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DNA sequencing reveal presence of the Lessepsian true limpet *Cellana rota* in the Syrian coasts of the Mediterranean

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Abstract

Many Red Sea species have entered the Eastern Mediterranean since the opening of the Suez Canal. Here, we report the presence of well-established populations of the Lessepsian true limpet *Cellana rota* (Gmelin, 1791) on the Syrian coasts of the Eastern Mediterranean. The collected specimens exhibited four different morphs and were 98.97-100% identical in Cytochrome Oxidase I (COI) sequence with *C. rota*. Six unique COI haplotypes, having 98.93-99.86% similarity, were noticed with variation at one to eight sites. The observed genetic divergence and dissimilarity were possibly low to assign four morphs to different species. In consistence with the phylogenetic data, our haplotype network exhibited that some alien individuals shared similar COI haplotype with those previously reported for indigenous populations from Eilat, Sinai and Hurghada, indicating the common origin from a single source in the Red Sea. In the present study, some implications of our findings for the systematics of *Cellana* species are also discussed. Based on our results, there is still some nomenclature and taxonomic confusion within *Cellana* species. Further multifaceted studies should be conducted to evaluate the taxonomy of this morphologically variable group. However, our data presented here could be a reliable reference for future investigations.

Keywords: *Cellana*; Eastern Mediterranean; Lessepsian species; Red Sea.

Introduction

The Mediterranean Sea is known as a biodiversity hotspot with high species richness and endemism (Lejeusne *et al.,* 2010). It has also been considered as one of the most impacted marine ecoregions primarily due to human activities (Korpinen *et al*., 2019). Since 1869, with the opening of the Suez Canal, many Indo-Pacific species have established prosperous populations in the Eastern Mediterranean (Galanidi *et al*., 2023), a process known as Lessepsian invasion. By December 2021, out of 1001 alien species in the Mediterranean, 759 were reported to be established (Zenetos *et al.*, 2022a, b). Here, we report the first record of an alien patellogastropod on the Syrian coasts of the Mediterranean.

Patellogastropods (true limpets) are known as some of the most ubiquitous molluscs in marine inter- and subtidal communities which perform critical ecological functions on hard-substrate shores through the world (Virgin & Schiel, 2023). Based on the latest classification, Patellogastropoda includes eight families: Eoacmaeidae, Pectinodontidae, Acmaeidae, Lottidae, Patellidae, Neolepetopsidae, Lepetidae and Nacellidae (Bouchet *et al.,* 2017). The family Nacellidae comprises Antarctic and sub-Antarctic *Nacella* and temperate/tropical *Cellana* (Nakano & Sasaki, 2011). *Cellana* includes many species and subspecies with obviously disjoint distribution across the Indo-Pacific (Powell, 1973; Gonzalez-Wevar *et al.,* 2017). *Cellana* species are known to have phenotypic plasticity with high variability in shell morphology, related to ecotype attributes of peculiar microhabitats or host associations (Joseph *et al.,* 2016a; Echeverry *et al.,* 2020). This has led to misidentification of some species and taxonomic confusion (Lindberg, 2008; Nakano & Sasaki, 2011). Moreover, adult limpets surprisingly differ from the immature forms, causing more complications for species identification. Recent molecular studies, however, have challenged the taxonomic designations of the *Cellana* limpets (Nakano & Ozawa, 2004; Zafar *et al.,* 2015).

Cellana limpets have moderate dispersion capacity but some species are broadly distributed in many regions worldwide (Gonzalez-Wevar *et al.,* 2017). The precise distribution of *C. rota* is unclear due to confusion with some other species or subspecies of *Cellana*, though its native range is believed to be the Red Sea and Indian

Ocean (Christiaens, 1987; Reisser, 2012). This species has been reported as an ~~alien~~ species in the Akko coast (Israel) (Christiaens, 1967), Egypt (Giannuzzi-Savelli *et al.*, 1994), Saronikos Gulf (Greece) (Fontoulakis & Sabelli, 1999), Gulf of Gabès (Tunisia), Libya (Zaouali *et al*., 2007), Albania (Dhora & Dhora, 2020) and Lebanon (Crocetta *et al*., 2020). The species is said to have spread along the Israel coasts by 2000 (Galil, 2007). However, these reports were mainly based on morphology and molecular studies. To date the presence of *C. rota* has only been confirmed on the Red Sea coasts, including Hurghada (Nakano & Ozawa, 2007; Gonzalez-Wevar *et al.,* 2010; Bird *et al.,* 2011), Eilat (Schnytzer *et al.,* 2018) and Sinai (Bird *et al.,* 2011).

In the present study, we describe shell morphological attributes and assess the probable affinities of the alien individuals of *C. rota* from the Eastern Mediterranean using COI partial sequencing. Our study was not designed as a taxonomic research. However, considering several misidentifications and the taxonomic confusion surrounding the *Cellana* complex, we reconstructed the phylogenetic tree and briefly discuss on systematics of *Cellana* species using a dataset generated with new and previously recorded COI sequences of the genus.

Materials and Methods

Cellana limpets are widely distributed through rocky intertidal areas of the Syrian coasts of the Mediterranean. We collected 60 limpet individuals with different shell exterior views from Al-ahlam Coast, south of Tartous (34.86305° N, 35.88554° E) and Ras Al-bassit Coast, north of Latakia (35.86583° N, 35.86661° E) during September 2021 (Fig. 1a, c, d). The specimens from each region were separately preserved in ethanol (96%). For shell morphological analysis, the limpet's body (30 individuals from each region) was thoroughly removed from the shell. Shells were cleaned and dried. The specimens were then classified in four groups (A, B, C and D) based on their shell exterior views. Shell biometric parameters, including height (H), length (L) and width (W), were evaluated to the nearest 0.1 mm . The flatness (I_F = ln L / H) and oblongness (I_O = L / W) indices were also calculated.

