dye, (Qiagen), 2 µl of GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific) for the electrophoresis. PCR products were purified using QIAquick® PCR Purification Kit (https://www.qiagen.com/cn/resources/download.aspx?id=e0fab087-ea52-4c16-b79f-c224bf760c39&lang=en).

Sequencing of mitochondrial COI genes

Sanger sequencing (Sanger *et al.* 1977) was carried out on both DNA strands using the same primers as in the PCR protocols, via an Applied Biosystems 3730 DNA Analyzer 48 capillaries (Life Technologies).

Phylogenetic analysis

A majority of the DNA sequence data (343 COI sequences) used in this study was obtained from the Genbank (https://www.ncbi.nlm.nih.gov/nuccore). Many of COI sequences were checked and removed because the low-quality reading. A 506 bp long sequence was trimmed and performed using the MEGA-X v10.0.5 (S Kumar, 2018). In order to constructing a phylogenetic tree. The phylogenetic tree was initially checked using the standard Neighbor-joining method of Saitou and Nei (1987) with the Kimura 2-parameter (K2P) substitution model. To further validate resultsmedian joining network (by Network software v10.2.0.0 and Network publisher 1.2.0.0) and a maximum likelihood (ML) method were used to represent more clear patterning of divergence between species. In all chosen subgroups of fish,bootstrapping for ML method was employed with 1000 replications.

RESULTS & DISCUSSION

The specimens (3 males and 5 females) matched the reported descriptions of P. forsskali (Al-Absy 1988; Ben-Tuvia and Kissil 1988; Khalaf and Disi 1997; Çinar *et al.* 2006; Sonin *et al.* 2013; and Bariche *et al.* 2013; Golani *et al.*, 2021). The overall weight of the fish ranged from 92.4 to 191.4 g and the total length ranged between 20.2 and 26 cm. Meristic counts resulted in: two dorsal fins, the first with seven to eight spines and the second with eight to nine rays (D1, VII - VIII; D2, 8-

9); anal fin with one spine and six rays for all the specimens (A, I+6); pectoral fin with twelve to fourteen rays (P, 12-14); Pelvic fin with one spine and five rays (V, I+5) (Table 1). Each jaw comprised a single row of teeth, with canines found solely on the upper jaw. The dark longitudinal stripe runs from the snout through the eye and down to the base of the second dorsal fin on the specimens, and there is a dark patch on the upper caudal peduncle. The background color of the specimens wasreddish. The first dorsal, pectoral, and pelvic fins were pink, while the anal, caudal, and second dorsal fins were yellowish.

Table 1.The morphometric measurements and meristic counts of Parupeneus forsskali specimensfrom Benghazi, Libya (southern Mediterranean).

Morphometric characters (cm)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Total length	20.2	24.6	24.8	24.2	26	22	24.6	24.9
Forked length	18	22	21.5	21	23.5	19	21.1	21.5
Standard length	16.5	19.5	20.0	19.2	21	17	19.8	20
Pre anal length	9.5	12.5	13.0	12.0	12.6	11.5	12.5	13
Pre dorsal length	5.5	8.0	7.5	8.1	8.9	7	8	8.5
Pre pelvic length	5	6.2	6.0	7	7.2	5.7	6.4	6.9
pre pectoral length	5	6.7	6.5	6.6	7.2	5.9	6.5	6.9
Max body depth	4.5	5.5	5.0	5.8	5.9	4.5	6.0	6
Caudal peduncle depth	1.8	2.2	2.2	2.5	2.4	1.9	2.2	2.3
Head length	5.2	6.3	6.5	6.5	6.6	5.2	6.0	6.8
Preorbital length	3.1	3.5	3.5	3.9	4	3.2	3.6	4
Eye diameter	1.0	1.3	1	1	1	0.9	1.0	1.1
Barbel length	3.0	3.6	3.8	3.8	4.4	3.2	4.0	3.6
inter-orbital width	1.5	2.0	2.0	2.0	2.0	1.8	2.0	2
Total weight	92.4	151.3	165.3	156.4	191.4	106	181	180
Sex	M	M	F	F	F	M	F	F
Meristic data								
First dorsal fin	VIII	VII	VII	VII	VII	VII	VII	VII
Second dorsal fin	8	9	8	9	8	8	8	8
Anal fin	I + 6	I + 6	I + 6	I + 6	I + 6	I + 6	I + 6	I + 6
Pectoral fin	14	13	13	14	14	13	13	13
Pelvic fin	I + 5	I + 5	I + 5	I + 5	I + 5	I + 5	I + 5	I + 5
Lateral line scales	28	30	29	28	28	27	27	28
Gill rackers on first arch (upper limb + lower limb)	**5+18**	**5+18**	**6+17**	**5+17**	**5+17**	**5+18**	**5+17**	**5+17**