For molecular analysis, DNA was extracted from foot musclesof alcohol-preserved samples (14 individuals) through a standard high-salt method (Sambrook *et al.,* 1989) with a slight modification. The quality and concentration of DNA were assessed by agarose gel electrophoresis (1%) and a Biophotometer (Eppendorf, Germany), respectively. The primers of LCO22me2 (5'-GGTCAACAAAYCATAARGATATTGG-3′) and HCO700dy2 (5'-TCAGGGTGACCAAAAAAYCA-3′) were applied to amplify cytochrome c oxidase I (COI) partial sequence (Walker *et al.,* 2006; 2007). A 25 µL reaction mixture was prepared using 1µL DNA (20-160 ng/µL), Taq 2x Master Mix Red-$MgCl_2$: 1.5 mM (15 µL) (Amplicon), primers (1 µL) and ddH_2O (7 µL). Polymerase chain reaction included 4 min at 94°C, 40 cycles at 94°C (30 s), 50°C (40 s) and 72°C (60 S), followed by 10 min at 72°C. The obtained products were then checked through electrophoresis in

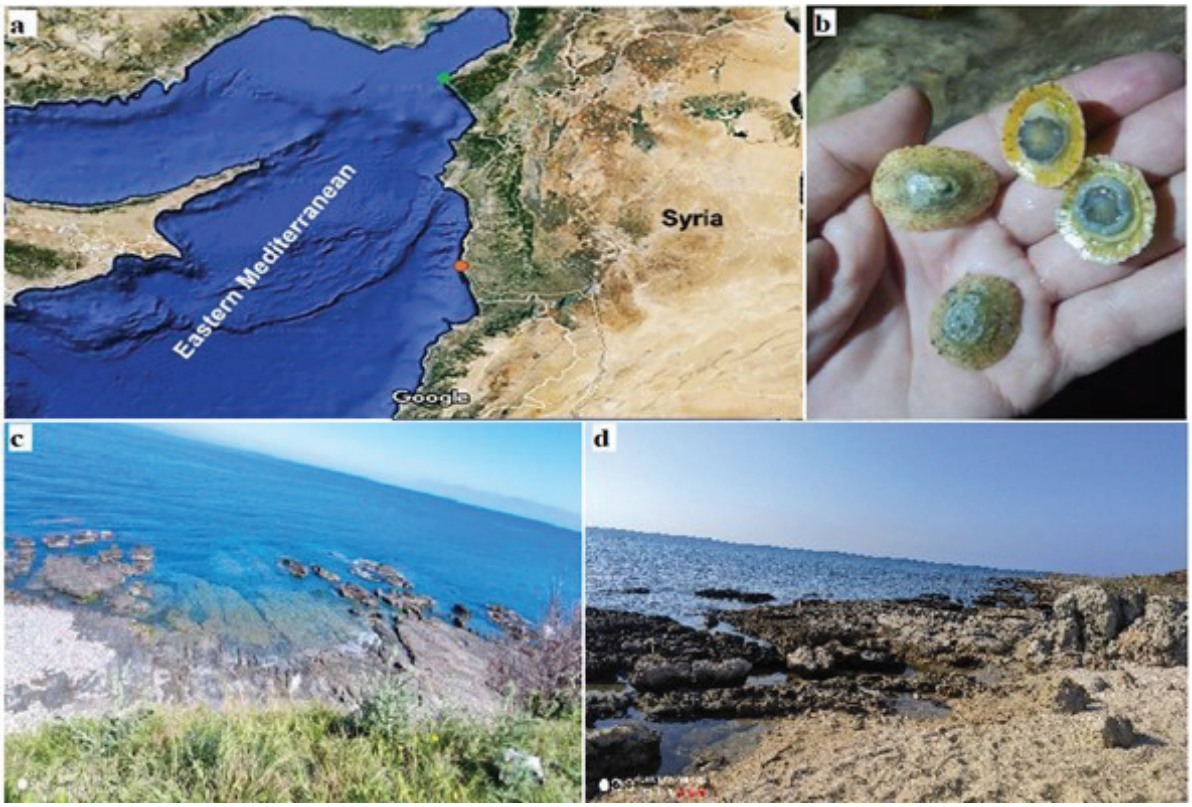

Fig. 1: Cellana rota: a) the study area's location: Green and red circles represent Ras Al-bassit and Al-ahlam coasts, respectively; b) Live samples of the limpets; c and d) Location view of the Al-ahlam and Ras Al-basit coasts, respectively (9th and 11th September 2021).

1.5% agarose gel in 1X Tris-borate-EDTA. High quality amplicons were finally sequenced by an ABI 3730XL automatic sequencer (Applied Biosystems, 3730/3730xl DNA Analyzers Sequencing, Bioneer, Korea).

A sequence alignment editor in the program BioEdit 7.2.5 (Hall, 1999) was used to assess the sequences. All the previously recorded COI sequences of *Cellana* species were extracted from the national center for biotechnology information's GenBank. A data set including 14 new and 1336 previously recorded sequences was prepared. Aligning Multiple sequence by ClustalW was done in the software BioEdit. The sequences were trimmed, and a 585-bp fragment was obtained. Identical sequences were deleted using FaBox (1.41) (Villesen, 2007). The Phylogenetic tree was then constructed using 318 unique sequences. *Nacella concinna* (Strebel, 1908) (MN941970 and MN941962) was used as the outgroup. Phylogenetic relationships among the studied taxa were reconstructed using the Bayesian inference method implemented in the MrBayes program (3.2.2) (Huelsenbeck & Ronquist, 2001). The best-fitting models for substitution of nucleotide on the basis of information criterion (Akaike, 1973) were assessed through MrModelTest (3.7) (Posada & Crandall, 1998) in the software PAUP (4.0) (Swofford, 2003). Two parallel runs were independently conducted. Each run contained one cold and three heated Metropolis coupled Markov Chain Monte Carlo. The program was conducted for 10 million generations and sampled once every 10,000 generations (a 20% burn-in fraction). The phylogenetic tree was finally visualized by the software FigTree (1.4.2) (Rambaut, 2008). Genetic divergences on the basis of *P*-distances were determined through the software MEGA (6.0) (Tamura *et al*., 2013). A median joining network (MJN) was also created using all COI sequences of the species *C. rota* (14 new and 16 previously reported sequences) by the software PopArt (1.7) (Leigh & Bryant, 2015) for assessing the relationships between the *C. rota* haplotypes.