Molecular analysis

The investigated P.forsskali sequence of COI mitochondrial gene (with good quality read lengths 506bp) was aligned with 343 Parupeneus sequences (21 different species ;Table S1) to construct ML phylogenetic tree. No deletions, insertions or stop codons were detected in any sequence.

The alignment of investigated P.forsskali sequence is identical to the sequences of all the P.forsskali that were recorded in the Mediterranean and Red Seas' countries; west of Suadi Arabia, Egypt, Israel and Lebanon (figure 3).

ML phylogenetic tree (figure 3) shows three major Parupeneus clades (symbolized into; A, B and C) that most of them were branched and distributed geographically in the Indo-Pacific Ocean. The first clade (A) includes 6 species (Table S1), clade B has just two sequences of P.fraserorum located in South Africa, while 14 species are clustered in clade C which consist of three sub-clades C1,C2 and C3. the latter sub-clade (C3) is branched into C3a (P.cyclostomus) and C3b (P.margaritatus and P.forsskali). phylogeographically speaking, The paraphyletic clade C3b with a high bootstrap support (100%; figure 3) is divided into two sub-branches out of Indo-Pacific ocean; the first one is clustered the P.margaritatus in the Arabian gulf, and the second sub-branch includes the P. forsskali in the Mediterranean and Red Seas.