Results

First record of the alien limpet Cellana rota *in Syria*

Well-established populations of a non-native limpet were detected on the Syrian coasts of the Mediterranean. The fringe of the respiratory tentacle in the collected limpets was interrupted by the head, indicating the genus *Cellana*. As different shell morphologies were observed between the collected specimens, the molecular study was carried out to determine the species. Based on genetic data, *C. rota* was recorded from Al-ahlam and Ras Al-bassit coasts, Syria.

Morphological data

Morphological studies based on shell exterior of the collected samples showed four morphs, here named as morph A, B, C and D (Fig. 2). Details of the shell characteristics are presented in Table 1. The shells were platiform but there were differences on the exterior views. Morphometric measurements of the limpets are also given in Table 2. The largest and smallest limpets were 33.11 and 21.7 mm long, respectively. The flatness and oblongness indices ranged from 0.96 to 1.67 and 1.12 to 1.31, respectively (Table 2).

Molecular data

The genus Cellana

A total of 318 unique haplotypes were obtained within 1350 COI sequences in the population data set of *Cellana* species (14 new and 1336 previously reported sequences). Alignment of the sequences contained 585 nucleotide positions. As expected for a coding region, no stop codon or indel were noticed and there was no significant saturation in the data set. The phylogenetic tree was reconstructed

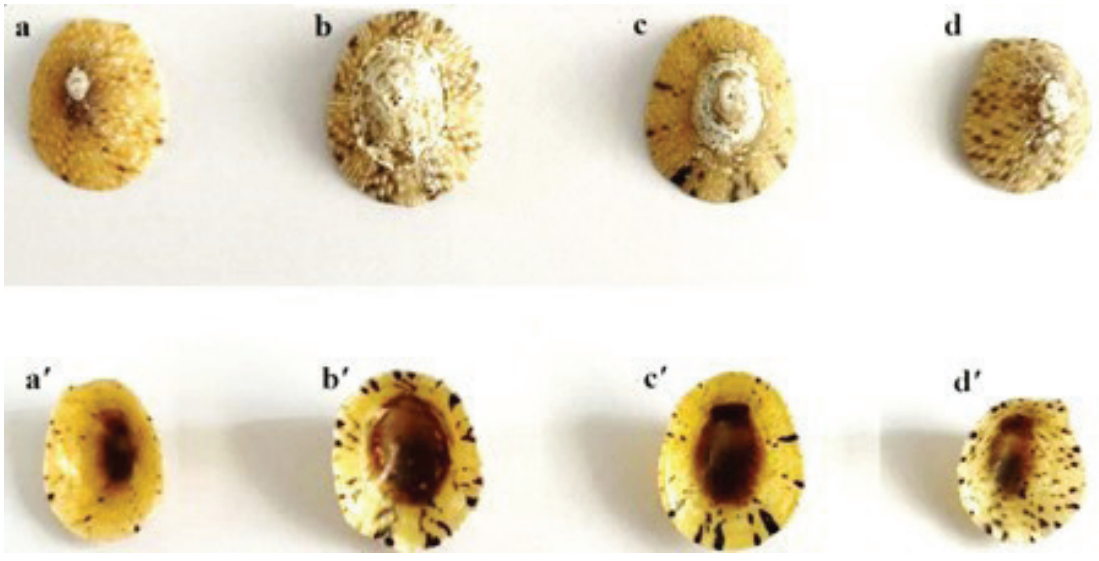

Fig. 2: *Cellana rota*: a-d and a′-d′ represent exterior and interior view of the shells, respectively.

Table 1. Shell characteristics for different morphs of *Cellana rota* in Syrian coasts of the Mediterranean.

Morph	Shape	Exterior view of shell	Interior view of shell	Muscle scar coloration
A	Round to slightly elliptic/rather flat, slightly elevated/ sharp pointed apex at anterior	Pale orangish brown with white radiating dashed lines on the entire surface and two dark dotted lines on the posterior part	Yellow with shine	Brown
B	Round to oblong ovate / moderately elevated/ apex central to slightly anterior	Pale brown with dominate white radiating dashed lines on the entire surface and alternate dark radial dashed lines (more complete on the posterior part)	Creamish pale yellow	Brown with a gray tinge
C	Round to oblong ovate / moderately elevated/sharp pointed apex central to slightly anterior	Light brown with two double and some single dark lines on the posterior and other parts, respectively	Light yellow with shine	Dark brown
D	Round to elliptic/slightly elevated/sharp pointed apex sub-central (rather anterior)	Pale brown with dominated radiating dashed lines on the entire surface and dark radiating dotted lines on the shell	Creamish yellow with glaze	Brown with a gray tinge

Table 2. Morphometric features of *Cellana rota* on Syrian coasts of the Mediterranean. I_O = oblongness index; I_F= flatness index.

		Morph-A	Morph-B	Morph-C	Morph-D
Length	Min-Max	21.7-26.46	27.79-33.11	22.18-30.66	22.19-31.28
	Mean	25.08	29.94	26.47	25.59
Width	Min-Max	17.1-21.94	22.0-27.32	19.68-25.88	19.8-25.45
	mean	19.54	24.04	22.04	21.37
Height	Min-Max	4.34-6.81	9.76-10.88	7.32-9.82	6.23-9.18
	mean	5.92	10.43	8.6	7.38
I_O	Mean-Max	1.17-1.31	1.21-1.28	1.13-1.25	1.12-1.26
	Mean	1.2	1.25	1.22	1.2
I_F	Mean-Max	1.21-1.67	0.96-1.08	1.08-1.17	1.17-1.33
	Mean	1.43	1.03	1.12	1.25

under the TIM+I+G model (Fig. 3), Phylogenetic reconstruction using COI sequences represented 33 well-supported lineages (here named as A-AG, Fig. 3) within the genus *Cellana*, each of them could correspond to a species based on the genetic divergences observed among them (Table 3); A: *C. testudinaria* (Linnaeus, 1758); B: *C. solida* (Blainville, 1825); C: *C. tramoserica* (Holten, 1802); D: *C.* strigilis (Hombron & Jacquinot, 1841); E: *C. denticulata* (Martyn, 1784); F: *C. radians* (Gmelin, 1791); G: *C. flava* (Hutton, 1873); H: *C. ardosiaea* (Hombron & Jacquinot, 1841); I: *C. stellifera* (Gmelin, 1791); J-M: previously termed the *C.* toreuma (Reeve, 1854) complex by Wang *et al.* (2016); N-R: previously termed the *C. radiata* (Born, 1778) complex by Nakano & Ozawa (2007); S: *C. karachiensis* (Winckworth, 1930); T: previously assigned to *C. radiata capensis* (Gmelin, 1791); U: *C. rota* ; V: *Cellana* complex; W: *C. dira* (Reeve, 1855); X: *C. tatiensis* (Röding, 1798); Y: *C. pricei* (Powell, 1973); Z: *C. ornata* (Dillwyn, 1817); AA: *C. nigrolineata* (Reeve, 1854); AB-AC: here named *C. grata* (Gould, 1859) complex; AD: *C. mazatlanica* (Sowerby, 1839); AE: *C. exarata* (Reeve, 1854); AF: *C. talcosa* (Gould, 1846) and AG: *C. sandwicensis* (Pease, 1861). The mean values of *P*-distances between the 33 lineages are given in Table 3. The lowest divergence was observed among lineages G (*C. flava*) and H (*C. ardosiaea*), while the highest was among lineages I (*C. stellifera*) and AC (*C. grata*).

Cellana rota

Fourteen COI sequences were obtained from the Syrian samples and deposited to the NCBI's GenBank (Table 4). These specimens exhibited six unique haplotypes (U1-U6). The morph-haplotype combinations of all the studied samples are presented in Table 4. Morphs A and C exhibited the haplotypes U_2 and U_1, respectively. Morph B represented U_6 and U_4 and morph D exhibited U_3- U_5 haplotypes (Table 4).