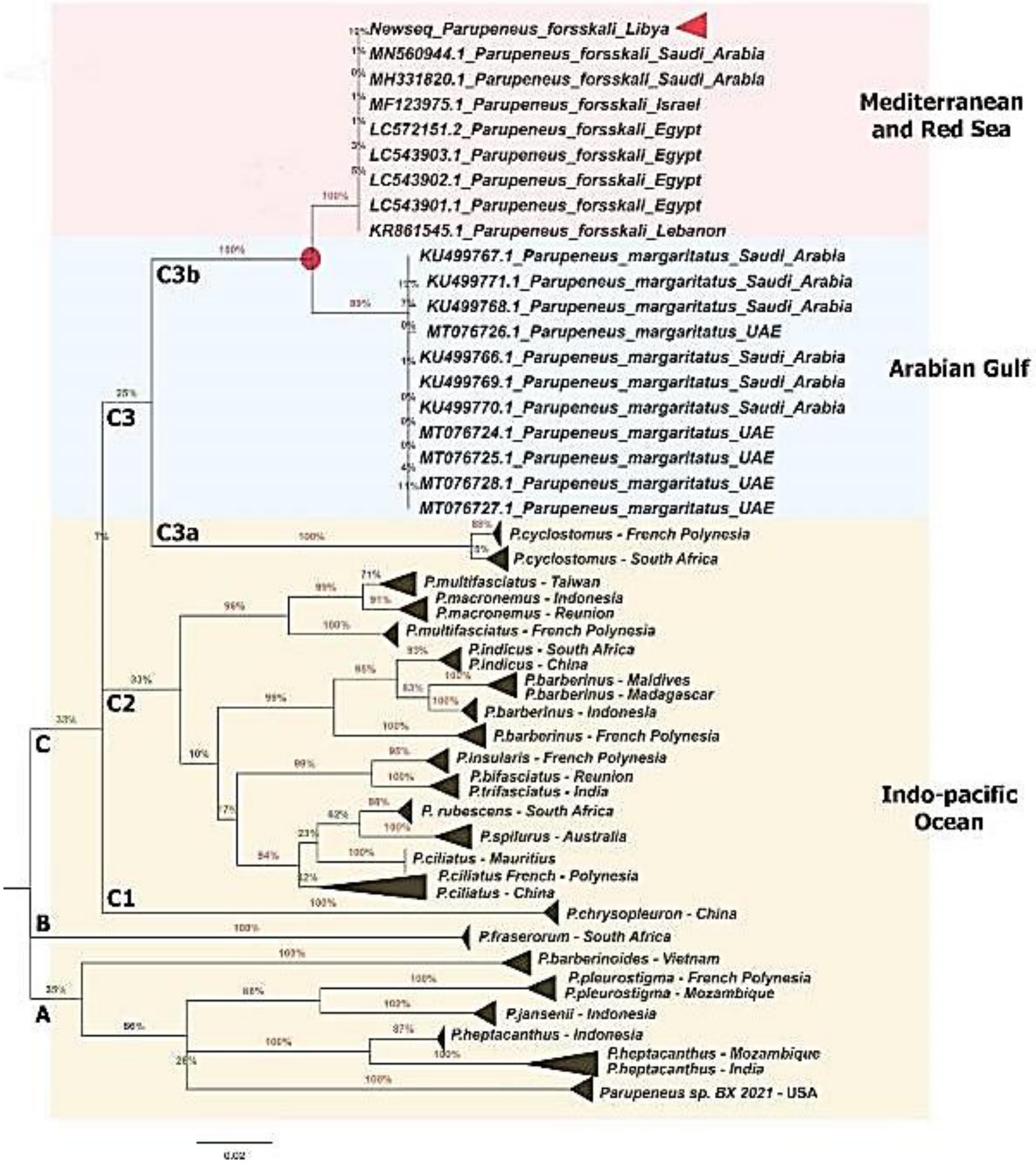

Figure 3. Phylogenetic tree based on the COI gene mitochondrial gene constructed by using the Maximum Likelihood method based on the Kimura 2-parameter model. All the bootstrap values (%) are shown next to the tree nodes. Assumed phylogenetic nomenclatures (A, B and C) were used to namethe clades. A red arrow refer to the Libyan sample that was sequenced in this study, while a red circle is pointed the ancestor node of P.forsskali.

Topologically, all the COI sequences (506bp) in the sub-clade C3 were reconstructed by using the median joining network method (figure 4), and they are rooted to the P.chrysopleuron (NC_029247.1) as a reference sequence. A total 8 informative positions cluster together the Libyan P.forsskali with the rest of P.forsskali present in the Mediterranean and Red Seas. phylogenetically speaking, The median joining network showsthat the P.forsskali in Mediterranean and red seas share the same ancestor (25 informative positions) with the P.margaritatus in the gulf sea. The P.margaritatus sub-branch is derived from their ancestor node (C3b) by 15 informative positions, while P.forsskali has a shortersub-branch with 8 informative positions (4 of informative positions are back mutations; figure S1).

Discussion

The Red Sea goatfishwas first observed in the Mediterranean in 2002 along Turkey's Levantine coast (Çinar *et al.* 2006), and subsequent sightings in Lebanon in 2012 and Israel in 2013 were confirmed. By 2014, the species had spread to Cyprus, Egypt, further west along the Turkish coast, and, more recently, Tunisia in 2016, Greece in 2017 (Evagelopoulos *et al.* 2020), and) in Libya, based on a photo taken by a fisherman, one specimen of P. forskalime measuring 20 cm total length (TL) was captured in Al-Tamimi on December 5, 2018 (Bariche *et al.* 2020)

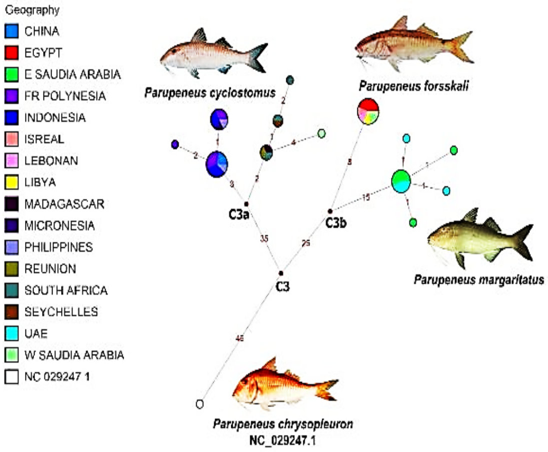

Figure 4. phylogenetic network based on the mitochondrial COI gene (for 43 sequences with read lengths 506pb) constructed by using the Median joining method, and rooted to the P.chrysopleuron (NC_029247.1) as a reference sequence. Each branch was labeled with total number of informative positions in red color.