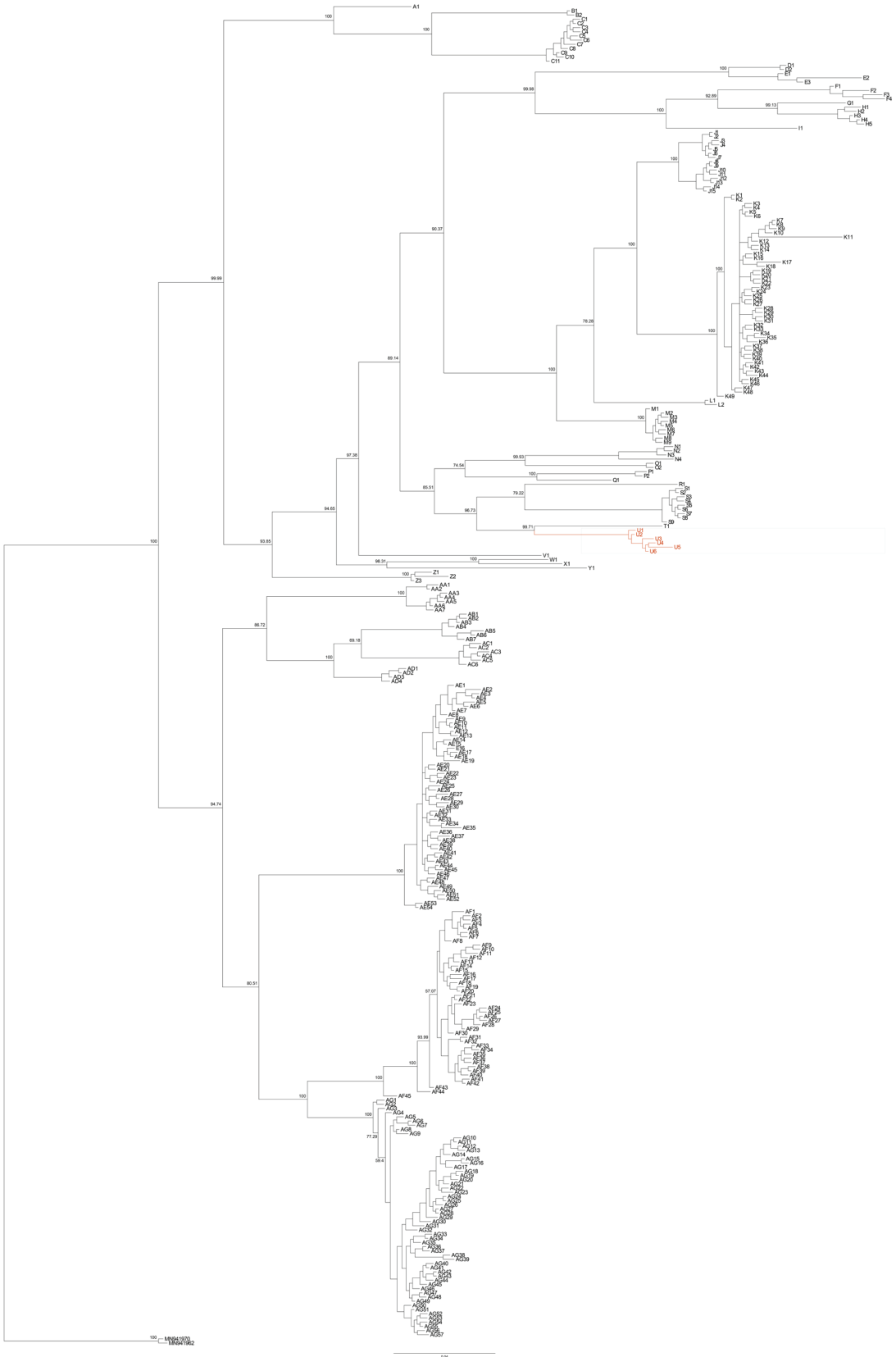

Fig. 3: The Bayesian phylogenetic tree on the basis of 318 unique COI sequences of *Cellana* spp.: A1: *C. testudinaria*; B1-B2: *C. solida*; C1-C11: *C. tramoserica*; D1-D2: *C. strigilis*; E1-E3: *C. denticulate*; F1-F4: *C. radians*; G1: *C. flava*; H1-H5: *C. arosiaea*; I1: *C. stellifera*; J1-J15, K1-K49, L1-L2 and M1-M9: previously termed the *C. toreuma* complex by Wang *et al.* (2016); N1-N4, O1-O2, P1-P2, Q1 and R1: previously termed the *C. radiata* complex by Nakano & Ozawa (2007); S1-S9: *C. karachiensis*; T1: previously assigned to *C. radiata capensis*; U1-U6: *C. rota*; V1: *Cellana* sp.; W1: *C. dira*; X1: *C. tatiensis*; Y1: *C. pricei*; Z1-Z3: *C. ornata*; AA1-AA7: *C. nigrolineata*; AB1-AB7 and AC1-AC6: here named *C. grata* complex; AD1-AD4: *C. mazatlanica*; AE1-AE54: *C. exarata*; AF1-AF45: *C. talcosa* and AG1-AG57: *C. sandwicensis*; Two sequences of *Nacella concinna* (MN941970 and MN941962) are the outgroups. The numbers on branches represent the bootstrap support values. The scale bar indicates the branch lengths.

Table 3. Genetic divergences based on mean uncorrected *P*-distance between the *Cellana* spp. lineages.

	AG	AF	AE	AA	AB	AC	AD	A	D	E	J	K	W	X	Z	B	F	C	N	R	I	Y	V	P	S	M	G	L	O	H	T	Q
U	15.3	15.3	16.2	15.9	16.8	17.1	15.5	14.4	16.6	17.0	13.5	13.6	12.8	12.2	14.3	15.4	16.2	16.7	12.6	10.7	15.4	12.7	12.3	11.6	9.7	12.8	14.5	13.2	12.0	15.0	8.4	10.3
Q	14.0	13.6	15.3	15.6	15.5	15.2	13.9	12.1	14.3	15.8	12.9	13.8	12.3	13.0	12.2	14.6	15.0	15.1	9.8	10.3	14.9	12.6	12.8	6.6	11.9	11.1	13.2	12.1	9.6	13.6	11.1	
T	14.5	15.6	16.4	15.6	16.6	15.3	15.1	14.2	16.7	17.6	14.7	14.8	13.2	13.2	11.9	16.7	15.9	15.7	11.7	11.5	16.8	13.0	12.1	12.9	11.6	14.0	15.6	13.7	10.9	16.4		
H	17.0	16.7	19.0	17.9	18.6	18.2	16.5	15.0	14.9	14.7	17.0	16.8	16.8	16.8	15.6	14.9	8.4	15.2	14.2	14.6	9.6	14.9	14.9	15.1	17.7	15.6	5.3	16.4	16.2			
O	14.1	16.0	15.8	14.0	16.5	15.8	14.7	12.4	16.0	17.5	13.0	13.6	12.6	12.0	12.7	15.6	17.8	15.3	9.4	12.2	17.1	11.5	13.0	11.0	12.7	13.6	15.6	12.8				
L	15.8	15.1	16.3	16.8	17.0	16.8	15.7	14.7	17.4	18.4	7.7	8.3	13.2	13.5	12.8	16.5	17.4	17.6	13.9	13.8	15.7	13.5	13.7	13.8	13.0	8.3	15.9					
G	16.9	16.8	17.9	17.9	19.2	19.2	17.1	14.4	14.6	15.4	17.2	16.5	16.4	16.2	15.8	15.5	8.6	15.1	14.7	14.4	9.2	14.7	14.2	14.6	15.6	15						
M	16.3	16.2	17.3	17.6	17.5	17.3	15.1	15.5	16.0	16.9	7.1	8.2	12	13.6	14.1	15.6	15.7	16.8	13.2	13.5	14.2	14.6	15.2	13.3	14.2							
S	16.6	16.0	16.5	16.5	17.4	17.3	16.1	13.5	17.1	17.2	13.9	14.5	13.7	14.7	14.3	14.8	16.3	15.5	12.7	10.7	15.6	13.7	14.0	11.1								
P	15.5	15.1	16.0	15.6	16.8	16.9	15.7	13.6	15.4	16.2	14.5	13.7	13.1	13.4	14.1	15.9	15.4	16.3	11.0	11.9	14.8	13.6	12.9									
V	14.6	15.2	15.7	16.1	15.3	15.3	14.5	15.2	16.7	17.2	14.6	14.1	12.1	12.6	13.0	16.8	15.1	17.1	12.9	13.5	16.6	13.2										
Y	17.0	16.5	17.0	16.5	17.5	17.0	14.9	14.9	17.5	19.0	15.2	15.4	12.5	12.5	13.8	15.0	17.0	16.0	13.8	13.5	16.2											
I	18.0	17.2	18.7	19.2	19.9	20.0	18.3	16.1	15.8	15.9	15.3	15.7	15.7	16.2	17.1	16.8	10.1	17.2	14.7	14.9												
R	13.3	13.2	15.3	16.0	16.5	17.6	14.3	13.0	17.0	17.7	14.6	14.6	12.6	13.0	13.1	15.0	16.1	15.6	10.7													
N	13.1	13.6	15.9	16.2	15.6	16.5	14.2	13.6	15.0	15.3	13.5	12.9	12.8	12.3	13.4	16.8	14.8	15.3														
C	16.9	17.0	16.8	16.2	18.0	17.3	16.1	10.3	14.1	14.5	17.9	16.8	15.4	15.6	13.8	9.3	14.7															
F	18.3	18.4	18.7	18.2	19.2	19.2	16.9	14.7	15.9	15.8	16.9	16.6	17.3	18.5	16.8	15.7																
B	16.3	16.7	15.8	16.1	17.0	16.5	15.6	10.3	16.4	16.6	17.3	17.2	15.8	16.0	14.7																	
Z	12.2	12.8	14.2	13.1	13.9	12.5	11.6	12.2	15.8	16.6	13.0	13.7	12.0	13.1																		
X	14.1	14.3	15.8	14.4	16.1	15.7	13.7	14.2	15.6	17.1	13.7	13.6	5.6																			
W	14.8	14.8	16.0	16.0	15.6	16.6	13.6	13.7	15.1	16.8	11.9	12.1																				
K	15.6	16.3	17.6	18.7	16.9	18.2	15.9	15.4	16.6	17.4	5.3																					
J	15.0	15.9	15.9	16.4	16.6	16.8	14.3	15.6	17.1	17.8																						
E	16.8	17.3	19.5	19.5	18.9	17.1	17.6	15.6	4.7																							
D	15.9	16.7	18.8	17.6	17.8	16.3	16.7	15.3																								
A	12.8	12.8	13.1	14.1	15.5	13.6	12.3																									
AD	10.0	10.0	10.4	9.8	5.7	6.7																										
AC	10.7	11.8	11.2	10.0	7.3																											
AB	10.6	11.7	12.3	11.4																												
AA	10.6	12.1	10.9																													
AE	9.9	10.2																														
AF	6.9																															

Table 4. Morph-haplotype combinations of *Cellana rota* in Syrian Coasts.

Sampling site	Accession no.	Haplotype	Morph-Haplotype
	OR512887	U_2	A-U_2
	OR512888	U_2	A- U_2
	OR512889	U_2	A- U_2
	OR512890	U_6	B-U_6
	OR512897	U_4	B-U_4
Al-Ahlam	OR512891	U_1	C- U_1
	OR512892	U_1	C-U_1
	OR512893	U_1	C-U_1
	OR512894	U_3	D-U_3
	OR512898	U_4	D-U_4
	OR512895	U_3	D-U_3
	OR512896	U_3	D-U_3
	OR512899	U_5	D-U_5
Al-Basit	OR512900	U_5	D-U_5

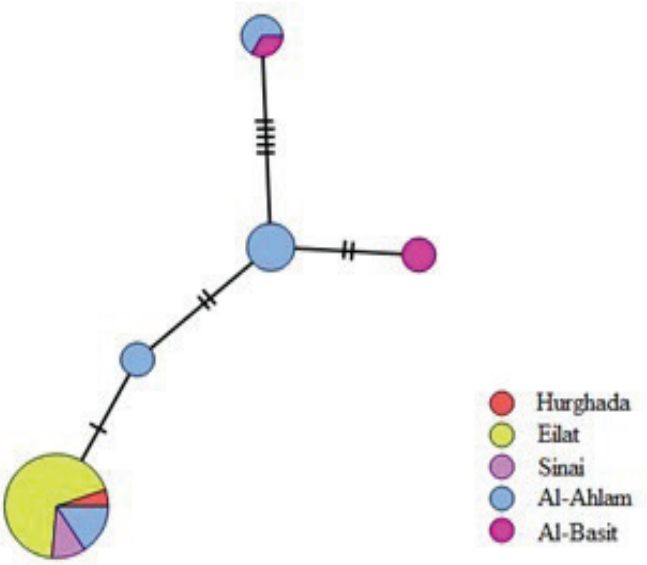

Fig. 4: Median joining network of *Cellana rota* (No. of COI sequences=30; 14 new sequences in this study and 16 previously reported sequences from the NCBI's GenBank); Short lines between the haplotypes represent the number of mutations.

The Syrian specimens were clustered together with previously reported individuals of *C. rota* from the Red Sea coasts of Israel and Egypt (lineage U; Hap U_1-U_6) with strong bootstrap support (above 99%) (Fig. 3). Some of our samples represented the same haplotype (Hap U_2) as recorded for indigenous populations in Eilat, Sinai (Israel) and Hurghada (Egypt). The intraspecific mean distance within this lineage was 0.6%. The analysis indicated that *C. rota* is more closely related to the lineage previously identified as *C. radiata capensis* from Japan (Lineage T). The highest distance between the lineage comprising the Syrian specimens and the others was also 17.1% (*C. grata*, lineage AC) (Table 3). In studying intraspecific diversity, low dissimilarities were noticed among the unique haplotypes: 98.93% (between U_3-U_6; U_3-U_5), 99.24% (between U_3-U_1; U_3-U_4), 99.40% (U_2-U_1; U_6-U_5), 99.55% (U_2-U_4; U_6-U_1), 99.71% (U_6-U_4; U_1-U_5; U_4-U_5) and 99.86% (U_2-U_6; U_1-U_4) similarity, corresponding to nucleotide diversity at seven, five, four, three, two and one site respectively.

The MJN was also constructed using 30 COI haplotypes of *C. rota* (Fig. 4). Consistent with our phylogenetic data, the MJN indicated different haplotypes for the Syrian specimens. Some Syrian individuals were lumped together with those previously reported from the Red Sea (Hurghada, Sinai and Eilat) into a same haplotype (Hap U_2). This was at the centre of the network probably applying to the ancestral haplotype and weakly differentiated from the Hap U_6 (1 substitution). There were also two mutation sites between Hap U_6 and Hap U_4. The individuals from Al-Basit belonged to two different haplotypes; one specimen was clustered with the haplotype U1 from Al-Ahlam (Hap U_3; the most divergent haplotype), and other individuals showed a single haplotype (Hap U_5).