The discovery of this species in Benghazi, Libya, strengthens the existence of a southern Mediterranean population and expands the known range of the species in the basin. Additionally, the presence of P. forsskali in Libya supports Evagelopoulos *et al.*, 2020 conclusions, that is the species westernmost records were recently made along both the southern and northern Mediterranean coasts, indicating that P. forsskali has recently expanded its range and depth distribution in the Mediterranean.

To the best of our knowledge, these observations of the Red Sea goatfish, P. forsskali, are the first ones confirmed physically presence of the species from the Libyan coast, but also the eighth Mediterranean record. These findings add the Red Sea goatfish to the Libyan ichthyofauna, which currently includes about 300 species (Elbaraasi *et al.*, 2019), twenty of which are of Red Sea origin (Lessepsian migrants). Notably, Libya is regarded as one of the Mediterranean's least explored regions (Coll *et al.*, 2010), its ichthyofauna is poorly known (Quignard and Tomasini, 2000), and information on the occurrence and distribution of exotic species is limited when compared to other sectors of the basin. In this regard, the entire country has been defined as a "nearly 2000 km gap in the southern Mediterranean coastline" (Shakman, 2008).

The Red Sea goatfish is expected to increase its abundance and distribution under the climate warming scenarios (D'Amen and Azzurro, 2020). The species, like the majority of goatfishes, is commercially valuable in its native habitat (Al-Absy 1988; Khalaf and Disi 1997) and could be exploited by the Mediterranean fishery, as Upeneusmoluccensis and U. porihave been in the Levant (Golani 1994), or as in the case of some little known Mediterranean thermophilic species such as Caranx rhonchusGeoffroy Saint-Hilaire, 1817 (Coco *et al.*, 2022). However, the ecological implications of the speciespotential success should be investigated further, especially in terms of competition and niche partitioning with indigenous and non-indigenous goatfishes. We also underline the importance of citizen science projects for monitoring and early detection of non-indigenous fishes (Tiralongo *et al.*, 2020). Additionally, studies of the fish species populations across its non-native range using contemporary molecular genetics methods (Bariche *et al.*, 2015; Stern *et al.* 2019) would provide critical insights into the mechanisms and evolution of P. forsskali's ongoing spread in the Mediterranean (Evagelopoulos *et al.* 2020).

Based on the well-differentiated haplotype networks of P. forsskali mitochondrial DNA, which did not separate into Mediterranean (Libya, Lebanon, and Israel) and Red Sea (Egypt and Saudi Arabia) populations, this may explain the high levels of gene flow between the two seas. This finding is consistent with several studies of invasive species conducted in various locations throughout the

Mediterranean Sea. Shakman *et al.* (2006) and Azzurro *et al* (2008)

Furthermore, no significant difference in genetic differentiation at mitochondrial DNA was found between the Libyan population and the Red population, the Arabian Gulf population, or all Mediterranean Sea populations (P. forsskali). This data is consistent with many studies conducted by Bonhomne *et al* (2003).

The analysis of P. forrsskail populations on the Libyan coast led us to the conclusion that mitochondrial diversity is preserved during the colonization process, as our data show that P. forrsskail mitochondrial DNA diversity was preserved during the colonization of the Libyan coast. As a result, the diversity of mitochondrial DNA in P. forrsskail may reveal a wide range of ecophysiological plasticity.

In conclusion, the multiple records of P. forsskali from Libya support the view that this species could have established a permanent population in the country. Additionally, this study provided a quick overview of the levels of genetic variation that resulted from P. forsskail's adaptation process in the new habitats of Libyan waters. These findings show that these species were not isolated from their source and that the flow of these species is still ongoing, implying that there has been no genetic asphyxia or geographical isolation of these species in their new environment.

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