Discussion

There is a conflict among the molecular and morphological studies on true limpets and the current taxonomic status is still under discussion. During recent decades, molecular studies have recognized new limpet species, declined/confirmed species validity, and elucidated no-

menclature confusion between species with morphological similarity (Crummett & Eernisse, 2007; Joseph *et al.,* 2016 a,b). Although molecular markers have started to elucidate phylogenetic relationships of *Cellana* species (Nakano & Ozawa, 2007; Nakano & Espinosa, 2010; Dong *et al.*, 2012; Wang *et al.,* 2016) there are still problems in the case of morphological identification and nomenclature. Here, we analysed all COI haplotypes within the genus *Cellana*, containing previously reported and new sequences. According to our molecular results, each clade in the Bayesian tree could correspond to a distinct species, indicating at least 33 species-level lineages with some geographically related haplogroups. As previously illustrated by Wang *et al.* (2016), the specimens characterized as *C. toreuma* should be considered as a complex since these fell in four well-separated clades with significant difference in their COI sequences. The clades comprising *C. toreuma* from Japan and China (K-M) differed by 8.2-8.3% in their sequences (more than 16 times the maximum divergence observed within them (0.5%); 10× threshold rule; Hebert *et al.,* 2004), indicating each clade could be assigned to a separate species. In the case of the lineage J, including individuals previously characterized as *C. radiata enneagona* (Reeve, 1854) by Nakano *et al.* (2009) and *C. toreuma* from Southeast Asia, there were two sub clades with 1.8% divergence between them that could correspond to two sub-species in this clade. A sister relationship was previously reported among *C. radiata enneagona* from Bonin Islands and *C. toreuma* from Japan (Nakano & Ozawa, 2007; Nakano *et al.,* 2009) and it was declared that *C. enneagona* could be considered at specific level (Nakano & Ozawa, 2007). However, consistent with the previous report by Wang *et al.* (2016), our phylogenetic data revealed that these individuals were separated from the others characterized by Powell (1973) as a sub-species of *C. radiata*. Based on Powell (1973), *C. radiata* comprises a complex including four sub-species of *C. radiata radiata* (Born, 1778), *C. radiata orientalis* (Pilsbry, 1891), *C. radiata enneagona* and *C. radiata capensis*. Further molecular studies have also resolved six distinct lineages, including four nominal subspecies and two unnamed lineages (Nakano & Ozawa, 2007; Nakano *et al.,* 2009). Our study indicated that the individuals previously identified as different sub-species of *C. radiata* fell in five well-supported clades (N-R) (Fig. 3). Hebert *et al*. (2004) recommended a "ten times threshold rule" for identification of likely cases of species-level divergence (10 times greater genetic divergence between than within species). The N-R clades differed by 6.6% (between the clades P and Q) to 12.2% (between the clades O and R) in their COI sequences that could be assigned to five separate species based on the "ten times threshold rule". In this case, the individual identified as *C. radiata orientalis* from Japan was distinct from an individual reported from the Indo-Pacific (6.6% divergence), indicating *C. radiata orientalis* is also a species complex. This, together with previous reports, underlines that the taxonomic status of nominal *C. radiata* needs to be revised and may deserve specific recognition. In the case of ~~the~~ lineage T, the individual previously assigned to *C. radiata capensis* was separated from the other taxa within the *C. radiata* complex. There was also 7.3% difference between ~~the~~ lineages AB and AC comprising the individuals recognized to be *C. grata*. We therefore considered these individuals as *C. grata* complex. In the case of ~~the~~ lineage AF, the specimen previously assigned to *C. talcosa* (EF620967; Hap AF45) exhibited 2.4% difference with other individuals of *C. talcosa*, suggesting this specimen could be considered as a sub-species of *C. talcosa*. Overall, further studies and extensive sampling seem necessary for a complete taxonomic revision of the individuals within the genus *Cellana* and for elucidating the relationships between them. However, precious steps have been taken in the right direction by Goldstein *et al.* (2006), Nakano & Ozawa (2007), Nakano & Sasaki (2011), Wang *et al.* (2016) and Nakashima *et al.* (2021).

COI sequences of the Syrian specimens matched with those of *C. rota*. In the case of *C. rota*, there is a confusion with *C. eucosmia* (Pilsbry, 1892), which some authors have referred to as a junior synonym of *C. rota* (Zuschin *et al.,* 2009; Schnytzer *et al.,* 2018). *Cellana rota* also showed a close relationship with an individual previously assigned to *C. radiata capensis* (lineage T) and the individuals of *C. karachiensis* (lineage S), compared to other *Cellana* species (Table 3). The Syrian specimens also exhibited six different COI haplotypes with low diversity and dissimilarity (98.93-99.86% similarity) between them.

Historical taxonomic studies have applied shell morphology but high level of plasticity in shell morphology of true limpets has resulted in taxonomic confusion. In fact, using shell morphology on its own could be highly misleading while inclusion of morphology into molecular data can produce better resolved phylogenetic trees (Teske *et al*., 2007). In this regard, molecular taxonomy using mtDNA COI sequencing was established to complement morphological studies and solve taxonomic misidentification. As an alien species, *C. rota* was previously reported from the Mediterranean coasts of Egypt, Israel, Greece, Tunisia, Libya, Albania and Lebanon), but these records were mainly based on morphological studies. In fact, Crocetta *et al.* (2017) rejected the Greek record as unsubstantiated. Considering morphological similarity and confusion with other species or subspecies, together with the morph diversity observed in the present study, it is difficult to assess the actual distribution and thus the previous records should be verified using molecular markers. *Cellana rota* has been reported from Pakistan coasts, but phylogenetic relationships between three morphs of the genus *Cellana* (assigned to different species of *C. rota*, *C. radiata* and *Cellana* sp. by earlier work (Hameed & Ahmed, 2000; Nasreen *et al.,* 2000; Rahman & Barkati, 2012)) exhibited a single haplotype belonging to the species of *C. karachiensis* (Zafar *et al.,* 2015). In another study by Joseph *et al.* (2016b), three types of limpets with various colour band patterns on the shells in Indian coasts exhibited 99.59% identity with the species *C. karachiensis* based on COI sequencing results. To date, molecular markers have only confirmed the presence of *C. rota* on the Red Sea coasts of Hurghada, Eilat and

Sinai (Nakano & Ozawa, 2007; Schnytzer *et al.,* 2018; Bird *et al.,* 2011). The present study also documents the first molecularly confirmed record of the alien *C. rota* on the Syrian coasts of the Mediterranean. The Syrian specimens showed 99.03-100% sequence identity with previously recorded sequences of *C. rota* in the Red Sea. Based on the molecular analysis, some individuals shared similar COI haplotype with those previously reported for indigenous populations from Eilat, Sinai and Hurghada, indicating the common origin from a single source in the Red Sea. There were also five more haplotypes with 1-8 nucleotide substitutions within the Syrian specimens, probably because of several introduction events, but due to limited molecular sequence data on native populations available on online databases, it is hard to discuss on the mutation sites observed in the present study. The Eastern Mediterranean is considered to be highly prone to biological invasions (Galanidi *et al*., 2023). The major driver of invasion is consecutive enlargements of the Suez Canal since 1960s, establishing a constant sea-level waterway. Like other invertebrates, *C. rota* is possibly introduced to the Mediterranean Sea via the Suez Canal by shipping and range expansion is probably mediated through ballast transport. Mariculture, including oyster farms, has also been considered as a gateway into the Mediterranean Sea for some limpets (Katsanevakis *et al*., 2013), but in the case of *C. rota*, shipping is the most probable pathway of introduction since there is no shellfish farm in the vicinity of the sampling locations. However, species introductions can adversely impact biodiversity, ecosystem services and also fishery benefits (Tsirintanis *et al*., 2022). In this regard, although there is no obvious evidence on direct competition among Lessepsian and native species, there are many cases of considerable change in abundance (Galil & Zenetos, 2002). Replacement of indigenous limpets by lessepsian *C. rota* has been previously reported on the Mediterranean coasts of Israel (Mienis, 2002). However, at a Mediterranean scale, communities are continuously changing and further studies at a large scale are needed to detect the impacts of limpets' invasion in the Eastern Mediterranean Sea.

Different morphs of the *Cellana* complex sympatrically occurred on the Mediterranean rocky shores of Syria. The evolutionary divergence observed between these morphs was very minor despite having differences in their shell attributes. The observed discordance between molecular and morphological results raises the question of whether these four morphs are cryptic or different species or they are really a single species. In a study on different morphs of *C. karachiensis* from Northern Arabian Sea, Joseph *et al.* (2016a) reported a divergence in the genetic lineage of the species and the beginning of a speciation process. In another study by Joseph *et al.* (2016b), three types of limpets with various colour band patterns on the shells on Indian coasts were suggested to be cryptic species of *C. karachiensis*. Cryptic species are considered as distinct evolutionary lineages with considerable genetic disparity and little or no morphological change (Korshunova *et al*., 2019). In the present study, the observed low divergence is not sufficient enough to presume these morphs as separate species. In our study, of the six D-morph individuals, three exhibited U_3-haplotype, two represented U_5-haplotype, and one had U_4- haplotype. Of the two B-morph individuals, one exhibited U_6-haplotype while the other showed U_4-haplotype (nucleotide variation at two positions). This suggests unidirectional or random breeding among these two morphs (Joseph *et al*., 2016a). Morphs A and C were characterised by unique haplotypes (U_2 and U_1, respectively), which were not seen in the other morphs. However, an increased association of haplotypes with special morphs was observed in most cases, suggesting the probability of a partial reproductive barrier and the onset of a speciation process. There may be also weak interaction among dissimilar individuals so long as differences get bigger enough to block the interaction among different morphs (Joseph *et al*., 2016a). Different morphs of *C. rota* may have arisen prior to genetic divergence. However, morphological change does not always accompany speciation, as many apparently identical groups are, in fact, reproductively isolated. In this regard, there may be some assumptions without any obvious conclusion. All this may completely be as a result of randomness and incipient speciation is just an assumption that needs further studies to answer the question if the observed association is purely coincidental or not.

In the present study, based on the observed minor genetic divergence between the morphs, we suggest that these probably belong to a single species. In a study by Zafar *et al*. (2015), three morphs of *C. karachiensis* showing a single haplotype were considered as a single species and the presence of different morphs was attributed to the phenotype plasticity of the species. Teske *et al.* (2007) also reported that four previously identified limpet species of *Siphonaria* (Sowerby, 1823) in South Africa and southern Mozambique are in fact various morphotypes of a single species (0.6-1.2% difference in their COI sequences). Intraspecific phenotypic plasticity of shell morphology is common in many marine molluscs, especially in species with high invasion capacity (Echeverry *et al*., 2020). This may produce ecophenotypic variants where a genotype can cause multiple phenotypes (Keogh *et al*., 2025). Phenotypic plasticity may quickly evolve from genetic diversity maintained by selection if the population has experienced immigration from populations under different selection regimes and may help population persistence under low mutation rates and unlike or impossible constitutive adaptation (Promy *et al*., 2023). However, in our study, different morphs of the Syrian individuals were found contiguous to each other in the same habitat, suggesting no directional selection on shell morphological characters. On the other hand, the individuals with different morphs observed in the present study may come from different regions of the Red Sea and also probably during several introduction events. Therefore, the alien individuals with different morphs may originally belong to native populations of different areas. But to prove this assumption, more morphological and molecular studies are required on native populations.

Overall, further molecular studies seem necessary to verify whether various morphotypes are single species

with different morphs, as suggested in this study, or belong to separate species, since the presence of common haplotypes between species could be related to a recent hybridization or incomplete lineage sorting. Interbreeding may cause sharing similar haplotypes between species, as previously reported for two divergent bat species sympatrically occurring through broad regions in Europe, where Berthier *et al.* (2006) reported replacement of *Myotis blythii* (Tomes, 1857) mitochondrial genome by that in *M. myotis* (Borkhausen, 1797). In contrast, Kemppainen *et al.* (2009) reported absence of any divergence between two *Littorina* (Reeve, 1857) species sympatrically occurring in the North Sea, suggesting two distinct species. The authors stated that species divergence could be related to incomplete lineage sorting. In another study by de Aranzamendi *et al.* (2011), no divergence was observed among two limpets, *Nacella deaurata* (Gmelin, 1791) and *N. magellanica* (Gmelin, 1791), sympatrically occurring through Argentina, in which the divergence was suggested to have started more than 250000 years ago with too low level or absence of gene flow after split. Here, we do not have enough data to reach a precise conclusion about different findings in the previous reports and the present one, and a multifaceted study based on molecular, morphological, ecological and physiological aspects seems to be necessary to answer the question whether they are different morphs, the same, cryptic or different species. However, the data presented here could be a reliable reference for future investigations.

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